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Chitosan for Biomaterials I

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The series *Advances in Polymer Science* presents critical reviews of the present and future trends in polymer and biopolymer science including chemistry, physical chemistry, physics and material science. It is addressed to all scientists at universities and in industry who wish to keep abreast of advances in the topics covered.

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Preface

Chitin and chitosan are known for their excellent biological properties, among which the biocompatibility with human cells, the ordered regeneration of wounded tissues, the immunoenhancing activity, the induction of immediate hemostasis, the radical scavenging activity, and the antimicrobial activity. Recent studies indicate that chitin and chitosan are most versatile in drug and gene delivery, elaborated diagnostics, devices for selective recognition of tumor cells, and surgical aids ranging from anti-adhesion gels to coated sterile stents.

The present volumes entitled "Chitosan for Biomaterials I and II" were conceived to provide broad and thorough knowledge for highly advanced applications of chitosan and its derivatives in the form of micro- and nanoparticles, nanocomposites, membranes, and scaffolds. The books consist of 15 chapters written in a manner that meets the expectations of scientists in various disciplines.

Chapter 1 deals with the use of chitosan and its derivatives in gene therapy. The effect of several parameters on transfection efficiency of DNA (or gene silencing of siRNA) has been discussed. Moreover, specific ligand and pH-sensitive modifications of chitosan for improvement of cell specificity and transfection efficiency (or gene silencing) are explained. Chapter 2 discusses the recent applications of chitosan nano/microparticles in oral/buccal delivery, stomach-specific drug delivery, intestinal delivery, colon-specific drug delivery, and gene delivery. Chapter 3 is focused on the recent developments of chitosan nanoparticles in bladder, breast, colon, lung, melanoma, prostate, pancreatic, and ovarian cancer therapy. Chapter 4 reviews the design of chitosan-based thiomers and their mechanism of adhesion. In addition, delivery systems comprising of thiolated chitosans and their in vivo performance are discussed. The importance of chitosan in particulate systems for vaccine delivery is emphasized in Chap. 5 according to administration routes, particularly the noninvasive routes involving the oral and pulmonary mucosae. Chapter 6 explains various multifunctional chitosan nanoparticles and their recent applications in tumor diagnosis and therapy. Chapter 7 discusses the current advances and challenges in the synthesis of chitosan-coated iron oxide nanoparticles, and their subsequent surface modifications for applications in cancer diagnosis and therapy. Chapter 8 reviews the recent updates of chemical modifications of

chitosan matrices using the cross-linking agents and their applications as drug-eluting devices such as vascular stents, artificial skin, bone grafts, and nerve guidance conduits. Chapter 9 discusses current efforts and key research challenges in the development of chitosan and other polymeric bio-nanocomposite materials for use in drug delivery applications. Chapter 10 provides an overview of chitosan and its derivatives as drug delivery carriers. Here, a special emphasis has been given on the chemical modifications of chitosan in order to achieve a specific application in biomedical fields. Chapter 11 highlights different fabrication methods to produce chitosan-based scaffolds. Moreover, the suitability of chitosan-based scaffolds for bone, cartilage, skin, liver, cornea, and nerve tissue engineering applications is discussed in this chapter. Chapter 12 discusses about chitosan and its derivatives as biomaterials for tissue repair and regeneration. In addition, integration with cell growth factors, genes, and stem cells, applications of the chitosan-based biomaterials in the repair of skin, cartilage, bone, and other tissues are dealt with. Chapter 13 examines the different mechanisms and bond strengths of chitosan coatings to implant alloys, coating composition and physiochemical properties, degradation, delivery of therapeutic agents such as growth factors and antibiotics, and in vitro and in vivo compatibilities. Chapter 14 highlights the beneficial activities of chitosan, and then it directs attention to the important developments of certain technologies capable to expand the surface area of chitosans, with impressive performance improvements in various applications such as drug delivery and orthopedic scaffolds. Finally, Chap. 15 discusses production, properties, and applications of fungal cell wall polysaccharides such as chitosan and glucan.

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Polymeric Nanoparticles of Chitosan Derivatives as DNA and siRNA Carriers

Y.K. Kim, H.L. Jiang, Y.J. Choi, I.K. Park, M.H. Cho, and C.S. Cho

Abstract The success of gene therapy is dependent on finding effective carrier systems. Viral vectors have been widely used in vivo and in clinical trials due to their high transfection efficiency; however, they have several disadvantages, including immunogenicity, potential infectivity, inflammation, and complicated production. Therefore, non-viral vectors have recently been tried as an alternative. Among non-viral vectors, chitosan and chitosan derivatives have been investigated due to several advantages, including biocompatibility, biodegradability, and low toxicity. However, the low transfection efficiency of DNA (or low gene silencing of siRNA) and the low cell specificity of chitosan as a gene carrier need to be overcome before clinical trials. The objective of this review is to discuss the use of chitosan and chitosan derivatives in gene therapy, and the effect of several parameters on transfection efficiency of DNA (or gene silencing of siRNA). Also, specific ligand and pH-sensitive modifications of chitosan for improvement of cell specificity and transfection efficiency (or gene silencing) are explained.

Keywords Cell specificity · Chitosan · Gene therapy · pH-sensitive · Small interfering RNA

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1 Introduction

Gene therapy has the potential to treat inherited and acquired diseases. Clinical therapies are the most ambitious applications of gene delivery [1]. The lack of effective vectors is a major limitation to progress in clinical trials, even though animal viral vectors have been commonly used in vivo and in clinical trials owing to their high transfection efficiency. However, the use of viral vectors in the human body is often accompanied by concerns about immunogenicity, potential infectivity, complicated production, and inflammation [2]. Therefore, non-viral vectors such as liposome [3, 4], diethylaminoethyl dextran [5], poly(L-lysine) [6], polyethylenimine (PEI) [7], polyamidoamine dendrimer [8], poly(β-amino ester) [9], and chitosan [10] have been widely sought as alternatives.

Among non-viral vectors, chitosan and chitosan derivatives are good gene carrier candidates because they are biocompatible and biodegradable, with low immunogenicity and low toxicity [11]. On the other hand, the low transfection efficiency of DNA (or low gene silencing of small interfering RNA (siRNA)] and low cell specificity should be improved for use in clinical trials. The objective of this review is to summarize the use of chitosan and chitosan derivatives in gene therapy, and particularly several parameters affecting the transfection efficiency of DNA (or gene silencing of siRNA). Also, strategies to overcome biological and cellular barriers to gene delivery are discussed. In addition, the roles of chemical modification of chitosan using specific ligands and pH-sensitive groups for enhancement of cell specificity and transfection efficiency (or gene silencing) are covered.

2 Parameters Affecting Transfection Efficiency of Chitosan/DNA Complexes or Gene Silencing of Chitosan/siRNA Complexes

Optimum conditions for transfection efficiency of chitosan/DNA complexes or gene silencing of chitosan/siRNA complexes should be clarified for clinical trials. A schematic illustration of the parameters affecting transfection efficiency of chitosan/DNA complexes has already been reported by Kim et al. [12].

Kiang et al. reported that DNA binding with chitosan decreased with decreasing degree of deacetylation (DDA) in chitosan, therefore requiring an increased N:P charge ratio for achievement of complete DNA complexation. A decrease in DDA resulted in a decrease of transfection efficiency due to destabilization of chitosan/DNA complexes by bulky acetyl groups in the chitosan chains; however, the transfection efficiency in vivo for chitosan with 70% DDA was two orders of magnitude higher than that of chitosan with 90% DDA, indicating differences of transfection efficiency in vitro and in vivo [13]. Lavertu et al. also reported that maximum transfection efficiency was obtained by simultaneous lowering of chitosan molecular weight (MW) and increase of DDA, or lowering of DDA and increase of MW, suggesting that the stability of chitosan/DNA complexes through co-operative electrostatic binding plays a dominant role in obtaining high transfection efficiency [14]. Liu et al. reported that higher gene silencing was obtained using chitosan/siRNA complexes with higher DDA (84%) than with lower DDA (45% and 65%) because chitosan with low DDA has less charge interaction with siRNA, resulting in unstable chitosan/siRNA complexes [15]. The situation is similar for chitosan/DNA complexes.

Huang et al. reported that low-MW chitosan was less efficient at retaining DNA upon dilution; therefore, it was less capable of protecting condensed DNA from degradation by DNase, resulting in low transfection efficiency [16]. Chitosan oligomer (18 monomer units)/DNA complexes formed at an N:P ratio of 60 mediated higher transfection efficiency than high MW (162 kDa) chitosan/DNA complexes formed at an N:P ratio of 2.4, owing to a better ability to release DNA from chitosan/DNA complexes [17]. This result is an indication that a good balance between extracellular DNA protection (better with high MW) versus efficient intracellular unpackaging (better with low MW) to obtain high transfection efficiency by chitosan should be considered [18]. Thibault et al. used fluorescent resonant energy transfer analysis to demonstrate that the most transfection efficient chitosan polyplexes showed an intermediate stability and a kinetics of dissociation that was in synchrony with lysosomal escape [19]. Liu et al. reported that chitosan of higher MW mediated siRNA gene silencing more efficiently than lower MW chitosan because longer chitosan chains probably favor hydrophobic interactions or hydrogen bonds between the sugar residues of chitosan and organic bases of the gene [15].

Techaarpornkul et al. compared gene silencing efficiency in HeLa cells with different chitosan salt forms, including chitosan aspartate, chitosan glutamate,

chitosan acetate, and chitosan hydrochloride because different acids might affect the physicochemical properties of the respective chitosans [20]. The results indicated that the salt form has little effect on gene silencing but depends on the weight ratio of chitosan to siRNA and the MW of chitosan.

Sato et al. studied the effect of pH on transfection of A549 cells because the pH of the culture medium affects the transfection efficiency of chitosan/DNA complexes [21]. The results showed that the transfection efficiency at pH 6.9 was higher than that of pH 7.6 because chitosan/DNA complexes at pH 6.9 are positively charged (due to the chitosan pK_a value of 6–6.8) and can bind to negatively charged cells. Nimesh et al. reported enhanced transfection efficiency in HEK293 cells when transfection was initiated at pH 6.5 with 10% serum for 8–24 h and continued at pH 7.4 with 10% serum for an additional 24–40 h [22].

Sato et al. also investigated the effect of serum on transfection of cells because the serum stability of the gene carrier is very important in vivo [21]. The transfection efficiency of chitosan/DNA complexes in the presence of serum was increased about two- to three-fold compared to transfection efficiency without serum due to increased cell function, although addition of 50% serum resulted in decreased transfection efficiency owing to cell damage.

Ishii et al. studied the effect of charge ratio on transfection of cells [23]. The results indicated that the transfection efficiency of chitosan/DNA complexes increased at charge ratios (N:P) of 3 and 5, and decreased at higher charge ratios, suggesting that there is an optimal range of the N:P ratio for high transfection efficiency. The effect is due to the relationship between cellular uptake and transfection efficiency.

Although the mechanism is not clear, chitosan- or chitosan-derivative-mediated transfection depends on the cell type. Among cells used, HEK293 cells are the most efficiently transfected [24].

Parameters affecting the transfection efficiency of DNA and the silencing effect of siRNA in complexes with chitosan are summarized in Table 1.

3 Biological and Cellular Barriers to Gene Delivery

To reach to the cellular nucleus or cytosol, DNA or siRNA has to be delivered aggressively through barriers. There are two barriers to gene delivery: one at the extracellular level and the other at the intracellular level, as shown in Table 2 [26]. In this section, we discuss strategies for overcoming these barriers.

3.1 DNA or siRNA Condensation and Protection

After administration into the body, a gene delivery system must protect DNA or siRNA and retain its functionality until it reaches the target cell nucleus for DNA or

Table 1 Parameters affecting transfection efficiency (TE) or silencing efficiency (SE) of chitosan polyplexes

Parameter		Optimum condition of TE (or SE)	Character assessment	References
DNA	DDA	Increase	Destabilization of chitosan polyplex by low DDA	[13, 14]
		Decrease of up to 70%	Twofold increase of TE in vivo	[13]
		Decrease	Dissocation of low DDA chitosan from polyplex	[19, 20]
	MW	High	Less protection of DNA by low-MW chitosan	[14]
		Oligomer (18 mer)	Easy release of DNA from complexes	[17, 19]
		Number-average degree of polymerization of 31–42	Optimum balance between polyplex stability and polyplex unpacking	[18]
	pH	6.8–7.0	More protonation of chitosan at acidic pH	[21]
		Change of pH from 6.5 to 7.4	Control of physicochemical and biological condition by pH change	[22]
	Serum	10–20 wt%	Increased cell function. Decrease of TE above 50% serum	[21]
	N:P ratio	3–5	Decrease of TE at higher N:P ratios due to cytotoxicity	[23]
	Cell type	HEK293 cells	Low TE of primary and differentiated cells	[24]
siRNA	DDA	Increase	Instability of chitosan polyplex with low DDA	[15]
	MW	High	Favoring of hydrophobic and hydrogen interactions	[15]
		Low	Easy entanglement of high-MW chitosan polyplex	[25]
	N:P ratio	High	Decrease of particle size with an increase of N:P ratio	[25]

Table 2 Strategies for overcoming extracellular and intracellular barriers [26]

Identified barrier	Strategy employed
Degradation of DNA by serum nucleases	Complexation with cationic liposomes
	Complexation with cationic polymers
Targeting of DNA to particular tissue types	Targeting ligands, e.g., asialoorosomucoid, transferrin, mannose, folate
DNA uptake by cells	Electroporation
	Complexation with cationic liposomes
	Complexation with cationic polymers
Endosomal escape	Endosomolytic peptides
	PEI
Transport from the cytoplasm to the nucleus	Nuclear localization peptides
	Nuclear localization nucleotide sequences

the cytosol for siRNA. DNA for therapeutic purposes is very large (2,000–20,000 kDa), and siRNA is 13 kDa and elongated in structure. Nucleases rapidly degrade genes during circulation and in various tissues and expel them from the body in less than 10 min. Protection against nucleases can be achieved by complexation of the DNA or siRNA with gene carriers such as liposomes or polymers. In addition to nuclease protection, complexes should be compact enough (<300 nm) to protect genes from elimination by the reticuloendothelial system in liver, spleen, and lung. The delivery system must retain its physicochemical properties during circulation in the body because negatively charged proteins compete with gene binding and induce aggregation of complexes. Use of hydrophilic groups, such as dextran [27], poly(ethylene glycol) (PEG) [28], and poly(vinyl pyrrolidone) (PVP) [29], has been used to increase the circulation of the complexes by masking the surface charge and by preventing protein opsonization.

3.2 Uptake by Target Cells

Another challenge for the delivery system is to leave the bloodstream and gain access to extravascular targets, traverse through the dense extracellular matrix while avoiding immune surveillance, and reach the target cells. Cell surface membrane is negatively charged because of the abundant presence of glycoproteins and glycolipids. This negatively charged membrane is a good target for cationic complexes, to induce cellular uptake, although this nonspecific interaction is also a hurdle for cell-specific delivery of genes after local or systemic administration. On the other hand, targeted drug delivery systems promise to expand the therapeutic window of a drug by increasing delivery to target cells [26]. Cell-specific gene delivery can be achieved by use of specific ligands that are specifically recognized by the target cells through a receptor-mediated endocytosis mechanism [26]. Among these ligands, sugar moieties have been extensively used. Detailed examples will be mentioned in the Sect. 4.

3.3 Endosomal Escape

Once the complexes are taken up into endocytic vesicles, they are largely retained in the endosomes or lysosomes and are either degraded or inactivated. Therefore, the ability of the complexes to avoid these fates is an important requirement for enhancement of gene transfer upon internalization. One approach for enhancement of the release of the gene from the endosome involves use of a vector with a high buffering capacity and the flexibility to swell when protonated [30]. Among nonviral vectors, PEI reduces acidification of the endosome, induces an extensive inflow of ions and water, and subsequently leads to rupture of the endosomal membrane [7].

3.4 Nuclear Targeting

Once DNA has escaped from endocytic vesicles, its transportation to the nucleus is vital for nuclear delivery of DNA, although the exact mechanism of nuclear transport remains unclear. Treatment of DNA with specific nuclease inhibitors can increase gene expression, although the benefit is marginal for synthetic vectors. For synthetic vectors, the precise timing of the unpacking of complexes to release DNA is crucial. If unpacking occurs too early then it is likely that the DNA will be susceptible to nuclease degradation, and inadequate unpacking will result in lack of access of the DNA to the transcription machinery. Nuclear membrane proteins form a highly organized supramolecular assembly, known as the nuclear pore complex, and this interacts with a group of cytoplasmic proteins that recognize macromolecules destined for the nucleus. Recognition of nucleoproteins is mediated by nuclear localization signals (NLS) that trigger translocation of DNA to the nucleus [31], and results in increased gene expression.

4 Specific Ligand Modification of Chitosan

One strategy for overcoming the extracellular barriers in non-viral gene delivery is receptor-mediated endocytosis for specific enhancement of cellular uptake. Table 3 summarizes information on the specific ligands incorporated into chitosan for cell specificity.

4.1 Galactose

Galactose is one of most studied ligands for targeting liver parenchymal cells because hepatocytes are the only cells that have large numbers of high-affinity cell surface receptors that can bind asialoglycoproteins (via their exposed galactose residues) [32, 34].

Murata et al. first incorporated antennary galactose residues to trimethylated chitosan for specific targeting to HepG2 cells [49]. The results showed that the cellular recognition ability increased with an increase of galactose residues, although the authors did not perform transfection in cells.

Kim et al. introduced galactose-bearing lactobionic acid to water-soluble chitosan (WSC) through an amide linkage in order to give hepatocyte specificity [33]. Galactosylated chitosan (GC)/DNA complexes were found to show much higher transfection efficiency than WSC/DNA complexes on HepG2 cells, whereas the transfection efficiency of WSC/DNA and GC/DNA complexes did not show any difference on HeLa cells without asialoglycoprotein receptors (ASGPRs).

Jiang et al. coupled galactose with chitosan-*graft*-PEI for hepatocyte targeting, as shown in Fig. 1. The results indicated that GC-*graft*-PEI/DNA complexes showed much

Table 3 Specific ligands incorporated into chitosan for cell specificity

Targeting ligand	Target cells	Character assessment	References
Galactose	HepG2	General chitosan (1,000 K) was used. PEG was grafted to GC for stability in water and enhancement of cell permeability	[28]
		Low-MW chitosan was used. The transfection efficiency of galactosylated low-MW chitosan increased with an increase of galactosylation degree	[32]
		Water-soluble chitosan was used. Particle sizes of the complexes decreased with increasing charge ratio of GC to DNA	[33]
		Lactose as a specific ligand was used. Lactose-chitosan/DNA complexes traversed endocytic compartments more rapidly than did chitosan/DNA complexes	[34]
	HepG2, liver cells	Low-MW PEI was grafted to GC to improve transfection efficiency. GC-*graft*-PEI/DNA complexes transfected liver cells in vivo after intraperitoneal administration more effectively than did PEI 25K	[35]
		Gal-PEG-chitosan-*graft*-PEI/DNA complexes transfected liver cells in vivo after intravenous administration more than PEI 25K and chitosan-*graft*-PEI	[36]
	Kupffer cells, hepatocytes	In vitro results suggested more affinity of galactosylated low-MW chitosan to Kupffer cells than to hepatocytes. More complexes were taken up by Kupffer cells in liver	[37]
	HEK293	Galactose was coupled to the hydroxyl group in the 6-position of the chitosan. HEK293 cells without ASGPRs were used in transfection	[38]
Mannose	Raw 264.7	Water-soluble chitosan was used. The transfection efficiency of MC/DNA was decreased in the presence of mannose	[39]
	Mice	IL-12 gene for the cytokine gene delivery was used. Intratumoral injection of MC/IL-12 complexes into BALB/C mice bearing tumors was carried out in vivo	[40]
	Macrophage	Formylmethyl mannoside was coupled with chitosan by reductive alkylation in the presence of cyanoborohydride. The recognition of the MC/DNA complexes depended on the degree of mannose in chitosan	[41]
	Raw 264.7	Mannose was introduced to chitosan-*graft*-PEI. The MC-*graft*-PEI showed higher transfection than chitosan-*graft*-PEI	[42]
Folic acid	Rat	IL-1 receptor antagonist (IL-1 Ra) gene was used. The folate-chitosan/IL-1 Ra gene complexes showed protection against bone damage and inflammation in a rat adjuvant-induced arthritis model	[43] [44]

(*continued*)

Table 3 (continued)

Targeting ligand	Target cells	Character assessment	References
	KB cells, SKOV3 cells	Transfection efficiencies of folate-TMC/DNA complexes in KB and SKOV3 cells were enhanced to 1.6- and 1.4-fold compared with those of TMC/DNA complexes	
	KB cells	Transfection efficiency of folate- and histidine- (or arginine) TMC/DNA complexes was comparable to that of PEI 25K	[45, 46]
	A549 cells, mice	Folate was introduced to the chitosan-*graft*-PEI. Folate-chitosan-*graft*-PEI/*Akt 1* shRNA complexes suppressed lung tumorigenesis in a mouse lung cancer model through aerosol administration	[47]
Transferrin	HEK293 cells, HeLa cells	Transferrin was conjugated to chitosan nanoparticles. The transferrin conjugation showed a fourfold increase in transfection efficiency compared with chitosan/DNA complexes	[48]

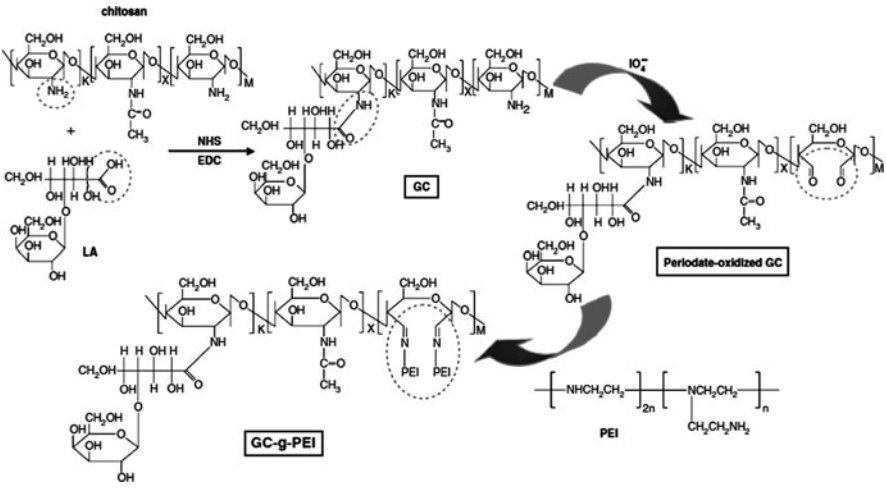

Fig. 1 Proposed reaction scheme for synthesis of GC-*graft*-PEI [35]. *LA* lactobionic acid, *NHS* *N*-hydroxysuccinimide, *EDC* 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide

higher transfection efficiency than chitosan-*graft*-PEI/DNA complexes. Also, when in vivo distribution of ^{99m}Tc-labeled GC-*graft*-PEI/DNA complexes was checked after intraperitoneal injection in mice, more ^{99m}Tc-GC-*graft*-PEI/DNA complexes were accumulated in the liver over time than ^{99m}Tc-labeled PEI/DNA complexes and ^{99m}Tc-labeled CHI-*graft*-PEI/DNA complexes. By contrast, accumulation of ^{99m}Tc-chitosan-*graft*-PEI/DNA complexes was decreased after 180 min [35]. Jiang et al. also synthesized Gal-PEG-chitosan-*graft*-PEI, as shown in Fig. 2 [36], and evaluated it for hepatocyte specificity in vitro and in vivo. The results showed that the transfection efficiency of Gal-PEG-chitosan-*graft*-PEI/DNA complexes on HepG2 was higher than that of PEG-chitosan-*graft*-PEI/DNA complexes, although the transfection

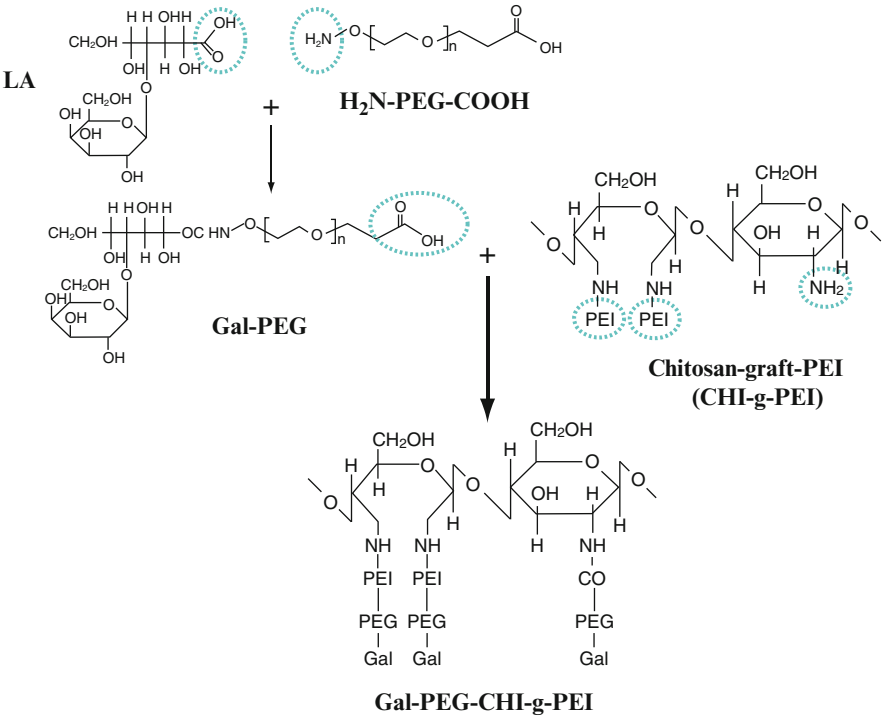

Fig. 2 Proposed reaction scheme for synthesis of Gal-PEG-chitosan-*graft*-PEI [36]. Circle residues: reaction groups

efficiency of Gal-PEG-chitosan-*graft*-PEI in vitro was lower than that of PEI 25K (PEI of 25 kDa) and Lipofectamine due to incorporation of PEG into the carrier. On the other hand, Gal-PEG-chitosan-*graft*-PEI/DNA complexes transfected liver cells more effectively than did PEI 25K in vivo after intravenous administration [36].

Song et al. prepared GC by coupling of galactose to the hydroxyl group in the 6-position of chitosan; however, they performed transfection of HEK293 cells, without ASGPRs [38].

Recently, Dong et al. reported that galactosylated low-MW chitosan/oligodeoxynucleotide complexes had higher affinity to macrophages than to hepatocytes in vitro, although the mechanism is not clear. Also, more galactosylated low-MW chitosan/oligodeoxynucleotide complexes were taken up by Kupffer cells in liver than by hepatocytes, indicating that there might be other lectins involved in endocytosis [37].

4.2 Mannose

Macrophages and immature dendritic cells (DCs) as antigen presenting cells (APCs) express high levels of mannose receptors that are used for endocytosis and phagocytosis of a variety of antigens that expose mannose [50].

Kim et al. prepared mannosylated chitosan (MC) to obtain receptor-mediated endocytosis for targeting to APCs [39]. The results indicated that MC/DNA complexes showed higher transfection efficiency compared with WSC/DNA complexes in Raw 264.7 cells, owing to the receptor-mediated endocytosis mechanism [39]. They also performed interleukin 12 (IL-12) gene delivery through intratumoral injection of MC/IL-12 complexes into BALB/C mice bearing tumors at the injected sites [40]. Intratumoral delivery of MC/IL-12 complexes suppressed more tumor growth than the control due to the mechanism of apoptosis and cell cycle arrest.

Hashimoto et al. synthesized MC by reductive ozonolyzation of allyl mannoside with chitosan [41]. MC/DNA complexes showed higher transfection efficiency than chitosan/DNA complexes.

Recently, Jiang et al. prepared MC-*graft*-PEI for Raw 264.7 cell targeting, as shown in Fig. 3 [42]. The results indicated that the transfection efficiency of MC-*graft*-PEI/DNA complexes into Raw 264.7 cells was higher than that of chitosan-*graft*-PEI and had lower cytotoxicity, suggesting that the MC-*graft*-PEI can be used as an APC-targeting gene carrier.

4.3 *Folate*

Folate receptors (FRs), known as a high-affinity membrane folate-binding protein and overexpressed in various human cancer cells, have been reported to bind to folic acid with high affinity ($K_d \sim 10^{-10}$ M) [51].

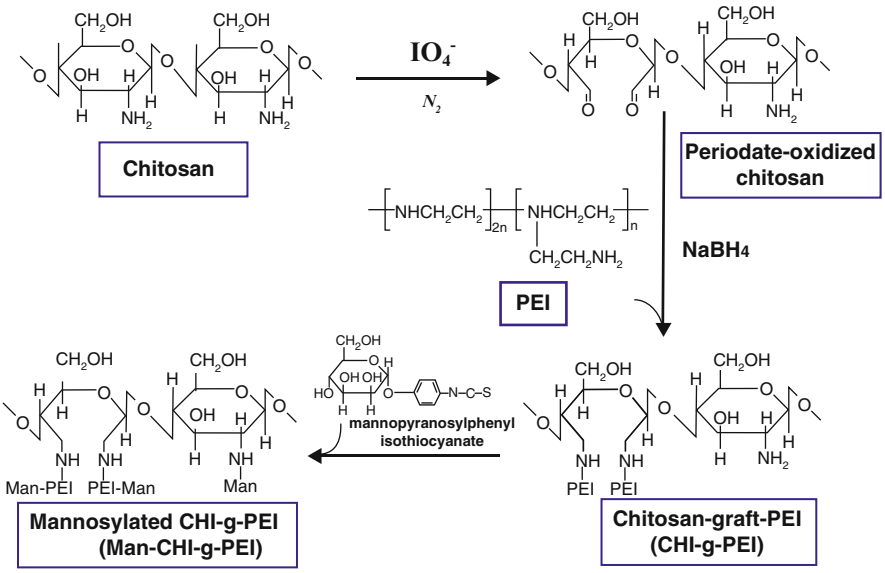

Fig. 3 Proposed reaction scheme for synthesis of MC-*graft*-PEI [42]

Mansouri et al. [52] and Chan et al. [53] introduced folic acid to chitosan and chitosan-*graft*-PEG, respectively, for folate-mediated endocytosis; however, they did not check transfection efficiency in vitro.

Lee et al. also introduced folic acid to chitosan for specific delivery of DNA to FR-overexpressing cancer cells [54]. Folic-acid-coupled chitosan was found to exhibit enhanced transfection efficiency in FR-overexpressing cancer cells, compared to chitosan itself. Fernandes et al. studied the protective effects of IL-1 receptor antagonists in bone metabolism in vivo using folate-coupled chitosan [43]. Folate-chitosan/DNA complexes enhanced IL-1 Ra protein synthesis in vitro and indicated better protection against inflammation and abnormal bone metabolism in vivo, compared to chitosan/DNA complexes. Zheng et al. coupled folate to *N*-trimethyl chitosan (TMC) [44]. The results indicated that the transfection efficiency of folate-TMC/DNA complexes in KB and SKOV3 cells (both folate receptor overexpressing cells) were enhanced up to 1.6- and 1.4-fold compared with those of TMC/DNA complexes; however, there was no significant difference between transfection efficiency of the two complexes in A549 and NIH/3T3 cells (both folate receptor deficient cells). Recently, Morris et al. introduced folate into histidine-modified TMC [45] and arginine-modified TMC [46] for enhancement of cellular and nuclear uptake. The transfection efficiency of modified chitosans was comparable to that of PEI 25K, owing to the conjugation of both folate and histidine (or arginine) to chitosan; however, they did not check the function of histidine and arginine in the carriers.

As shown in Fig. 4, Jiang et al. prepared folate-chitosan-*graft*-PEI for in vitro and in vivo delivery of *Akt1* shRNA [47]. Folate-chitosan-*graft*-PEI/*Akt1* shRNA

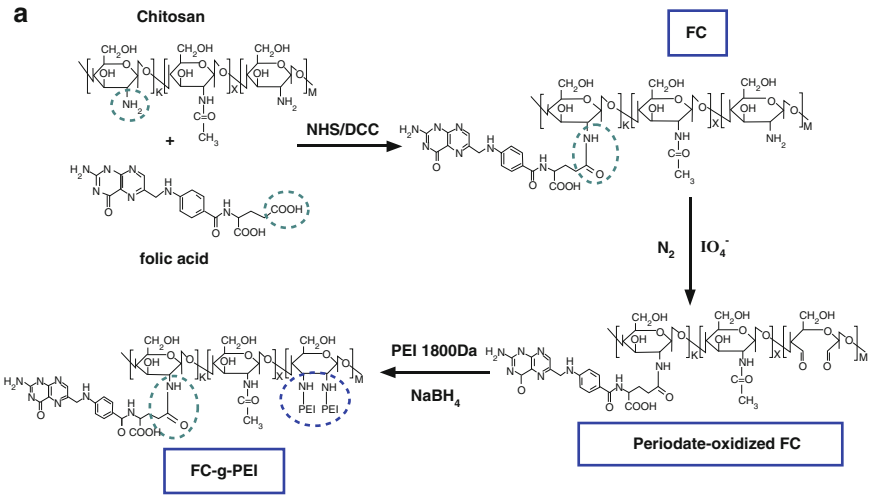

Fig. 4 Proposed reaction scheme for synthesis of FC-*graft*-PEI [47]. *NHS N*-hydroxysuccinimide, *DCC N,N'*-dicyclohexylcarbodiimide. Circle residues: reaction groups

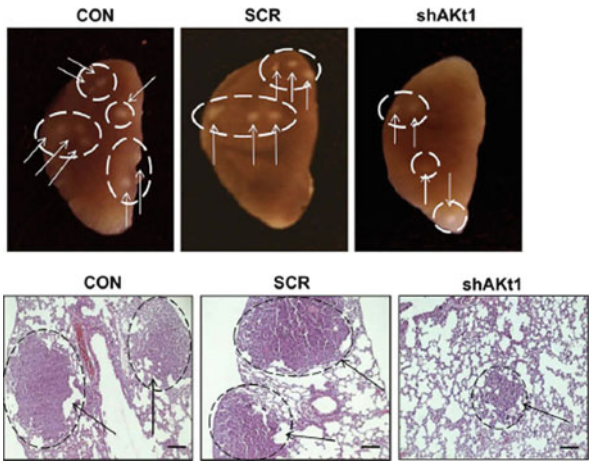

Fig. 5 Efficiency of FC-*graft*-PEI as an aerosol carrier for delivery of gene-silencing shRNA. *Upper panel*: Lungs show numerous visible lesions (*arrows* and *dotted circles*). *Lower panel*: Histological characteristics. *Arrows* indicate the incidence of lesions in the lungs. *CON* untreated control, *SCR* after treatment with scrambled shRNA, *shAkt1* after treatment with *Akt1* shRNA. 100× magnification, scale bars: 100 mm [47]

complexes showed more gene silencing in A549 cells in vitro than did chitosan-*graft*-PEI/*Akt1* shRNA complexes and showed greater suppression of lung tumorigenesis in a mouse urethane-induced lung cancer model after aerosol delivery in vivo through the Akt signaling pathway, as shown in Fig. 5.

4.4 Transferrin

Transferrin receptors are found on many mammalian cells and transferrin as a ligand is used for delivery of drugs, liposomes, and macromolecules [55]; however, use of transferrin in chitosan has rarely been reported.

Mao et al. only introduced transferrin to chitosan [48]. Compared with chitosan itself, transferrin-coupled chitosan showed a fourfold increase of transfection efficiency in HEK293 and HeLa cells.

4.5 Epidermal Growth Factor and RGD

Epidermal growth factor (EGF) can bind with receptors of tumor cells with high affinity; therefore, Supaprutsakul et al. recently conjugated EGF to chitosan for tumor-targeted gene delivery [56]. However, compared with chitosan/DNA complexes, EGF-conjugated chitosan/DNA complexes did not improve transfection efficiency due to aggregation of the complexes. Han et al. also coupled RGD (peptide Arg-Gly-Asp) with chitosan for tumor-targeted gene delivery [57]. The results indicated that RGD-chitosan/siRNA complexes significantly increased selective intratumoral delivery in orthotopic animal models of ovarian

cancer. Compared with controls, RGD-chitosan/*PLXDC1* siRNA complexes in αβ3-integrin-positive tumor endothelial cells of tumor-bearing mice significantly inhibited tumor growth.

5 Modification of Buffering Capacity

One of the primary causes of poor transfection efficiency by chitosan across intracellular delivery is inefficient release of DNA in chitosan/DNA complexes from endosomes into the cytoplasm [58]. Therefore, chitosan was modified with pH-sensitive groups having buffering capacity to overcome the intracellular barrier. Table 4 summarizes pH-sensitive groups incorporated into chitosan for buffering capacity.

5.1 Imidazole

Imidazole-containing polymers designed on the basis of histidine have been reported to have a proton-sponge property and enhanced release of complexes into the cytoplasm following adsorptive pinocytosis [62].

Kim et al. introduced urocanic acid (UA) bearing an imidazole ring to have a proton sponge effect into modified chitosan [59]. Transfection efficiency in 293T cells by UAC/DNA complexes was increased with an increase of UA in the UAC owing to the buffering property in the endosomal compartment, which is similar to that for PEI. They also delivered UAC/programmed cell death protein 4 (PDCD4) complexes into a *K-ras* null lung cancer mouse model through nose-only inhalation [60]. The results indicated that UAC/PCD4 complexes suppressed tumor angiogenesis, facilitated apoptosis, and inhibited pathways important for cell proliferation, compared with vector control. In addition, they delivered UAC/phosphatase and tensin homolog deleted on chromosome 10 (PTEN) complexes into a *K-ras* lung cancer mouse model through an aerosol route [61]. UAC/PTEN complexes were found to significantly suppress lung tumors by nuclear complex formation between PTEN and p53 through Akt-related signal pathways as well as cell cycle arrest.

Wang et al. transfected UAC/p53 gene complexes into HepG2 in vitro and into BALB/C nude mice through intratumoral injection in vivo [62]. UAC/p53 gene complexes induced apoptosis by inhibiting the growth of HepG2 cells in vitro and suppressed tumor growth in vivo.

Ghosn et al. introduced imidazole acetic acid (IAA) into chitosan to improve its endosomal buffering and water solubility [63]. The results indicated that IAA-modified chitosan mediated a 100-fold increase in transfection efficiency, compared to chitosan itself.

Moreira et al. also introduced IAA into chitosan [64]. According to the results, IAA-chitosan with 22.1% of substitution was found to enhance β-galactosidase

Table 4 pH-sensitive groups incorporated into chitosan to give buffering capacity

pH-sensitive group	Gene	Character assessment	References
Imidazole	Plasmid DNA	Urocanic acid (UA) bearing an imidazole ring was introduced to chitosan. The transfection efficiency increased with increasing substitution value of UA	[59]
	PDCD4	A mouse *K-ras* null lung cancer model was used in vivo. Nose-only inhalation was administered	[60]
	PTEN	Lung tumor was suppressed by the UAC/PTEN complexes through Akt-related signal pathways as well as through cell cycle arrest	[61]
	p53	UAC/p53 gene complexes were injected intratumorally. The complexes induced apoptosis in vivo	[62]
	Plasmid DNA	Imidazole acetic acid (IAA) was introduced to chitosan. Water-solubility was also improved by introduction of IAA to chitosan	[63–65]
	siRNA	PEG-IAA-chitosan/siRNA complexes significantly knocked down GAPDH enzyme in lung and liver after intravenous delivery and silenced GAPDH protein expression in the lungs after intranasal delivery	[66]
Low-MW PEI	Plasmid DNA	Cationic polymerization of aziridine was performed by water-soluble oligo-chitosan. The complexes were injected into the bile duct of rat liver	[67]
		Chitosan-*graft*-PEI was obtained by imine reaction between periodate-oxidized chitosan and low-MW PEI	[68]
		Low-MW PEI was grafted to *N*-maleated chitosan. The particle size of the complexes was 200–400 nm	[69]
		Low-MW PEI was grafted to *N*-succinyl-chitosan. The transfection efficiency was not affected by serum	[70]
		Ethylene glycol diglycidyl ether was used as a spacer to improve the water solubility of chitosan. The transfection efficiency of the PEI-*graft*-chitosan was increased as compared with chitosan	[71]
		1,1′-carbonyldiimidazole was used as a linking agent between chitosan and PEI. Long-term transfection efficiency of the chitosan-*graft*-PEI was higher than for PEI 25K in vitro	[72]
	Akt1 siRNA	Chitosan-*graft*-PEI efficiently delivered *Akt1* siRNA and thereby silenced onco-protein Akt1 in A549 cells	[73]
MAA	*RhoA* siRNA	MAA/TMC/siRNA complexes exhibited higher inhibition of *RhoA* mRNA expression than did TMC/siRNA complexes	[74]

expression in 293T and HepG2 cells due to escape of the complexes from the endosomes.

Recently, Chang et al. introduced histidine to chitosan in order to increase cellular uptake and impart a buffering effect [65]. Cellular uptake of histidine-chitosan/DNA complexes was higher than that of chitosan/DNA complexes. A broader buffering range of histidine-chitosan was observed and resulted in high gene expression of histidine-chitosan/DNA complexes, even though only 3–4% of histidine was introduced to chitosan.

Ghosn et al. [66] used IAA-chitosan to mediate gene silencing after administration via either intravenous or intranasal routes. PEGylated IAA-chitosan/siRNA complexes significantly knocked down glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme in both lung and liver after intravenous delivery. Significant silencing of GAPDH protein expression was found in the lungs over 3 days after intranasal delivery.

5.2 Low-MW PEI

PEI has been one of the most widely used polymeric carriers because it has an excellent buffering capacity in an endosomal environment and facilitates endosomal escape to the cytoplasm. However, PEI has a high cytotoxicity in vitro and in vivo, although the cytotoxicity of PEI depends on the molecular weight of PEI. Low-MW PEI has less cytotoxicity, with low transfection efficiency.

Wong et al. first prepared PEI-*graft*-chitosan through cationic polymerization of aziridine by water-soluble oligo-chitosan to obtain a synergistic effect of PEI (having a proton sponge effect) and chitosan (having biocompatibility) [67]. PEI-*graft*-chitosan was found to show higher transfection efficiency than that of PEI 25K in vitro and in vivo, with lower cytotoxicity.

Jiang et al. also synthesized chitosan-*graft*-PEI through an imine reaction between periodate-oxidized chitosan and low-MW PEI (1,800 Da) [68]. Chitosan-*graft*-PEI/DNA complexes showed higher transfection efficiency than PEI 25K in three different cell lines (HeLa, 293T, and HepG2), with lower cytotoxicity owing to low-MW PEI.

Lu et al. grafted low-MW PEI (800 Da) to *N*-maleated chitosan (NMC-*graft*-PEI) by a Michael addition reaction [69]. NMC-*graft*-PEI/DNA complexes showed high transfection efficiency in 293T and HeLa cells, compared to PEK 25K; however, the particle sizes of the NMC-*graft*-PEI/DNA complexes were 200–400 nm. They also grafted low-MW PEI (800 Da) to *N*-succinyl-chitosan (NSC) as a serum-resistant gene carrier [70]. The transfection efficiency of NSC-*graft*-PEI/DNA complexes was found to be higher than that of PEI/DNA complexes, and the transfection efficiency of NSC-*graft*-PEI/DNA complexes was not affected by the presence of serum. Lou et al. synthesized PEI-*graft*-chitosan using ethylene glycol diglycidyl ether as a spacer in order to increase the water solubility and transfection efficiency of chitosan [71]. The transfection efficiency of

PEI-*graft*-chitosan/DNA complexes was significantly higher than that of chitosan/DNA complexes at high weight ratios, with lower cytotoxicity.

Recently, Gao et al. also prepared chitosan-*graft*-PEI using 1,1′-carbonyldiimidazole as a linking agent between chitosan and PEI (1.8 kDa) and evaluated it as a gene carrier in vitro and in vivo [72]. The results indicated that long-term transfection efficiency of chitosan-*graft*-PEI in three different cancer cells was higher than that of PEI 25 kDa, with lower cytotoxicity. The tumor growth rate was significantly decreased when mice were inoculated with the *CCL22* gene (using chitosan-*graft*-PEI as gene carrier) due to the rapid escape of the carrier from endosomes into the cytoplasm.

Jere et al. prepared chitosan-*graft*-PEI and evaluated it for delivery of *Akt1* siRNA to A549 cells [73]. Chitosan-*graft*-PEI efficiently delivered *Akt1* siRNA and thereby silenced onco-protein Akt1 in A549 cells. Also, silencing of cell survival protein significantly reduced lung cancer proliferation and decreased malignancy and metastasis of A549 cells.

5.3 Methacrylic Acid

Dehousse et al. added pH-sensitive methacrylic acid (MAA) copolymer to TMC/siRNA complexes to promote escape of complexes from the endosome to the cytoplasm [74]. MAA/TMC/siRNA complexes were able to transfect L929 cells with greater efficiency of inhibition of *RhoA* mRNA expression than were TMC/siRNA complexes owing to the membrane-destabilizing property of MAA in the endosome.

6 Summary

As discussed in this review, chitosan and chitosan derivatives have been utilized as non-viral vectors for DNA and siRNA delivery systems. The efficiency of transfection or gene silencing using chitosan as carrier depends on the DDA and MW of chitosan, the pH and serum content of the medium, charge ratio of chitosan to DNA (or siRNA), and cell type. A number of in vitro and in vivo studies on chitosan and chitosan derivatives as non-viral carriers have been performed. Continued development of structure–gene function relationships and fundamental studies on cellular processes in vitro and in vivo are necessary for future development of chitosan and chitosan derivatives as gene carriers. Also, most studies carried out so far have only been conducted using in vitro and animal models; therefore, additional preclinical studies need to be conducted for approval in clinical trials.

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Chitosan and Its Derivatives for Drug Delivery Perspective

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Abstract Biopolymers are promising materials in the delivery of protein drugs due to their compatibility, degradation behavior, and nontoxic nature on administration. On suitable chemical modification, these polymers can provide better materials for drug delivery systems. Nanostructured drug carriers allow the delivery of not only small-molecule drugs but also of nucleic acids and proteins. The use of biopolymers like dextran, starch, alginate, and pullulan nanoparticles in drug delivery are briefly discussed. Being the only cationic polysaccharide of natural origin, chitosan, a versatile biopolymer of the aminoglucopyran family is being extensively explored for various biomedical and pharmaceutical applications such as drug delivery. In this review, we aim to comprehensively integrate the recent applications of chitosan nano/microparticles in oral and/or buccal delivery, stomach-specific drug delivery, intestinal delivery, colon-specific drug delivery, and gene delivery, giving special emphasis to oral drug delivery.

Keywords Chitosan · Drug delivery · Mucoadhesion · Nanoparticles · Thiolated chitosan

Contents

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1 Introduction

Nanotechnology is being increasingly explored in science and industry for widely different applications. Nanotechnology and polymers have captivated a tremendous interest in many areas such as the pharmaceutical industry and therapeutic innovation among others. Natural and synthetic polymers have been used as a promising tool for nanoscale drug carrier systems, especially in oral administration of poorly absorbed therapeutic drugs [1]. In recent years, great developments have been made in the field of mucoadhesive polymer systems in formulations that increase the residence time of drugs on mucosal membranes and subsequently, enhance the bioavailability of drugs with poor oral absorption [2, 3]. In this review, we will be emphasizing the importance of the mucoadhesive polymer chitosan and its derivatives as a drug carrier, giving special attention to oral protein delivery.

2 Nanomaterials

The rapid expansion of nanotechnology promises to have great benefits for society. Nanomaterial is matter at dimensions of roughly 1–100 nm, where a unique phenomenon offers novel applications. The major advantages of nanoparticles as a delivery system are in controlling particle size, surface properties, and release of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen. Although nanoparticles offer many advantages as drug carrier systems, there are still many limitations to be solved such as poor oral bioavailability, instability in circulation, inadequate tissue distribution, and toxicity [4]. For example, their small size and large surface area can lead to particle aggregation, making physical handling of nanoparticles difficult in liquid and dry forms. In addition, small particle size and large surface area readily result in limited drug loading and burst release. These practical problems have to be overcome before nanoparticles can be used clinically or made commercially available. Nanotechnology is making significant advances in biomedical applications, including newer drug delivery techniques. There has been considerable research in the developing of biodegradable nanoparticles as effective drug delivery systems [5]. The drug is dissolved, entrapped, adsorbed, attached, or encapsulated into the nanoparticle matrix. The nanoparticle matrix can be of biodegradable materials such as polymers or proteins. Depending on the method of preparation, nanoparticles can be obtained with different properties and release characteristics for the encapsulated therapeutic agents [6].

2.1 Nanomaterials in Drug Delivery

Nanoparticles applied as drug delivery systems are submicron-sized particles (3–200 nm), devices, or systems that can be made using a variety of materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes), viruses (viral nanoparticles), and even organometallic compounds (nanotubes) [7]. Nanoparticles are engineered structures with at least one dimension of 100 nm or less. These novel materials are increasingly used for commercial products, including developing new designs for medicinal application. We are facing tremendous opportunities and challenges in combining emerging nanotechnology with cellular and molecular techniques to develop better diagnosis and therapeutics for diseases such as cancer. However, before these applications are used in clinical practice and commercialized, we have to ensure that they are safe. A number of studies have demonstrated that nanoparticle toxicity is complex and multifactorial, potentially being regulated by a variety of physicochemical properties such as size, chemical composition, and shape, as well as surface properties

such as charge, area, and reactivity [8]. However, there is no regulation or guideline, particularly in toxicology, to guide the research of medicinal application of nanomaterials.

3 Biopolymers in Drug Delivery

During the past few decades there has been an increasing interest in the development of biodegradable nanoparticles for effective drug, peptide, protein, and DNA delivery [9]. Incorporation of the drug into a particulate carrier can protect the active substance against degradation in vivo and in vitro, improve therapeutic effect, prolong biological activity, control drug release rate, and decrease administration frequency [10]. At present, biodegradable polymers like starch, dextran, pullulan, chitosan, and alginate (Fig. 1) are being used to encapsulate proteins and peptides. In this section we will discuss the use of these biodegradable polymers as drug carriers. Although a number of synthetic biodegradable polymers have been developed for biomedical applications, the use of natural biodegradable polymers remains attractive because of their abundance in nature, good biocompatibility, and ability to be readily modified by simple chemistry [11]. A majority of drug delivery systems using natural polymers have been based on proteins (e.g., collagen, gelatin, and albumin) and polysaccharides (e.g., starch, dextran, hyaluronic acid, and chitosan). Polysaccharide-based microparticles have gained much attention in developing controlled-release microparticulate systems because of their flexibility in obtaining a desirable drug release profile, cost-effectiveness, ease of modification by simple chemical reactions for specific applications, broad range of physicochemical properties, and broad regulatory acceptance [12]. Further, they display biocompatibility and biodegradability, which are the basic characteristics for polymers used as biomaterials. Therefore, they are widely used in pharmaceutical formulations and, in several cases, they play a fundamental role in determining the release rate from the dosage form. Polysaccharide biodegradable matrices are of interest because the degradation of natural products like starch occurs naturally in the human body [13, 14]. Applications of proteins to delivery of protein drugs have been limited due to their poor mechanical properties, low elasticity, possible occurrence of an antigenic response, and high cost [15].

3.1 Alginate

Alginate is a nonbranched, high molecular weight binary copolymer of (1-4) glycosidically linked β-D-mannuronic acid and α-L-guluronic acid monomers (Fig. 1b) [16, 17]. The high acid content allows alginic acid to undergo spontaneous and mild gelling in the presence of divalent cations, such as calcium ions.

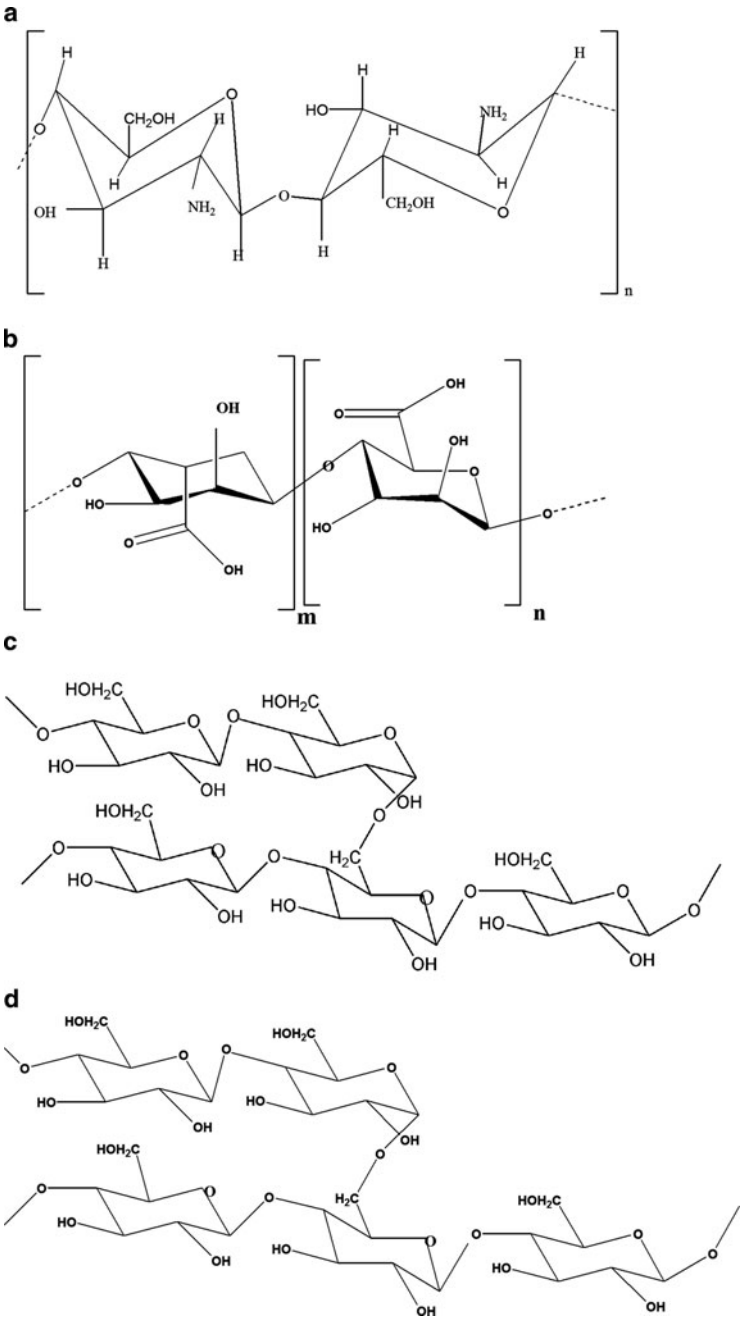

Fig. 1 Natural polymers used in drug delivery: (**a**) chitosan, (**b**) alginate, (**c**) starch, and (**d**) dextran

This mild gelling property allows the encapsulation of various molecules or even cells within alginate gels with minimal negative impact. Furthermore, the carboxylic acid groups of alginic acid are highly reactive and can be appropriately modified for various applications. Alginate has also been extensively investigated as a drug delivery device [18–20] wherein the rate of drug release can be varied by varying the drug polymer interaction as well as by chemically immobilizing the drug to the polymer back bone using the reactive carboxylate groups. Hydrophobically modified alginates are also used for drug delivery applications [21]. The encapsulation of proteins and bioactive factors within ionically crosslinked alginate gels are known to greatly enhance their efficiency and targetability and, as a result, extensive investigation has been undertaken to develop protein delivery systems based on alginate gels [18, 22]. A disadvantage of using alginate-based gels, apart from their poor degradability, is poor cell adhesion on alginate gel. However, the encapsulation efficiency of this system is low, due to its porous nature [23]. The unique property of these linear copolymers is that their surface is negatively charged. When positively charged polymers are added to the alginate solution, they can form a polycation–polyanion complex, which will enhance the overall stability of the microcapsules. Several natural polymers such as chitosan [24] have been used in combination with sodium alginate in order to increase the encapsulation efficiency and hence the protein release profiles. At low pH (gastric environment), alginate shrinks and the encapsulated contents are not released. The payload of, e.g., insulin, could be increased by liposome encapsulation and this lipoinsulin can be entrapped in an alginate system. The aqueous interior of the liposome will preserve the structure and conformation of insulin, while the lipid exterior may help improve absorption across biological barriers. Oral administration of lipoinsulin-loaded alginate–chitosan capsules was found to reduce blood glucose level in diabetic rats. The effect could be observed within 6 h and was prolonged when compared with insulin-loaded alginate–chitosan capsules [25]. It has been reported that purified alginate is nontoxic and biodegradable when given orally.

3.2 Starch

Starch, $(C_6H_{10}O_5)_n$, consists mainly of two glucosidic macromolecules, amylose and amylopectin (Fig. 1c). Due to the substantial swelling and rapid enzymatic degradation of native starch in biological systems, it is not suitable in some controlled drug delivery systems. Starch is a potentially useful polymer for thermoplastic biodegradable materials because of its low cost, availability, and production from renewable resources [26]. However, it has some limitations like low moisture resistance, poor processability (high viscosity), and incompatibility with some hydrophobic polymers. Various strategies like physical or chemical modification of starch granules have been created to overcome these problems [27, 28]. The hydrophilic nature of starch due to the abundance of hydroxyl

groups, is a major constraint that seriously limits the development of starch-based material for industrial applications. Chemical modification has been studied as a way to solve this problem and to produce water-resistant material. Esterification with organic acid is known to result in thermoplastic and hydrophobic starch material.

Mahkam et al. modified chitosan crosslinked starch polymers for oral insulin delivery. Increasing the chitosan content in the copolymer enhanced the hydrolysis in the SIF and thus led to slower release in intestinal pH [29].

3.3 Dextran

Dextran is a polysaccharide consisting of glucose molecules coupled into long branched chains, mainly through the 1,6- and partly through the 1,3-glucosidic linkages (Fig. 1d). Dextrans are colloidal, hydrophilic and water-soluble substances, which are inert in biological systems and do not affect cell viability [30]. Dextrans have been used for many years as blood expanders, whose role is to maintain or replace blood volume and their use as a carrier system for a variety of therapeutic agents have also been investigated, including for antidiabetics, antibiotics, anticancer drugs, peptides and enzyme [31]. Since dextran can be degraded by the enzyme dextranase in the colon, it has been possible to design polymeric prodrugs or nanoparticles for colonic drug delivery based on dextran [32]. Dextran molecules could be degraded by microbial dextranases, which make the ester bond accessible to hydrolysis, thereby releasing the drugs.

Dextran and its derivatives are among the main promising candidates for the preparation of networks capable of giving a sustained release of proteins. Poly (lactide-*co*-glycolide) (PLGA)-grafted dextran was used as a nanoparticulate oral drug carrier. It was expected that dextran would form the hydrophilic outer shell, due to its solubility in water, while PLGA would form the inner core of the nanoparticle, due to its hydrophobic properties. Because the dextran domain can degrade in the colon, nanoparticles of PLGA-grafted dextran can be used as a drug carrier for oral targeting [33].

Sarmento et al. developed nanoparticle insulin delivery system by complexing negatively charged dextran sulfate and positively charged chitosan [34]. The author states that release profile of insulin suggest a dissociation-driven, pH-dependent release mechanism. This was further illustrated by a slower release from particles with a higher ratio of dextran sulfate to chitosan. Increasing the ratio from 1:1 to 2:1 decreased the total insulin release after 24 h from 76 to 59%. Vitamin B12-coated dextran nanoparticle conjugates (150–300 nm) showed profound (70–75% blood glucose reductions) and prolonged (54 h) antidiabetic effects with biphasic behavior in streptozotocin-induced diabetic rats [35]. Modified dextran is also used in gene delivery applications [36, 37].

3.4 Pullulan

Pullulan is a linear bacterial homopolysaccharide formed by glycosidic linkages of α-(1 → 6) D-glucopyranose and α-(1 → 4) D-glucopyranose units in a 1:2 ratio, originating from *Aurebasidium pullulans* [38]. This polysaccharide has numerous uses: in foods and beverages as a filler; in pharmaceuticals as a coating agent; in manufacturing and electronics where it is used because of its film and fiber-forming properties. The backbone structure of pullulan resembles dextran with both of them lending themselves as plasma expanders. Like dextran, pullulan can also be easily derivatized in order to impart new physicochemical properties, e.g., to increase the solubility in organic solvents or to introduce reactive groups.

Anionic and/or amphiphilic pullulan hydrogel microparticles have also been obtained using epichlorohydrin or sodium trimetaphosphate as crosslinking agents [39]. The interactions between lysozyme and various substrates were studied. Such nanoparticles were able to protect the loaded enzyme in acidic environment, thus suggesting its possible use for oral administration of gastro-sensitive drugs. The presence of anionic charges in nanogels crosslinked by trisodium trimetaphosphate also allowed the entrapment of cationic compounds such as methylene blue and polyethylenimine, the model drug's release being affected by the ionic strength of the medium. Cationized pullulan is also used in gene delivery [40].

Though pullulan is not a natural gelling polysaccharide, an appropriate chemical derivatization of its backbone can actually lead to a polymeric system capable of forming hydrogel. Hydrophobized pullulan, i.e., cholesterol-bearing pullulan nanogels were capable of binding various hydrophobic substances and various soluble proteins [41, 42] and has been used as a drug delivery carrier for hydrophobic substances, such as anticancer drugs like adriamycin, doxorubicin etc. and also for gene delivery [43–45].

3.5 Chitosan

Chitosan is a natural, cationic aminopolysaccharide (p*K*a 6.5) copolymer of glucosamine and *N*-acetylglucosamine obtained by the alkaline, partial deacetylation of chitin. It is the second most abundant natural polysaccharide and originates from shells of crustaceans. Chitosan is a biodegradable, biocompatible, positively charged nontoxic mucoadhesive biopolymer. Since chitosan contains primary amino groups in the main backbone that make the surfaces positively charged in biological fluids, biodegradable nano/microparticles can be readily prepared by treating chitosan with a variety of biocompatible polyanionic substances such as sulfate, citrate, and tripolyphosphate [46]. These unique features of chitosan have stimulated development of delivery systems for a wide range of biological agents [47]. Its natural mucoadhesive properties allows design of bioadhesive drug carrier systems that can bind to the intestinal mucosa, and thus improve the residence time

of drugs in the intestinal lumen and, consequently, their bioavailability [48]. Chitosan has been reported to enhance drug permeation across the intestinal, nasal, and buccal mucosa [49]. Chitosan microspheres have arisen as a promising candidate in oral or other mucosal administration for improving the transport of biomacromolecules such as peptides, proteins, oligonucleotides, and plasmids across biological surfaces. This is because chitosan microspheres can improve the drug adsorption via the paracellular route. Chitosan nanoparticles were first prepared in 1997 by Alonso et al. [50] and the same group later used chitosan–insulin nanoparticles for the nasal delivery of insulin in rabbits. Chitosan nanoparticles have been developed to encapsulate proteins and nucleic acids [51]. Chitosan considerably enhanced the absorption of peptides such as insulin and calcitonin across the nasal epithelium. Chitosan is generally considered nontoxic and biodegradable, with an oral LD50 in mice of over 16 g/kg [52].

4 Chitosan Sources and Chemical Structure

Chitin is found in the exoskeleton of some anthropods, insects, and some fungi. Commercial sources of chitin are the shell wastes of crab, shrimp, lobster, etc. Chitosan is usually prepared by the deacetylation of chitin. The conditions used for deacetylation will determine the average molecular weight (Mw) and degree of deacetylation (DD). The structure of chitosan is very similar to that of cellulose [made up of β (1-4)-linked D-glucose units], in which there are hydroxyl groups at C-2 positions of glucose rings. Chitosan is a linear copolymer polysaccharide consisting of β (1-4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) units (see Fig. 1a and graphical abstract) The properties, biodegradability, and biological role of chitosan is frequently dependent on the relative proportions of *N*-acetyl-D-glucosamine and D-glucosamine residues. The term chitosan is used to describe a series of polymers of different Mw and DD, defined in terms of the percentage of primary amino groups in the polymer backbone [53]. The DD of typical commercial chitosan is usually between 70 and 95%, and the Mw between 10 and 1,000 kDa.

4.1 Chemical Methodology for the Preparation of Chitosan

Preparation of chitosan involves four steps: deproteinization, demineralization, decoloration, and deacetylation. Deproteinization is carried out by alkaline treatment using 3–5% NaOH (w/v) aqueous solution at room temperature overnight. Other inorganic constituents remaining are removed by treatment with 3–5% aqueous HCl (w/v) solution at room temperature for 5 h. The product is again reacted with 40–45% NaOH solution at 120°C for 4–5 h. This treatment gives the crude sample of chitosan. The crude sample is purified by precipitating the chitosan

from its aqueous acetic acid solution to NaOH and washing with distilled water until neutralized [54].

4.2 Physicochemical and Biological Properties of Chitosan

Chitosan is a semicrystalline polymer that exhibits polymorphism. Chitosan belongs to a series of polymers with different DD and Mw [55], which are the two important physicochemical properties of chitosan. DD is defined as of the percentage of primary amino groups in the polymer backbone. The DD and Mw of chitosan can be altered by changing the reaction conditions during the manufacture of chitosan from chitin (typical commercial chitosan has a DD of 66–95%). Chitosan appears as colorless, odorless flakes. It is readily soluble in aqueous acidic solution. The solubilization occurs through protonation of amino groups on the C-2 position of D-glucosamine residues, whereby polysaccharide is converted into polycation in acidic media. Chitosan has a low solubility at physiological pH of 7.4 as it is a weak base (p*K*a 6.2–7). Adjusting solution pH to approximately 7.5 induces flocculation due to deprotonation and insolubility of the polymer [56]. Higher Mw chitosan of approximately 1,400 kDa demonstrates a stronger level of mucoadhesion than low Mw chitosan of 500–800 kDa, because the former has a higher level of viscosity. The viscosity of chitosan solution increases with an increase in chitosan concentration and DD but with a decrease in solution temperature and pH. It is known to possess a good complexing capacity. Chitosan can also complex with an oppositely charged polymer such as poly(acrylic acid), sodium salt of poly(acrylic acid), carboxymethyl cellulose, xanthan, carrageenan, alginate, pectin etc. The biological properties of chitosan are illustrated in Fig. 2.

4.3 Limitations

Chitosan suffers from low solubility at a physiological pH of 7.4, limiting its use as absorption enhancer in, for example, nasal or peroral delivery systems [57]. Another limitation of chitosan for the preparation of sustained release systems arises from its rapidly adsorbing water and higher swelling degree in aqueous environments, leading to fast drug release [58]. In order to overcome these problems, a number of chemically modified chitosan derivatives have been synthesized.

4.4 Modification of Chitosan

Most chemical modifications of chitosan are performed at the free amino groups of the glucosamine units. There are also reports on modifications of chitosan hydroxyl

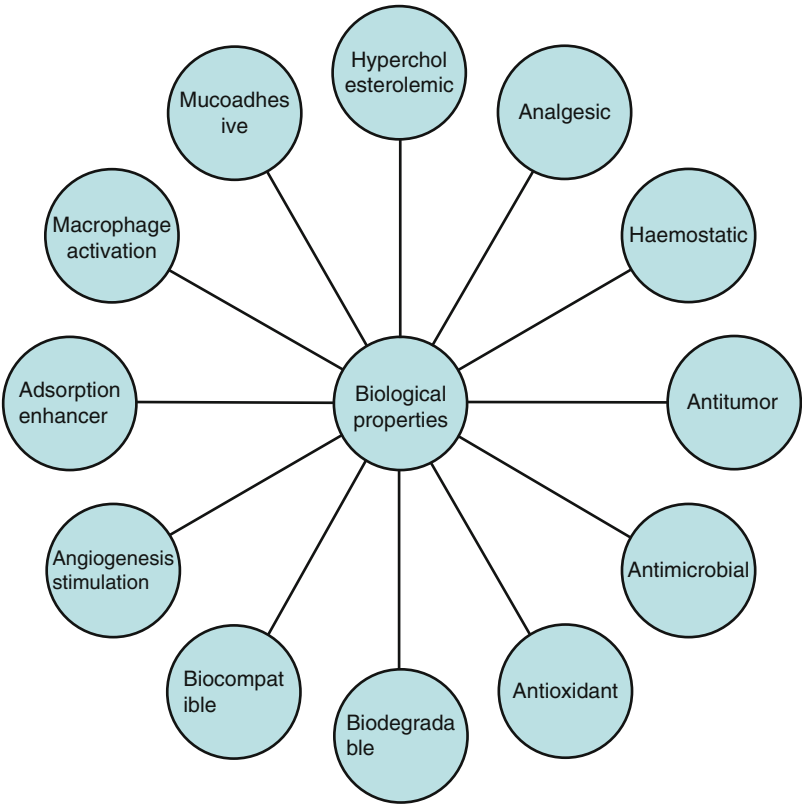

Fig. 2 Biological properties of chitosan

groups [59]. For example, the formation of amide bonds between these amino groups and activated carboxylic groups can be initiated easily. Purification of modified chitosans can often be done by simple dialysis. A great number of chitosan derivatives have been obtained by grafting new functional groups on the chitosan backbone. The chemical modifications (quaternization, acylation, cyclodextrin encapsulation, thiolation etc.) afford a wide range of derivatives with modified properties for specific applications in biomedical and biotechnological fields [60]. Modification does not change the fundamental skeleton of chitosan but brings new or improved properties for, e.g., mucoadhesion and permeation enhancement. The advantage of chitosan over other polysaccharides is that its chemical structure allows specific modifications without too many difficulties at C-2 position. Specific groups can be introduced to design polymers for selected applications. The main reaction easily performed involving the C-2 position is the quaternization of the amino group or a reaction in which an aldehydic function reacts with $–NH_2$ by reductive amination. This latter reaction can be performed in aqueous solution under very mild conditions to obtain randomly distributed substituents in a controlled

amount along the chitosan chain. This method has been proposed for introduction of different functional groups on chitosan using acryl reagents in an aqueous medium; introduction of *N*-cyanoethyl groups is said to produce some crosslinking through a reaction between the nitrile group and the amine group [61]. In addition, it is important to note that more regular and reproducible derivatives should be obtained from highly deacetylated chitin [62]. Assuring control of the quality of the initial material is essential before modification, especially when biological applications are to be explored.

4.4.1 Covalent Modifications

Covalent modification of chitosan includes thiolation and hydrophobic modifications like acylation and quaternization, which will be discussed in later sections (Sections 7.1, 7.2 & 7.3).

4.4.2 Polyelectrolyte Complexes

Polyelectrolyte complex formation occurs when two oppositely charged polymers (polycations and polyanions) in solution phase separate to form a dense polymer phase, known as the coacervate, and a supernatant, which typically has very low concentrations of polymer. A nanoparticle system, composed of hydrophilic chitosan and poly(γ-glutamic) acid (γ-PGA) hydrogels, was prepared by Lin et al. by a simple ionic-gelation method [63]. The prepared nanoparticles with chitosan on the surfaces could efficiently open the tight junctions between Caco-2 cell monolayers. This suggests that these nanoparticles can be an effective intestinal delivery system for peptide and protein drugs and other large hydrophilic molecules. Lin et al. demonstrated that positively charged chitosan PGA nanoparticles (size 110–150 nm) transiently opened the tight junctions between Caco-2 cells and thus increased the paracellular permeability. After loading of insulin, the nanoparticles remained spherical and the insulin release profiles were significantly affected by their stability in distinct pH environments. The in vivo results clearly indicated that the insulin-loaded nanoparticles could effectively reduce the blood glucose level in a diabetic rat model [64].

Sonaje et al. reported the biodistribution of aspart-insulin, a rapid-acting insulin analog, following oral or subcutaneous (SC) administration to rats using a pH-responsive nanoparticle system composed of chitosan and poly(γ-glutamic acid) (CS/γ-PGA NPs). The pharmacodynamic (PD) and pharmacokinetic (PK) evaluation of these nanoparticles in a diabetic rat model produced a slower hypoglycemic response for a prolonged period of time, whereas the SC injection of aspart-insulin produced a more pronounced hypoglycemic effect for a relatively shorter duration. These nanoparticles could effectively adhere on the mucosal surface, increase the

intestinal absorption of insulin, and produce a slower, but prolonged hypoglycemic effect [65].

Transepithelial electrical resistance (TEER) measurements and transport studies implied that CS/γ-PGA NPs can be effective as an insulin carrier only in a limited area of the intestinal lumen where the pH values are close to the p*K*a of chitosan. So, a pH-responsive nanoparticle system was self-assembled by TMC and γ-PGA for oral delivery of insulin. In contrast, TMC(40% Degree of Quaternisation) / γ-PGA NPs may be a suitable carrier for transmucosal delivery of insulin within the entire intestinal tract. The loading efficiency and loading content of insulin in TMC/ γ-PGA NPs were 73.8 ± 2.9% and 23.5 ± 2.1%, respectively. TMC/γ-PGA NPs had superior stability in a broader pH range to CS/γ-PGA NPs; the in vitro release profiles of insulin from both test nanoparticles were significantly affected by their stability at distinct pH environments. TEER experiments showed that TMC/γ-PGA NPs were able to open the tight junctions between Caco-2 cells, and this was further confirmed by confocal microscopy [66].

A biodistribution study in a rat model showed that some of the orally administered CS/γ-PGA NPs were retained in the stomach for a long duration, which might lead to the disintegration of nanoparticles and degradation of insulin [67]. To overcome these problems, nanoparticles were freeze-dried and used to fill an enteric-coated capsule. Upon oral administration, the enteric-coated capsule remained intact in the acidic environment of the stomach, but dissolved rapidly in the proximal segment of the small intestine. In another study, a nanoparticle delivery system self-assembled by the positively charged chitosan and the negatively charged γ-PGA for oral administration of insulin was prepared by mixing the anionic γ-PGA solution with the cationic chitosan solution in presence of $MgSO_4$ and sodium tripolyphosphate [68]. The in vitro results showed that the transport of insulin across Caco-2 cell monolayers by nanoparticles appeared to be pH-dependent; with increasing pH, the amount of insulin transported decreased significantly. The in vivo data indicated that these nanoparticles could effectively adhere on the mucosal surface and their constituted components were able to infiltrate into the mucosal cell membrane. Oral administration of insulin-loaded nanoparticles demonstrated a significant hypoglycemic action for at least 10 h in diabetic rats, and the corresponding relative bioavailability of insulin was found to be 15.1%. Polyelectrolyte complexes of alginate/chitosan nanoparticles were found to be effective for oral insulin delivery [69].

5 Methods for the Preparation of Nano/Microparticles of Chitosan

Various methods for preparation of chitosan nano/microparticles are emulsification/solvent evaporation, spray drying, ionotropic gelation and coacervation, emulsion crosslinking, sieving etc.

5.1 Emulsion Crosslinking

In this method, a water-in-oil emulsion is prepared by emulsifying the chitosan aqueous solution in the oil phase. It is then stabilized by the addition of surfactants. The emulsion thus obtained is crosslinked using crosslinking agents such as glutaraldehyde to harden the droplets. Finally, microspheres are filtered and washed repeatedly with *n*-hexane followed by alcohol, and then dried. The main disadvantage of this method is that the unreacted crosslinking agent cannot be removed completely from the system [70].

5.2 Coacervation/Precipitation

In this method, sodium sulfate solution is added dropwise to an aqueous acidic chitosan soluion containing a surfactant. It is then ultrasonicated for 10 min. Microparticles thus obtained are separated by centrifugation and resuspended in demineralized water. The particles are then crosslinked with glutaraldehyde [71].

5.3 Spray Drying

Drug is first dispersed in an aqueous acidic solution of chitosan. The next step is the addition of a suitable crosslinking agent. This solution is then atomized in a stream of hot air. This leads to the formation of small droplets from which solvent evaporates, leading to the formation of free flowing particles [72].

5.4 Emulsion Droplet Coalescence Method

This method utilizes the principle of emulsion crosslinking and precipitation. In the first step, a stable emulsion containing aqueous solution of chitosan along with the drug is produced in liquid paraffin oil. In the second step, another stable emulsion containing aqueous solution of chitosan in NaOH is prepared. Then both emulsions are mixed under high speed stirring, whereby the droplets of each emulsion collide at random and coalesce, thereby precipitating chitosan droplets to give small-sized particles [73].

5.5 Ionic Gelation

An aqueous acidic solution of chitosan is added dropwise under constant stirring to sodium tripolyphosphate solution. Chitosan undergoes gelation due to the

complexation between oppositely charged species and precipitates to form spherical particles [74].

5.6 Reverse Micellar Method

The surfactant is first dissolved in an organic solvent to produce reverse micelles. To this, an aqueous solution of chitosan and drug are added with constant vortexing to avoid any turbidity. The aqueous solution is kept in such a way as to keep the entire mixture in an optically transparent microemulsion phase. Additional amount of water may be added to obtain nanoparticles of larger size. To this solution, a crosslinking agent is added and the mixture kept overnight under constant stirring. The organic solvent is then evaporated to obtain the transparent dry mass. The material is dispersed in water, followed by the addition of a suitable salt, which helps to precipitates the surfactant out. It is then centrifuged and the supernatant decanted, which contains the drug-loaded nanoparticles. The aqueous dispersion is immediately dialysed through dialysis membrane for about 1 h and the liquid is lyophilized to dry powder [75].

5.7 Seiving Method

An aqueous acidic solution of chitosan is crosslinked using glutaraldehyde. The crosslinked chitosan is then passed through a sieve with a suitable mesh size to obtain microparticles. The microparticles are then washed with 0.1 N sodium hydroxide solution to remove the unreacted glutataraldehyde and dried at 40°C [76].

6 Applications in Drug Delivery

Chitosan is widely used for dental, buccal, gastrointestinal, colon-specific, and gene delivery applications due to its favorable biological properties. It is used in the form of tablets, gels etc. (Fig. 3).

6.1 Drug Delivery for Dental Diseases

Chitosan has been widely used as an effective medicament in various fields of medicine and dentistry. In endodontics, it could be used as an anti-inflammatory root canal dressing material for periapical lesions. Chitosan stimulates the fibroblastic

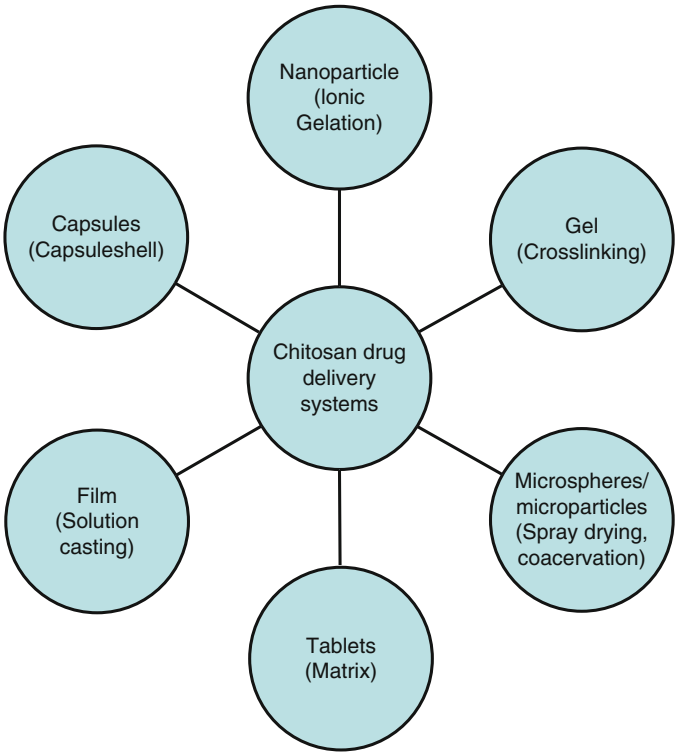

Fig. 3 Different types of chitosan-based drug delivery systems

cells to release chemotactic inflammatory cytokines, especially interleukin 8 (IL-8) [77, 78]. Histological findings indicate that chitosan induces the migration of polymorphonuclear leukocytes and macrophages in the applied tissue at the early stage [79]. At the final stage of wound healing using chitosan, angiogenesis, reorganization of the extracellular matrix, and granulation tissue have been demonstrated [80]. In the case of direct pulp capping as a biological pulp treatment, chitosan produced severe inflammation in the pulp at the early stage [81]. Furthermore, chitosan oligomer (the mixture ranged from dimer to octamer) has been used on the dental pulp tissue and similar initial inflammation was observed. The pulp is surrounded by the hard tissue, dentine. The inflammatory reactions are thought to cause fatal damage to the pulp tissue, and must be overcome before the clinical application of chitosan to dental pulp. D-Glucosamine hydrochloride as a chitosan monomer is easily produced by totally hydrolyzing chitin with hydrochloride, and is the simplest form of chitosan [79]. Chitosan monomer accelerates cell proliferation and differentiation in vitro at a very low concentration, and the ideal tissue regeneration in pulp wounds occurs where there is a minimal initial inflammation reaction [82].

6.2 Buccal Delivery Systems

Drug administration through the buccal mucosa in the mouth provides unique advantages such as avoidance of the hepatic first-pass metabolism, and of the acidity and proteolytic activity of the rest of the gastrointestinal (GI) tract [83]. An ideal buccal delivery system should stay in the oral cavity for few hours and release the drug in a unidirectional way toward the mucosa in a controlled or sustained-release fashion. Mucoadhesive polymers prolong the residence time of the device in the oral cavity whereas bilayered devices ensure that drug release occurs in a unidirectional way [84]. Buccal delivery can be used to treat a number of diseases, such as periodontal disease, stomatitis, fungal and viral infections, and oral cavity cancers. Chitosan is an excellent polymer to be used for buccal delivery because it has muco/bioadhesive properties and it can act as an absorption enhancer [85]. Directly compressible bioadhesive tablets of ketoprofen containing chitosan and sodium alginate in the weight ratio of 1:4 showed sustained release 3 h after intraoral (into sublingual site of rabbits) drug administration [86]. Buccal tablets based on chitosan microspheres containing chlorhexidine diacetate give prolonged release of the drug in the buccal cavity, thus improving the antimicrobial activity of the drug. Chitosan solutions have been evaluated for buccal delivery of proteins; but in order to prolong drug release, films or hydrogels would be more suitable. Chitosan films should degrade slowly under physiological conditions, and for this reason they need to be crosslinked with crosslinking agents like tripolyphosphate. Chitosan hydrogels and films were able to limit adhesion of the common pathogen *Candida albicans* to human buccal cells. These drug delivery systems were also able to sustain drug release (chlorhexidine gluconate) from a hydrogel as well as from film formulations [87]. Chitosan hydrogels were also able to deliver ipriflavone, a lipophilic drug that promotes bone density, into the periodontal pockets. For this purpose, mono- and multilayer composite systems consisting of chitosan and PLGA were designed, and were shown to prolong drug release for 20 days in vitro. Chitosan integrated into bilayered films and tablets with the oral drugs nifedipine and propranolol hydrochloride showed effective buccal membrane adhesion. These complexes were used with and without polyelectrolyte complex (PEC)-forming polymers, such as polycarbophil, sodium alginate, and gellan gum [88].

6.3 Gastrointestinal Delivery

Gastroretentive drug delivery systems increase the retention of a per-oral dosage form in the stomach and offer numerous advantages for drugs exhibiting an absorption window in the GI tract, drugs that are poorly soluble in the alkaline medium (Verapamil), and drugs that are intended for local action on the gastroduodenal wall [89]. Chitosan has a high potential in the development of a successful gastroretentive drug delivery system because it combines both bioadhesion and

floating capabilities [90], especially for drugs that are poorly soluble in intestinal medium and readily soluble in acidic medium. Chitosan could be ideal for use in formulations intended to release drugs slowly in the stomach, since the gel formation by cationic chitosan that is pronounced at acidic pH levels results in marked retardant effects on drug release. Orally administered formulations are initially exposed to the acidic milieu of the stomach, especially if they have been administered to subjects in fasted states, in whom gastric pH is likely to range from approximately 1 to 2. Mucoadhesive ability could result in formulations containing chitosan being retained in the stomach. Adhesion would be expected to be particularly marked under the acidic conditions in the stomach, where cationic chitosan would be highly charged. The chitosan beads can serve as depot reservoir that allows the continuous gradual release of small amounts of Verapamil in solution to the upper part of the small intestine (the main site of absorption), leading to higher and more uniform blood levels of the drug. Thus, reduced adverse effects are highly expected. Floating hollow microcapsules of melatonin showed gastroretentive controlled-release delivery. Release of the drug from these microcapsules is greatly retarded, with release lasting for 1.75–6.7 h in simulated gastric fluid. Most of the mucoadhesive microcapsules are retained in the stomach for more than 10 h (e.g., Metoclopramide- and glipizide-loaded chitosan microspheres) [91].

6.4 Colon-Specific Drug Delivery

Due to its high solubility in gastric fluids, chitosan is widely used for colon-specific drug delivery. Similarly to other polysaccharides, it shows degradation in the colon. Although chitosan can be insoluble in acidic fluids through chemical crosslinking of the microsphere with aldehydes, it is not effective in preventing the release of the encapsulated drugs. To overcome this problem, microencapsulated chitosan microspheres coated with enteric coating materials were developed [92]. The potential of this microsphere was evaluated using sodium diclofenac, an anti-inflammatory drug. Sodium diclofenac was entrapped into the chitosan cores by the spray drying method, after which the chitosan cores were microencapsulated into Eudragit L-100 and Eudragit S-100 using an oil-in-oil solvent evaporation method. The in vitro release studies revealed that no sodium diclofenac was released at gastric pH; however, when the microspheres reached the colonic environment, a continuous release was observed for a variable time (8–12 h). Eudragit S-100-coated chitosan beads developed by Jain et al. exhibited pH-sensitive properties and specific biodegradability for colon-targeted delivery of satranidazole. Eudragit S-100 coating on the chitosan beads prevented premature drug release in simulated upper gastrointestinal conditions and most of the loaded drugs was released in the colon, an environment rich in bacterial enzymes that degrade the chitosan [93]. Chourasia et al. prepared a similar multiparticulate system, by coating crosslinked chitosan microspheres with Eudragit L-100 and S-100 as pH-sensitive polymers, for targeted delivery of the broad-spectrum antibacterial agent metronidazole [94]. The results

showed a pH-dependent release of the drug that was attributable to the presence of Eudragit coating. Moreover, the release of drug was found to be higher in the presence of rat caecal contents, indicating the susceptibility of the chitosan matrix to colonic enzymes. Similar nanoparticular systems for colon-specific delivery of metronidazole were reported by Elzatahry and Eldin [95]. For the treatment of 2,4,6-trinitrobenzene sulfonic acid sodium salt (TNBS)-induced colitis in rats, 5-aminosaliycylic acid (5-ASA) was orally administered using chitosan capsules or a carboxymethyl cellulose (CMC) suspension. Better therapeutic effects were obtained with chitosan capsules than with the CMC suspension. The release of 5-ASA from the chitosan capsule was markedly increased in the presence of rat cecal contents [96]. Degradation of chitosan–tripolyphosphate hydrogel beads in the presence of rat cecal and colonic enzymes indicated the potential of this microparticulate system for colon targeting. The ability of rat cecal and colonic enzymes to degrade chitosan hydrogel beads was independent of pretreatment conditions [97]. Chitosan salts mixed with anti-inflammatory drugs, such as sodium diclofenac, were evaluated for their in vitro release behavior. Chitosan salts, such as glutamic and aspartic salts, provided good controlled release of sodium diclofenac. Slower drug release was obtained with physical mixtures compared to the pure drug both in acidic and alkaline pHs. Interaction with β-glucosidase at pH 7.0 enhanced the release rate [98].

6.5 Mucosal Vaccination

Chitosan is a mucoadhesive polymer that is able to open tight junctions and allow the paracellular transport of molecules across mucosal epithelium, and is therefore suitable for the mucosal delivery of vaccines [99]. Chitosan microparticles were able to associate large amounts of ovalbumin (model vaccine) and diphtheria toxoid (DT). They are not disintegrated in an acidic environment and protect the antigen against degradation by entrapping it into their porous structure [100]. The chitosan microparticles were only taken up by the follicle-associated epithelium (FAE), in which microfold cells (M-cells) are present. This result was from oral delivery studies in mice, in which chitosan microparticles were found to transport associated ovalbumin into the Peyer's patches [101]. Oral efficacy studies with DT associated to chitosan microparticles demonstrated that they were able to induce strong DT-specific systemic and local immune responses (IgG and IgA titers, respectively) [102]. The amount of neutralizing antibodies in mice vaccinated with DT-loaded chitosan microparticles was also high; the levels were considered to be protective in man. Chitosan is shown to be a promising candidate in mucosal vaccine delivery for protein vaccines such as *Bacillus anthracis* [103], diphtheria [104], and influenza [105]. Chitosan derivative trimethylated chitosan/tripolyphosphate/ovalbumin (TMC/TPP/OVA) nanoparticles have previously been shown to be very effective nasal vaccine carriers. Replacing TPP by CpG as a crosslinking agent to obtain TMC/CpG/OVA nanoparticles modulated the immune response towards a Th1

(T helper cell 1) response after nasal vaccination, while maintaining the strong systemic and local antibody responses observed with TMC/TPP nanoparticles. TMC/CpG nanoparticles are therefore an interesting nasal delivery system for vaccines requiring broad humoral as well as strong Th1-type cellular immune responses [106]. In another study, ovalbumin was encapsulated in alginate-coated chitosan nanoparticles and delivered via the oral route to Peyer's patches [107]. Jain et al. [108] observed high secretory IgA concentrations and better immune response with nanoparticulate vesicular formulation upon oral administration. Chitosan microparticles show suitable in vitro and in vivo characteristics for oral vaccination and are therefore a promising carrier system for this particular purpose. Fluorescently labeled chitosan microparticles can be taken up by the epithelium of murine Peyer's patches. Since uptake by Peyer's patches is an essential step in oral vaccination, these results show that the presently developed porous chitosan microparticles are a very promising vaccine delivery system. Chitosan particles have been described as a potential oral vaccine carrier [109] and transport of these particles by M-cells has been observed. A drawback of chitosan is its water solubility. With a pK_a of 6.2, at physiological pH the primary amine groups of chitosan are protonated and, consequently, OVA/chitosan nanoparticles lose their repulsive surface charge and show colloidal instability. The slightly acid environment of the jejunum will promote the stability of chitosan nanoparticles, but as soon as these particles are transported to the subepithelial space to interact with immune cells, the physiological pH will be deleterious for its stability. Because TMC carries a permanent positive charge, OVA/TMC nanoparticles will not be affected by small pH shifts and may be a more suitable carrier for mucosal vaccination [110]. An intraduodenal immunization study with OVA/chitosan nanoparticles, OVA/TMC nanoparticles, and unencapsulated OVA was performed to analyze the extent and type of immune response elicited.

6.6 Gene Delivery

Chitosan is the most prominent among natural polymer-based gene delivery vectors, due to is biodegradability, biocompatibility, and degradation potential [111]. Its role in gene delivery is supported by its ability to protonate in acidic solution and to form a complex with DNA through electrostatic interactions [112]. Mumper et al. first described chitosan as a delivery system for plasmids [113]. Chitosan/DNA nanoparticles may be readily formed by coacervation between the positively charged amine groups on chitosan and negatively charged phosphate groups on DNA. The transfection efficiency of chitosan depends on Mw, DD, pH of the transfecting medium, and cell type. As the Mw increased, transfection efficiency also improved [114]. Moderate DD increases the transfection efficiency. A pH of 6.8–7.0 is found to be optimum to achieve a high level of transfection [115]. Higher gene transfer efficiency was achieved in HEK293 cells as compared to other cell types [116]. But, the major drawback of chitosan is its low transfection efficiency.

Numerous chemical modification like quaternization [117], deoxycholic acid modification [118], galactosylation [119], PEI grafting [120], and thiolation [121] have been carried out by various groups in an attempt to improve transfection efficiency.

7 Chitosan and Its Derivatives

Chemical modification is a powerful tool for controlling the interaction between polymer and drug and thus for enhancing the loading capacity and controlling drug release from the matrix. Modified chitosan improves the bulk properties for the sustained release and has a wide range of pharmaceutical applications.

7.1 Hydrophobic Modification

The hydrophobic character of chitosan can be increased by the covalent attachment of hydrophobic excipients. Hydrophobic interactions are believed to enhance the stability of substituted chitosan by reducing the hydration of the matrix and thereby increasing resistance to degradation by gastric enzymes [122]. Introduction of carboxylic acid groups to chitosan makes chitosan pH-sensitive. Under acidic conditions, the carboxylic groups exist in non-ionized form and are therefore poorly hydrophilic. By contrast, in alkaline conditions, the polymer is ionized and is considerably more hydrophilic. Chitosan succinate with an average diameter of around 50 micrometer, exhibited a loading capacity of around 60% for insulin. Only a small amount of drug was released in simulated gastric fluid, whereas insulin was quickly released in simulated intestinal fluid of pH 7.4. By using chitosan succinate microspheres, the relative pharmacological efficacy was about fourfold improved in comparison to chitosan succinate solution [123]. Chitosan phthalate microspheres (46.34 μm) prepared by emulsion phase separation exhibited a loading capacity of 62%. They protect the insulin from degradation by gastric enzymes [124].

Phthalyl chitosan–poly(ethylene oxide) (PCP) semi-interpenetrating network microparticles developed by Rekha and Sharma showed improved release properties of insulin compared to chitosan [125]. The particle size was about 1.3 μm, with a ζ-potential of about -28.6 ± 12.6 mV and an insulin loading efficiency of 89.6. Insulin release in acidic media was minimized because of the presence of the phthalate group. In another work, Rekha and Sharma tried to establish the role of the hydrophilic/hydrophobic balance on gastrointestinal absorption of peptide drugs along with surface charge of the particles [126]. Lauryl succinyl chitosan particles developed by Rekha and Sharma were found to be highly mucoadhesive, which could be due to the hydrophobic interaction of the lauryl groups with the hydrophobic domains of mucosa, as well as to the negative ζ-potential of the particles. Negatively charged mucoadhesive lauryl succinyl chitosan nano/microparticles (size 315 nm to 1.090 μm) with both hydrophilic (succinyl) and

hydrophobic (lauryl) moieties improved the release characteristics, mucoadhesivity, and the permeability of insulin compared to the native chitosan particles. The presence of hydrophobic moieties controlled the release of the loaded insulin from the particles at intestinal pH. The particles were capable of reducing blood glucose levels in diabetic rats for the duration of about 6 h. The hydrophobic moiety is expected to enhance mucoadhesivity through hydrophobic interactions and also the permeability by loosening the tight junctions.

One of the facile approaches that has drawn much attention is the introduction of hydrophobic groups onto chitosan by acylation (Fig. 4). Fatty acids have been shown to enhance the permeability of peptide drugs [127]. They act primarily on the phospholipid component of the membrane, thereby creating disorder and leading to increased permeability. It has been established by various groups that sodium salts of medium chain fatty acids such as caprylate (C_8), caprate (C_{10}), and laurate (C_{12}) are able to enhance the paracellular permeability of hydrophilic compounds. N-Acylation of chitosan with various fatty acid (C_6–C_{16}) chlorides increased its hydrophobic character and made important changes in its structural features. Tein et al. described the N-acylation of chitosan with fatty acylchlorides (C_8–C_{16}) to introduce hydrophobicity for use as matrix for drug delivery [128]. Anacardoylated chitosan exhibited sustained release of insulin in the intestinal environment, and the released insulin was stable and retained its conformation [129]. The bioadhesive property of chitosan was enhanced by N-acylation with fatty acid chlorides. Chitosan modified with oleoyl chloride showed better mucoadhesion properties than chitosan modified with lower fatty acid groups [130]. Oleoyl chitosan exhibited less swelling than octanoyl chitosan at acidic pH and was capable of maintaining the biological activity of insulin. Compared to octanoyl chitosan, oleoyl chitosan can better resist degradation by gastric enzymes, enhance mucoadhesivity through

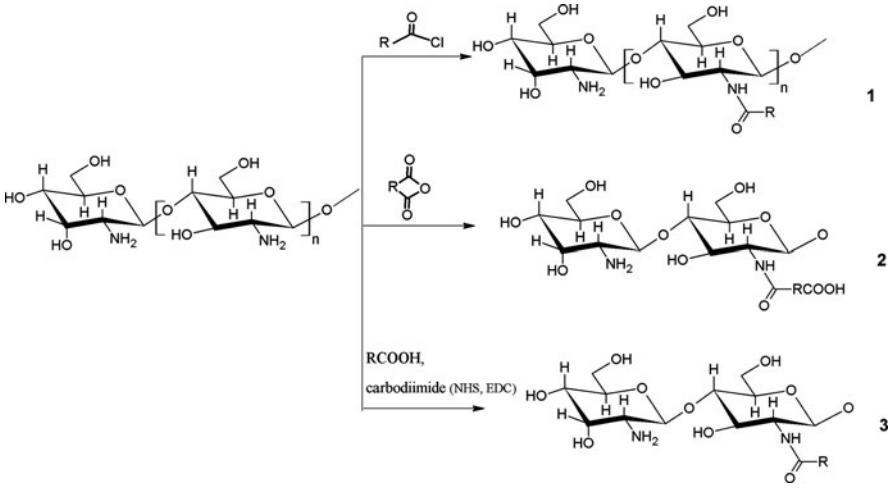

Fig. 4 Acylated chitosan derivatives obtained by the reaction of chitosan with (*1*) acid chloride, (*2*) lactones, and (*3*) carboxylic acids

hydrophobic interactions, and improve permeability by loosening the tight junctions. The oleoyl chitosan derivative exhibited more significant uptake than octanoyl chitosan [131].

7.2 Thiolation

Thiolated polymers/thiomers are hydrophilic macromolecules exhibiting free thiol groups on the polymeric backbone and represent new promise in the field of mucoadhesive polymers. Nowadays, thiolated chitosans are gaining popularity because of their high mucoadhesiveness and extended drug release properties [132]. They are obtained by the derivatization of the primary amino groups of chitosan with coupling reagents. Figure 5 depicts thiolated chitosans obtained by the reaction of chitosan with cysteine, thioglycolic acid, 2-iminothiolane, and thiobutylamidine. The permeation-enhancing effect of thiolated chitosan has been studied with the permeation mediator glutathione, and results indicate that chitosan–TBA/GSH is a potentially valuable tool for inhibiting the ATPase activity of permeability glycoprotein (P-gp) in the intestine [133]. The permeation-enhancing effect also seems to be based on the inhibition of protein tyrosine phosphatase,

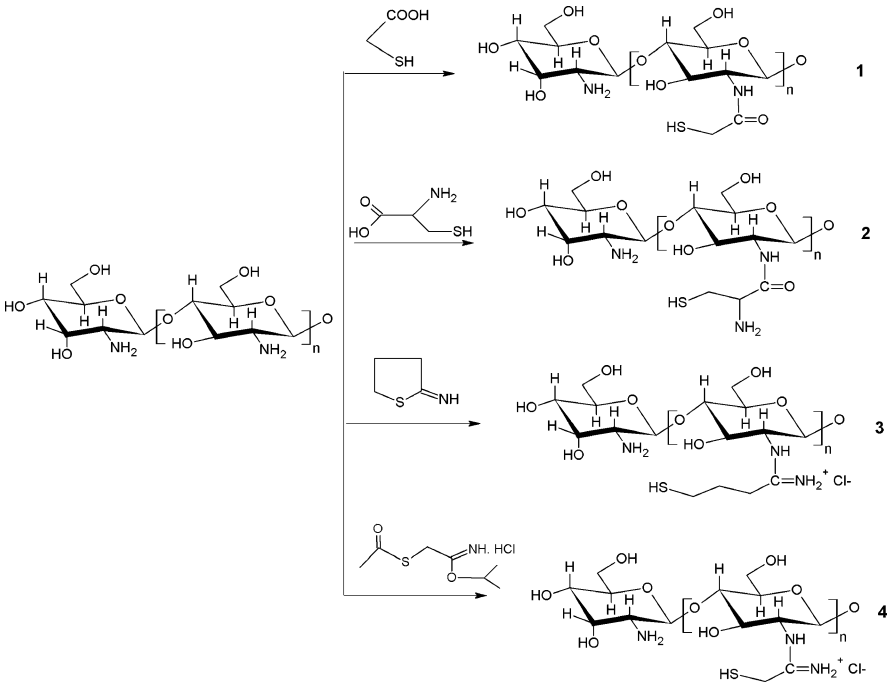

Fig. 5 Thiolated chitosan derivatives obtained by the reaction of chitosan with (*1*) cysteine, (*2*) thioglycolicacid, (*3*) 2-iminothiolane, and (*4*) thiobutylamidine

resulting in an opening of the tight junctions for hydrophilic macromolecules. This theory is supported by various in vitro and in vivo studies in which significantly improved pharmacological efficacy and/or bioavailability of model drugs was seen. Due to the inter- and intramolecular formation of disulfide bonds, a tight three-dimensional network is formed, which leads to high cohesiveness and allows a controlled drug release. Mucoadhesiveness is due to the formation of disulfide bonds with mucus glycoproteins. These thiolated polymers interact with cysteine-rich subdomains of mucus glycoproteins via disulfide exchange reactions. The permeation of paracellular markers through mucosa can be enhanced by utilizing thiolated instead of unmodified chitosan. Moreover, thiolated chitosans display in situ gelling features due to the pH-dependent formation of inter- as well as intramolecular disulfide bonds. This latter process provides strong cohesion and stability of carrier matrices, being based on thiolated chitosans. Moreover, it could be demonstrated that a reversible opening of tight junctions occurs in the presence of thiomers, leading to a more pronounced permeation-enhancing effect of thiomers in comparison to the unmodified polymer. Recently, it has been demonstrated that thiomers are capable of inhibiting efflux pumps such as P-gp, which renders them likely as promising tools for oral delivery of efflux pump substrates.

Trimethyl chitosan–cysteine conjugate (TMC-Cys) was synthesized in an attempt to combine the mucoadhesion and the permeation-enhancing effects of TMC and thiolated polymers related to different mechanisms for oral absorption [134]. TMC-Cys with various Mw (30, 200, and 500 kDa) and quaternization degrees (15 and 30%) was allowed to form polyelectrolyte nanoparticles with insulin through self-assembly, which demonstrated particle size of 100–200 nm, ζ-potential of +12 to +18 mV, and high encapsulation efficiency. TMC-Cys/insulin nanoparticles (TMC-Cys NP) showed a 2.1- to 4.7-fold increase in mucoadhesion compared to TMC/insulin nanoparticles (TMC NP), which might be partly attributed to disulfide bond formation between TMC-Cys and mucin. Insulin solution and TMC NP induced increased insulin transport through rat intestine by 3.3- to 11.7-fold, whereas TMC-Cys NP increased transport by 1.7- to 2.6-fold. Oral and ileal administration of TMC-Cys (200kDa(Mw), 30%(Degree of Quaternization)) NP led to notable hypoglycemic effects as compared to insulin solution, which lasted until 8 h and 7 h post-administration, respectively, with the maximum blood glucose depression of 35 and 70%, respectively.

7.3 Quaternized Chitosan

Quaternized derivatives of chitosan, obtained by introducing various alkyl groups to the amino groups of chitosan molecule structure, were extensively studied for oral insulin delivery [135]. These derivatives are drastically more soluble in neutral and alkaline environments of the intestine and, hence, are more efficient than chitosan for drug delivery and absorption across the intestinal epithelium of the jejunum and ileum. TMC is prepared by reacting chitosan with trimethyliodide

bond. The permeation-enhancing properties of these chitosan derivatives have been attributed to their ionic interactions with the tight junctions and cellular membrane components to increase the paracellular permeation of hydrophilic compounds. The chitosan derivatives in nanoparticle form have less positive surface charge because part of the basic group is involved in the crosslinking reaction with TPP. Their interactions with tight junction are therefore limited and, hence, drug transport across the monolayer more likely occurs through the transcellular pathway rather than by tight junction opening. Recently, nanoparticles based on TMC were proved to interact with intestinal tissue (rat jejunum) and with Caco-2 cell monolayers. In particular, the increase in the quaternization degree of TMC favored mucoadhesion [136]. TMC nanosystems with 35% quaternization combined good penetration-enhancement properties and mucoadhesion. Even if the mucoadhesive properties slowed down the absorption of nanoparticles through the mucus layer into the cell, the increased contact with the intestinal epithelium offered more possibilities for nanoparticle internalization [137]. Chitosan and TMC were demonstrated to be biocompatible both as solutions and in nanoparticulate form. The in vitro model (Caco-2 cells) showed that chitosan and TMC were able to enhance insulin permeation both as nanoparticles and as solutions. Chitosan nanoparticles were still able to widen paracellular pathways, and TMC slightly interfered with substrate integrity. Another quaternized derivative, *N*-hydroxypropyltrimethylammonium chitosan chloride (HTCC), obtained by reacting chitosan with glycidyltrimethylammoniumchloride, was found to be mucoadhesive and was successfully used for oral insulin delivery [138]. Figure 6 depicts two quaternized derivatives of chitosan: TMC and HTCC.

TMC NP was more effective than chitosan nanoparticles and polymer solutions in enhancing permeation across jejunum tissue because the pH of the microclimate mucus environment (pH 6–6.5) was disadvantageous to chitosan and TMC NP has high mucoadhesive potential. Both TMC and chitosan failed penetration enhancement across the ileum, probably due to a thicker mucus layer with barrier properties

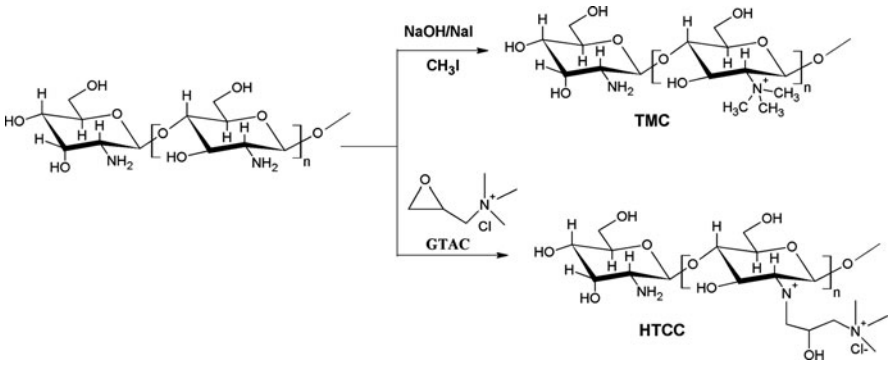

Fig. 6 Quaternized chitosan derivatives: *TMC* trimethylchitosan, HTCC *N*-hydroxypropyltrimethylammoniumchitosan

and a rich enzyme pool. Values for the apparent permeability coefficient (P_{app}) determined by means of an in vitro model (Caco-2) were lower than those observed in ex vivo experiments because the tissue enzymes cause nanoparticle digestion and consequent drug adsorption, and the mucus favors a deep interaction with epithelial cells. The mechanism of penetration enhancement involves the paracellular pathway, with an enlargement of tight junctions for polymer solutions and chitosan nanoparticles, while endocytosis/internalization into duodenum and jejunum epithelial cells was confirmed only for the nanoparticulate form [139].

Jelvehgari et al. investigated the efficiency of nanoparticles formed by a complex coacervation method using chitosan of various Mw and Eudragit L100-55 polymers [140]. As the Mw of chitosan increased, the amount of insulin released increased with respect to time. A new oral delivery system for insulin was developed by Lee et al. aiming to improve bioavailability based on a conjugate between insulin and low Mw chitosans (LMWC; of 3, 6, 9, and 13 kDa) of narrow Mw distribution. LMWC–insulin conjugates after oral administration to diabetic rat models could control blood glucose levels effectively for several hours. Of those conjugates, LMWC(9 kDa)–insulin exhibited the highest pharmacodynamic bioavailability of 3.7 (0.3% relative to that of subcutaneously injected insulin (100%) [141].

7.4 Chemical Grafting of Chitosan

Graft copolymerization onto chitosan is an important field of study for the functionalization and practical use of chitosan [142]. It is an attractive technique for modifying the chemical and physical properties of chitosan. A graft copolymer is a macromolecular chain with one or more species of block connected to the main chain as side chain(s). The properties of the resulting graft copolymers are broadly controlled by the characteristics of the side chains, including molecular structure, length, and number [143]. Grafted chitosan bearing a short graft chain formed a ring structure (40–60 nm in diameter) unimolecularly, that bearing middle chain length was monodisperse spherical (30–40 nm), whereas the longer chain aggregated intermolecularly and led to larger particles (100–400 nm). These studies should be useful for determining a strategy for regulating the molecular design and guest-binding properties of water-soluble grafted chitosan. Grafted chitosans have great utility in controlled release and targeting studies of almost all class of bioactive molecules. The literature suggests that graft copolymerized chitosan is a good candidate in the fields of drug delivery, tissue engineering, antibacterial, biomedical (cardiovascular applications, wound healing), metal adsorption, and dye removal. Grafting can be carried out using free radicals, radiation, or enzymatic and cationic graft copolymerization onto chitosan.

Chitosan graft copolymer nanoparticles (CM, CDM and CTM, based on monomer methyl methacrylate, *N*-dimethylaminoethyl methacrylate hydrochloride, and

N-trimethylaminoethyl methacrylate chloride, respectively) enhanced the absorption and improved the bioavailability of insulin via the GI tract of normal male Sprague-Dawley rats to a greater extent than that of a phosphate-buffered solution of insulin. Increasing the surface charge of the nanoparticles improves insulin encapsulation efficiency and slows the release [144]. The in vivo studies indicate that CDM and CTM nanoparticles seem to be a very promising vehicle for oral administration of hydrophilic proteins and peptides.

8 Conclusion

So far, numerous promising chitosan derivatives exhibiting a better solubility, stronger mucoadhesive capabilities, and enzyme inhibitory properties have been generated. Because of its permeation-enhancing effect, mucoadhesive properties, controlled release properties, and ability to open tight junctions, chitosan is a promising tool for various peptide delivery systems. These properties can be further improved by simple chemical modifications on its primary amino group. These unique features make chitosan and, in particular, its derivatives valuable excipients for drug delivery. Chitosan is still being explored for a variety of applications. The safety of chitosan, its ability to prolong residence time in the GI tract through mucoadhesion, and its ability to enhance absorption by increasing cellular permeability have all been major factors contributing to its widespread evaluation as a component of oral dosage forms.

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Chitosan-Based Nanoparticles in Cancer Therapy

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Abstract In recent years, many nanotechnology platforms in the area of medical biology, including cancer therapy, have attracted remarkable attention. In particular, research in targeted, polymeric nanoparticles for cancer therapy has increased dramatically in the past 5–10 years. However, the potential success of nanoparticles in the clinic relies on consideration of important parameters such as nanoparticle fabrication strategies, their physical properties, drug loading efficiencies, drug release potential, and, most importantly, minimum toxicity of the carrier itself. Recent work has suggested that chitosan materials hold much promise in advancing nanoparticle-based therapeutics. The field of oncology could soon be revolutionized by novel strategies for therapy employing chitosan-based nanotherapeutics. Several aspects of cancer therapy would be involved. Chitosans can also be applied to a variety of cancer therapies to improve their safety and efficacy. Further applications of chitosans in cancer therapy are being examined. This review focuses on providing brief updates on chitosan nanoparticles for cancer therapy.

Keywords Cancer therapy · Chitosan · Drug delivery · Nanoparticles

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Abbreviations

5-ALA	5-Aminolaevulinic acid
CNP	Chitosan-based nanoparticle
CPT	Camptothecin
CS	Chitosan
DOX	Doxorubicin hydrochloride
DTX	Docetaxel
EA	Ellagic acid
ePC	Egg phosphatidylcholine
FACN	Folic acid conjugated chitosan nanoparticle
GC	Glycol chitosan
Gd-NCT	Gadolinium neutron capture therapy
GMO	Glyceryl monooleate
HACTNP	Hyaluronic acid-coupled chitosan nanoparticles
HGC	Hydrophobically modified glycol chitosan
HPMC	Hydroxypropyl methylcellulose
IL-12	Interleukin 12
IO	Iron oxide
MMC	Mitomycin C
MMP2	Matrix metalloprotease-2
MTX	Methotrexate
NFBG	Nonfasting blood glucose
NP	Nanoparticle
OCH	Oleoyl chitosan
PLGA	Poly(lactide-*co*-glycolide)
PTX	Paclitaxel
QD	Quantum dot
RGD-CNP	Arg-Gly-Asp peptide-labeled chitosan nanoparticle

siRNA	Small interfering RNA
SNAP	Sertoli cell nanoparticle protocol
SPION	Superparamagnetic iron oxide nanoparticle
TC	6-*N*,*N*,*N*-Trimethyltriazole–chitosan
TMC	*N*-Trimethyl chtiosan

1 Introduction

Recently, interdisciplinary research in cancer diagnosis and therapy has evolved to the point where nanoscale particles can be monitored in real-time and noninvasively at a molecular level in live animals by using imaging modalities, such as optical imaging, magnetic resonance imaging, and nuclear imaging [1]. Nanoparticle size allows for interactions with biomolecules on the cell surfaces and within the cells in ways that do not necessarily alter the behavior and biochemical properties of those molecules [2]. With the advances in polymer science, the field of polymeric nanoparticles has been expanding and has attracted significant interest in recent years, with most focus on biodegradable nanoparticles for drug delivery. In addition, noninvasive live animal imaging technology is providing new research opportunities in the preclinical and clinical development of nanosized drug carriers in cancer therapy [3]. Biodegradable nanoparticles are frequently used to improve the therapeutic value of various water-soluble and insoluble medicinal drugs and bioactive molecules by improving bioavailability, solubility, and retention time [4]. These nanoparticle–drug formulations reduce the patient expenses and risks of toxicity [5]. Nanoencapsulation of medicinal drugs (nanomedicines) increases drug efficacy, specificity, tolerability, and therapeutic index of corresponding drugs [6–11]. Several disease-related drugs and bioactive molecules have been successfully encapsulated to improve bioavailability, bioactivity, and controlled delivery [12–14]. Nanomedicines are being developed for dreadful diseases like cancer [15], AIDS [16], diabetes [17], malaria [18], prion disease [19] and tuberculosis [20].

2 Chitosan-Based Nanotechnology

Polysaccharides, lipids, surfactants, and dendrimers have received increasing attention because of their outstanding physical and biological properties [21]. Natural cationic polysaccharides from crab and shrimp have drawn increasing attention for pharmaceutical and biomedical applications, due to their abundant availability, unique mucoadhesivity, inherent pharmacological properties, and other beneficial biological properties such as biocompatibility, biodegradability, nontoxicity, and low-immunogenicity [22, 23]. Chitosan (deacetylated chitin) is a

nontoxic biodegradable, cationic polysaccharide with randomly distributed *b*(1,4)-linked *N*-actyl-D-glucosamines and D-glucosamines. Chitosan was introduced as a potential gene carrier in 1995 and various nanosystems based on chitosans and chitosan derivatives have been investigated, including unmodified chitosans with different molecular weights and degrees of deacetylation, quaternized chitosans, bile acid-modified chitosan, PEGylated chitosan, and chitosans bearing specific ligands [24]. Chitosan-based nanodelivery systems have been extensively studied for organ-specific applications (e.g., for colon, kidney, lung, and liver) [25].

3 Chitosan Nanoparticles in Cancer Therapy

There are a variety of nanoparticle systems currently being explored for cancer therapeutics [26]. The types of nanoparticles currently used in research for cancer therapeutic applications include dendrimers [27], liposomes [28], polymeric nanoparticles [29], micelles [30], protein nanoparticles [31], ceramic nanoparticles [32], viral nanoparticles [33], metallic nanoparticles [34], and carbon nanotubes [35]. These nanoparticles have been investigated in order to minimize the side effects of anticancer drugs and enhance the antitumoral drug efficacy in cancer therapy since they have shown a high tumor targeting ability, leading to high antitumoral therapeutic efficacies [36–43].

To elevate the local concentrations of anticancer drugs at tumor sites, various drug delivery systems involving intratumoral administration of anticancer drugs have been investigated [44–46]. For example, glycol chitosan (GC) nanoparticles that had been modified with hydrophobic bile acid analogs were shown to self-assemble into polymeric nanoparticles with hydrophilic shells of GC and hydrophobic cores of bile acid derivatives [47–54]. These self-assembling hydrophobically modified GC–5β cholanic acid conjugates were shown to encapsulate water-insoluble anticancer drugs and to have enhanced tumor-targeting properties in tumor-bearing mice [53, 55–57]. It was concluded that these amphiphilic chitosan-based nanoparticles (CNPs) can efficiently take up various hydrophobic anticancer drugs into their hydrophobic inner cores, which are covered with biocompatible and biodegradable GC shells. Compared to a free drug formulation, the biodegradable and biocompatible anticancer drug-encapsulating CNPs showed prolonged blood circulation time in vivo, enhanced tumor specificity, and improved therapeutic efficacy [58, 59].

Recent years have witnessed the development of new nanoscale drug carriers such as CNPs that can be used for photodynamic therapy [60]. In the context of nanomedicine-based therapeutics, effective cancer therapy requires drug delivery to cancer tissues, meaning that a drug delivery system should hold the anticancer drug in the blood and then allow a burst or continuous drug release at the cancer site.

3.1 Breast Cancer

It was estimated by the American Cancer Society that 15% of cancer-related deaths in women were due to breast cancer, making breast cancer the most common type of cancer among women. This mortality rate is due, in part, to the high rate of tumor metastasis to bone and lymph nodes that develops during the progression of the disease in patients. Thus, early diagnosis is crucial to improve the survival rates of breast cancer patients. To this end, there has been much research on the design of nanosized and tumor-targeting polymeric drug carriers, focusing on improving drug solubility, prolonging drug circulation time, and reducing drug toxicity. A number of studies have developed hydrophobically modified glycol chitosan (HGC) nanoparticles composed of a hydrophilic shell of GC and hydrophobic multicores of bile acid analogs [47, 48, 50, 51, 61–64]. It has been reported that anticancer drug-encapsulated HGC nanoparticles present a promising therapeutic drug delivery system for cancer therapy [63, 64]. HGC nanoparticles protect the active camptothecin (CPT) lactone drug from hydrolysis and target solid tumors with high specificity. Hence, HGC nanoparticles preferentially localized in tumor tissues can supply active CPT molecules for extended times through a sustained release (the so-called metronomic effect) pattern, thereby exerting an antitumor effect in the MDA-MB231 human breast cancer xenograft model [65]. Because of their hydrophobic inner cores, HGC nanoparticles can efficiently imbibe various hydrophobic anticancer drugs, and show a sustained drug release profile. Also, near infrared fluorescence-labeled HGC nanoparticles were shown by noninvasive and in real-time imaging system in tumor-bearing mice to have reasonably prolonged blood circulation time and tumor targeting ability, compared to control nanoparticles [65]. Examination of a nanosized drug delivery system for encapsulating CPT into prolonged circulating and tumor-homing HGC nanoparticles were addressed [66]. The in vivo tissue distribution, tumor targeting ability, and time-dependent excretion profile were also confirmed by noninvasive and live animal imaging systems, suggesting the anticancer efficacy of CPT-HGC nanoparticles in a human breast cancer xenograft model, compared to free CPT-treated mice [66].

Targeted delivery of small interfering (siRNA) against HER2 to HER2-overexpressing SKBR3 breast cancer cells was shown to be specific, with chitosan/quantum dot (QD) nanoparticles labeled on their surface with HER2 antibody targeting the HER2 receptors on SKBR3 breast cancer cells [67]. These experiments demonstrated that the chitosan/QD nanoparticles labeled with suitable surface ligands can be specifically targeted to cells with a high degree of precision. In vitro confocal microscopy and flow cytometry studies using MCF-7 and SKBR3 cells also showed that chitosan/QD nanoparticles may be readily internalized into cells [67]. A recent insight into overexpression of RhoA in cancer suggests that this indicates a poor prognosis, because of increased tumor cell proliferation and invasion and tumor angiogenesis. Researchers reported that anti-RhoA siRNA inhibited aggressive breast cancer more effectively than conventional blockers of Rho-mediated signaling pathways [68]. The study reports the efficacy and lack of

toxicity of intravenously administered anti-RhoA siRNA encapsulated in chitosan-coated poly(isohexylcyanoacrylate) (PIHCA) nanoparticles in xenografted aggressive breast cancers (MDA-MB-231) [68]. A recent study reported that a system incorporating methotrexate (MTX) covalently conjugated to chitosan nanoparticles has potential as a delivery system for MTX [69]. By conjugating MTX to chitosan using glutaraldehyde as crosslinking agent, in vitro release tests showed that the stable covalent bonding of chitosan and MTX was beneficial for providing slow release of MTX. In vitro studies using MCF-7 cancer cells further demonstrated the effective anticancer efficacy of this new type of delivery for MTX [69].

As an alternative approach, investigators tried the use of an in situ implant incorporating superparamagnetic iron oxide nanoparticles (SPIONs) as a form of minimally invasive treatment of cancer lesions by magnetically induced local hyperthermia [70]. Studies showed that injectable formulations that form gels are capable of entrapping magnetic particles when injected into a tumor [70]. Using SPIONs embedded in silica microparticles to favor injectability, a chitosan gel incorporated the highest proportion possible to allow large heating capacities. The hydrogel (a single-solvent organogel and low-toxicity hydrophilic cosolvent organogel) formulation was injected into human cancer tumors xenografted in mice. Unfortunately, the thermoreversible hydrogels (poloxamer, chitosan), which accommodated 20% w/v of the magnetic microparticles, proved to be inadequate at reducing tumors [70].

Using "click chemistry," the selective introduction of a trimethylammonium cationic group into the C-6 position of chitosan was successfully performed for the first time and the 6-*N*,*N*,*N*-trimethyltriazole–chitosans (TCs) showed good solubility in water. The introduction of a trimethyltriazole group led to significantly increased cellular uptake compared with unmodified chitosan, which resulted in higher transfection efficiency in HEK 293 and MDA-MB-468 cells [71]. Further studies also showed that chitosan colloidal carriers, which consist of an oily core and a chitosan coating, facilitated a controlled intracellular delivery of docetaxel (DTX) [72]. Fluorescence was observed in more than 80% of MCF7 (human breast adenocarcinoma) cells after only 2 h of treatment with fluorescent chitosan carriers. As a result, the DTX-loaded chitosan carriers had an effect on cell proliferation that was significantly greater than that of free DTX. These results suggest that DTX remains fully active upon its encapsulation into the colloidal carriers and that these systems actively transport DTX into cancer cells and, thus, result in a significant increase in its antiproliferative effect [72].

To achieve a therapeutic effect at lower doses of paclitaxel (PTX), to minimizing the adverse side effects, chitosan/glyceryl monooleate (GMO) nanoparticles loaded with PTX were characterized and their safety and efficacy evaluated by MTT cytotoxicity assay in human breast cancer cells (MDA-MB-231) [73]. This nanoparticle formulation showed evidence of mucoadhesive properties, a fourfold increased cellular uptake, and a 1,000-fold reduction in the half-inhibitory concentration (IC50) of PTX [73].

Two polymers, chitosan and poly(lactide-*co*-glycolide) (PLGA) copolymer, were developed in the form of nanoparticles for delivery of protein drug substances into tumor cells [74]. Cystatin was selected as a model protein drug due to its high

potential to inhibit cysteine proteases, known to trigger the invasive process. As a result, the cellular uptake of the nanoparticles was tested on a transformed human breast epithelial cell line, MCF-10A neoT, a cell line characterized by an increased expression of cysteine proteases and a highly invasive cell phenotype. The results suggested that cystatin delivery by nanoparticles effectively inhibits intracellular proteolytic activity of cathepsin B [74]. Another strategy to develop biodegradable nanoparticles suitable for cellular delivery of chemotherapeutic drugs has been to modify the surface of PLGA nanoparticles with chitosan [75]. Chitosan-modified PLGA nanoparticles showed significant uptake by neoplastic 4 T1 cells, and were distributed to several major organs that are frequently seen as sites of cancer metastasis in mice [75]. Poly(methyl methacrylate) nanoparticles coated with chitosan–glutathione conjugate were studied for anticancer drugs encapsulation [76]. Nanoparticles were synthesized through radical polymerization of methyl methacrylate initiated by cerium (IV) ammonium nitrate and then PTX, a model anticancer drug, was encapsulated in the nanoparticles with a maximal encapsulation efficiency of 98.27%. The PTX-loaded nanoparticles showed cytotoxicity for NIH 3 T3 and T47D breast carcinoma cells [76].

3.2 Prostate Cancer

According to the American Cancer Society, prostate cancer is the most common cancer in men, and the second most deadly. As a result of slow tumor growth, the disease is usually not detected early, which results in spreading of the disease via metastasis in 30–50% of patients diagnosed with prostate cancer. Thus, improved techniques are urgently needed for detection of the disease in its early stages in order to improve the survival rates of patients. Downregulation of Relaxin (RLN) family peptide receptor 1 (RXFP1) expression using siRNA reduces cancer growth and metastasis in a xenograft model of prostate cancer. One group has shown that intratumoral injections of siRNAs loaded on biodegradable chitosan nanoparticles led to a downregulation of RXFP1 receptor expression and a dramatic reduction in tumor growth [77]. In the xenograft models treated with siRNA against RXFP1, the smaller tumor size was associated with decreased cell proliferation and increased apoptosis suggesting that the suppression of RLN/RXFP1 might have potential therapeutic benefits in prostate cancer [77]. Magnetofluorescent polymeric nanoparticles for prostate cancer imaging in vivo have been extensively studied using GC chemically modified with *N*-acetyl histidine (NAHis) as a hydrophobic moiety. Bombesin (BBN) was conjugated to the hydrophobically modified GC for use in targeting gastric-releasing peptide receptors (GRPR) overexpressed in prostate cancer cells [78]. BBN-conjugated chitosan-NAHis-GC nanoparticles (BC-NAHis-GC nanoparticles) showed significantly higher binding to the PC3 cell surface than nanoparticles without BBN, and the cellular binding was clearly inhibited by BBN [78]. Further, iron oxide nanoparticles (IO) were loaded into BC-NAHis-GC nanoparticles to investigate the possibility of their use as a probe

for Magentic resonance imaging (MRI). IO-BC-NAHis-GC nanoparticles were well observed in the PC3 cells, and the blocking with BBN significantly reduced the cellular binding of the nanoparticles. Altogether the results show that BBN conjugation to NAHis-GC nanoparticles improves their tumor accumulation in PC3-bearing mice in comparison to nanoparticles without BBN, suggesting that BC-NAHis-GC nanoparticles may be useful for prostate cancer imaging [78]. Springate et al. reported an injectable, intratumoral, controlled release delivery system for clusterin antisense oligonucleotide (clusterin ASO) [79]. They showed that when clusterin ASO complexed with chitosan microparticles (CC complexes) and then blended with a biodegradable polymeric paste (CC in paste), there was a 52–62% inhibition of the expression of clusterin protein in PC-3 cells in vitro as compared to clusterin ASO alone [79]. The authors concluded that the amount of clusterin ASO loaded into microparticulate chitosan was dependent on the amount of chitosan present and the pH of the environment, and that clusterin ASO was released from the various formulations in a controlled manner and in a bioactive form [79]. Other researchers studied an injectable, controlled release delivery system for a phosphorothioate ASO for intratumoral treatment of solid tumors that was based on complexed ASO–chitosan dispersed in a biodegradable polymeric paste [80]. Results were similar to those for the clusterin ASO complexed with a chitosan particle system. When injected intratumorally with or without PTX or DTX, the tumor volumes and serum prostate specific antigen (PSA) levels in tumor-bearing mice decreased over the 4 week period. Complexation of clusterin ASO with chitosan and incorporation into polymeric paste with PTX or DTX produced in vitro controlled release of the ASO [80].

Studies using a 166Ho–chitosan complex (DW-166HC) in rats showed its absorption, distribution, and excretion after administration into the prostate [81]. DW-166HC administered into the prostate of male rats resulted in three- to fourfold higher levels of radioactivity in the main tissues, including liver, spleen, kidney cortex, and bone, 24 h after administration as compared to controls administered 166Ho nitrate alone. It was concluded that the chitosan complex may be used to retain 166Ho within a limited area in cancer of the prostate [81]. Studies on the treatment of metastatic prostate tumors using GC as immunoadjuvant and indocyanine green (ICG) showed that this treatment significantly reduced the growth of primary tumors and lung metastases. Long-term survival of the rats bearing the prostate tumors was observed after the laser immunotherapy treatment [82].

3.3 Lung Cancer

Lung cancer is the leading cause of cancer death among both men and women, with 5-year survival rates of only 50–80% after pathologically complete resection of stage I or II disease, in particular. Nanotechnology is an exciting and rapidly progressing field that offers potential solutions to multiple challenges in the diagnosis and treatment of lung cancer, with the potential for improving imaging and

mapping techniques, drug delivery, and ablative therapy. In studies performed to overcome drug delivery limitations inhibiting the optimization of deep lung therapy, isolated rat Sertoli cells were preloaded with chitosan nanoparticles to obtain a high density distribution and concentration (92%) of the nanoparticles in the lungs of mice [83]. In a similar study, Sertoli cells loaded with chitosan nanoparticles coupled with the anti-inflammatory compound curcumin, were injected intravenously into control or experimental mice with deep lung inflammation [83]. Mice model studies showed the therapeutic effect achieved 24 h following curcumin treatment delivered by this Sertoli cell nanoparticle protocol (SNAP), suggesting an efficient protocol for targeted delivery of drugs to the deep lung mediated by extratesticular Sertoli cells [83].

Zhang et al. devised a process for forming self-assembled oleoyl-chitosan (OCH) nanoparticles based on chitosan with different molecular weights (5, 38, and 300 kDa) [84]. They loaded their OCH nanoparticles with doxorubicin hydrochloride (DOX) and showed that the drug had a sustained release in phosphate-buffered saline (PBS), and that loading efficiency and the DOX release rate increased as the molecular weight of chitosan decreased [84]. Zhang et al. proposed the utilization of DOX-loaded OCH nanoparticles for treatment of human lung cancer, since DOX-loaded OCH nanoparticles exhibited significant inhibitory effects on A549 cells as compared to DOX solution. They concluded that low molecular weight OCH nanoparticles have potential as carriers for antitumor agents. The blank OCH nanoparticles showed no cytotoxicity to mouse embryo fibroblasts or human lung cancer cell line A549 [84]. Beisner et al. developed chitosan-coated PLGA nanoparticles to mediate efficient delivery of 2′-*O*-methyl-RNA into human lung cancer cells [85]. Cellular uptake of the inhibitor mediated by chitosan-coated PLGA nanoparticles was greatly enhanced compared to the uptake of ASO alone, as shown by flow cytometry analysis. Results clearly showed that the chitosan-coated PLGA nanoparticles were suitable for delivery of 2′-*O*-methyl-RNA and induced effective telomerase inhibition and telomere shortening in human lung cancer cells [85]. Also, complexes such as chitosan-modified PLGA Nanospheres (CS-PLGA NS) were designed to be preferentially taken up by human lung adenocarcinoma cells (A549) [86]. Cellular uptake of CS-PLGA NS by lung cells (A549) was energy-dependent, suggesting a clathrin-mediated endocytic process. It was also observed that CS-PLGA NS showed low cytotoxicity, similar to non-PLGA NS [86]. In a further exploration of the usefulness of chitosan nanoparticles, Yang et al. developed a method of encapsulating PTX-loaded CNPs (PTX-CNPs) to understand the mechanism of the preferential accumulation of PTX in lung tumors [87]. Parameters such as ζ-potential and in vitro cellular cytotoxicity (A549 cells and CT-26 cells) of PTX-CNPs, and in vitro uptake of coumarin 6 to lung cells from chitosan-modified PLGA nanoparticles containing coumarin 6 (coumarin 6-CNPs) were examined. However, the in vitro uptake of coumarin 6 by A549 cells and CT-26 cells was enhanced at lower pH for coumarin-6-CNPs. Analyses showed that PTX-CNPs showed enhanced cytotoxicity as the pH became more acidic. Therefore, enhanced electrostatic interaction between chitosan-modified PLGA nanoparticles and the acidic microenvironment of tumor

cells appears to be an underlying mechanism of lung tumor-specific accumulation of PTX from PTX-CNPs [87].

Methods for preparing polymeric amphiphilic nanoparticles based on oleoyl-chitosan (OCH) loaded with DOX were developed by Zhang et al. and involved envelopment of the OCH nanoparticles efficiently loaded with DOX and provided a sustained released after a burst release in PBS [88]. Nanoparticles alone showed no cytotoxicity to mouse embryonic fibroblasts (MEF) and low hemolysis rates (<5%). Human lung carcinoma cells (A549) and mouse macrophages (RAW264.7) were used to determine the time and concentration dependence of cellular uptake by incubating with fluorescently labeled OCH nanoparticles. Their findings revealed that the activity of OCH nanoparticles increases cancer cell uptake of DOX, resulting in significant improvement for therapeutic efficiency [88]. Novel Pluronic/chitosan nanocapsules encapsulating iron oxide nanoparticles were produced by Bae et al., resulting shell crosslinked nanocapsules with a unique core–shell nanoreservoir architecture: an inner core encapsulating magnetic nanoparticles and a hydrophilic Pluronic/chitosan polymer shell layer [89]. Confocal laser scanning microscopy of rhodamine-labeled nanocapsules clearly showed efficient internalization of the nanocapsules by human lung carcinoma cells upon exposure to an external magnetic field. These novel Pluronic/chitosan nanocapsules encapsulating iron oxide nanoparticles were suitable for the magnetically triggered delivery of various anticancer agents and for successful cancer diagnosis using magnetic resonance imaging [89].

Taetz et al. developed a method utilizing tailorable cationic chitosan–PLGA nanoparticles for the delivery of an antisense 2′-*O*-methyl-RNA (2OMR) directed against the RNA template of human telomerase [90]. The efficacy of the nanoplex system was determined by measurements of binding efficiency, complex stability, and uptake in different human lung cell types. Results of flow cytometry analysis clearly showed that the uptake of 2OMR into A549 lung cancer cells was considerably higher in combination with nanoparticles than alone, and was dependent on the amount of chitosan. Furthermore, the uptake into A549 cells was confirmed by confocal laser scanning microscopy and suggested that uptake is mediated via complexes of 2OMR and chitosan–PLGA nanoparticles [90]. To produce nanoparticles as vehicles for anticancer compounds to lung, Yang et al. developed a method of preparing chitosan-modified PTX-loaded PLGA nanoparticles [91]. Their findings revealed that the loading of drug-encapsulating nanoparticles was significantly high. Data also demonstrated that a lung-specific increase in the distribution index of PTX [i.e., AUC (lung)/AUC (plasma)] was observed for chitosan-modified nanoparticles (e.g., 99.9 for nanoparticles versus 5.4 for Taxol) when nanoparticles were administered to lung-metastasized mice via the tail vein at a PTX dose of 10 mg/kg. These findings revealed the transient formation of aggregates in the blood stream followed by enhanced trapping in the lung capillaries. An electrically mediated interaction appeared to be responsible for the lung-tumor-specific distribution of the chitosan-modified nanoparticles via an enhanced uptake across the endothelial cells of the lung tumor capillary [91].

Another chitosan colloidal, consisting of oily core and a chitosan coating, was utilized for targeting human lung carcinoma cells for anticancer drug delivery.

Lozano et al. developed a method of preparing biodegradable colloidal carrier loaded with DTX to facilitate a controlled intracellular delivery of the drug [72]. Flow cytometry analysis in a human lung cells showed that the fluorescent chitosan carriers were rapidly internalized and that almost 100% of A549 cells were fluorescent. Data revealed that for 24 h after treatment, DTX-loaded chitosan carriers had a greater effect on cell proliferation than free DTX. With this delivery system, the DTX anticancer agent remains fully active upon its encapsulation into the colloidal chitosan carrier and resulted in active delivery of drug into cancer cells and increasing treatment specificity [72]. Hydrophobically modified GC (HGC) nanoparticles, a new nanosized drug carrier, were prepared by Hwang et al. by introducing a hydrophobic molecule, cholanic acid, to water-soluble GC [92]. The HGC nanoparticles were formulated comprising DTX-loaded self-assembled aggregates with a mean diameter of 350 nm in aqueous condition using a dialysis method. The DTX–HGC nanoparticles were well dispersed and stable for 2 weeks under physiological conditions (pH 7.4 and 37°C) and showed a sustained drug release profile in vitro. In addition, the DTX–HGC nanoparticles were found to be stable in the presence of excess bovine serum albumin, which suggests that the DTX–HGC nanoparticles might also be stable in the blood stream. Furthermore, noninvasive live animal imaging technology showed the time-dependent excretion profile, in vivo biodistribution, prolonged circulation time, and tumor targeting ability of the DTX–HGC nanoparticles. Finally, under optimal conditions for cancer therapy, the DTX–HGC nanoparticles clearly showed a higher antitumor efficacy (e.g., reduced tumor volume and increased survival rate) in mice bearing A549 lung cancer cells, and strongly reduced the anticancer drug toxicity compared to free DTX in tumor-bearing mice. The studies on encapsulating DTX illustrated the efficacy of nanosized drug carriers and demonstrated a promising nanosized drug formulation for cancer therapy [92].

Nafee et al. studied chitosan-coated PLGA nanoparticles utilizing AOS to lung cancer cells [93]. Biodegradable nanoparticles of 130 nm were formed and the adjustable surface charge tailored by controlling the process parameters. As a proof of concept, the overall potential of these particulate carriers to bind the AOS 2′-*O*-methyl-RNA and improve its cellular uptake was demonstrated. The study proved the efficacy of chitosan-coated PLGA nanoparticles as a flexible and efficient delivery system for AOS to lung cancer cells [93].

Cafaggi et al. developed a technique utilizing an anionic cisplatin–alginate complex with a cationic polyelectrolyte, i.e., chitosan or *N*-trimethyl chitosan nanoparticles were prepared through electrostatic interaction [94]. Particles of 180–350 nm mean diameter were formed, and cisplatin–alginate complex release studies showed that almost all the drug was released in saline-buffered solution at pH 7.4, i.e., approximately 40% w/w of total cisplatin was released from negative nanoparticles and roughly 50% w/w from positive nanoparticles. Furthermore, the drug loading of nanoparticles with a positive ζ-potential (43 mV–60 mV) ranged from 13 to 21% w/w and particle yield (referred to total polymers) was about 15% w/w (50% w/w if referred to the cisplatin–alginate complex). On A549 human cells, the nanoparticles with the smallest size and the lowest positive ζ-potential were

more active than cisplatin and showed a similar capability to induce apoptosis in other human cancer cells (A2780). These results indicate that cisplatin complexes with polycarboxylate polymers can be transformed into cisplatin particulate carriers for anticancer therapy [94]. Another interesting study demonstrates that use of biomaterial chitosan to form chitosan–siRNA nanoparticles for gene silencing protocols. Liu et al. directed much effort towards the application of chitosan as a nonviral carrier for siRNA and the importance of polymeric properties for the optimization of gene silencing using chitosan–siRNA nanoparticles [95]. The studies showed that the physicochemical properties (size, ζ-potential, morphology, and complex stability) and in vitro gene silencing of chitosan–siRNA nanoparticles are strongly dependent on chitosan molecular weight (Mw) and degree of deacetylation (DD). High Mw and DD of chitosan resulted in the formation of discrete stable nanoparticles of approximately 200 nm in size. Data revealed that chitosan–siRNA formulations (N:P 50) prepared with low Mw (approximately 10 kDa) chitosan showed almost no knockdown of endogenous enhanced green fluorescent protein (EGFP) in H1299 human lung carcinoma cells, whereas those prepared from chitosan of higher Mw (64.8–170 kDa) and DD (approximately 80%) showed greater gene silencing that ranged between 45 and 65%. Interesting, the study achieved the highest gene silencing efficiency (80%) using chitosan–siRNA nanoparticles at an N: P ratio of 150 using higher Mw (114 and 170 kDa) and DD (84%) that correlated with formation of stable nanoparticles of approximately 200 nm [95].

3.4 Ovarian Cancer

Ovarian carcinoma is the leading cause of death from gynecologic malignancy, which is due to its late initial diagnosis in addition to recurrence of ovarian cancer associated with resistance to therapy. To date, several depot systems have been designed for local delivery for ovarian cancer therapy. Nanotechnology has great promise in addressing existing problems and could improve diagnosis and therapy of ovarian cancer. As an example, Han et al. developed an Arg-Gly-Asp (RGD) peptide-labeled chitosan nanoparticle (RGD-CNP) by thiolation reaction [96]. In vitro studies examined the binding of RGD-CNP with αvβ3 integrin by flow cytometry and fluorescence microscopy. A human ovarian cancer-bearing orthotopic animal model utilizing RGD-CNPs loaded with siRNA showed significant increased selective intratumoral delivery. In addition, the same group showed targeted silencing of multiple growth-promoting genes (*POSTN*, *FAK*, and *PLXDC1*) along with therapeutic efficacy in the SKOV3ip1, HeyA8, and A2780 models using siRNA incorporated into RGD-CNP (siRNA/RGD-CNP) [96]. Furthermore, the in vivo tumor vascular targets using RGD-CNP by delivering PLXDC1-targeted siRNA into the αvβ3 integrin-positive tumor endothelial cells in A2780 tumor-bearing mice [96]. These studies showed that RGD-CNP is a novel and highly selective delivery system for siRNA, with the potential for broad

applications in human disease and especially ovarian cancer [96]. Liu et al. developed a method of target diagnosis of ovarian cancer using magnetic resonance imaging and used prepared anti-Sperm protein 17 (Sp17) immunomagnetic nanoparticles (IMNPs) [97]. To assess the anti-Sp17 IMNPs for study of ovarian cancer target therapy, the group developed a method involving complex formation between the anti-human Sp17 IMNPs by grafting anti-Sp17 antibodies on the surface of chitosan-coated magnetic nanoparticles (MNPs) using the linker EDC/NHS [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide]. In addition, the immunologic activity of IMNPs was evaluated by enzyme-linked immunosorbent assay (ELISA). The efficacy was determined using ovarian cancer HO-8910 cells by transfecting IMNPs with human Sp17 gene. Results clearly showed that the MNPs grafted with anti-Sp17 antibody and the IMNPs kept good bioactivity and a significant targeting of cells was observed [97]. Yang et al. developed a method of targeting Multi drug resistant 1 genes (MDR1) that can effectively reverse the PTX resistance in A2780/TS cells in a time-dependent manner using PTX-loaded chitosan/pshRNA plasmid nanoparticle preparations [98]. Nanoparticles were synthesized by means of a complex coacervation technique and A2780/TS cells transfected with MDR1-targeted chitosan/pshRNA plasmid nanoparticles were examined. Cells transfected with chitosan/pGPU6/GFP/Neo no-load plasmid nanoparticles served as negative control cells. Furthermore, the MDR1 mRNA in the transfected cells was significantly decreased by 17.6, 27.8, and 52.6% on post-transfection days 2, 4, and 7, respectively, when compared with that in A2780/TS control cells ($P < 0.05$). Data clearly suggest that the IC50 of PTX for A2780/TS cells, as determined by the MTT method, revealed that the relative reversal efficiency increased over time and was 29.6, 51.2, and 61.3% in the transfected cells at 2, 4, and 7 days after transfection, respectively, and that IC50 values (0.197 ± 0.003, 0.144 ± 0.001, and 0.120 ± 0.004) decreased, with the difference being significant ($P < 0.05$) as compared to A2780/TS control cells (0.269 ± 0.003) [98].

Localized and sustained delivery of anticancer agents to the tumor site has great potential for the treatment of solid tumors. Lim Soo et al. proposed the utilization of PLA-*b*-PEG/PLA nanoparticles containing a chitosan–egg phosphatidylcholine (chitosan-ePC) implant system for the delivery of PTX to treat ovarian cancer [99]. Overproduction of ascites fluid in the peritoneal cavity is a physical manifestation of ovarian cancer. The efficacy of PTX was determined by in vitro release studies from the implant system and were conducted in various fluids including human ascites fluid. The experimental data showed a strong correlation ($r^2 = 0.977$) between the release of PTX in ascites fluid and in PBS containing lysozyme (pH 7.4) at 37°C. In addition, the swelling, degradation, and morphology data suggested that the drug release mechanism. Furthermore, healthy Balb/C mice were used to confirm that the in vitro release of PTX was a good indicator of the in vivo release profile (correlation between release rates: $r^2 = 0.965$). Healthy Balb/C mice were also used to determine the efficiency of the release of PTX over a 4-week period following implantation of the chitosan-ePC system into the peritoneal cavity of mice. Further results

indicated that there was constant level of PTX concentration in both plasma and tissues (e.g., liver, kidney, and small intestine) [99].

Nanoparticle carriers of encapsulated anionic cisplatin–alginate complex with a cationic polyelectrolyte (known as chitosan or *N*-trimethyl chitosan) may also be modified to provide efficient drug release in the human ovarian carcinoma cell lines [94]. Cafaggi et al. developed a technique utilizing anionic cisplatin–alginate complex with chitosan or *N*-trimethyl chitosan nanoparticles for efficient drug release [94]. Mean particle diameter ranged from 180 to 350 nm and cisplatin–alginate complex release studies showed almost all the drug in saline-buffered solution at pH 7.4: approximately 40% w/w of total cisplatin was released from negative nanoparticles and roughly 50% w/w from positive ones. Furthermore, the drug loading of nanoparticles with a positive ζ-potential (43–60 mV) ranged from 13 to 21% w/w, and the particle yield (referred to total polymers) was about 15% w/w (50% w/w if referred to cisplatin–alginate complex). Human and murine model studies showed that the cytotoxic activity of the positive nanoparticles was similar to or lower than that of cisplatin, probably depending on the combination of size and ζ-potential values, in P388 murine and A2780 human cells. These results indicate that cisplatin complexes with polycarboxylate polymers can be transformed into cisplatin particulate carriers for anticancer therapy [94].

Grant et al. developed a method of reducing therapeutic difficulties associated with PTX preparation and use by encapsulating PTX into chitosan and ePC [100]. The method involved preparation of composite films for localized drug delivery using chitosan and ePC. Therapeutic efficacy was determined using chitosan-ePC film as a matrix for the localized delivery of PTX. Nanoparticles containing PTX were dispersed throughout the chitosan-ePC film to result in a drug:material ratio of 1:8 (wt/wt). Results clearly showed that the composite film provided a sustained release of PTX over a 4-month period in biologically relevant media. It was proposed that utilization of PTX loaded in the chitosan-ePC film could provide sustained release of the drug in SKOV-3 human ovarian cancer cells [100].

Therapeutic efficiency of the delivery of camptothecin is limited by the low aqueous solubility and high toxicity of the drug. Zhou et al. developed a method of preparing camptothecin encapsulation in *N*-trimethyl chitosan (CPT-TMC) to increase its water-solubility and lower its side effects, and tested it on a high potential lymphogenous metastatic model of human ovarian cancer [101]. In that study, SKOV3 cells were transfected with VEGF-D recombinant plasmid DNA (pcDNA3.1(±)/VEGF-D) and stable SKOV3/VEGF-D cell lines were established to study the lymphogenous metastatic model. In vivo nude mice model studies looked at the antitumor and antimetastatic activities of CPT-TMC after subcutaneous injection with SKOV3/VEGF-D cells. In contrast to controls, the results clearly showed that the administration of CPT-TMC achieved effective inhibition of primary tumor volume and lymphogenous metastasis, yet without apparent systemic toxic effects. These effects were associated with simultaneously down-regulated VEGF-D and MMP-9 expression, significantly decreased tumor-associated lymphatic and blood sprouts, tremendously reduced systemic toxic effects, and dramatically increased tumor apoptotic index. The investigator

proposed that CPT-TMC is superior to CPT because of maximizing its anticancer and antimetastatic activities with minimal toxicity on hosts, suggesting that treatment with CPT-TMC can be a highly efficient and effective therapeutic strategy against advanced human ovarian cancer [101].

In a further exploration of the usefulness of nanocarriers, Li et al. developed a method of encapsulating tCPT to overcome the problem of low water solubility and severe toxicity of CPT after intravenous administration [102]. The method of preparation is based on using chitosan and dibasic sodium phosphate (DSP) to encapsulate CPT intended for local administration. Encapsulation of CPT nanocolloids had a large effect on the gelling time as well as the micro-structure of the hydrogel. Data from both in vitro and in vivo degradation studies revealed that the chitosan/DSP hydrogel was biodegradable and biocompatible. In vitro release studies revealed that CPT was released from chitosan/DSP hydrogel over an extended period, with about 70% of total CPT released from hydrogel after 18 days. Furthermore, nearly 90% of CPT in the chitosan hydrogels could be preserved in the lactone form (active form) even after 7 days of storage at 37°C. Furthermore, in vitro cytotoxicity of CPT nanocolloids on SKOV3 human ovarian cancer cells suggested that good antitumor cell efficiency could be gained at a lower concentration [102]. A series of chitosan derivatives (mPEGOSC) with hydrophobic moieties of octyl and hydrophilic moieties of sulfate and polyethylene glycol monomethyl ether (mPEG) groups were synthesized by the group of Qu et al. [103]. The method involved preparation of PTX-loaded micelles into a chitosan complex. The in vivo–in vitro correlations of PTX-loaded micelles was studied, including interaction of the drug-loaded micelles with protein and the kinetics of PTX-loaded micelles with O-carboxymethylated chitosan (CMC). Data from tissue distribution studies in mice showed that PTX-mPEGOSC2000M micelles were phagocytozed less than PTX-OSC micelles by the reticuloendothelial system (RES). Furthermore, a higher targeting efficiency of PTX-mPEGOSC2000M to the uterus (including ovary) was estimated. Together, the results of Qu et al. suggested that PTX-encapsulated formulations could be used for the chemotherapy of ovarian cancer [103]. Anticancer agents known to have a high effect in anticancer treatment, such as PTX, are either unable to be formulated safely or do not have a well-tolerated method of administration [104].

Vassileva et al. developed a method of preparing a novel chitosan-ePC implantable drug delivery system that is safe, less toxic, biocompatibile, and with antitumor efficacy [104]. The novel PTX-loaded ePC formulation was used in their experiment for providing controlled and sustained release of PTX (ePC) compared with commercial PTX formulated in Cremophor EL, PTX (CrEL). Therapeutic efficacy of PTX-loaded ePC was determined utilizing a human ovarian carcinoma SKOV-3-bearing xenograft model. Results clearly showed that the enhanced antitumor efficacy was achieved with PTX (ePC) in contrast to PTX (CrEL) with the same total dose of 60 mg/kg PTX. Furthermore, to determine the safety and toleration, toxicity studies were conducted using healthy CD-1 female mice. Data showed that the drug-free or PTX (ePC) formulations did not exhibit observable toxicity, local inflammation, or fibrous encapsulation of the implant. In contrast, mice receiving

PTX (CrEL) or CrEL encountered significant toxicity, lethality, abnormal peritoneal organ morphology, and hepatic inflammation. The maximum tolerable dose (MTD) of PTX (CrEL) was 20 mg/kg/week, whereas PTX doses of up to 280 mg/kg/week were well tolerated when administered as PTX (ePC) [104].

Gene therapy is a method used to stimulate the body's immune response so that it attacks cancer cells by introducing genetic material (DNA or RNA) to activate cellular processes [105]. Chitosan-mediated gene delivery has gained increasing interest due to its ability to treat cancers and genetic diseases [105]. Lee et al. developed a method utilizing folic acid covalently conjugated to chitosan (FACN) as a targeting ligand in an attempt to specifically deliver DNA to folate receptor-overexpressing ovarian and breast cancer cells. Cells from human epithelial ovarian cancer OV2008 and human breast cancer MCF-7 were used to determine the in vitro gene transfer potential of FACN. The FACN formed nanoparticles that, at a weight ratio of 10:1, exhibited significantly ($P<0.01$) enhanced gene transfer potential in folate receptor-overexpressing cancer cells as compared to unmodified chitosan. Transfection efficiency of FACN/pDNA nanocomplexes was shown to be competitively inhibited by free folic acid, suggesting that the specific gene delivery of FACN/pDNA nanocomplexes was achieved through folate receptor-mediated endocytosis. These results showed that the formed nanocomplex of FACN had a high gene delivery efficiency and is a promising carrier for cancer gene therapy [105].

3.5 Pancreatic Cancer

Pancreatic cancer is sometimes called a "silent killer" because early pancreatic cancer often does not cause symptoms, and the later symptoms are usually nonspecific and varied. Therefore, pancreatic cancer is often not diagnosed until it is advanced. Therefore, new treatment strategies are urgently required to combat this deadly disease. In cancer therapeutics, nanoparticle-mediated targeted delivery systems might significantly reduce the low toxicities and improve bioavailability. Zaharoff et al. developed techniques for investigating biodegradable polysaccharide chitosan co-formulated with intratumoral (i.t.) administration of interleukin (IL)-12 to enhance the antitumor activity of IL-12 while limiting its systemic toxicity [106]. IL-12 is a potent antitumor cytokine that exhibits significant clinical toxicities after systemic administration. Noninvasive imaging studies monitored local retention of IL-12, with and without chitosan co-formulation, after i.t. injection. Antitumor efficacy of IL-12 alone and of IL-12 co-formulated with chitosan (chitosan/IL-12) was assessed in mice bearing established colorectal (MC32a) and pancreatic (Panc02) tumors. Additional studies involving depletion of immune cell subsets, tumor rechallenge, and CTL activity were designed to elucidate mechanisms of regression and tumor-specific immunity. Data clearly indicated that co-formulation with chitosan increased local IL-12 retention from 1–2 days to 5–6 days. Weekly i.t. injections of IL-12 alone eradicated $\leq$10% of established MC32a and Panc02 tumors, while i.t. chitosan/IL-12 immunotherapy caused

complete tumor regression in 80–100% of mice. Depletion of CD4(±) or Gr-1(±) cells had no impact on chitosan/IL-12-mediated tumor regression. However, CD8(±) or natural killer cell depletion completely abrogated antitumor activity. chitosan/IL-12 immunotherapy generated systemic tumor-specific immunity, as >80% of mice cured with i.t. chitosan/IL-12 immunotherapy were at least partially protected from tumor rechallenge. Furthermore, CTLs from spleens of cured mice lysed MC32a and gp70 peptide-loaded targets. Chitosan/IL-12 immunotherapy increased local retention of IL-12 in the tumor microenvironment, eradicated established aggressive murine tumors, and generated systemic tumor-specific protective immunity. Chitosan/IL-12 administration is a well-tolerated, effective immunotherapy with considerable potential for clinical translation [106]. Trickler et al. studied whether the drug gemcitabine loaded into chitosan/GMO nanostructures was able to enhance cellular accumulation and provide significant increase in cell death of human pancreatic cancer cells in vitro [107]. The delivery system was prepared by a multiple emulsion solvent evaporation method. The cellular accumulation, cellular internalization, and cytotoxicity of the nanostructures were evaluated by HPLC, confocal microscopy, or MTT assay in Mia PaCa-2 and BxPC-3 cells. The MTT cytotoxicity dose–response studies revealed that placebo at/or below 1 mg/mL has no effect on MIA PaCa-2 or BxPC-3 cells. The delivery system demonstrated a significant decrease in the IC50 (3–4 log unit shift) for gemcitabine nanostructures at 72 and 96 h post-treatment compared with treatment with a solution of gemcitabine alone. The nanostructure reported here can be resuspended in an aqueous medium, and demonstrated increased effective treatment compared with gemcitabine treatment alone in an in vitro model of human pancreatic cancer. The drug delivery system demonstrates the capability to entrap both hydrophilic and hydrophobic compounds to potentially provide an effective treatment option for human pancreatic cancer [107]. Chitosan/gelatin hydrogels were utilized for the functional longevity of microencapsulated islet cells during xenotransplantation [108]. Yang et al. demonstrated that the hydrogel acts as an immunoisolative matrix to provide additional protection to the microencapsulated islet cells [108]. This study assessed whether chitosan/gelatin hydrogels provide protection for the microencapsulated islet cells during xenotransplantation. Their data demonstrated the biocompatibility of the chitosan/gelatin hydrogel with the mouse insulinoma cell line, NIT-1. Insulinoma/agarose microspheres macroencapsulated in hydrogel revealed functional activity and secreted insulin continually for 60 days in vitro. Data clearly indicated that chitosan/gelatin hydrogel was not cytotoxic to islet cells, and in contrast, the hydrogel showed cytoprotective effects against cytokine-mediated cytotoxicity. In vivo, an insulinoma/agarose microsphere with chitosan/gelatin solution was applied as an injectable bioartificial pancreas (BAPs). As an experimental approach, an insulinoma/agarose microspheres suspended in phosphate-buffered saline or in chitosan/gelatin solution were injected into the subcutaneous layer of diabetic rats. Data clearly showed that the non-fasting blood glucose (NFBG) of diabetic rats transplanted with free insulinoma/agarose microspheres was decreased to euglycemia but restored to hyperglycemia in 15 days. By contrast,

the NFBG of rats transplanted with insulinoma/agarose microspheres with hydrogel remained euglycemic for 42 days. After predetermined intervals, the histologic sections revealed that the fibrous tissue envelopment and the infiltrated immune-related cells contributed to the dysfunction of BAPs [108]. Similarly, the group of Yang et al. demonstrated again that chitosan/gelatin hydrogel can be used as a cell carrier for an injectable bioartificial pancreas; the hydrogel prolonged the function of cells encapsulated in agarose microspheres during xenogenic transplantation for protections [109]. The NFBG of diabetic rats injected with chitosan/gelatin hydrogel, which contained insulinoma/agarose microspheres, was maintained at less than 200 mg/dL for 25 days. The histological section revealed immune cell infiltration and accumulation within the hydrogel and around the insulinoma/agarose microspheres that may have contributed to the slowly increasing NFBG after day 25. Together, the data suggest that chitosan/gelatin hydrogel can provide an injectable bioartificial pancreas and achieve prolonged function of cells encapsulated in agarose microspheres during xenogenic transplantation [109].

Recently, RNase inhibitors, either synthetic or natural, have been intensively sought because they appeared to be promising for therapy of cancer and allergy [110]. However, there are only a limited number of efficient RNase inhibitors. Yakovlev et al. developed a system utilizing the low molecular weight chitosan [M(r) approximately 6 kDa] that inhibits the activity of pancreatic RNase A and some bacterial RNases, with inhibition constants in the range of 30–220 nM at pH 7.0 and ionic strength 0.14 M. The contribution to the chitosan complex formation with RNases is due to establishment of five or six ion pairs. RNase inhibitors are commonly used to block the RNase activity in manipulations with RNA-containing preparations. The results of this work show that polycations may efficiently inhibit ribonuclease activities [110].

3.6 Colon Cancer

Colorectal cancer is one of the leading causes of malignant death because it often remains undetected until later stages of the disease [111]. Kanthamneni et al. produced drug formulations using controlled release polymers chitosan, pectin, and hydroxypropyl methylcellulose (HPMC) for the targeted delivery of combinatorial regimens to the colon for the chemoprevention of colon cancer [111]. Furthermore, the use of aspirin in combination with calcium and folic acid was assessed for synergistic inhibitory responses of a novel chemopreventive combination regimen of drugs on two human colon cancer cell lines, HT-29 and SW-480. Chemopreventive combination regimens demonstrated significant synergistic efficacy in both cell lines using XTT assay studies, when compared to the effects of individual agents. With combinations of aspirin (5 mM) and folic acid (1.5 mM), HT-29 cells demonstrated a 30% decrease in cell viability compared to approximately 38% decrease in the SW-480 cell line. Overall, all drug combinations demonstrated significant synergistic responses in the cell lines tested, with the

SW-480 cell line being more significantly affected by the drug regimens than the HT-29 cell line. Drug-encapsulating nanoparticles demonstrated a spherical morphology, <125 nm average particle size (aspirin and folic acid) of nanoparticles, and encapsulation efficiencies in the range of 80–91%. Drug release from nanoparticles was controlled, with approximately 60% of the original amount released over a 96 h period. Overall, the cell line studies demonstrated, for the first time, the ability of novel chemopreventive combinations to inhibit the growth of colon cancer cells, whereas the nanotechnology-based drug delivery system provides valuable evidence for targeted therapy towards colon cancer chemoprevention [111]. Akhlaghi et al. devised a process of forming nanoparticles containing insoluble anticancer drugs [76]. The method of preparation involved a complex formation of poly(methyl methacrylate) nanoparticles coated by chitosan–glutathione conjugate to encapsulate insoluble anticancer drugs. Nanoparticles were synthesized through radical polymerization of methyl methacrylate, initiated by cerium (IV) ammonium nitrate. PTX was encapsulated in nanoparticles with a maximal encapsulation efficiency of 98.27%. These nanoparticles showed sustained in vitro release of the incorporated PTX (75% of the loaded dose was released in 10 days). All nanoparticles had positive charge and were spherical, with a size range of about 130–250 nm. Data revealed that the PTX-loaded nanoparticles showed cytotoxicity for NIH 3 T3 and T47D breast carcinoma cells, and no cytotoxicity for two colon cell lines (HT29, Caco2) [76].

Researchers have also studied a nanoparticle system to deliver oxaliplatin to colorectal tumors. Jain et al. developed methods of preparing hyaluronic acid (HA)-coupled chitosan nanoparticles (HACTNP) bearing oxaliplatin (L-OHP) encapsulated in Eudragit S100-coated pellets for effective delivery to colon tumors [112]. The in vitro drug release was investigated using a United States Pharmacopoeia (USP) dissolution rate test paddle-type apparatus in different simulated gastrointestinal tract fluids. In a murine model, the pellets of free drug, and HA-coupled and uncoupled chitosan nanoparticles bearing L-OHP were administered orally at the dose of 10 mg L-OHP/kg body weight to tumor-bearing Balb/c mice. In vivo data showed that HACTNP delivered 1.99 ± 0.82 and 9.36 ± 1.10 μg of L-OHP/g of tissue in the colon and tumor, respectively, after 12 h, reflecting their targeting potential to the colon and tumor. These drug delivery systems show relatively high local drug concentration in the colonic milieu and colonic tumors with prolonged exposure time, which provides a potential to enhance antitumor efficacy with low systemic toxicity for the treatment of colon cancer [112]. Similarly, the group also investigated the use of HACTNP [113]. The method of preparation of HACTNP nanoparticles encapsulated 5-flurouracil (5FU) drug by an ionotropic gelation method, for the effective delivery of drug to the colon tumors. HACTNP appeared to be spherical in shape and mean size was found to be around 150 ± 3.4 nm, with low polydispersity index. The in vitro drug release was investigated in different simulated gastrointestinal tract fluids. The biocompatibility of nanoparticle formulations were evaluated for in vitro cytotoxicity by MTT assay using HT-29 cell lines, and cell uptake was assessed by fluorescent microscopy. In addition, the data demonstrated cellular uptake of HACTNP by

incorporating calcein as a fluorescent marker. The cellular uptake of fluorescent HACTNP was clearly evidenced by fluorescence microscopy. HACTNP nanoparticles showed significant higher uptake by cancer cells as compared to uncoupled nanoparticles, and the uptake of HACTNPs by HT-29 colon cancer cells was observed to be 7.9 times more than uncoupled CTNPs at the end of 4 h. The cytotoxicity of 5FU incorporated in HACTNPs was higher compared to the conventional 5FU solution, even at lower concentrations. 5FU in HACTNP was about 2.60-fold more effective than free 5FU on HT-29 cells [113].

A study by Yang et al. designed an oral form of nanoparticle to encapsulate 5-aminolaevulinic acid (5-ALA) to improve the detection of colorectal cancer cells in vivo [114]. The nanoparticle should escape bacterial uptake in the gastrointestinal tract, which seriously interferes with the results of endoscopic observation. In this study, chitosan was mixed with sodium tripolyphosphate (STPP) and 5-ALA to prepare chitosan nanoparticles and 5-ALA-loaded chitosan nanoparticles (CNA) by adding 5-ALA solutions of different pH values and concentrations. The average particle size and ζ-potential of chitosan nanoparticles and CNA were measured by the Zetasizer-3000. The results revealed that particle size with different ζ-potential could be manipulated just by changing 5-ALA concentrations and pH values. CNA particles prepared at pH 7.4 and pH 9 of 5-ALA solutions with a concentration higher than 0.5 mg/mL showed a promising loading efficiency of up to 75% and an optimum average particle size of 100 nm. Fluorescence microscope examination showed that CNA could be engulfed by Caco-2 colon cancer cells, but showed no evidence of being taken up by *Escherichia coli*. This result implies that CNA could avoid the influence of normal flora inside the gut and serve as an adequate tool for fluorescent endoscopic detection of colorectal cancer cells in vivo [114].

Park et al. developed all-*trans* retinoic acid (ATRA)-incorporated nanoparticles of methoxy poly(ethylene glycol) (MPEG)-grafted chitosan through ion-complex formation between ATRA and chitosan preparation [115]. This nanoparticle was around 100 nm in diameter and had favorable reconstitution properties. An ATRA-incorporated nanoparticle has almost similar cytotoxicity against CT-26 tumor cells as free ATRA. But, nanoparticles were more effective at inhibiting invasion of tumor cells than free ATRA in an invasion test using matrigel. Apoptosis analysis revealed that tumor cells treated with free ATRA or ATRA-incorporated nanoparticles shown progressed cell death [115]. Studies also performed on chitosan nanoparticles and copper (II)-loaded chitosan nanoparticles were prepared on the basis of ionic gelation of chitosan with tripolyphosphate anions and copper ion sorption [116]. In their study, the cytotoxic activities of the chitosan nanoparticles and copper (II)-loaded chitosan nanoparticles was investigated and a relationship between physiochemical properties and activity is suggested. Data clearly demonstrates that the chitosan nanoparticles and copper (II)-loaded chitosan nanoparticles elicited dose-dependent inhibitory effects on the proliferation of tumor cell lines [116]. Folic acid can be covalently conjugated to chitosan molecules via its gamma-carboxyl moiety and thus retain a high affinity for colorectal cancer cells bearing folate receptor overexpression [117]. Colorectal cancer is one of the leading causes of malignant death and often goes undetected

with current colonoscopy practices. Improved methods of detecting dysplasia and tumors during colonoscopy will improve mortality. Yang et al. studied the effect of folic acid-conjugated chitosan nanoparticles as a suitable vehicle for carrying 5-aminolaevulinic acid (5-ALA) to enhance the detection of colorectal cancer cells in vivo after a short-term uptake period [117]. The method involves preparation of chitosan conjugated with folic acid to produce folic acid-chitosan conjugate, which is then loaded with 5-ALA to create nanoparticles (fCNA). The loading efficiency of 5-ALA in fCNA particles and the z-average diameter were in the range 35–40% and 100 nm, respectively. The ζ-potential for fCNA was 20 mV, enough to keep the nanoparticle stable without aggregation. The fCNA is then incubated with HT29 and Caco-2 colorectal cancer cell lines overexpressing folate receptor on the surface of the cell membrane to determine the rate of accumulation of protoporphyrin IX (PpIX). The results show that fCNA can be taken up more easily by HT29 and Caco-2 cell lines after a short-term uptake period, most likely via receptor-mediated endocytosis, and that the PpIX accumulates in cancer cells as a function of the folate receptor expression and the folic acid modification. The work by Yang et al., suggested that the folic acid–chitosan conjugate could be an ideal vector for colorectal-specific delivery of 5-ALA for fluorescent endoscopic detection [117].

Another interesting approach has been studied by Ji et al. using chitosan nanoparticle-incorporated siRNA to knock down FHL2 gene expression [118]. Furthermore, the knockdown effect of the chitosan/siRNA nanoparticles on gene expression in FHL2 overexpressed human colorectal cancer Lovo cells was investigated. The results showed that FHL2 siRNA formulated within chitosan nanoparticles could knock down about 69.6% of FHL2 gene expression, which is very similar to the 68.8% reduced gene expression when siRNA was transfected with liposome Lipofectamine. Interestingly, protein expression was shown by western blot analysis to be reduced by the chitosan/siRNA nanoparticles. The results also showed that blocking FHL2 expression by siRNA could inhibit the growth and proliferation of human colorectal cancer Lovo cells. Yang et al., proposed that chitosan-based siRNA nanoparticles could be a very efficient delivery system for siRNA in vivo to colon cancer therapy [118]. Guo et al. investigated multifunctional nanocarriers based on chitosan/gold nanorod (CS-AuNR) hybrid nanospheres, which have been successfully fabricated by a simple nonsolvent-aided counterion complexation method [119]. Therapeutic efficiency of the delivery of anticancer drug cisplatin was investigated by loading into the obtained hybrid nanospheres. These CS-AuNR hybrid nanospheres were utilized for real-time cell imaging and as a near-infrared thermotherapy nanodevice to achieve irradiation-induced cancer cell death owing to the unique optical properties endowed by the encapsulated gold nanorods. The efficiency of the loaded anticancer drug cisplatin was studied in the cancer cells. The nanocarriers were an all-in-one system possessing drug delivery, cell imaging, and photothermal therapy functionalities [119]. Yang et al. developed high-performance nanoparticle for photodynamic detection of colorectal cancer, whereby alginate is physically complexed with folic acid-modified chitosan to form nanoparticles with improved drug release in the cellular lysosome [120]. The incorporated alginate molecules could complex

stably with chitosan via electrostatic attraction, and the z-average diameter and ζ-potential of the prepared fCAN nanoparticles was 115 nm and 22 mV, respectively, enough to keep the nanoparticles stable in aqueous suspension without aggregation. Using this combined approach, efficiency of 5-ALA loading was assessed and 27% loading efficiency reported. The nanoparticles (fCANA) displayed no differences in particle size or ζ-potential compared to fCAN. Moreover, the fCANA nanoparticles showed uptake of nanoparticles by colorectal cancer cells via folate receptor-mediated endocytosis. Subsequently, the loaded 5-ALA was released in the lysosome, and this was promoted by the reduced intensity of attraction between chitosan and 5-ALA via the deprotonated alginate, resulting in a higher intracellular PpIX accumulation for the photodynamic detection. Together, these studies demonstrate that the alginate incorporated- and folic acid-conjugated chitosan nanoparticles are excellent vectors for colorectal-specific delivery of 5-ALA for fluorescent endoscopic detection [120].

3.7 Melanoma Cancer

Melanoma is the least common but most dangerous and serious type of skin cancer. Melanoma affects people of all ages but the chance of developing it increases with age. Some of the research has focused on tumor therapy and delivery systems using nanotechnology. Examples such as antiangiogenic peptide drugs have received much attention in the fields of tumor therapy and tumor imaging because they show promise in the targeting of integrins such as αvβ3 on angiogenic endothelial cells [121]. However, systemic antiangiogenic peptide drugs have short half-lives in vivo, resulting in fast serum clearance via the kidney, and thus the therapeutic effects of such drugs remain modest. Kim et al. developed techniques of preparation of self-assembled GC nanoparticles and explored whether this construct might function as a prolonged and sustained drug delivery system for RGD peptide, used as an antiangiogenic model drug in cancer therapy [121]. GC hydrophobically modified with 5β-cholanic acid formed nanoparticles with a diameter of 230 nm, and RGD peptide was easily encapsulated into HGC nanoparticles (yielding RGD-HGC nanoparticles) with a high loading efficiency (>85%). Data clearly demonstrated that, in vitro, RGD-HGC have prolonged and sustained release of RGD, lasting for 1 week. RGD-HGC also inhibited HUVEC adhesion to a βig-h3 protein-coated surface, indicating an antiangiogenic effect of the RGD peptide in the HGC nanoparticles. In additional in vivo studies, the antiangiogenic peptide drug formulation of RGD-HGC markedly inhibited bFGF-induced angiogenesis and decreased hemoglobin content in Matrigel plugs. Intratumoral administration of RGD-HGC resulted in significantly decreased tumor growth and microvessel density compared to native RGD peptide injected either intravenously or intratumorally, indicating that the RGD-HGC formulation strongly enhanced the antiangiogenic and antitumoral efficacy of RGD peptide by affording prolonged and sustained RGD peptide delivery locally and regionally in solid tumors [121].

Shikata et al. designed gadolinium loaded as gadopentetic acid (Gd-DTPA) in chitosan nanoparticles (Gd-CNPs), and studied their accumulation in vitro in cultured cells as an evaluation of their use gadolinium neutron-capture therapy (Gd-NCT) for cancer [122]. Using L929 fibroblast cells, the Gd accumulation for 12 h at 37°C was investigated at Gd concentrations lower than 40 ppm. The accumulation leveled above 20 ppm and reached 18.0 ± 2.7 (mean ± S.D.) μg $Gd/10^6$ cells at 40 ppm. Furthermore, the corresponding accumulations in B16F10 melanoma cells and SCC-VII squamous cell carcinoma, which were used in the previous Gd-NCT trials in vivo, were 27.1 ± 2.9 and 59.8 ± 9.8 μg $Gd/10^6$ cells, respectively, hence explaining the superior growth-suppression in the in vivo trials using SCC-VII cells. The accumulation of Gd-NCPs in these cells was 100–200 times higher than accumulation of Gd from dimeglumine gadopentetate aqueous solution (Magnevist), a magnetic resonance imaging contrast agent. The endocytic uptake of Gd-NCPs, strongly holding Gd-DTPA, was suggested from transmission electron microscopy and comparative studies at 4°C and with the solution system. These findings indicated that Gd-NCPs had a high affinity to the cells, probably contributing to the long retention of Gd in tumor tissue and leading to the significant suppression of tumor growth [122]. Interestingly, new class of chitosan-based hybrid nanogels by in-situ immobilization of CdSe quantum dots (QDs) in the chitosan–poly(methacrylic acid) (chitosan-PMAA) networks were developed by Wu et al. [123]. The method of preparation uses covalently crosslinked hybrid nanogels with chitosan chains semi-interpenetrating in the crosslinked PMAA. The networks exhibit excellent colloidal and structural stability as well as reversible physical property changes in response to a pH variation. In contrast, the hybrid nanogels formed by noncovalent physical association exhibit a significant change in the structure and composition upon exposure to physiological pH [123]. This distinction in the structural stability of hybrid nanogels produces very different outcomes for their biomedical applications. The covalently crosslinked hybrid nanogels have low cytotoxicity and could illuminate the B16F10 cells, sense environmental pH changes, and regulate the release of anticancer drug in the typical abnormal pH range of 5–7.4 found in the pathological zone, thus successfully combining multiple functionality into a single nanoobject. However, the physically associated hybrid nanogels exhibit a nonreversible pH-sensitive PL property and a significant cytotoxicity after 24 h treatment. It is crucial to construct a highly stable biopolymer–QD hybrid nanogel, via a rational design for safe bionanomaterials, to simultaneously combine the biosensing, bioimaging, and effective therapy functions [123].

Delivery nanoparticles system 200–300 nm of apoptin gene with *O*-carboxymethylated chitosan (CMC) to human melanoma cells A375 in vitro [124]. The ratio of chitosan to apoptin DNA (N:P ratio) was 5.5:1. The apoptin gene in chitosan/apoptin nanoparticles could be protected from DNase degradation and could be used as the model in the process of replication. Data clearly showed that, in human melanoma, A375 cells are transiently transformed by nanoparticles containing the apoptin gene and could induce apoptosis of A375 cells in a dose-dependent manner in vitro at 48 h after transformation. The investigator proposed

that the chitosan vector and apoptin gene could be combined to be a safe nanoparticle gene delivery system, which could induce apoptosis in human melanoma A375 cells [124]. The studies from Kim et al. used drug-loaded HGC nanoparticles as a carrier system for PTX [63]. These self-assembled HGC conjugates were prepared by chemically linking 5β-cholanic acid to GC chains using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide chemistry. The efficacy of PTX drug loaded into HGC nanoparticles was determined using a dialysis method. The nanoparticles were 400 nm in diameter and were stable in PBS for 10 days. Results clearly demonstrated that PTX-HGC nanoparticles showed sustained release of the incorporated of PTX (80% of the loaded dose was released in 8 days at 37°C in PBS). Owing to sustained release, the PTX-HGC nanoparticles were less cytotoxic to B16F10 melanoma cells than free PTX formulated in Cremophor EL. Injection of PTX-HGC nanoparticles into the tail vein of tumor-bearing mice prevented increases in tumor volume for 8 days. Kim et al. used this proposed system and showed that PTX was less toxic to the tumor-bearing mice when formulated in HGC nanoparticles than when formulated with Cremophor EL [63]. Self-assembled nanoparticles, formed by polymeric amphiphiles, have been demonstrated to accumulate in solid tumors by the enhanced permeability and retention effect, following intravenous administration [125]. Hyung Park et al. prepared hydrophobically modified GCs capable of forming nanosized self-aggregates by chemical conjugation of fluorescein isothiocyanate or DOX to the backbone of GC [125]. The biodistribution of self-aggregates (300 nm in diameter) was evaluated using tissues obtained from tumor-bearing mice, to which self-aggregates were systemically administered via the tail vein. Irrespective of the dose, a negligible quantity of self-aggregates was found in heart and lung, whereas a small amount (3.6–3.8% of dose) was detected in liver for 3 days after intravenous injection of self-aggregates. The distributed amount of self-aggregates gradually increased in tumor as blood circulation time increased. The concentration of self-aggregates in blood was as high as 14% of the dose at 1 day after intravenous injection and was still higher than 8% even at 3 days. Furthermore, the author investigated nanocomplexes loaded with DOX administered into tumor-bearing mice via the tail vein, and found that they exhibited lower toxicity than free DOX but comparable antitumor activity. These results revealed the promising potential of self-aggregates on the basis of GC as a carrier for hydrophobic antitumor agents [125]. Kabbaj et al. developed chitosan-DNA nanoparticles and determined their DNase susceptibility, and its possible influence on their antiproliferative activity [126].

Incorporation of DNA within chitosan nanoparticles significantly decreased degradation by DNase. *Mycobacterium phlei* DNA inhibits cancer cell division but is susceptible to degradation by DNase. To address the ability of *M. phlei* DNA-chitosan nanoparticles to inhibit melanoma cell division, the group studied *M. phlei* DNA and a cationic liposomal *M. phlei* DNA formulation. *M. phlei* DNA had antiproliferative activity (MTT reduction, IC50 = 0.9 mg/mL) without intrinsic cytotoxicity (LDH release, ED50 > 50 μg/mL). Cationic polyphosphate chitosan nanoparticles were inert (antiproliferative IC50 > 1 mg/mL, ED50 > 1 mg/mL). As a result, *M. phlei* DNA-chitosan nanoparticles were 20-fold more potent

than *M. phlei* DNA. Cationic DOTAP/DOPE liposomes were cytostatic (IC50 = 49 μg/mL) and cytotoxic (ED50 = 87 μg/mL), and complexation of *M. phlei* DNA resulted in a significant reduction of antiproliferative activity. The author proposed that chitosan nanoparticles may therefore be appropriate delivery vehicles for *M. phlei* DNA [126].

Another interesting potential of gadolinium neutron-capture therapy (Gd-NCT) for cancer was evaluated using chitosan nanoparticles as a novel gadolinium device [127]. Tokumitsu et al. developed nanoparticle synthesis by incorporating 1,200 μg of natural gadolinium and studied the effect of intratumoral administration twice in mice bearing subcutaneous B16F10 melanoma. The thermal neutron irradiation was performed for the tumor site and demonstrated fluorescence of 6. 32×10^{12} neutrons/cm^2, 8 h after the second gadolinium administration. The data clearly demonstrate that the tumor growth in the nanoparticle-administered group was significantly suppressed compared to that in the gadopentetate solution-administered group after the irradiation. This study demonstrated the potential usefulness of Gd-NCT using gadolinium-loaded nanoparticles [127]. This same group had also previously reported that Gd-CNPs) may be used for Gd-NCT [128]. Through emulsion-droplet coalescence technique, the group could show in vitro Gd-DTPA release from the Gd-CNPs in plasma over 24 h. In vivo data in mice bearing subcutaneous B16F10 melanoma on the releasing properties of Gd-CNPs and their ability for long-term retention of Gd-DTPA in the tumor indicated that Gd-CNPs might be useful as an i.t. injectable device for Gd-NCT [128]. Wang et al. studied chitosan sulfated derivatives to address the common structural requirement for the sulfate pattern to block P-selectin-mediated tumor cell adhesion [129]. Interestingly, the 6-O-sulfation of chitosan is indispensable for inhibition of P-selectin binding to human melanoma A375 cells and additional N-sulfation or 3-O-sulfation dramatically enhanced the inhibitory activity of 6-O-sulfated chitosan. The author proposed that efficient anti-P-selectin adhesion activity of sulfated saccharides needs the synergy of 6-O-sulfation and N- or 3-O-sulfation in glucosamine units [129].

Camptothecin (CPT) has recently attracted increasing attention as a promising anticancer agent for a variety of tumors. Liu et al. developed a technique to incorporate CPT with *N*-trimethyl chitosan (CPT-TMC) through microprecipitation and sonication. In this study, Liu et al. were able to demonstrate the inhibition effect on B16-F10 cell proliferation and induction of apoptosis in vitro [130]. Furthermore, the antitumor activity of CPT-TMC in C57BL/6 mice bearing B16-F10 melanoma showed significant inhibition compared with the group treated with free CPT (81.3% vs. 56.9%) in the growth of B16-F10 melanoma xenografts, and prolonged the survival time of the treated mice ($P < 0.05$). Data clearly showed decreased cell proliferation, increased tumor apoptosis as well as a reduction in angiogenesis. The group proposed that *N*-trimethyl chitosan-encapsulated camptothecin is superior to free CPT by overcoming its insolubility, and finally raises the potential of its application in melanoma therapy [130].

Antitumor efficacy and systemic toxicity of chitosan-based plumbagin microspheres in comparison to free plumbagin were proposed by Mandala Rayabandla

et al. [131]. The optimized formulation had a mean particle size of 106.35 μm with an encapsulation efficiency of 80.12%. Pharmacokinetic studies showed a 22.2-fold increase in elimination half-life, $t_{1/2}$, of plumbagin from chitosan microspheres as compared to free plumbagin. In vivo data showed that administration of plumbagin microspheres resulted in a significant tumor growth inhibition and reduced systemic toxicity. Chitosan-based microspheres could be a promising strategy for the systemic delivery of anticancer agents like plumbagin [131]. The study by Kim et al. developed a local chemotherapy device using chitosan as a local drug carrier [132]. The anticancer drug ellagic acid (EA) was complexed with chitosan in the form of chitosan-EA films with concentrations of 0, 0.05, 0.1, 0.5, and 1% (w/v) of EA, and the films characterized. Studying a skin cancer model, the investigator used WM115 human melanoma cell line and demonstrated the cell response to the films with an antiproliferative effect through induction of apoptotic cell death compare to control HS68 human newborn fibroblast cells. In addition, the effect of composites on cell behaviors has been clearly demonstrated for use of local chemotherapy [132].

Nanosized and stable chitosan-*g*-PEG/heparin polyelectrolyte complexes were developed by Bae et al. [133]. The nanocomplex was utilized to study apoptotic death of cancer cells. The prepared polyelectrolyte complex micelles had a spherical shape with an average diameter of 162.8 ± 18.9 nm. They were highly stable and well dispersed even in the presence of serum due to the presence of a hydrophilic PEG shell layer surrounding the micelles. The polyelectrolyte complex micelles were internalized by cancer cells to a greater extent than free heparin alone, indicating that the dramatic cell death was attributed to the increased cellular uptake of heparin. The internalized heparin was shown to induce apoptotic death of the cancer cells via a caspase-dependent pathway. The proposed chitosan-*g*-PEG/heparin polyelectrolyte complex micelles facilitated the intracellular delivery of heparin, triggered the caspase activation, and consequently promoted apoptotic death of cancer cells [133].

The crucial event in metastasis is tumor invasion, which in the case of melanoma cells is dependent on matrix metalloprotease 2 (MMP2) [134]. Gorzelanny et al. studied chitosan-based attenuation to the invasive activity of melanoma cells in a cell-based invasion assay and reduced MMP2 activity in the supernatant of melanoma cells [134]. Investigator found that the expression level of MMP2 was not affected. The amount of MMP2 in the cell supernatant was reduced, indicating a post-transcriptional effect of chitosan on MMP2. Data from atomic force microscopy clearly demonstrated a direct molecular interaction between MMP2 and chitosan, forming a complex with a diameter of 349.0 ± 69.06 nm and a height of 26.5 ± 11.50 nm. The high binding specificity of MMP2 to chitosan, shown by affinity chromatography and a colorimetric MMP2 activity assay, suggests a noncompetitive inhibition of MMP2 by chitosan [134]. Laminin, a cell adhesion protein, consists of three peptide chains (α1, β1 and γ1) [135]. The β1 chain contains a Tyr-Ile-Gly-Ser-Arg (YIGSR) sequence that has been found to inhibit experimental metastasis in mice. Hojo et al. developed a method of preparation

using a hybrid of a water-soluble chitosan and a laminin-related peptide [135]. A method involving the laminin-related peptide, acetyl-Tyr-Ile-Gly-Ser-Arg-βAla-OH (Ac-YIGSRβA-OH) reacted with a water-soluble chitosan. A small spacer, *tert*-butyloxycarbonyl-Gly, was intercalated in chitosan, using the TBTU method, to facilitate its coupling with the peptide. After removal of the protecting group, the Gly-chitosan was coupled with Ac-YIGSR βA-OH by the water-soluble carbodiimide method to give Ac-YIGSR βAG-chitosan. Conjugation of the peptide with the larger chitosan molecule did not reduce the inhibitory effect of the peptide on experimental metastasis in mice; it actually potentiated the antimetastatic effect, demonstrating that chitosan may be effective as a drug carrier for peptides [135].

3.8 Bladder Cancer

Bladder cancer is the fourth leading cause of cancer and it occurs about four times more frequently in men than in women. At the time of diagnosis, approximately 75% of bladder tumors are superficial, 20% are invasive, and up to 5% are metastatic. Superficial bladder cancer is initially managed by transurethral resection (TUR) to allow accurate tumor staging and grading. However, the recurrence rate of superficial bladder transitional cell carcinoma is reported to be between 50 and 80% and has a 15% chance of progression after TUR [136]. Therefore, intravesical chemotherapy or immunotherapy is required. However, intravesical drug delivery is challenged by the difficulty of establishment of a suitable and effective drug concentration because of periodical discharge of the bladder. In this context, researchers looked at different nanosystem deliveries to bladder. In the following examples, the clinical applicability of antibodies and plasmonic nanosensors as topically applied, molecule-specific optical diagnostic agents for noninvasive early detection of cancer and precancer is severely limited by our inability to efficiently deliver macromolecules and nanoparticles through mucosal tissues [137].

Ghosn et al. developed an imidazole-functionalized conjugate of the polysaccharide chitosan (chitosan-IAA) to enhance topical delivery of contrast agents, ranging from small molecules and antibodies to gold nanoparticles up to 44 nm in average diameter [137]. A result shown on contrast agent uptake and localization in freshly resected mucosal tissues was monitored using confocal microscopy. Chitosan-IAA was found to reversibly enhance mucosal permeability in a rapid, reproducible manner, facilitating transepithelial delivery of optical contrast agents. Permeation enhancement occurred through an active process, resulting in the delivery of contrast agents via a paracellular or a combined paracellular/transcellular route depending on size. In addition, co-administration of epidermal growth factor receptor-targeted antibodies with chitosan-IAA facilitated specific labeling and discrimination between paired normal and malignant human oral biopsies. Together, these data suggest that chitosan-IAA is a promising topical permeation enhancer for mucosal delivery of optical contrast agents [137].

Bilensoy et al. prepared cationic nanoparticles of chitosan, poly(ε-caprolactone) coated with chitosan (CS-PCL) and poly(ε-caprolactone) coated with poly-L-lysine (PLL-PCL) to encapsulate the intravesical chemotherapeutic agent Mitomycin C (MMC) for longer residence time, higher local drug concentration, and prevention of drug loss during bladder discharge [136]. Nanoparticle diameters varied between 180 and 340 nm depending on polymer used for preparation and coating. Zeta-potential values demonstrated the positive charge expected from cationic nanoparticles. The determination of MMC encapsulation efficiency was increased twofold for CS-PCL and threefold for PLL-PCL as a consequence of the hydrophilic coating. The cellular interaction was studied in CS-PCL nanoparticles loaded with Rhodamine123, sharing hydrophilic properties with MMC, which were selectively incorporated by the bladder cancer cell line, but not by normal bladder epithelial cells. The author proposed that the CS-PCL nanoparticles seem to be promising for MMC delivery with respect to anticancer efficacy against the MB49 bladder carcinoma cell line [136].

Interleukin-12 (IL-12) is a potent T_H1 cytokine with robust antitumor activity and the ability to potentiate immunologic memory [138]. Unfortunately, intravesical IL-12 did not show antitumor efficacy in a recent clinical study of patients with recurrent superficial bladder cancer. Zaharoff et al. developed a co-formulation of IL-12 with chitosan, which demonstrated that 88–100% of mice bearing orthotopic bladder tumors were cured after four intravesical treatments with chitosan/IL-12 [138]. Antitumor responses following chitosan/IL-12 treatments were durable and provided complete protection from intravesical tumor rechallenge. Furthermore, urinary cytokine analysis showed that chitosan/IL-12 induced multiple T_H1 cytokines at levels significantly higher than either IL-12 alone or BCG. Immunohistochemistry revealed moderate to intense tumor infiltration by T cells and macrophages following chitosan/IL-12 treatments. Bladder submucosa from cured mice contained residual populations of immune cells that returned to baseline levels after several months. The co-formulation of IL-12 with chitosan was proposed in this study because chitosan is a biocompatible, mucoadhesive polysaccharide that could improve intravesical IL-12 delivery and thus provide an effective and durable alternative for the treatment of superficial bladder cancer [138]. Oztürk et al. proposed pharmaco-therapeutic agent delivery systems for treating bladder cancer in order to supply a suitable dosage of the agent for a certain time period to solve problems due to the recurrency of tumoral tissues after TUR, when further treatment is necessary, usually in the form of intravesical chemotherapy or immunotherapy [139]. Oztürk et al. developed Mitomycin-C-loaded alginate and chitosan carriers to use as an alternative system in postoperative chemotherapy in bladder cancer. The carriers were prepared in the form of cylindrical geometries to facilitate the insertion of the carrier in in vivo studies. The effects of some parameters (i.e., polymer molecular weight, crosslinker concentration, Mitomycin-C to polymer ratio etc.) on the morphology, swelling behavior, bioadhesion, and in vitro drug release rate of the carriers indicated a promising approach for cancer treatment, especially bladder[139]. Similar work was also proposed by Eroğlu et al. for treating bladder cancer by a pharmacotherapeutic agent (i.e., Mytomycin-C) delivery system prepared using a mucoadhesive polymer (i.e.,

chitosan) in the form of cylindrical geometry to facilitate the insertion of the carrier for in vivo studies [140]. The chitosan carriers were prepared by crosslinking during the solvent evaporation technique. In the preparation of the chitosan carriers, chitosan polymers with different molecular weights, different amounts of crosslinker (i.e., glutaraldehyde), and different amounts of pharmacotherapeutic agent were used to obtain desired attachment onto the bladder wall and optimum release rate of the agent. On the other hand, because of the gelatinous structure of the chitosan, the swelling behavior of the polymer was periodically evaluated by gravimetric determinations in aqueous media [140].

Lekka et al. studied the effect of chitosan on the stiffness of the nonmalignant transitional epithelial cells of ureter (HCV 29) and the transitional cell cancer of urine bladder (T24) [141]. The investigations were performed in culture medium (RPMI 1640) containing 10% fetal calf serum in the presence of the microcrystalline chitosan of the three different deacetylation degrees. In parallel, the effect of chitosan on production of lactate and ATP levels was determined. The results showed a strong correlation between the decrease in energy production and the increase in Young's modulus values obtained for the cancer cells treated with chitosan in living cells [141].

4 Conclusions

In this review, research has been highlighted on the directions and development of specific tumor targeting gene, protein, and local drug delivery systems using chitosan nanoparticles. These approaches show much promise for improving the therapeutic outcomes of cancer treatments. This review has also presented information on how nanoparticles take advantage of chitosan's special features to act as effective carriers of agents for delivery to specific tumor sites. Nanotechnology has already advanced cancer detection and treatment. Abraxane is a major success story in the treatment of metastatic breast cancer, and has extended the scope of nanomedicine to other cancers and chemotherapeutic drugs. Nanotechnology has already begun shaping the way disease, particularly cancer, is diagnosed, treated, and monitored [142]. As technologies are perfected, side effects due to healthy cell damage will decrease, enabling patients to have a better quality of life and lengthening survival. A similar approach may be used in the near future to develop chitosan-based nanoparticle delivery for treatment of different human diseases, mainly in the field of cancer.

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Chitosan and Thiolated Chitosan

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Abstract Thiolated chitosans constitute an integral part of designated "thiomers", which are thiolated polymers widely investigated for non-invasive drug delivery. In brief, thiomers display thiol-group-bearing ligands on their polymer backbone. Through thiol/disulfide exchange reactions and/or a simple oxidation process, disulfide bonds are formed between such polymers and the cysteine-rich subdomains of mucus glycoproteins, thus building up the mucus gel layer. Most chemical modifications of chitosan are performed at the free amino groups of the glucosamine units. So far, the alkyl thiomers chitosan–cysteine, chitosan–thiobutylamidine, chitosan–thioglycolic acid, chitosan–*N*-acetylcysteine, and chitosan–thioethylamidine and the aryl thiomers chitosan–6-mercaptonicotinic acid and chitosan–4-mercaptobenzoic acid have been generated. Due to the immobilization of thiol groups on the chitosan backbone, its mucoadhesive, permeation enhancing, in situ gelling, efflux pump inhibitory, and controlled drug release properties are improved. The great benefits of this new generation of chitosans in comparison to the corresponding unmodified polymers has been verified via numerous in vivo studies on various mucosal membranes. A proof of concept for oral, nasal and buccal drug delivery is provided. This chapter includes an overview of the mechanism of adhesion and the design of thiomers as well as of delivery systems comprising thiolated chitosans and their in vivo performance.

Keywords Chitosan · Mucoadhesion · Particles · Permeation enhancement · Thiomers

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Contents

1 Introduction

Chitosan is a cationic polysaccharide obtained by the alkaline deacetylation of chitin, which is derived from crustaceans. It is composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit). The degree of deacetylation has a direct impact on the solubility of the polymer. Chitosan is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0. Therefore, it is not soluble at neutral and alkaline pH values. However, when it is in acid medium such as hydrochloric acid, acetic acid, glutamic acid, and lactic acid, the amine groups of chitosan are protonated and thus promote its solubility [1]. The amino groups on the chitosan backbone undoubtedly play an essential role because they are the main target for the immobilization of thiol groups.

Recently, attention has been paid to chitosan because of its favorable biological properties, such as biodegradability, biocompatibility, and non-toxicity, as well as its physiochemical properties [2, 3]. Moreover, it has been reported that chitosan can accelerate gastric ulcer healing [4], that pretreatment with chitosan prevents ulcerogenic effect in rats [5], and that chitosan displays antimicrobial activity [6, 7].

Chitosan has been widely used as an absorption enhancer [3], drug carrier [8], mucoadhesive, and permeation-enhancing polymer [9] in formulations for buccal/sublingual, nasal, gastrointestinal, vaginal, and colonic drug delivery [2] as well as for gene delivery [10, 11]. For gene delivery, in particular, chitosan nanoparticles offer the advantage of an easy preparation by mixing negatively charged DNA or RNA

with the cationic chitosan to produce stable nanoparticles [12]. In the case of oral drug delivery, chitosan offers various advantages. For instance, due to its cohesive properties it can be used in matrix tablets in order to release the drug in a controlled manner [13]. Moreover, being capable of opening tight junctions of mucosal membranes and providing protection towards proteolytic digestion of drugs in the gastrointestinal (GI) tract, it can be used to improve the oral uptake of therapeutic peptides [14].

The mucoadhesive properties of chitosan can be mainly attributed to ionic interactions, in particular between the cationic amino groups of chitosan and the negative substructures of the mucus, such as sialic acid moieties.

As previously mentioned, the amino groups are target moieties for the modification of the backbone in order to gain certain additional advantageous properties. For instance, various chitosan derivatives such as chitosan–EDTA conjugates [15], *N*-trimethylated chitosan [16], mono-*N*-carboxymethyl chitosan [17] and *N*-sulfo-chitosan [18] have been introduced in the pharmaceutical field in the last decade. A further modification of chitosan is based on the immobilization of thiol groups, leading to so-called thiolated chitosans (Fig. 1).

Thiolated chitosans show various promising properties such as mucoadhesion, efflux pump inhibition, permeation enhancement, in situ gelling capacity, and controlled drug release. In contrast to unmodified chitosans, these novel polymers are capable of forming covalent bonds with the mucus-forming constituents. In fact, through thiol/disulfide exchange reactions and/or a simple oxidation process, disulfide bonds are formed between such polymers and the cysteine-rich subdomains of mucus glycoproteins (Fig. 2). Hence, thiolated chitosans mimic the natural

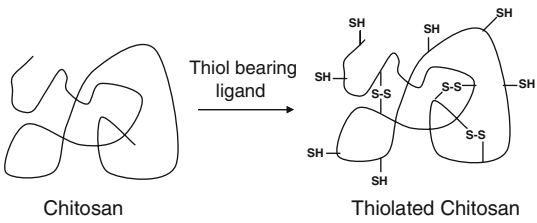

Fig. 1 Thiolated polymer "thiomers"

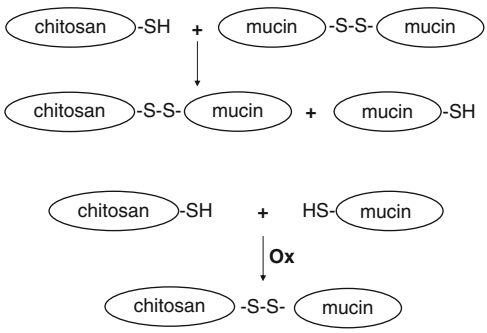

Fig. 2 Mechanism of disulfide bond formation between thiomers and mucus glycoproteins (mucins)

behaviour of secreted mucus glycoproteins, which are also covalently anchored in the mucus layer by disulfide bonds. So far, numerous thiolated chitosan derivatives have been synthesized, including the alkyl thiolated chitosans chitosan–cysteine conjugate (chitosan-Cys) [19], chitosan–4-thio-butyl-amidine conjugate (chitosan-TBA) [20], chitosan–thioglycolic acid conjugate (chitosan-TGA) [19], chitosan–*N*-acetylcysteine conjugate (chitosan-NAC) [21], chitosan–2-thio-ethyl-amidine conjugate (chitosan-TEA) [22], and chitosan–glutathione conjugate (chitosan-GSH) [23] and the aryl thiolated chitosans chitosan–6-mercaptonicotinic acid (chitosan-MNA) [24] and chitosan–4-mercaptobenzoic acid (chitosan-MBA) [25] (Fig. 3).

2 Synthesis of Thiolated Chitosans

Most chemical modifications on the chitosan backbone are performed at the free amino groups of the glucosamine units. For example, the formation of amide or amidine bonds between these primary amino groups and the activated carboxylic groups of a low molecular mass thiol-bearing compound can be initiated easily. A typical procedure can be summarized as follows: *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) is added to a solution of the low molecular mass thiol-bearing compound in order to activate the carboxylic groups. After a certain incubation period, the mixture is added to a chitosan solution. The reaction mixture is stirred and incubated at room temperature for several hours. Finally, this is dialyzed to remove unbound reagents and lyophilized [26]. The initial reason for the introduction of thiol groups was based on the hypothesis that these thiol groups would form disulfide bonds with the thiol groups of the mucus, consequently leading to high mucoadhesive properties [20, 27]. In fact, the GI mucus comprises around 80% glycoproteins in which cysteine-rich subdomains occur.

2.1 Alkyl Thiolated Chitosans

Examples of alkyl sulfhydryl-bearing compounds that can covalently attach to a chitosan backbone are cysteine, *N*-acetylcysteine, thioglycolic acid, and glutathione. These molecules become linked to the chitosan primary amino group via an amide bond.

By contrast, ligands such as thioethylamidine and thiobuthylamidine form amidine bonds with chitosan. Most of these sulfhydryl-bearing compounds have thiol groups whose pK_a value is in the range of 8–10 [28]. During the synthesis process, an unintended oxidation of thiol moieties can be avoided by performing the polymer synthesis at a pH < 5. At this pH, the concentration of the reactive form

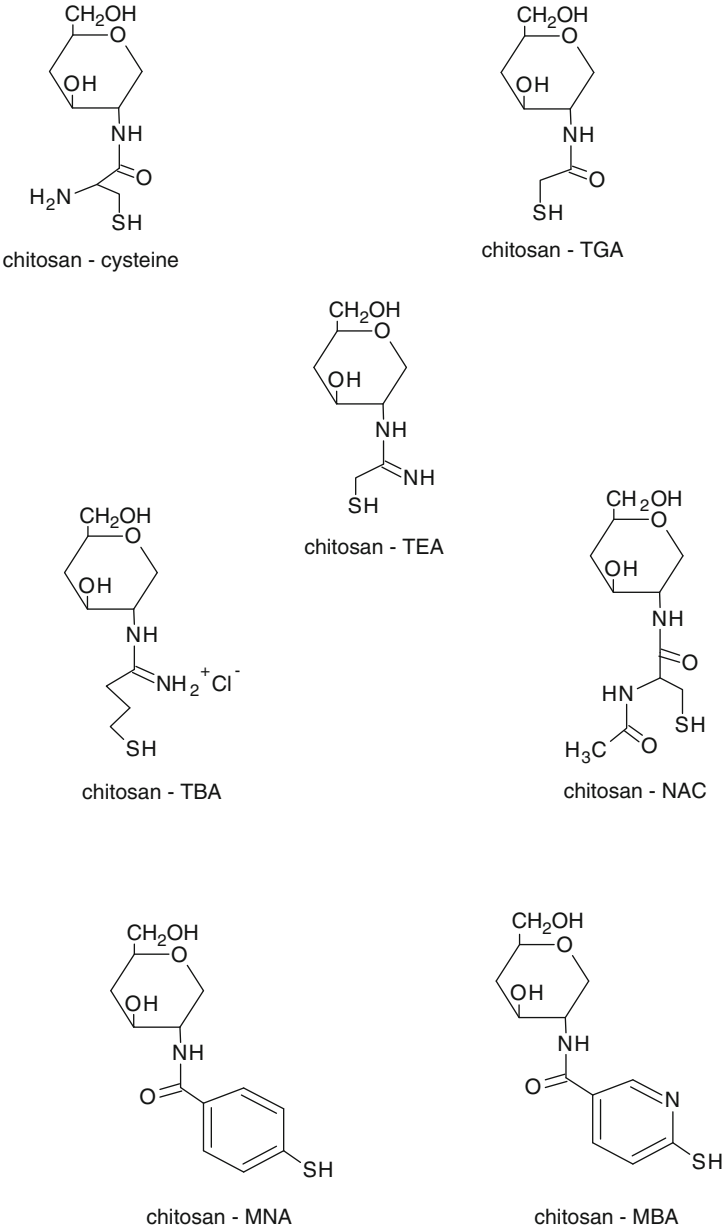

Fig. 3 Structure of established thiolated chitosans

(S^-) is marginal. Therefore, low formation of disulfide bonds is observed. The amount of thiol groups remaining on the polymer can be finally determined by Ellman's method [29].

Table 1 Comparison of the amount of thiol groups on various thiolated chitosans

Polymer	Reagent	Thiol groups (μmol/g)
Chitosan-TBA	Thiobutylamidine	290.3 ± 15.9
Chitosan-TGA	Thioglycolic acid	189.7 ± 50.9
Chitosan-Cys	Cysteine	100
Chitosan-NAC	*N*-Acetylcysteine	325.5 ± 41.8
Chitosan-TEA	Thioethylamidine	138.5 ± 71.1
Chitosan-MNA	6-Mercaptonicotinic acid	1,856.6
Chitosan-MBA	6-Mercaptobenzoic acid	176.27

Values are means of at least three experiments

2.2 Aryl Thiolated Chitosans

It is known that aryl thiols are more reactive than alkyl thiols due to their lower pK_a values (5–7) [28]. Aromatic thiols, for example, have already been shown to increase the folding rate of disulfide-containing proteins more than aliphatic thiols [30, 31]. For a pharmaceutical excipient such as thiolated chitosans, the increased reactivity of the thiol group would further increase its beneficial properties over alkyl thiol chitosan and non-thiolated polymers. Examples of aryl sulfhydryl-bearing compounds covalently attached to chitosan via an amide bond are 6-mercaptonicotinic acid and 4-mercaptobenzoic acid. As mentioned for alkyl thiol chitosan, the amount of free and oxidized thiol groups can be determined by Ellman's method. Examples of developed thiolated chitosans and their degrees of modification are listed in Table 1.

3 Properties of Thiolated Chitosans

3.1 Mucoadhesive Properties

Thiolated chitosans represent an advantage in drug delivery since they are mucoadhesive and therefore able to prolong the residence time of drugs on various mucosa. This allows an increase in the extent of drug absorption. Moreover, thiolated chitosans can improve the specific localization of drug delivery systems [32]. Mucoadhesion is gained by the formation of both non-covalent bonds (such as hydrogen bonds) and ionic interaction or physical interpenetration effects between the mucus gel layers and polymers. In the case of thiolated chitosans, thiol groups on the polymer form disulfide bonds with cysteine-rich subdomains of glycoproteins in the mucus through thiol/disulfide exchange reactions, an oxidation process, and disulfide bond formation within the polymer itself [27]. The formation of disulfide bonds can be determined by both an increase in viscosity of the polymer and a decrease in free thiol groups [20]. The strong mucoadhesive properties of thiolated chitosans in comparison to unmodified chitosans were demonstrated by

Table 2 Comparison of the mucoadhesive properties of thiolated chitosans

Polymer	Total work of adhesion (μJ)	Adhesion time (h)
Chitosan-TBA	408 ± 68	20 ± 0.2
Chitosan-TGA	200 ± 17	4 ± 0.1
Chitosan-NAC	345 ± 6	100 ± 10
Chitosan-TEA	105 ± 39	24 ± 2
Chitosan-MNA	130 ± 5	>200
Chitosan-MBA	–	170 ± 12

Values are means of at least three experiments

in vitro studies such as those performed via the rotating cylinder method and/or via tensile studies. The adhesive properties of polymers were expressed either in terms of adhesion time or in terms of total work of adhesion. Examples of the improved mucoadhesion of thiolated chitosans are given in Table 2.

Among these thiolated chitosans, chitosan-MNA showed the highest mucoadhesive features [33]. Even though thiolated chitosans have shown excellent mucoadhesive properties, the adhesion of the polymers is sloughed off by the natural mucus turnover due to a continuous process of mucus secretion. In the human intestine, for instance, the mucus turnover rate is typically about 12–24 h; this is very rapid and makes it difficult for the polymers to interact with the mucosa for a prolonged period of time [34].

3.2 In Situ Gelling Properties

Since the concept of in situ gelling polymers was introduced to the pharmaceutical literature, various formulations showing in situ gelling characteristics have been developed. In fact, a reversible phase shift within a few minutes under physiological conditions is favorable for improving the bioavailability of drugs. The sol–gel transition can be activated by several process changes including pH [35], temperature [36, 37], light [38, 39], and/or ion concentration [40].

The in situ gelling properties of thiolated chitosans are significantly better than those of unmodified chitosan. The improvement is based on the formation of inter- and/or intramolecular disulfide bonds within the polymeric matrix under physiological pH. These in situ gelling properties have been characterized in vitro by rheological measurements. For example, it was observed that after 6 h, the elastic modulus of chitosan-TGA was 168-fold increased compared to unmodified chitosan. The elastic modulus of unmodified chitosan remained unchanged over the whole observation period [41]. Nevertheless, the phase transition time of chitosan-TGA is extensively long. When oxidizing agents such as hydrogen peroxide (H_2O_2) were added to chitosan-TGA, gelation took place within a couple of minutes. The dynamic viscosity value at time zero was not showed in the research article 42 and therefore in Fig. 4. However, as this value is close to 0, a 16,000-fold improvement after 20 minutes was calculated by the author of article

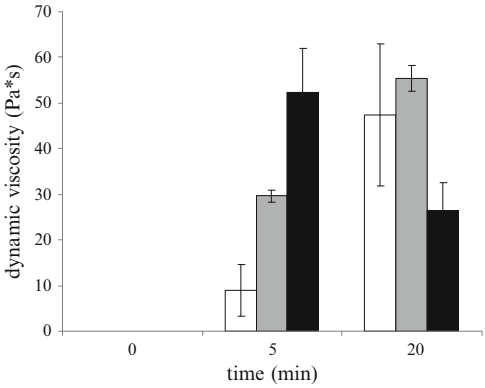

Fig. 4 Comparison of the dynamic viscosity (η^*) of a chitosan-TGA/H_2O_2 system at pH 6.0 and 37°C. The molar ratios of SH groups: oxidizing agent were 1:0.28, 1:0.63, and 1:1.9 and are represented by *white*, *black*, and *gray bars*, respectively. The means ± SD of three experiments are shown (Adopted from [42])

42 [42]. Over an observation period of 20 min, the thiol moieties of the chitosan-TGA/H_2O_2 system were not completely oxidized; more than 60% of thiol groups remained stable. Moreover, evidence for an increase in the crosslinking of thiolated chitosans as a function of time was provided by frequency sweep measurements.

In conclusion, the capacity of a polymer to achieve an in situ sol–gel transition at pH values above 5 makes the polymer suitable for application to vaginal, nasal, and ocular mucosa.

3.3 Permeation-Enhancing Effect

The use of permeation enhancers in drug delivery systems has gained great importance in overcoming the absorption barrier on mucosal membranes. Auxiliary agents such as low molecular mass sodium salicylates and medium chain glycerides have proven to improve the permeation of drugs across epithelial membranes. However, they can be rapidly absorbed from mucosal tissues, leading to systemic toxic side effects [43]. Thiolated chitosans have demonstrated great benefit over those types of enhancer. They can remain concentrated at the target site because of their mucoadhesive properties and increase the paracellular permeability of drugs due to the opening of tight junctions. This has been shown for various routes of delivery, such as for nasal [44] or intestinal drug delivery [45]. In addition, the systemic side effects of thiolated chitosans are comparatively low, owing to lack of toxicity even after intravenous application [46]. Unabsorbed portions of the polymer may hardly have some side effects.

The influence of thiolated chitosans on the permeation of hydrophilic compounds (such as peptide drugs) across freshly excised rat intestinal mucosa was evaluated in an Ussing-type chamber. For example, 0.5% (m/v) chitosan-TGA combined with the permeation mediator reduced glutathione (GSH) led to a 4.2-fold improvement of rhodamine-123 (Rho-123) permeation across rat intestinal mucosa [48]. In addition, the permeation-enhancing properties of thiolated chitosans was proven

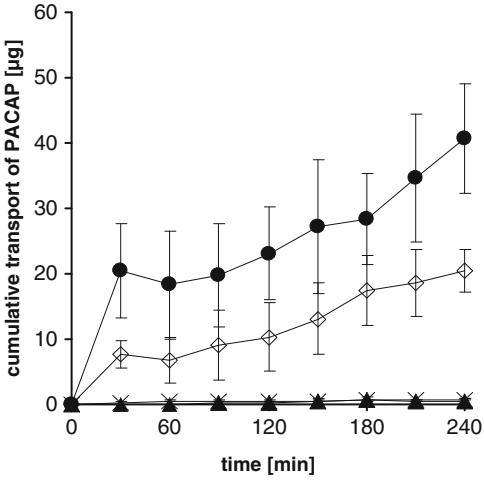

Fig. 5 In vitro permeation profile of PACAP across the buccal mucosa of pigs. Control without polymer (*times*); 1% (m/v) chitosan (*filled triangle*); 1% (m/v) chitosan-TBA (*open diamond*); 1% (m/v) chitosan-TBA and 2% (m/v) GSH (*filled circle*) (Adopted from [49])

by studies on permeation of pituitary cyclase-activating polypeptide (PACAP). It has been reported that 1% (m/v) chitosan-TBA enhanced the permeation of PACAP across buccal tissue up to 38-fold compared to unmodified chitosan. Moreover, with 1% (m/v) chitosan-TBA combined with 2% (m/v) GSH the highest permeation was observed as drug uptake was enhanced approximately 80-fold (Fig. 5) [49]. The underlying mechanism of the permeation-enhancing effect of the system is based on the inhibition of the enzyme tyrosine phosphatase (PTP, 60–65 kDa). This protein is capable of dephosphorylating tyrosine residues of occludine, which is believed to be involved in the opening process of tight junctions. When dephosphorylation occurs, the tight junctions are closed, resulting in a decreased permeation of hydrophilic macromolecules. According to this mechanism, the inhibition of PTP by reduced glutathione avoids the phosphorylation process, with consequent opening of tight junctions.

Thiolated chitosans demonstrated the ability to shift the balance between oxidized glutathione and reduced glutathione [50].

Another example of a thiolated chitosan that has permeation-enhancing properties is chitosan-NAC, which showed an improvement in the uptake of tobramycin sulfate on Caco-2 cell monolayers of up to 2.7-fold in comparison to control tobramycin sulphate in buffer. A more pronounced permeation-enhancing effect could be achieved by the combination of 0.5% (m/v) chitosan-NAC and 0.5% GSH, giving an improvement ratio of 3.3 [51].

3.4 Efflux Pump Inhibition

In the human body, the multidrug resistance proteins (MDRs) and the multidrug resistance-associated proteins MRP1 and MRP2, members of the ATP-binding

cassette (ABC) transporter superfamily, are membrane-associated proteins that carry out a detoxification role. In fact, they are responsible for molecule efflux, especially xenobiotics, out of the cells thus limiting drug accumulation within cells. These proteins are efflux pumps located in several tissues such as kidney, brain, liver, and intestine [52]. Although their physiological function guarantees protection of the body from harmful compounds, the efflux pump transporters also reduce the bioavailability of a variety of drugs.

The most important transporter in this group is probably P-glycoprotein (P-gp), which is primarily expressed at the apical side of epithelial cells of the intestine and in tumor cells. A broad range of compounds like β-adrenergic agonists, vinca alkaloids, digoxin, and antracyclines are known to be P-gp substrates [50]. Efflux pumps can be inhibited through several mechanisms; however, blocking the drug-binding site both via competition and allosteric regulation is the most important [53]. In the case of thiolated chitosans, the inhibition of efflux pumps is based on the interaction of the thiol moieties expressed on the polymeric backbone with the cysteine-rich subunit of the transmembrane region of P-gp [54].

The P-gp inhibition effect of chitosan-TBA was shown in an in vitro study using Rho-123 as representative substrate. It has been reported that chitosan-TBA improves the transport of substrates from the apical to basolateral site of freshly excised guinea pig ileal mucosa and that it decreases the basolateral to apical transport. Furthermore, chitosan-TGA/GSH, labeled with cationic fluorescence marker in order to avoid unintended ionic interaction between the polymer and the marker, showed higher permeation-enhancing properties than the unmodified chitosan control. The permeation of Rho-123 across freshly excised rat small intestinal mucosa in the presence of 0.5% (m/v) chitosan-TGA was 4.2-fold improved in comparison to unmodified chitosan [48].

The efficacy of chitosan-TGA as permeation enhancer was also proven in vivo. Oral administration to rats of enteric-coated tablets containing chitosan-TBA/GSH significantly increased the area under the plasma concentration versus time curve (AUC_{0-12}) of Rho-123 compared to Pluronic P85 and Myrj 52, as shown in Fig. 6 [55].

3.5 Transfection-Enhancing Properties

In gene delivery, a site-specific carrier system has to release active compounds such as nucleic acids in a way that they are readily available for translation or transcription. Viral vectors have been widely used because of their high transfection efficacy. However, they cause oncogenic and inflammatory responses of the human body. To overcome the drawbacks of viral vectors, non-viral gene delivery systems such as cationic phospholipids and cationic polymers are currently adopted. Polyplexes, complexes of DNA and polymer, are one example of delivery systems that offer advantages such as high stability and high transfection efficiencies [56]. Regarding thiolated chitosans, it was demonstrated that the complexation of

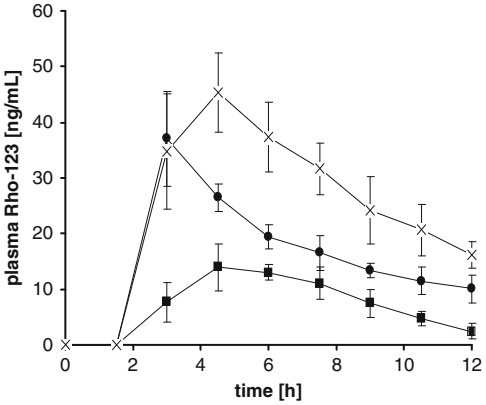

Fig. 6 Plasma concentration of Rho-123 after oral administration of 1.5 mg Rho-123 in Pluronic P85-tablets (*filled square*), Myrj 52-tablets (*filled circle*), and chitosan-TBA/GSH-tablets (*times*). Indicated values are means ± SD of five experiments. Values for Rho-123 at 4.5, 6, 7.5, 9, 10.5, and 12 h differ from corresponding values for Myrj 52-tablets with significances of $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.007$, $p < 0.006$, and $p < 0.01$, respectively (Adopted from [55])

chitosan-TGA with pDNA leads to a transfection rate in Caco-2 cells that was fivefold higher than that gained with unmodified chitosan/pDNA [57]. Thiolated chitosan/pDNA complexes were prepared at pH 4.0 and 5.0 and were resistant to degradation during incubation with DNase I for 30 min. This result was consistent with a previous study with thiolated chitosan/DNA nanocomplexes [58]. The transfection efficiency of chitosan-TGA/DNA nanocomplexes was assessed in HEK293, MDCK, and Hep-2 cell lines, in which the nanocomplexes induced significantly higher green fluorescent protein gene expression in comparison to unmodified chitosan. In particular, when chitosan-TGA/pDNA nanoparticles were crosslinked by oxidation with H_2O_2, the expression of green fluorescence protein was improved in comparison to non-crosslinked nanoparticles [58].

3.6 Controlled Drug Release Properties

Controlled drug delivery technology represents one of the most rapidly advancing areas of science. Such delivery systems offer numerous advantages over conventional dosage forms, including improved efficacy, reduced toxicity, improved patient compliance, and convenience. Therefore, all controlled release systems aim to improve the effectiveness of drug therapy and can be achieved via temporal and/or distribution control of drug release.

Controlled release over an extended duration is highly beneficial for drugs that are rapidly metabolized and eliminated from the body after administration. Requirements for these delivery systems are the cohesion and the stability of the drug formulation. Chitosan is a polymer that can fulfill such requirements,

especially in its thiolated form. In fact, model compounds like salmon calcitonin and pDNA have been formulated in controlled drug release systems using thiolated chitosans as a carrier matrix [59–61].

It was reported that in simulated gastric fluids the release profile of salmon calcitonin out of mucoadhesive tablets based on chitosan-TBA was about 87% after 3 h [59]. Over the whole observation period, a pseudo-zero-order release profile of salmon calcitonin in artificial gastric fluid was detected. Tablets exhibited good cohesiveness and released the active agent via a controlled diffusion process.

Additionally, controlled release profiles of pDNA out of a chitosan-TEA matrix system have been demonstrated. The study was performed under simulated physiological conditions and the results showed that within 10 h pDNA was totally released from chitosan/pDNA particles, whereas only 12% of it was liberated from the chitosan-TEA/pDNA particles [62].

3.7 Safety and Stability

To investigate the safety of thiolated chitosans, several studies have been performed measuring its potential cytotoxicity on various cell lines. The toxicity profiles of chitosan-TBA, for example, were assessed using the red blood cell lysis test. The results showed that thiolated chitosan had a lower membrane damaging effect than the corresponding unmodified control. The safety of thiolated chitosan was further confirmed by metabolic activity (cleavage of dimethyl-thiazolyl-diphenyltetrazolium bromide) (MTT) and DNA synthesis (incorporation of 5-bromo-2′-deoxyuridine [BrdU]) assay on L-929 mouse fibroblast cells. It was found that the toxicity of chitosan-TBA was concentration-dependent [63]. Another thiolated chitosan investigated for cytotoxicity was chitosan-MBA, which was found to be nontoxic because Caco-2 cell viability was around 100% after 4 and 24 h of incubation [25].

The stability of thiolated chitosans was tested under different storage conditions. Chitosan-TGA was chosen as a model polymer and stored in the form of freeze-dried powders and matrix tablets for 6 months. The free thiol groups of chitosan-TGA were found to be stable against oxidation processes when stored at −20°C and at 4°C. In the case of storage at 20°C and 70% relative humidity, a decrease of 25% of the initial amount of free thiol groups was found after 6 months [64].

4 Formulations

4.1 Microparticles and Nanoparticles

For non-invasive drug administration, particulate delivery systems offer the advantage of providing a prolonged residence time on mucosal membranes [65] and the possibility to reach greater mucosal surface areas, leading to a comparatively higher

drug uptake. Delivery systems such as liposomes, and micro- and nanoparticles have attracted great attention in the last few decades. For example, nanoparticulate delivery systems based on polymers such as polyacrylates, poly(lactic-*co*-glycolic acid), or chitosans have been developed and widely investigated. Among these polymers, chitosan has shown numerous advantages and, due to the presence of thiol groups on their polymeric backbone, micro- and nanoparticles guarantee strong mucoadhesive properties and a high permeation-enhancing effect [66]. Micro- and nanoparticulate thiolated chitosans can be produced via different techniques such as ionic gelation, emulsification/solvent evaporation, radical emulsion polymerization, and air jet milling.

The mucoadhesive features of chitosan- and chitosan-TBA-coated poly(isobutylcyanoacrylate) (PIBCA) nanoparticles were evaluated under physiological conditions. The results obtained by ex vivo studies showed that the presence of chitosan-TBA on the particle surface significantly enhanced the mucoadhesive properties of carrier systems [67]. In particular, the higher the amount of thiol groups immobilized on the surface of particles, the stronger the mucoadhesive properties and the stability of the particles [68]. The underlying mechanism is based on disulfide bond formation between mucus glycoproteins and the polymer, as already explained [27]. In addition, the permeation-enhancing effect of chitosan- and thiolated-chitosan-coated PIBCA as a potential carrier for mucosal administration was evaluated using the Ussing chamber technique [69]. Results showed that the paracellular permeability of intestinal epithelium was more pronounced in the presence of thiolated-chitosan-coated PIBCA than in the presence of chitosan-coated core–shell nanoparticles. The thiolated-chitosan-coated nanoparticulate core–shell structure offers advantageous effects because of its biocompatibility and harmless features.

Furthermore, chitosan-GSH-coated poly(hydroxyethyl methacrylate) nanoparticles prepared by radical polymerization showed an improvement in mucoadhesion and in the apparent permeability coefficient (P_{app}) compared to unmodified chitosan [70]. Finally, thiolated chitosan nanoparticles significantly enhanced the anti-inflammatory effect of theophylline, as evidenced by the decrease in parameters such as eosinophilis in bronchoalveolar lavege fluid and bronchial damage [71].

4.2 Matrix Tablets

Mucoadhesive matrix tablets are useful for intraoral, peroral, ocular, and vaginal local or systemic delivery. In oral administration, for example, approximately 40% of products on the market are tablets due to the convenience of such a dosage form [43]. Thiolated chitosan can be easily compressed to matrix tablets by simply incorporating the active pharmaceutical ingredient in the polymer. In addition, the in situ crosslinking properties of thiolated chitosans guarantee the cohesiveness and, subsequently, the stability of the swollen carrier matrix. Disintegration studies, for instance, performed with tablets comprising unmodified chitosan revealed a stability of less than 6 h, whereas tablets based on chitosan-MBA were stable for over 12 h [25].

In the case of using thiolated chitosans as drug carrier matrixes, the release rate of the active ingredient is mainly controlled by hydration and diffusion processes [9]. An enteric coating is beneficial for matrix tablets to protect them from the harsh environment in the gastrointestinal tract (e.g., the low pH) and from degradation mediated by enzymes, and to guarantee the swelling behavior of tablets on the intestinal mucosa.

5 In Vivo Studies: Proof of Concept

Several in vivo studies have demonstrated the potential of thiolated chitosans to improve the bioavailability of therapeutic compounds. In particular, thiolated chitosans have been used to develop carrier matrices for oral, nasal, and buccal delivery systems.

5.1 Oral Drug Delivery

Since the 1970s, a considerable number of publications in the field of peptide and protein delivery have been established [72]. Peptides and proteins are generally administered via parenteral routes, which involve the pain, fear, and risks associated with these types of administration. A conversion from "injectable formulation" to "oral formulations" is therefore in high demand [43]. The efficacy of thiolated chitosans in term of peptide delivery systems via oral administration has been demonstrated. In fact, after oral administration of chitosan-TBA matrix tablets containing salmon calcitonin to rats, the calcium plasma level as a primary indicator was measured and it was found that unmodified chitosan tablets showed no significant effect on plasma levels. By contrast, the calcium plasma level obtained with chitosan-TBA tablets containing calcitonin decreased by over 5% [59]. In addition, oral insulin delivery systems based on chitosan-TBA tablets have been developed. Blood glucose levels of non-diabetic rats were determined after oral administration of chitosan-TBA/insulin tablets. After 24 h, the relative pharmacological efficacy for chitosan-TBA/insulin tablets was 1.7 + 0.4% (Table 3) [72].

Recently, it has been shown that the AUC of human insulin plasma levels after oral administration of chitosan-MNA nanoparticles was increased fourfold compared to that after administration of unmodified chitosan nanoparticles (Fig. 7) [73].

Oral administration of the peptide drug antide in a liquid dosage form showed no detectable concentration of the drug in plasma at all. When a thiolated chitosan was introduced into the formulation, however, an improved uptake of the drug was found. The absolute and relative bioavailability of these formulations were calculated to be 1.1% and 3.2% increased, respectively [74].

Table 3 Pharmacokinetic parameters of chitosan-TBA/insulin tablets and control tablets of unmodified chitosan/insulin after oral administration to non-diabetic rats compared with subcutaneous insulin administration

Formulation	Oral administration		Subcutaneous injection
	Chitosan-TBA/insulin tablets	Control tablets	
Insulin dose (mg/kg)	11	11	0.0832
Minimum blood glucose level in % of initial	75.4 ± 5.2	87.8 ± 5.59	45.3 ± 4.8
Time point of minimum blood glucose level (h)	6	4	1
$AUC_{0–24}$/rat[a]	356.4 ± 88.5	120.6 ± 69.8	160.2 ± 42.9
Relative efficacy (%)	1.7 ± 0.4	0.6 ± 0.3	100
Absolute efficacy (%)	0.6 ± 0.2	0.2 ± 0.1	–

Indicated values are mean ± SD of 3–7 experiments. Adopted from [72]

[a]$AUC_{0–24}$ is the area under the curve obtained when plasma concentration is plotted against time for 24 h

Relative pharmacological efficacy was calculated referred to the s.c. injection results, whereas absolute pharmacological efficacy was calculated referred to i.v. injection results (data not shown)

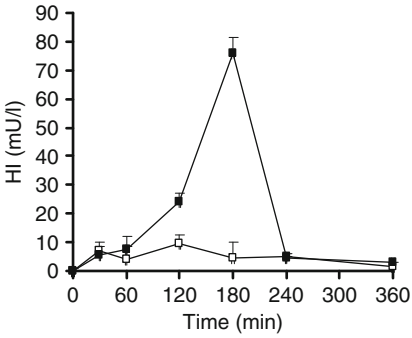

Fig. 7 Serum concentrations of human insulin (*HI*) after oral administration of formulations containing unmodified chitosan (*open square*) and chitosan-MNA (*closed square*). Indicated values are means ± SD of at least five rats (Adopted from [73])

A further benefit of oral drug uptake based on thiolated chitosan has been documented. The efflux pump inhibitory capacity of chitosan-TBA combined with GSH was proven by the significant improvements in Rho-123 plasma levels in rats. The improvement was higher than that obtained with tablets based on poloxamer or Myrj 52, which are well-known P-gp inhibitors [75]. Indeed, the applicability of thiolated chitosans for oral administration of other high molecular mass compounds would appear to be an interesting subject for ongoing studies.

5.2 Nasal Drug Delivery

Dealing with nasal applications means that obstacles such as low membrane permeability, short local residence time, and high turnover rate of secretion in nasal cavities have to be overcome. These limitations lead the bioavailability of nasally

administered drugs to be less than than 80% [76]. The most common approach to improve nasal drug absorption is to use permeation enhancers. The potential of thiomers in nasal drug delivery has already been demonstrated [77].

Thiolated chitosans, in particular, seem to be suitable for overcoming the above-mentioned drawbacks owing to their permeation-enhancing effect. To prove the efficacy of thiolated chitosans for nasal drug targeting, insulin was chosen as model drug. Insulin-loaded chitosan-TBA microparticles were administered to the nostrils of male Wister rats, using an intravenous injection of insulin as a positive control. It was found that the absolute bioavailability of insulin loaded in chitosan-TBA microparticles was 7.24 $\pm$ 0.76%, which was more than 3.5-fold higher than the absolute bioavailability obtained from the unmodified chitosan/insulin microparticles [78].

Moreover, intranasally delivered theophylline incorporated into thiolated chitosan nanoparticles as carrier matrix was developed and investigated for its capacity to relieve allergic asthma. In a mouse model of allergic asthma, the combination of theophylline with thiolated chitosan nanoparticles accelerated the efficacy of the drug in comparison to theophylline alone [70].

5.3 Buccal Drug Delivery

Bypassing degradation of drugs in the GI tract and the hepatic first-pass metabolism, the buccal route is an alternative choice for distributing drugs to the application site. Although highly accepted by patients as route of application, buccal administration has the drawback of poor drug permeation across the buccal mucosa [79]. With thiolated chitosans, however, a successful nasal drug delivery has been achieved. In fact, the capacity of chitosan-TBA combined with GSH to liberate a peptide via buccal administration has been demonstrated [80]. Pituitary adenylate-cyclase-activating polypeptide (PACAP), a peptide drug for treatment of type 2 diabetes, was chosen as model peptide. The absolute bioavailability of PACAP was evaluated after buccal administration and results revealed that the combination of chitosan-TBA/GSH led to an absolute bioavailability of 1%, whereas no systemic drug uptake was achieved at all with unmodified chitosan formulations. In the case of the thiolated chitosan system, the plasma level of glucose was maintained in the therapeutic range over the whole 6 h experimental period.

6 Conclusions

Despite the beneficial properties of chitosan in drug delivery, various thiolated chitosans have been synthesized and found to exhibit improved features in comparison to unmodified chitosan. This improvement is due to the covalent attachment of thiol-bearing compounds on the polymeric backbone.

In detail, thiolated chitosans, which constitute a group of the so-called thiomers, have shown strong mucoadhesive, in situ gelling, and controlled drug release

properties. In addition, these biopolymers proved efficacy as efflux pump inhibitors and permeation enhancers. The efficacy of this new generation of chitosan derivatives has already been demonstrated by various in vivo studies and their combination with innovative technologies such as micro- and nanotechnology is a promising choice in drug delivery.

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Chitosan-Based Particulate Systems for Non-Invasive Vaccine Delivery

Sevda Şenel

Abstract The use of particulate systems is considered very promising for the delivery of antigenic molecules via parenteral and non-parenteral routes. They provide improved protection and facilitated transport of the antigen as well as more effective antigen recognition by the immune cells, which results in enhanced immune responses. The natural cationic polysaccharide chitosan has been investigated extensively both as an adjuvant and delivery system for vaccines. It has been shown to enhance both humoral and cellular responses. From the formulation point of view, chitosan-based particulate systems offer advantages over the other polymers used by avoiding the harsh conditions of heat and/or organic solvents for encapsulation of the antigen. Furthermore, versatility in the physicochemical properties of chitosan provides an exceptional opportunity to engineer antigen-specific adjuvant/delivery systems. In this review, the importance of chitosan in particulate systems for vaccine delivery will be emphasized according to administration routes, particularly focusing on non-invasive (needle-free) routes including oral, mucosal and pulmonary mucosae as well as skin.

Keywords Adjuvant · Chitosan · Needle-free · Particulate systems · Vaccine

Contents

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1 Introduction

In the area of vaccine development, the crucial concern is to balance safety with efficacy. Most of the vaccines involve killed or attenuated microorganisms (bacteria, viruses, fungi, etc.) or chemically detoxified toxins (toxoids) from bacteria. Killed bacterial or viral vaccines often have residual toxicity following inactivation and might contain toxic components, such as lipopolysaccharides [1]. Therefore, despite their efficacy, these type of vaccines exert serious safety issues. Well-defined, immunogenic fragments of pathogens, known as subunit antigens (such as purified protective proteins or carbohydrates), provide a much cleaner, safer and more immunologically defined alternative to live or killed whole cell vaccines. However, these subunit vaccines are weakly immunogenic on their own, hence incorporation of an adjuvant is required to enhance their ability to evoke effective immune responses [2–5]. Adjuvants (the word coming from the latin word *adjuvare*, which means to help or to enhance) are defined as molecules, compounds or macromolecular complexes that boost the potency and longevity of a specific immune response to antigens, causing only minimal toxicity or long-lasting immune effects on their own [6]. Adjuvants act by a diverse series of pathways, which may involve changing the properties of the antigen to provide a slow-release antigen depot that targets innate immune pathways to selectively activate specific pathways of immunity [7–11]. The mechanism of action of an adjuvant is mainly either as an immunostimulant or as a delivery system [12]. In spite of the intense studies on adjuvant strategies, adjuvant toxicity still remains the major limitation to be surpassed.

Most vaccines today are given by parenteral injection, which stimulates the immune system to produce antibodies in the serum but fails to generate a mucosal antibody response [13]. In general, topical application of vaccine is required to induce a protective immune response. The use of vaccines that induce protective mucosal and dermal immunity thus becomes attractive when one considers that most infectious agents come into contact with the host at these surfaces. Furthermore, immunization at one mucosal site can induce specific responses at distant sites. The search for needle-free (non-invasive) delivery of vaccine not requiring a needle and syringe has been accelerated by recent concerns regarding pandemic disease, bioterrorism, and disease eradication campaigns. Hence in recent years, needle-free administration, which is possible via oral, nasal, pulmonary and dermal routes, has become an alternative to parenteral route [14, 15]. However, poor immunogenicity and impaired antigen delivery with these systems still remains to be improved. For this purpose, particulate systems have been investigated as the appropriate vaccine formulations to target topical inductive sites and appropriately stimulate the innate system in order to generate effective adaptive immunity as well

as to prevent the antigen from physical elimination and enzymatic degradation. From the formulation point of view, needle-free vaccination also offers several benefits over parenteral routes, including ease and speed of administration, possibility of self-administration, reduced side effects, reduced risk of infection and disease transmission, improved safety and compliance, and reduced costs.

When all the limitations mentioned above are taken into consideration, chitosan seems to be a very promising material for vaccine delivery, both as an adjuvant and as a delivery system due to its numerous favorable properties such as bioadhesivity, biocompatibility, immunostimulating activity, and mucosal penetration enhancing effect [12, 13]. Furthermore, its positive charge and ability to form particulate systems without requiring harsh conditions of heat and/or organic solvents for encapsulation of antigen offer advantages over other polymers used for this purpose.

In this paper after explaining why particulate systems are preferred for vaccine delivery, a brief introduction to chitosan will be given, and the importance of chitosan in particulate systems for vaccine delivery will be emphasized according to administration routes, particularly focused on needle-free routes including, oral, mucosal and pulmonary mucosa as well as skin. For comparison, examples on parenteral route will also be mentioned.

2 Particulate Systems for Vaccine Delivery

Particulate carrier systems such as immune-stimulating complexes (ISCOMs) [16–18], liposomes [3, 19], and polymeric micro/nanoparticles [20–27] have been proven to be highly efficacious for antigen delivery. These particles, due to their similar size to the pathogens that invade the host, can be efficiently internalized by antigen-presenting cells (APCs). The uptake of microparticles (<10 μm) by phagocytic cells has been well recognized, and uptake into APCs is likely to be important in the ability of particles to perform as vaccine adjuvants. Microparticles are phagocytosed by a variety of cells including macrophages and dendritic cells (DCs) [28–30]. Once taken up by these cells, antigen is released and subsequently selected for presentation via major histocompatibility complex (MHC) II. Phagocytosis of microparticles and presentation of incorporated antigens have been reported to be strongly influenced by the chemical and physical nature of the particulate system. Cationic microparticles have been shown to be particularly effective for uptake into macrophages and DC. This is attributed to the enhancement of binding to the negatively charged cell surface by the positively charged particle, which subsequently sets off internalization into the cell [31]. Particulate systems have been shown to create a depot effect that helps to increase the persistence of antigens for a longer time, which is important for the induction of efficient protective T-cell responses [32].

Various biodegradable polymers, including polyanhydrides, polyorthoesters, hyaluronic acid, alginate, chitosan, and starch as well as poloxamers (Pluronics), which self-assemble into particulates, and polyphosphazenes have been investigated

as vaccine delivery systems. Other adjuvants can also be incorporated into these systems to improve the immune response. With most of the polymers mentioned above, use of organic solvents is inevitable for particle preparation. Additionally, during the manufacturing process of the particles, the antigen may also be exposed to high shear stress and elevated temperatures. Hence, although these systems have advantage over other dosage forms in enhancing the immune response, there are limitations such as inefficient incorporation, limited antigen release, stability and integrity of the antigen during the formulation process as well as after administration or storage. The degradation and denaturation of proteins during encapsulation has been avoided by adsorbing the antigen onto the surface of the microparticles instead of encapsulating it [33]. Furthermore, there are potential limitations associated with regulatory and industrial issues related to the components of these systems, and also higher cost compared to other formulations [34].

It is well documented that the polymers used and formulation properties affect the success of the particulate systems for vaccine delivery [35]. Recently, Mohanan et al. [36] investigated the influence of the administration route of particulate systems on the type and strength of immune response elicited following immunization of mice by different routes, such as the subcutaneous, intradermal, intramuscular, and intralymphatic routes. Three delivery systems were used: ovalbumin-loaded liposomes, *N*-trimethyl chitosan (TMC) nanoparticles, and poly (lactide-*co*-glycolide) (PLGA) microparticles, all with and without specifically selected immune-response modifier (trehalose 6,6′-dibehenate, lipopolysaccharide, CpG). The route of administration was found to cause only minor differences in inducing an antibody response of the IgG1 subclass associated with Th2-type immune responses, and any such differences were leveled out after boosting. However, the administration route strongly affected both the kinetics and magnitude of the IgG2a response associated with Th1-type immune responses. A single intralymphatic administration of each of the delivery systems was found to induce a robust IgG2a response, whereas subcutaneous administration failed to elicit a substantial IgG2a response even after boosting, except with the adjuvanted nanoparticles. The intradermal and intramuscular routes generated intermediate IgG2a titers. It was suggested that all immunization routes mediated efficient drainage of all three delivery systems from peripheral non-lymphoid tissues to lymphoid organs, where strong immune responses were mounted against the antigen. No direct comparison was made between the three different formulations, stating that these systems differ in their chemistry, size, particle architecture, degradation rate, and erosion and antigen release kinetics.

3 Chitosan

Chitosan is a cationic polymer derived from chitin obtained from crustacean and insect skeletons. Structurally, it is a linear polysaccharide consisting of β (1-4)-linked D-glucosamine with randomly located *N*-acetylglucosamine groups

depending upon the degree of deacetylation of the polymer. The degree of acetylation represents the proportion of *N*-acetyl-D-glucosamine units with respect to the total number of units and can be employed to differentiate between chitin and chitosan. Chitin with a DD of 65–70% or above is generally known as chitosan. The DD is an important property of chitosan and defines its physicochemical and biological properties, hence determining its applications. Despite being the most abundant polymer in nature after cellulose, the utilization of chitin has been restricted by its intractability and insolubility. Chitosan has been favored more in a wide range of application owing to its better solubility and also to its free amine groups, which are an active site in many chemical reactions. Chitosan is readily soluble in dilute acidic solutions below pH 6.0. With increasing pH, the amino groups become deprotonated and the polymer loses its charge and becomes insoluble. The solubility of chitosan is dependent on the DD and the method of deacetylation used since the pK_a value is highly dependent on the degree of N-acetylation [37].

The positive surface charge of chitosan allows it to interact with macromolecules like exogenous nucleic acids, negatively charged mucosal surfaces, or even the plasma membrane [38, 39]. Chitosan is degraded by enzymes such as chitosanase and lysozyme. The rate of degradation of chitosan inversely depends on the degree of acetylation and crystallinity of the polymer. The highly deacetylated form exhibits the lowest degradation rates and may last several months in vivo. It is still not clearly known what the mechanism of degradation is when chitosan is injected intravenously. It has been reported that distribution, degradation, and elimination processes are strongly dependent on molecular weight and DD [40].

It is possible to prepare different forms of formulations such as aqueous dispersions, gels, sponges and micro/nanoparticles using chitosan and its derivatives. Furthermore, versatility in the physicochemical properties of chitosan allows the formulator an excellent opportunity to engineer antigen-specific adjuvant/delivery systems.

3.1 Mode of Action

Chitosan has been shown to induce both cellular and humoral responses when administered via parenteral, mucosal, or transcutaneous routes [41–43]. Various studies have demonstrated the activation of the DCs, macrophages, and lymphocytes by chitosan [44]. Maeda and Kimura [45] have reported that the presence of chitosan caused enhancement of the natural killer (NK) cell activity in intestinal intraepithelial lymphocytes and splenic lymphocytes. The NK activity of intraepithelial lymphocytes (IELs) or splenic lymphocytes treated with low molecular weight chitosan (LMWC; 21 and 46 kDa) was found to be stronger than that of lymphocytes treated with high molecular weight chitosan (HMWC; 130 and 650 kDa). In addition, IELs or splenic lymphocytes treated with LMWC were found to enhance the cytotoxic activity against sarcoma 180 cells. Hence, variations in

molecular weight or DD of chitosan can lead to different degrees of activation in cells from the immune system.

The uptake and distribution of chitosan, the phenotype of recruited APCs, the induction of cytokines tumor necrosis factor- α (TNF-α), interleukin (IL)-12, IL-4, IL-10, and transforming growth factor (TGF-β), and the activation of T lymphocytes were investigated in rats after oral feeding [46]. The orally administered chitosan in the absence of an antigen was found to enhance a naturally Th2/Th3-biased microenvironment at the mucosal level by stimulating the production of regulatory cytokines. McNeela et al. [47] showed in human as well that chitosan selectively enhanced the induction of Th2 cells following intranasal immunization with the genetically detoxified diphtheria toxin.

Recently, the effects of chitosan oligosaccharides (COS) with different polymeri zation degree (3–7 and 7–16) on the activation of spleen CD11c-positive ($CD11c^+$) dendritic cells (SDCs) and the role of Toll-like receptor 4 (TLR4) in this process was investigated by Dang et al. [48]. It was shown that SDCs could be induced to a mature state, secrete TNF-α, and promote the proliferation of $CD4^+$ T cells by B-COS. TLR4 may play a critical role in this process. The biological activity of COS was suggested to be dependent on the molecular size or polymeration degree of COS. But, the mechanisms of B-COS recognition and the B-COS-related signaling pathway have not yet been described.

The immunostimulating activity of chitosan and its derivatives have also been investigated in combination with other adjuvants. Combinations of chitosan and its trimethyl derivative with LTK63 mutant, which is a mucosal adjuvant, were investigated in an effort to enhance the immunogenicity and protective efficacy of the CRM-MenC conjugate vaccine given intranasally to mice [49, 50]. At very low doses of the LTK63 adjuvant, high bactericidal antibody titers were induced only in the presence of TMC. The quality of this protective immune response was reported to be modulated depending on the appropriate dosing of the mucosal adjuvants.

In recent years, new information about the functions of immunomodulatory cytokines and the discovery of TLRs have provided promising new alternatives for adjuvants [51]. Based on the delivery potential of chitosan and its compatibility with IL-12, Heffernan et al. [52] combined chitosan and IL-12 as an adjuvant system for subcutaneous administration of a model antigen, ovalbumin (OVA). The chitosan/IL-12/OVA vaccine was found to elicit greater antigen-specific $CD4^+$ and $CD8^+$ T-cell responses, as determined by $CD4^+$ splenocyte proliferation, Th1 cytokine release, $CD8^+$ T-cell interferon-γ release, and MHC class I peptide pentamer staining. The IgG2a and IgG2b antibody responses to OVA were also enhanced with a combination of chitosan and IL-12. Various TLR ligands including CpG-containing oligonucleotides, which have shown adjuvant activity when administered mucosally, were combined with chitosan. Recombinant hepatitis B surface antigen (HBsAg)-loaded nanoparticles were associated with Class B CpG ODN 1826 (5′-TCC ATG ACG TTC CTG ACG TT-3′) [74] . The generation of Th1-biased antigen-specific systemic antibodies was observed only when HBsAg-loaded nanoparticles were applied together with Class B CpG ODN.

Sometimes, conflicting results have been reported by different groups on stimulatory activity of chitosans; however, this is probably due to the differences in experimental conditions or parameters evaluated. Besides its immunostimulating activity, the bioadhesive and penetration enhancing properties of chitosan also contribute to the immune system stimulation by enhancing paracellular absorption at the mucosal site and allowing increased uptake of antigens [26, 53].

In the following sections, the application of chitosan-based particulate systems for vaccine delivery will be reviewed according to the administration routes (mucosal, dermal and parenteral) after giving a brief introduction to each route.

4 Mucosal Delivery

Mucosal immunization has been focused on oral, nasal, and aerosol vaccines. The use of vaccines that induce protective mucosal immunity becomes very attractive when one considers that most infectious agents come into contact with the host at mucosal surfaces. The mucosal immune system, which protects the host from pathogenic microorganisms at mucosal surfaces, is an integrated network that permits communication between the organized lymphoid tissues (inductive sites) and the diffuse mucosal tissues (effector sites) [54, 55]. Through innate and adaptive immunity, the mucosal immune system maintains immunological homeostasis along the vast expanse of the epithelial surface area, including oral and nasal cavities, respiratory, intestinal and genito-urinary tracts. The initiation of mucosal immune responses occurs with the transport of antigen through specialized epithelial cells (M cells) that are present in the organized mucosa-associated lymphoid tissues (MALT) (including Peyer's patches, mesenteric lymph nodes, solitary follicles in the intestine, appendix, tonsils and adenoids). After antigens are taken up via M cells, they are entrapped by APCs (DCs, B lymphocytes and macrophages) and presented to $CD4^+$ and $CD8^+$ T cells [56]. Upon sensitization by the antigens, B cells proliferate and switch to IgA-committed cells. These B cells eventually leave the MALT and migrate through the systemic circulation to various mucosal sites, including the initial induction site for terminal differentiation to sIgA-producing plasma cells. Recent evidence shows that TLRs recognize specific patterns of microbial components, especially those from pathogens, and regulate the activation of both innate and adaptive immunity. TLRs are membrane bound pattern recognition receptors (PRRs) responsible for detecting most antigen-mediated infections [57]. There are at least 13 different forms of TLR, each with its own characteristic ligand.

The oral route would be considered to be the most suitable route for mucosal immunization with regard to the ease and acceptability of administration. However, the acidic pH as well as the presence of the digestive enzymes limits the antigen's access to the mucosal epithelium, which makes oral immunization complicated. Hence, over the past two decades, new strategies for design of delivery systems and adjuvants have been investigated with the aim of enhancing immune responses

based on entrapping or adsorbing the selected potent antigens into a variety of micro- and nanoparticulate systems in the absence or presence of another adjuvant [58, 59]. Such particles can protect the antigens from degradation in the stomach and intestine, and deliver them efficiently to the gut-associated lymphoid tissue (GALT) located in the lower portion of the small intestine. When such microparticles reach the Peyer's patches in the GALT, they can be taken up by M cells. However, the ability of microparticles to perform as adjuvants or delivery systems following oral mucosal administration has not been very encouraging in either animals or human [60]. Due to these limiting issues in oral vaccination, nasal mucosa, which does not have the issues of instability in the gastrointestinal region and permits lower doses to be used, has been investigated as an alternative route for mucosal immunization [61–63]. The formulation of antigens in particulate delivery systems for intranasal administration protects the antigen from mucosal enzymes and facilitates the preferential uptake of the antigen by specialized M cells of the nasal-associated lymphoid tissue (NALT) [34]. Nasal immunization with particulate systems has provided more encouraging data than oral immunization thus far in both animal and human studies. Targeting of antigens to alveolar macrophages has also been explored using the pulmonary route [64]. Lungs are highly vascularized, have a large absorptive surface area because of the alveoli structure, and contain bronchoalveolar lymphoid tissue. Furthermore, local APCs are ideally located for antigen sampling and subsequent presentation to T cells. Pulmonary vaccination has also the advantage of inducing both systemic and local immunity (IgA and IgG) in the respiratory tract. However, for pulmonary protein delivery, poor deposition of protein formulations at the alveoli (the absorption site) is still a major limitation [65].

4.1 Oral Delivery

Due to the limitations with the gastrointestinal tract, there are few studies available on chitosan-based delivery systems for oral vaccine delivery (Table 1). Van der Lubben et al. [66] were among the first to demonstrate that chitosan microparticles with a particle size smaller than 10 μm, incorporated with the model protein OVA as well as diphtheria toxoid (DT), were taken up by the Peyer's patch after intragastric administration to mice. A dose-dependent immune reaction was observed for mice vaccinated with different doses of DT associated to chitosan microparticles [67]. It was also observed that the immune response started only 1 week after the boosting, indicating the formation of memory cells after priming.

Chitosan formulations administered mucosally have been shown to enhance tolerance induction in murine models of allergy. Besides protecting against antigenic entry to the systemic immune system, mucosal immune system also provides to be unresponsive to food antigens. Chitosan/DNA nanoparticles carrying the gene for a principal peanut allergen, Arah2 were prepared for oral immunization and their efficacy in modulating antigen-induced hypersensitivity in a murine model of peanut allergy was demonstrated [68]. Oral administration using chitosan/DNA

Table 1 Chitosan-based particulate systems for oral, dermal and parenteral delivery of vaccines

Antigen	Dosage form	Immunization route	Chitosan type	Adjuvants	Animal model	Immune response	References
Ovalbumin Diphteria toxoid	Microparticles 4.3 ± 0.7 μm	Oral	Chitosan (DD 93%) (Primex, Avaldsnes, Norway)	–	Female BALB/c mice	Taken up by the epithelium of the murine Peyer's patches; protective systemic and local immune response against diphteria toxoid	[66, 67]
DNA	Nanoparticles 150–300 nm	Oral	Chitosan (MW 390 KDa)	–	AKR/J mice	Modified immune system and protection against food allergen-induced hypersensitivity	[68]
Der p1. DNA	Nanoparticles 506.9 ± 9.7 nm	Oral	Chitosan C390 (MW 390 kDa), (DA 83.5%) (Vanson, Redmond, WA)	–	6- to 8-week-old female BALB/cJ mice	Increased immune responses	[69]
Ovalbumin	Microparticles 1–3 μm and 300–800 nm	Sublingual	Chitosan (310–375 kDa and 190–310 kDa), (references 419419 and 448877, Sigma)	–	BALB/c mice	Enhanced tolerance induction only with highly positively charged microparticles	[70]

(*continued*)

Table 1 (continued)

Antigen	Dosage form	Immunization route	Chitosan type	Adjuvants	Animal model	Immune response	References
Plasmid DNA	Chitosan oligomer/CMC nanoparticles 93–270 nm Chitosan/CMC nanoparticles 180–650 nm	Intradermal	Chitosan (MW 102 kDa; DD 80%) (Natural Biopolymer, Raymond, WA)	–	BALB/C mice	Quantifiable levels of luciferase expression in skin after 24 h; signicant antigen-specific IgG titer to expressed β-galactosidase 28 days after the first application	[42]
Diptheria toxoid Ovalbumin	Nanoparticles 100 nm Solution	Transcutaneous	TMC (quaternization: 15% and 30%)	– Before and after microneedle array used	Female BALB/c mice	Strong IgG and neutralising antibody titres No effect observed with microneedle application or type of microneedle array	[71, 72]
DNA-based respiratory syncytial virus (RSV)	Nanoparticles 80–150 nm	Parenteral (intravenous)	Chitosan	–		Higher level of RSV protein expression in mouse tissues	[73]
Hepatitis B surface antigen (HBsAg)	Alginate-coated chitosan nanoparticles 643 ± 171.7 nm	Parenteral (subcutaneous)	Chitosan (DD 95%) (Primex BioChemicals AS, Avaldsnes, Norway)	Class B, CpG ODN (1826) (5′-TCC ATG ACG TTC CTG ACG TT-3′)	7- week-old female BALB/cAnNHsd mice	High anti-HBsAg IgG titer, majority of antibodies being of Th2 type No significant differences in antigen-specific	[74]

						splenocyte proliferation and in secretion of IFN-γ and IL-4 Increased IgG2a/IgG1 ratio in presence of the adjuvant Increased IFN-γ production	
Ag85B–MPT64–Mtb8.4 (AMM) from *Mycobacterium tuberculosis* genes	Microparticles 5.78±0.65 μm	Parenteral (subcutaneous)	Chitosan (DD 85%) (Sigma–Aldrich, USA)	Incomplete Freund's adjuvant	6-week-old female C57BL/6 mice	Higher levels of IFN-γ Higher levels of Ag85B-specific IgG(H+L), IgG1 and IgG2a	[75]

DD degree of deacetylation, *TMC* trimethylated chitosan, *CMC* carboxymethylcellulose, *IFN-γ* interferon-γ, *IL* interleukin

nanoparticles for raising specific antibodies to native Der p1, which is regarded as the most important *Dermatophagoides pteronyssimus* allergen and is a major triggering factor for mite allergy worldwide, was investigated in mice [69]. The nanoparticle formulations were shown to prime Th1-skewed immune responses against both domains of Der p1. It was suggested that by resorting to an oral route of DNA delivery, it was possible to induce Der p1-specific immune responses that were undetectable using a parental route of immunization.

Chitosan has also been investigated for sublingual immunotherapy (SLIT), which is a noninvasive and efficacious treatment of type I respiratory allergies. Two types of chitosan microparticles with distinct size and charge characteristics were investigated for their ability to facilitate allergen uptake by various murine APC populations as well as to enhance allergen-specific tolerance when administered sublingually to OVA-sensitized mice with asthma [70]. Animals were treated sublingually with soluble or chitosan-formulated OVA twice a week for 2 months. Airway hyperresponsiveness (AHR), lung inflammation, and T-cell responses in cervical and mediastinal lymph nodes (LNs) were assessed. Only highly positively charged (which had the highest mucoadhesivity as well) and microparticulate form of chitosan was found to enhance OVA uptake, processing, and presentation by murine bone marrow DCs and oral APCs. Targeting OVA to DCs with this formulation increased specific T-cell proliferation and interferon γ (IFN-γ)/IL-10 secretion in vitro, as well as T-cell priming in cervical LNs in vivo. Sublingual administration of such chitosan-formulated OVA particles was reported to enhance tolerance induction in mice with established asthma, with a dramatic reduction of both AHR, lung inflammation, eosinophil numbers in bronchoalveolar lavages, as well as antigen-specific Th2 responses in mediastinal LNs. Due to both its mucoadhesive property and availability to form particles with different properties (size, charge), chitosan was suggested to be very promising for sublingual immunization. These results indicated that chitosan can mediate an adjuvant effect by itself that is dependent on the type and properties of the chitosan used as well as the route of administration.

4.2 Nasal Delivery

There are numerous studies demonstrating that chitosan and its derivatives enhance the immune response upon nasal immunization [12, 34]. Recent studies on chitosan-based particulate systems for nasal immunization are summarized in Table 2. The immune response has been shown to be affected by the particle size, DD, and molecular weight of the chitosan used. We have prepared nanoparticulate systems using differently charged chitosan derivatives, TMC (polycationic) and mono-*N*-carboxymethyl chitosan (MCC, polyampholytic), for nasal immunization [95]. This was the first time the negatively charged chitosan derivative, MCC, was investigated for mucosal immunization. Enhanced immune responses were obtained with intranasal (i.n.) application of tetanus toxoid (TT)-loaded nanoparticle formulations

Table 2 Chitosan-based particulate systems for nasal delivery of vaccines

Antigen	Dosage form	Chitosan type	Adjuvants	Animal model	Immune response	References
Bacillus anthracis PA and PA-conjugate	ChiSys Dry powder	Protasan UP G 213 (MW 200–600 kDa; DD 75–90%; Novamatrix, Norway)	MPL	Female New Zealand white rabbits	Induction of IgG levels	[76]
Recombinant Anthrax protective antigen (rPA)	ChiSys Dry powder	Protasan UP G 213 (MW 200–600 kDa; DD 75–90%; Novamatrix)	MPL	Female New Zealand white rabbits	Induction of IgG levels	[77]
BBD	Microspheres 0.29–5.3 μm	Chitosan (MW 10, 100 and 300 kDa; DD 90.8%; Jakwang, Korea)	–	BALB/c female mice	Induction of IgG titer in serum Induction of IgA in nasal wash	[78]
BBD	Microspheres 1.94–5.21 μm	Chitosan (MW 10 kDa; DD 80.4%; Jakwang)	Pluronic F127	Mice	Induction of IgG and IgA levels in serum, saliva and nasal wash Significant TNF-α values	[79]
BBD	Microspheres 5–6 μm	Water-soluble chitosan (MW 100 kDa; DD 95.4%; Sunchon National University) Mannosylated chitosan	–	Female mice	Induction of IgG and IgA levels in serum, saliva and nasal wash	[80]
BBD	Microspheres	Chitosan (MW 10 kDa; DD 80.4%; Jakwang)	–	Colostrum-deprived pigs	Induction of IgG and IgA levels in serum, IgA levels in nasal wash	[38]
FMDV DNA antigen	Nanoparticles 255 nm	Chitosan (MW 170 kDa; DD >85%; Dalian Xindie Chitin, China)	Provax IL-15	Female BALB/c mice, guinea pigs	Induction of IgG levels in serum, IgA levels in lung and vaginal wash Induction of cellular immune response (IL-4 and IFN-γ in $CD4^+$ cells and IFN-γ in $CD8^+$ T cells)	[81]
Plasmid DNA HbsAg	Glycol chitosan-coated liposomes 775 nm	Glycol chitosan-(MW 250 kDa; DD 88%; Sigma, USA)	–	Female BALB/c mice	Induction of IgG levels in serum and IgA levels in nasal, saliva, vaginal wash	[82]

(*continued*)

Table 2 (continued)

Antigen	Dosage form	Chitosan type	Adjuvants	Animal model	Immune response	References
					Induction of cellular immune response (IL-2 and IFN-γ)	
Plasmid DNA HbsAg	Nanoparticles 300–400 nm	Chitosan (MW 400 kDa; DD 85%; Fluka, Switzerland)	–	Female BALB/c mice	Induction of IgG levels in serum and IgA levels in nasal, saliva, vaginal wash Induction of cellular immune response (IL-2 and IFN-γ)	[83]
Recombinant HbsAg	Alginate-coated chitosan nanoparticles 300–600 nm	Chitosan (DD 95%; Primex BioChemicals)	CpG ODN	Female BALB/cAnNHsd mice	Induction of IgA levels in feces, nasal and vaginal wash Induction of cellular immune response (IFN-γ) Induction of Th2-type immune response (IgG1/IgG2a > 1)	[74, 84]
Plasmid DNA Hepatitis B core antigen	Nanoparticles 340–380 nm	Chitosan (MW 173 kDa; Shanghai KABO Trading Co., China)	–	Male New Zealand white rabbits	Induction of serum IgG levels	[85]
Inactivated influenza virus	Nanoparticles	Chitosan (DD 17%; MW 23 kDa, 43 kDa; Primex) TMC		Female C57-BL/6 mice (Charles River)	Induction of stronger total IgG, IgG1 and IgG2a/c responses	[86]
Inactivated influenza virus	Dry powder <100 μm	Chitosan (MW 161 kDa; DD 92%; Vanson HaloSource, USA)	–	Female brown Norway rats	Induction of IgG levels in serum and IgA levels in nasal wash	[87]
Subunit influenza antigen	Nanoparticles 850 nm	Chitosan (MW 177 kDa; DD 93%; Primex); TMC	–	Female C57BL/6 (B6) mice	Induction of Th2 type immune response (IgG1/IgG2a > 1) Induction of IgA immune response in nasal wash	[88]
CRM197	Microparticles or powder 4.5 μm (chitosan) 2.5 μm (TMC)	Chitosan (DD 94.5%; Primex) TMC	LTK63	Female BALB/c mice	Induction of IgG levels in serum and IgA levels in nasal and vaginal wash	[50]

MCP-CRM197	Powder	Chitosan glutamate Protasan UP G 213 (Novamatrix)	–	Female and male healthy volunteers	Induction of IgG2, IgG1, IgG4 Induction of IgA response in nasal wash	[89]
Tetanus toxoid	Nanoparticles Chitosan 300–400 nm MCC 40–90 nm TMC 300–400 nm	Chitosan glutamate Protasan UP G 113 (MW 150 kDa; DD >75–90%) (Novamatrix, Norway) MCC, TMC	–	BALB/c mice	Induction of Th2-type immune response (IgG2a/IgG1 < 1)	[95]
Tetanus toxoid	Nanoparticles 280 nm	MCC-TMC	–	BALB/c mice	MCC-TMC nanoparticles induced significantly higher immune responses than TMC, MCC, or chitosan nanoparticles	[90]
Bovine herpes virus	Microparticles 35–50 μm	Protasan UP Cl213 (Novamatrix, Norway) Chitopharm S, Chitopharm M, Chitopharm L (Cognis, Germany)		In vitro cell culture	Infectivity decreased with increasing molecular weight of chitosan	[91]
Ovalumin	Nanoparticles 380 nm	TMC	CpG DNA (ODN 2006)	Female BALB/c mice	Replacing TPP by CpG as acrosslinking agent modulated the immune response towards a Th1 response after nasal vaccination	[92]
Ovalbumin	Nanoparticles 450 nm (TMC-PLGA) 280 (TMC)	TMC TMC-coated PLGA	–	Female BALB/c (nu/nu) mice	TMC nanoparticles superior over PLGA and PLGA/TMC nanoparticles	[93]
Recombinant HbsAg	Nanoparticles 160–200 nm	Protasan UP Cl 113 (MW 125 kDa; DD 14%; Novamatrix)	–	Female BALB/c mice	Induction of anti-HBsAg IgG levels Prolonging antibody response	[94]

BBD Bordetella bronchiseptica dermonecrotoxin, *CpG* trehalose 6,6′-dibehenate, lipopolysaccharide, *CRM* cross-reacting material, *DD* deacetylation degree, *FMDV* foot and mouth disease virus, *HbsAg* hepatitis B surface antigen, *IFN-γ* interferon, *IL* interleukin, *MCC* mono-*N*-carboxymethyl chitosan, *MCP* meningococcal C-polysaccharide, *MPL* monophosphoryl lipid A, *MW* molecular weight, *ODN* oligodeoxynucleotide, *PA* protective antigen, *PLGA* poly (lactic-*co*-glycolic acid), *TMC N*-trimethyl chitosan, *TPP* tripolyphosphate

in BALB/c mice. Chitosan and TMC nanoparticles (300–400 nm), which have positively charged surfaces, induced higher serum IgG titres compared to those prepared with MCC, which are negatively charged and smaller in size (40–90 nm). The aqueous dispersions of chitosan, TMC, and MCC incorporating TT were also investigated in vivo. Significantly higher immune responses were obtained with aqueous dispersions following intranasal application compared to that of TT solution in phosphate-buffered saline, whereas with subcutaneous administration, no enhancement in immune response was observed. This is attributed to the bioadhesive property inherent to chitosan that prolong the contact time of the formulation on the nasal mucosa, thereby leading to higher antibody titres. Both dispersion and nanoparticle systems prepared by chitosan derivatives were found to enhance mucosal immune responses. Certainly, encapsulation of TT into the nanoparticles would bring advantages over a simple aqueous dispersion, especially in protecting the antigen from the potential undesired environment at the nasal surface and preventing the loss of the antigen before reaching the target M cells within the delivery system. Moreover, the retention time of the nanoparticles, which was longer than that of the solutions, would allow efficient uptake of antigen through the mucosa thus improving the immune response. Our results showed that the nature of the surface charge and particle size exert an important role in obtaining an enhanced immune response using carriers prepared with chitosan. We have also developed nanoparticles by means of TMC and MCC complexation without using any crosslinker, and also expected to enhance the immunostimulant effect by using two chitosan derivatives in the same formulation [90]. TT was incorporated into the nanoparticles (280 nm) and the immune responses were investigated after nasal immunization in mice model. The TMC/MCC complex nanoparticles were found to induce significantly higher immune responses than the nanoparticles prepared with TMC, MCC, or chitosan alone. These results indicated that the developed TMC/MCC nanoparticles with very mild processing and high loading efficiency, maintaining the protein integrity, are very promising both as an adjuvant and as a delivery system for antigens.

Recently, we have prepared chitosan microparticles for mucosal delivery of bovine herpes virus (BHV-1), which is a major pathogen of cattle causing serious infections including infectious bovine rhinotracheitis (IBR)/infectious pustular vulvovaginitis (IPV) and is still a big threat in Turkey [91]. The integrity and antigenicity of the virus incorporated into microparticles (35–50 μm) prepared with various types of chitosan with different molecular weight and solubility were evaluated through direct immunofluorescent staining in which the virus infected cells were fluorescently labeled with BHV-1-specific FITC antibody conjugate. The results showed that the solubility of chitosan did not have any significant effect on integrity and potency of the antigen, whereas the infectivity decreased with increasing molecular weight of chitosan. Cytopathic effect (CPE) plaques after inoculation with BHV-1 loaded microparticles were observed in 16 h with water soluble chitosan particles whereas with base chitosan particles the post-infection was observed in longer period of time (LMWC 23 h; medium and HMWC 42 h].

Vila et al. [96] have also investigated the immune responses of nanoparticles prepared using different molecular weight chitosans. The mode of action of chitosan nanoparticles loaded with TT was reported not to be affected significantly by the chitosan molecular weight. Greater response was observed with LMWC particles at early times (12 weeks), and for HMWC at late times (24 weeks). This was attributed to the different release rate of TT from LMWC and HMWC formulations.

The potential of LMWC (5 and 8 kDa) for DNA vaccine delivery via nasal mucosa was evaluated in vitro and in vivo [97]. The in vitro transfection efficiency of chitosan/DNA polyplexes were investigated, and the LMWC was found to have lower binding affinity to DNA and mediated higher transfection efficiency. The capabilities of the polyplexes based on LMWC to elicit serum IgG antibodies and to attenuate the development of atherosclerosis after intranasal vaccination were compared with the polyplexes based on HMWC (32, 173, and 425 kDa) in a rabbit model. Intranasal vaccination with LMWC/DNA polyplexes could elicit significant systemic immune responses, modulate the plasma lipoprotein profile, and attenuate the progression of atherosclerosis. Those aspects were comparable to those obtained by HMWC/DNA polyplexes. The LMWC/DNA polyplexes were shown to remain stable on the nasal mucosa, and were internalized by nasal epithelial cells, which was similar to the case of HMWC/DNA polyplexes.

Microspheres using different molecular weight chitosans were prepared for nasal delivery of *Bordetella bronchiseptica* dermonecrotoxin (BBD), a major virulence factor of a causative agent of atrophic rhinitis in pigs [78]. The highest BBD release was obtained with lower molecular weight of chitosan and at higher pH. TNF-α and nitric oxide from RAW264.7 cells exposed to BBD-loaded chitosan microspheres (BBD-CMs) were gradually secreted with time, showing immunostimulating activity in vitro. Furthermore, the effect of Pluronic F127-associated microparticles on immunostimulating activity was investigated in mice [79]. Protective immunity was measured by survival rate after challenge with *B. bronchiseptica* via the nasal cavity. The survival rate of the group treated with BBD-CMs/F127 was reported to be higher than those of the other groups. Pluronic F127-associated chitosan microparticles were suggested as an efficient adjuvant for nasal delivery of BBD.

For influenza vaccines, nasal administration has been regarded as a good alternative to parenteral injection because of the enhancement of the mucosal immune response and the ease of vaccine administration. Chitosan and its derivatives have also been investigated for nasal delivery of influenza virus. In a study performed in humans, standard inactivated trivalent influenza vaccine with chitosan was administered nasally and intramuscularly [98]. Although the induction of mucosal antibodies was not investigated, the vaccine was reported to be safe and well tolerated by the subjects. Overall, the geometric mean serum HI antibody titres induced by the vaccine were reported to meet the regulatory requirements, and the seroconversion rate and protective antibody levels achieved were not statistically different from those for saline vaccines given intramuscularly.

Intranasal vaccination studies with whole inactivated influenza virus (WIV) adjuvanted with *N,N,N*-trimethylchitosan (TMC-WIV) have shown promising

results [86] in mice. No significant differences in the nasal residence time between WIV and TMC-WIV were observed. However, a remarkable difference in the location and distribution of WIV in absence and presence of the TMC was observed, which was correlated with the observed differences in immunogenicity of these two formulations. TMC-WIV was reported to allow a much closer interaction of WIV with the epithelial surfaces, potentially leading to enhanced uptake and induction of immune responses. Both WIV and TMC-WIV formulations were shown to induce minimal local toxicity.

In a study performed in humans, *Neisseria meningitidis* serogroup C polysaccharide (MCP)-CRM197 conjugate vaccine mixed with chitosan was administered in two nasal insufflations 28 days apart and the effects compared to those of intramuscular injection with alum. Nasal immunization was reported to be well tolerated, with fewer symptoms reported than after intramuscular injection. Increases in CRM197-specific IgG and diphtheria toxin-neutralizing activity were observed after nasal or intramuscular immunization, with balanced IgG1/IgG2 and higher IgG4. Significant MCP-specific secretory IgA was detected in nasal wash only after nasal immunization and predominantly on the immunized side [89].

Recently, in another study performed in humans, intranasal vaccine against norovirus infection (known as the "stomach flu") incorporated in a dry powder formulation based on virus-like particle (VLP) antigens including Monophosphoryl Lipid A (GlaxoSmithKline) and chitosan was reported to show immunogenicity in clinical Phase I studies [99].

4.3 Pulmonary Delivery

Immunization by the pulmonary route in order to establish a mucosal as well as systemic immune response against airborne pathogens is an emerging field [100, 101]. The effect of chitosan particles as vaccine carriers in endotracheal immunization has been investigated by several groups (Table 1). An HLA-A2 transgenic mouse model was used to investigate the effects of pulmonary delivery of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of *Mycobacterium tuberculosis* formulated in chitosan nanoparticles [102]. It was shown that the chitosan/DNA formulation was able to induce the maturation of DCs while chitosan solution alone could not, indicating that the DNA released from the particles was able to stimulate DCs. Pulmonary administration of the DNA plasmid incorporated in chitosan nanoparticles was shown to induce increased levels of IFN-γ secretion compared to pulmonary delivery of plasmid in solution or the more frequently used intramuscular immunization route. These results indicated that pulmonary delivery of DNA vaccines against tuberculosis may provide an advantageous delivery route compared to intramuscular immunization, and that increased immunogenicity can be achieved by delivery of this DNA encapsulated in chitosan nanoparticles.

The potential of a chitosan derivative, TMC, for pulmonary delivery of DT was also investigated [88]. Pulmonary immunization with DT-TMC microparticles

containing 2 or 10 Lf units of DT resulted in a strong immunological response, as reflected by the induction of IgM, IgG, and IgG subclasses (IgG1 and IgG2) antibodies, as well as neutralizing antibody titers comparable to or significantly higher than those achieved after subcutaneous administration of alum-adsorbed DT (2 Lf). Moreover, the IgG2/IgG1 ratio after pulmonary immunization with DT-TMC microparticles was substantially higher than that of the subcutaneously administered alum-adsorbed DT. DT-TMC microparticles were also able to induce detectable pulmonary secretory IgA levels. Recently, Heuking et al. [103] has developed a new water-soluble chitosan derivative (6-*O*-carboxymethyl-*N*,*N*,*N*-trimethyl chitosan) that was functionalized with a TLR-2 agonist as a material for pulmonary vaccine delivery. Furthermore, TLR-2-decorated CM-TMC nanocarriers (400 nm) by complexation with pDNA were prepared and shown to protect pDNA against enzymatic degradation [104].

The kinetics and toxicity of the nanoparticles (350 nm) prepared using hydrophobically modified glycol chitosan (HGC) were evaluated after intratracheal instillation to mice [105]. The half-life of HGC nanoparticles in the lung was determined as 131.97 $\pm$ 50.51 h, showing rapid uptake into the systemic circulation and excretion via urine, which peaked at 6 h after instillation. HGC nanoparticles were found to be distributed to several extrapulmonary organs; however, the levels were extremely low and transient. Transient neutrophilic pulmonary inflammation was induced from 6 h to day 3 after instillation. Expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and chemokine (MIP-1α) in lung showed an increase after instillation and recovered thereafter. The HGC nanoparticles were suggested as successful candidates for pulmonary delivery owing to their excellent biocompatibility, transiency, low pulmonary toxicity, and property of rapid elimination without accumulation.

5 Transdermal and Intradermal Delivery

The skin is accepted to be a good target organ to generate both cellular and humoral immune responses, hence it is used as an alternative route for vaccine delivery [106–109]. Studies in several animal species and clinical trials in humans have established the proof of principle [14]. The skin is rich in DCs found at high density in the dermis and Langerhans cells (LCs), mostly localized in the epidermis. Application of vaccine antigens and adjuvants to the skin, also known as transcutaneous immunization (TCI) involves the topical application of the formulation directly onto the skin surface [110]. The quality and duration of the humoral and cellular responses to transcutaneous vaccination depend on the appropriate targeting to the APCs, vaccine dose, route of administration, and use of adjuvant. Various methods of vaccination have been designed to target the antigen to LCs and DCs for the induction of immune responses. Among the different parameters that have to be considered in formulation of particulate sytems for transcutaneous vaccine delivery, the size of the particles and their physical and chemical properties that allow

their internalization by APCs are important. Limitations in particle-based TC vaccination are imposed by skin layers and hair follicles.

In recent years, utilization of chitosan and its derivatives for TC immunization has been investigated and compared to to intradermal delivery (Table 1). Bal et al. [71] first studied the immunogenicity of both OVA-loaded TMC nanoparticles (200 nm) and TMC/OVA solutions after intradermal (ID) injection. TMC/OVA nanoparticles were shown to be taken up in vitro by DCs and the nanoparticles and mixtures were able to induce upregulation of MHC-II, CD83, and CD86 whereas a solution of plain OVA did not induce DC maturation or T cell proliferation. In vivo studies were also performed in mice by injecting three times with TMC-based formulations containing either OVA or DT. All TMC-containing formulations were shown to increase the IgG titres compared to antigen alone, and induced Th2-based immune responses. Hence TMC was suggested as an immune potentiator for antigens delivered via the skin. The same formulations were applied onto the skin in a solution before or after microneedle treatment with two different 300-μm microneedle arrays and also injected intradermally [72]. As a positive control, alum-adjuvanted DT (DT-alum) was injected subcutaneously. Microneedle-based application of a TMC/DT mixture was also shown to increase the antibody titres when compared to application of the DT solution. However, topically applied DT-loaded TMC nanoparticles were not able to enhance the immune response. Modifying the method of microneedle application or the type of microneedle array used was not helpful in improving the immunogenicity of the TMC nanoparticles.

In another study, reporter antigen expression and immune responses of plasmid DNA-condensed chitosan nanoparticles and pDNA-coated on preformed cationic chitosan/carboxymethylcellulose (CMC) nanoparticles were investigated following topical application onto the skin of shaved mice [42]. Significant levels of luciferase expression was obtained in skin after 24 h, and significant antigen-specific IgG titers to expressed β-galactosidase were seen 28 days after the initial application to the skin. No general correlation between the levels of gene expression in skin at 24 h and IgG titer on day 28 was found for the formulations studied except for chitosan oligomer/CMC nanoparticles with both the highest levels of gene expression and IgG titers.

6 Parenteral Delivery

Non-invasive immunization is a top priority for public health agencies due to the fact that the current immunization practices are unsafe, particularly in developing countries because of the widespread reuse of non-sterile syringes. However, parenteral delivery is still the most valid route. Hence, studies are also being carried out using chitosan for parenteral delivery (Table 1). The efficiency of chitosan-encapsulated DNA-based respiratory syncytial virus (RSV) vaccine was investigated after intravenous injection to mice [73]. The nanoparticles were found to be nontoxic to cells when used at concentrations $\leq$400 μg/mL. Immunohistochemical

and real-time polymerase chain reaction results showed a higher level of RSV protein expression in mouse tissues when nanoparticles were injected intravenously than after injection of naked DNA.

Borges et al. [84] have developed a nanoparticulate delivery system composed of a chitosan core to which HBsAg was adsorbed and subsequently coated with sodium alginate. A potent adjuvant, CpG ODN 1826, that was shown to induce a Th1-type immune response was also associated with the nanoparticles [74]. A high anti-HBsAg IgG titer (2271 ± 120 mIU/mL), with the majority of antibodies being of Th2 type, was observed with the coated nanoparticles. However, regarding cellular immune response, no significant differences were observed for antigen-specific splenocyte proliferation or for the secretion of IFN-γ and IL-4, when compared to the control group. An increase in IgG titers was obtained in the presence CpG ODN 1826 that was not statistically different from the group without the immunopotentiator, whereas a significant increase in the IgG2a/IgG1 ratio of the IFN-γ production by the splenocytes stimulated with the HBV antigen was observed [74]. Chitosan nanoparticles co-administered with the CpG ODN were reported to result in a mixed Th1/Th2-type immune response.

Chitosan microspheres to deliver a fusion protein, Ag85B–MPT64–Mtb8.4 (AMM), from three *Mycobacterium tuberculosis* genes were prepared and C57BL/6 mice were immunized at weeks 1, 3 and 5 subcutaneously with the AMM-loaded chitosan microspheres (6 μm), with AMM in incomplete Freund's adjuvant (IFA), or with AMM in PBS [75]. Splenocytes immunized with AMM loaded chitosan microspheres were found to produce higher levels of IFN-γ compared to that of control. The levels of Ag85B-specific IgG (H+L), IgG1, and IgG2a were also higher than those with AMM in PBS.

7 Concluding Remarks

Chitosan and its derivatives are very promising both as adjuvants and as delivery systems for vaccine delivery, especially in micro- and nanoparticulate forms. Amongst the non-invasive routes studied for antigen delivery, the nasal route seems to be the most suitable, particularly because of ease of administration, absence of degrading enzymes, and safety. However, it is important to maintain contact of the antigen with the nasal mucosa in order to guarantee an efficient antigen uptake. The bioadhesive property of chitosan provides prolonged contact of the antigen and hence a superior uptake, which results in an enhanced immune response. Furthermore, owing to its positive surface charge, chitosan increases the interaction of the antigen with the tissue, which also contributes to improvement of the immune response. These two latter properties make chitosan favorable for vaccine delivery. To date, there are very few studies reported on chitosan-based delivery systems for vaccine delivery in humans. Nevertheless, the results obtained are very promising which encourages researchers to continue their investigations in this field.

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Multifunctional Chitosan Nanoparticles for Tumor Imaging and Therapy

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Abstract Chitosan and its derivatives have been widely used for various biomedical applications because of their unique chemical and biological characters. The amine groups in the backbone of chitosan allow chemical modification to change the physical properties of chitosan. Based on hydrophobic or charge interactions with this chitosan polymer backbone, stable self-assembled nanoparticles can be fabricated in aqueous condition. These nanosized structures enable intravenous injection and show large accumulation in tumor tissue, indicating great potential in imaging and drug delivery. The main objective of this review is to introduce various chitosan nanoparticles and their recent applications for tumor diagnosis and therapy.

Keywords Chitosan · Molecular imaging · Nanoparticle · Tumor

Contents

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1 Introduction

For cancer diagnosis, many noninvasive imaging methods have been developed and applied to living subjects, and they are feasible for assessing the biological or biochemical processes related to cancer metabolism. Various nanoparticles have been developed especially for this purpose, and they have allowed fine tumor imaging in living systems at different time points, providing valuable information. These nanoparticles are also notable tools for drug and gene delivery and could increase the efficacy of therapy. Furthermore, they enable the "theragnosis," the combined diagnosis and therapy, of various diseases, including tumors, whereby imaging agent and drug are included in one nanoparticle. The building blocks of nanoparticles may consist of natural and synthetic polymers that are biocompatible and biodegradable [1–3]. Among them, natural polymers include nucleotides, proteins, and polysaccharides. Polysaccharides are especially privileged materials in nanoparticle fabrication because of their physical and biological properties, such as stability or the sufficiency of functional groups [4, 5].

Among the natural polysaccharides, chitin and chitosan are very widely used in biomedical fields. Chitin is a biodegradable polymer of high molecular weight, and is one of the most common polysaccharides found in nature. It originates from the abundant exoskeletons of crustaceans. Like cellulose, chitin is a fiber, and it presents unique chemical and biological qualities that can be used in various medical applications.

Chitin and chitosan have similar chemical structures, and they are made up of cationic polysaccharide linear chain of D-glucosamine and *N*-acetyl-D-glucosamine linked by glycosidic bonds. Chitosan is obtained by removing enough acetyl groups (CH_3–CO) for the molecule to be soluble in most diluted acids. This process, called deacetylation, releases amine groups (–NH) and gives the chitosan a cationic characteristic. This is especially interesting in an acid environment where the majority of polysaccharides are usually neutral or negatively charged, and these cationic charges of the amine groups enable chitosan molecules to be soluble in acidic aqueous condition. These primary amine groups in a deacetylated subunit of chitosan have a pK_a value of about 6.5. Therefore, chitosan is soluble in acidic solution such as acetic acid, citric acid, aspartic acid, glutamic acid, hydrochloric acid, and lactic acid. However, at neutral or alkaline pH it loses some of the surface charge and becomes insoluble, forming aggregates. In addition to pH, the solubility of chitosan is greatly influenced by its degree of deacetylation and molecular weight, and by the ionic strength of the solution.

This incomplete solubility of chitosan in aqueous condition could be a obstacle in handling for many uses, and therefore various chitosan derivatives were developed

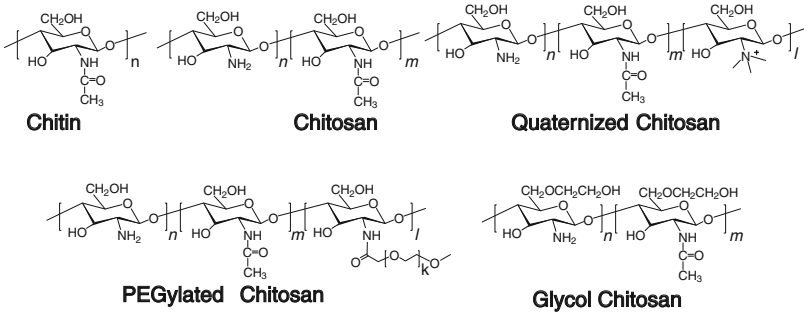

Fig. 1 Chemical structure of chitin, chitosan, and its derivatives

for improved solubility and other purpose (Fig. 1). Quaternized chitosan (*N*,*N*, *N*-trimethyl chitosan; TMC) and polyethelene-glycol-conjugated (PEGylated) chitosan are more soluble in water than the original form. The amine groups of quaternized chitosan are mainly protonated at neutral pH, therefore the overall charge of quaternized chitosan is positive. This positive charge has advantages for good solubility, enhanced cellular uptake, and condensing ability with gene drugs, but chitosan aggregates with anionic serum protein.

PEGylated chitosan is very stable even in serum because of its bioinert PEG group, but it also has lower efficiency of cellular uptake than natural chitosan. Glycol chitosan (GC) is a chitosan derivative containing short ethylene glycol groups in its backbone, and these glycol groups inhibit aggregation with anionic serum protein and provide good solubility, but they are not long enough to inhibit cellular uptake. These chitosan derivatives were developed to improve the properties of original chitosan, and have shown their usefulness in biomedical fields such as imaging and therapy [6–11].

In this article, we will review the application of chitosan-based nanoparticles in tumor imaging and therapy. The first part of the review is focused on the mechanism of efficient tumor accumulation using chitosan nanoparticle. The second part considers the recent applications of chitosan nanoparticles for tumor imaging, and the last part is concerned with antitumor therapy using drug- or gene-containing chitosan nanoparticles.

2 Tumor-Targeted Delivery of Chitosan Nanoparticles

Nanosized particles, with diameters of <100 nm to 1 μm, have been utilized for targeted delivery of imaging agents, drugs, or therapeutic genes. Nanoparticle delivery systems enable the prolonged circulation time of imaging agents or drugs, and can be controlled by targeting tactics [12]. Intravenously administered nanoparticles need to slip through the tumoral blood vessels to reach their target tumor site. Although targeting moieties can improve tumor-homing efficiency,

some nanoparticles show high accumulation at a tumor site without a targeting ligand. Nanosized particles show a tendency to extravasate from leaky blood vessels and to be easily retained in the tumor site. This effect is known as passive targeting for enhanced permeability and retention (EPR) [13]. However, chemically modified or targeted ligands conjugated to chitosan nanoparticles showed better performance for imaging or therapy in many studies. Therefore, localization of nanoparticles at tumor sites depends on a combination of the effects of various factors such as charge and size of particles, and the presence of a targeting ligand.

2.1 Passive Targeting (EPR Effects)

The origin of the EPR concept dates back to the late 1970s, when the selective accumulation of macromolecular drugs in tumor tissues was discovered [13]. The specific passive accumulation of macromolecules was attributed to defective tumor blood vessels with poorly organized endothelium at the tumor site and a poor lymphatic drainage system. Since then, researchers have capitalized on this concept for the delivery of various drugs by conjugating them with polymers or encapsulating them within nanoparticles. Nowadays, it is evident that long-circulating macromolecular polymer–drug conjugates and nanosized particulates such as micelles or liposomes accumulate passively in the tumor tissue due to the EPR effect. To maximize the EPR effect, the size, surface charge, and deformability of the nanoparticles should be considered carefully. Therefore, some chemical modifications have been developed to improve the EPR effect by changing the chemical or physical properties of chitosan nanoparticles.

The EPR effect has been confirmed in various kinds of biocompatible macromolecules, and chitosan-based nanoparticles are also able to passively accumulate in the tumor through this effect. Chitosan has a free amine group in a unit of hexosaminide residue. With this functional group, hydrophobic groups can be conjugated to hydrophilic chitosan or GC polymer to refine the self-assembling capabilities. These polymeric amphiphiles form self-assembled nanoparticles in an aqueous environment via hydrophobic interactions between the hydrophobic parts, primarily to minimize interfacial free energy. Therefore, chitosan-based nanoparticles can be readily obtained by chemically attaching the hydrophobic moiety to the backbone of chitosan and its derivatives. Various hydrophobic groups like oleic acid, cholesterol, stearic acid, deoxycholic acid, 5β-cholanic acid, and some hydrophobic drugs have been conjugated to chitosan and its derivatives. These chitosan conjugates have improved physicochemical and functional properties compared to native chitosan. Furthermore, these self-assembled hydrophobically modified chitosan nanoparticles can encapsulate a quantity of drugs or imaging agents inside the particles or present them on the surface by conjugation. Many studies using self-assembled nanoparticles have been carried out for imaging and drug delivery purposes.

These nanoparticles can circulate in the bloodstream for a relatively long time without recognition by phagocytes and can easily accumulate at the leaky vasculature throughout the EPR effect. In general, the results have demonstrated that nanoparticles based on chitosan and its derivatives are stable in a physiological solution for a long period of time without significant change in the particle size. When these nanoparticles are systemically administrated into tumor-bearing mice, they can circulate in the bloodstream for at least 1 day, thereby increasing the probability of the nanoparticles reaching the target site. It should be emphasized that a significant amount of chitosan-based nanoparticles have been reported to be selectively accumulated into the tumor site, primarily owing to the EPR effect (Fig. 2).

Chitosan conjugated with ethylene glycol is totally water soluble, regardless of pH values, and the GC retains a positive charge [14]. GC has been additionally modified with polymers or hydrophobic analogs to further improve its ability as a drug carrier [15–17]. Previously, Park et al. evaluated three different molecular weights (20, 100, and 250 kDa) of GC nanoparticles [16] to evaluate their effect on the EPR mechanism. The mean diameters, in vitro colloidal stability, and surface charges of the GC nanoparticles were not significantly different. However, fluorescence imaging data revealed that the GC with higher molecular weight (250 kDa) showed longer blood circulation time and higher tumor selectivity. In previous studies, GC nanoparticles modified with deoxycholic acid or bile acid were also characterized [6], and these GC nanoparticles, loaded with drugs such as paclitaxel, cisplatin, or doxorubicin, were applied for cancer therapy (reviewed in Sect. 4.1) [7–9].

PEG and PEGylated nanoparticles have many advantages for biomedical applications, and PEGylation is another representative method for enhanced passive targeting. PEGylation of chitosan nanoparticles also improves their physical stability and prolongs blood circulation time [16]. Paclitaxel- or camptothecin-loaded PEGylated chitosan nanoparticles were investigated and found to be phagocytized less than unconjugated nanoparticles [10, 11].

2.2 *Active Targeting*

The EPR effect is the basis of the tumor targeting and is generally accepted for selective delivery of many macromolecular anticancer drugs. However, drug accumulation in tumor tissue does not always mean a successful therapeutic effect,

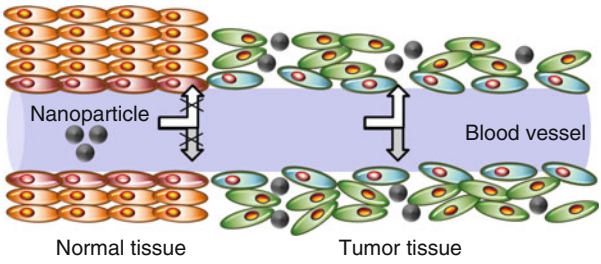

Fig. 2 Illustration of enhanced permeation and retention (EPR) effect and accumulation of nanoparticles in tumor tissue

e.g., for drugs or genes that cannot reach the target cells or inside organelles in their original form. Active targeting is a tactic that enhances the efficiency of a nanoparticle delivery system by using conjugation with targeting moieties, and receptor-mediated endocytosis is the major cellular mechanism used in active targeting. Consequently, overexpression of particular antigens or receptor proteins on the surface of tumor cells is often utilized in these targeting tactics, and the expression of specific receptors on the tumor cells may be helpful for successful active targeting. Ligand-targeted nanoparticles can recognize tumor cells with high efficiency and showed lower toxicity in nontargeted tissues in cases of drug delivery [18, 19]. Several targeting moieties, including specific ligands or antibodies, have been studied and utilized for chitosan-based nanoparticle systems.

Folic acid is a vitamin with a low molecular weight (441 Da), and the folate receptor is known to be overexpressed in various tumor cells, including lung epithelia and ovarian cancer [20–22]. The high binding affinity of folate to its receptors has been applied to modification of chitosan nanoparticles for receptor-mediated endocytosis, and folate is one of the most common molecules in use for active targeting of nanoparticles in cancer research. The improvement of transfection efficiency of delivered gene involves not only targeting ability or endocytosis, but also the nuclear localization ability of the arginine residue of the delivered gene [23]. Folate-conjugated chitosan nanoparticles loading paclitaxe, aminolevulinic acid (5-ALA), pyrrolidinedithiocarbamate, or doxorubicin were applied for cancer therapy [24–27]. Various studies on the active targeting of chitosan nanoparticles using folate and other moieties are summarized in Table 1.

Transferrin is a 80-kDa blood plasma protein that transports iron ions to cells carrying transferrin receptors. Transferin-conjugated chitosans are also used as tumor-targeting nanoparticles, because transferrin receptors are overexpressed in malignant tumor cells. Transferrin, as a targeting ligand, has the advantage that it can be internalized into the cells without the effects of the drug efflux system associated with p-glycoproteins [28]. Moreover, overexpression of the transferrin receptor is closely related to drug resistance in tumor cells [29]. Dufes et al. reported that nanoparticles based on transferrin-conjugated chitosan showed a significantly higher cellular uptake than those with unconjugated chitosan [30].

Glycyrrhetinic acid is the bioactive compound of licorice species, which are perennial herbs [31]. Glycyrrhetinic acid-modified chitosan nanoparticles have affinity to human hepatocellular carcinoma cells (QGY-7703 cells) and demonstrated a 19.0-fold increased uptake in vitro. Doxorubicin (DOX)-loaded glycyrrhetinic acid-modified chitosan nanoparticles could inhibited tumor growth successfully in H22 cell-bearing mice [32].

Short peptides are also favorable targeting moieties for many researchers. In previous studies, some peptides were conjugated to chitosan-based nanoparticles as targeting moieties. Arg-Gly-Asp (RGD) peptide showed increased selectivity for small interfering RNA (siRNA) delivery when linked to chitosan nanoparticles [33]. RGD-linked chitosan nanoparticles showed a high therapeutic efficacy against ovarian carcinoma in vivo. Moreover, bombesin, a 14-amino acid peptide binding to the bombesin receptor, has been studied for therapy of gastric-releasing peptide

Table 1 Cancer-targeted chitosan-based nanoparticles and their active targeting moieties

Targeting moiety	Drug or gene	Remarks	References
Folate	Paclitaxel	Cytotoxicity of paclitaxel-loaded folate-conjugated chitosan nanoparticles was evaluated in vitro	[24]
	δ-ALA	Folic acid–chitosan conjugates were used as a vector for colorectal-specific delivery of 5-ALA for fluorescent endoscopic detection	[25]
	PDTC, doxorubicin	Doxorubicin released from pH-sensitive folate–chitosan micellar nanoparticle. The particle showed the ability for co-delivery of PDTC and doxorubicin in vitro	[26]
	Paclitaxel	Folate-conjugated stearic acid-grafted chitosan oligosaccharides increased cellular uptake in cells expressing abundant folic acid receptors	[27]
Transferrin	Doxorubicin	Transferrin-conjugated chitosan nanoparticles showed an increased uptake efficiency in vitro and tumoricidal activity in vivo compared to the nontargeted chitosan	[30]
Glycyrrhetinic acid	–	Glycyrrhetinic acid-conjugated chitosan nanoparticles showed affinity to human hepatocellular carcinoma cells and could inhibit H22 tumor cells in vivo	[32]
Arg-Gly-Asp (RGD) peptide	siRNA specific for POSTN, FAK, and PLXDC1	RGD–chitosan conjugates, as an αvβ3 integrin-targeted delivery system, was used for targeted siRNA delivery. Enhanced antitumor therapeutic efficacy was confirmed	[33]
Bombesin peptide	–	Bombesin was conjugated to *N*-acetyl histidine-modified GC for targeting gastric-releasing peptide receptors overexpressed in prostate cancer cells. Iron oxide was loaded to be tested as a probe for MRI	[34]
Mannose	Murine IL-12 gene plasmid	Mannosylated chitosan/IL-12 plasmid complexes delivered the plasmid to the dendritic cells around tumor cells, and could inhibit tumor growth in vivo with higher efficiency	[35]

ALA Aminolevulinic acid, *GC* glycolchitosan, *FAK* focal adhesion kinase, *IL-12* interleukin-12, *PDTC* pyrrolidinedithiocarbamate, *PLXDC1* plexin domain-containing protein 1, *POSTN* periostin, *RGD* Arg-Gly-Asp peptide, *siRNA* short interfering RNA

receptor (GRPR)-positive tumors. Bombesin conjugated to *N*-acetyl histidine-modified GC nanoparticles were able to bind to prostate cancer cells (PC-3) over-expressing GRPR, and iron oxide-loaded nanoparticles could be applied as a probe for magnetic resonance imaging (MRI) [34].

Because immature dendritic cells display abundant mannose receptors for endocytosis or phagocytosis, mannose can be applied to active targeting of chitosan nanoparticles. For interleukin-12 (IL-12) gene delivery to dendritic cells around the tumor cells, mannose ligand was considered for modification of the chitosan nanoparticles [35]. The study showed that mannosylated chitosan and IL-12 plasmid complexes could inhibit tumor growth, and that biocompatible mannosylated chitosan nanoparticles were effective as a gene carrier for cancer immunotherapy.

3 In Vivo Tumor Imaging with Chitosan Nanoparticles

Nanoparticles have been studied for biomedical imaging with various imaging agents, which enables noninvasive in vivo live imaging. Molecular imaging modalities for this purpose include optical imaging, positron emission tomography (PET) or single photon emission computed tomography (SPECT), and MRI. Although conventional imaging modalities have been used for molecular imaging of biological targets, these modalities lack the target site specificity required for precise diagnosis of disease. Therefore, targeted delivery of imaging agent using nanoparticles could play an important role in molecular imaging. In addition, each modality has a different character with respect to sensitivity, resolution, time, and cost. Therefore, each imaging modality has its own advantages and limitations; consequently, appropriate choice or combination of these modalities is highly required according to the type and degree of disease.

3.1 Optical Imaging

Light is the representative property that can be applied to imaging, and the emission photons can be produced by bioluminescent proteins, fluorescent proteins, or fluorescent dyes coupled to nanoparticles. The most important advantage of optical imaging is the high sensitivity of visualizing target molecules such as peptides, drugs, DNA, or siRNA. For in vivo optical imaging, probes emitting in the near-infrared fluorescence (NIRF) spectrum are favored because NIRF has emission wavelengths of approximately 600–1,000 nm, which are not well absorbed in biological tissues. Moreover, autofluorescence from connective tissue and other biological fluorophores does not interfere strongly in the NIR [36]. Although some new NIRF probes with high photostability and low cytotoxicity have been developed, polymethines such as Cy5.5 are still the most common NIR fluorophores used for fluorescence imaging in vivo.

Whole-body NIR fluorescence intensity monitoring has been performed in tumor-bearing nude mice after administration of probe containing nanoparticles. Previous studies showed tissue distribution and tumor accumulation of chitosan nanoparticles in vivo. To assess biodistribution of nanoparticles, fluorescent images

are acquired at various time points up to 72 h post-injection using various photon counting scanners such as eXplore Optix system [9, 16], the IVIS Spectrum small-animal in vivo imaging system [34], or Kodak Image Station 4000 MM [37]. Although the tumor-homing efficiency of the nanoparticles was somewhat differentiated according to the different characteristics of each nanoparticle, many studies demonstrated that chitosan-based nanoparticles were accumulated in tumors. Tumor-bearing mice at the peak tumor-accumulation time point can be sacrificed to estimate the tissue distribution of nanoparticles ex vivo. NIRF images of dissected organs and tumors are obtained using cameras with a Cy5.5 filter set (680–720 nm) [9]. The chemical structure, noninvasive fluorescent optical imaging, and biodistribution of Cy 5.5-conjugated chitosan nanoparticles are shown in Fig. 3 [34].

Quantum-dots (QDs) are colloidal semiconductors. They exhibit unique optical and electronic properties that are closely related to the size and shape of each crystal. QDs are in the limelight for optical applications due to their high sensitivity and the stability towards photobleaching. However, they were not originally biocompatible because traditional QDs are made with toxic metal ions, such as cadmium. To increase the stability or the efficacy of delivery, chitosan derivatives have been utilized for coating or encapsulating QDs. Tan et al. developed chitosan

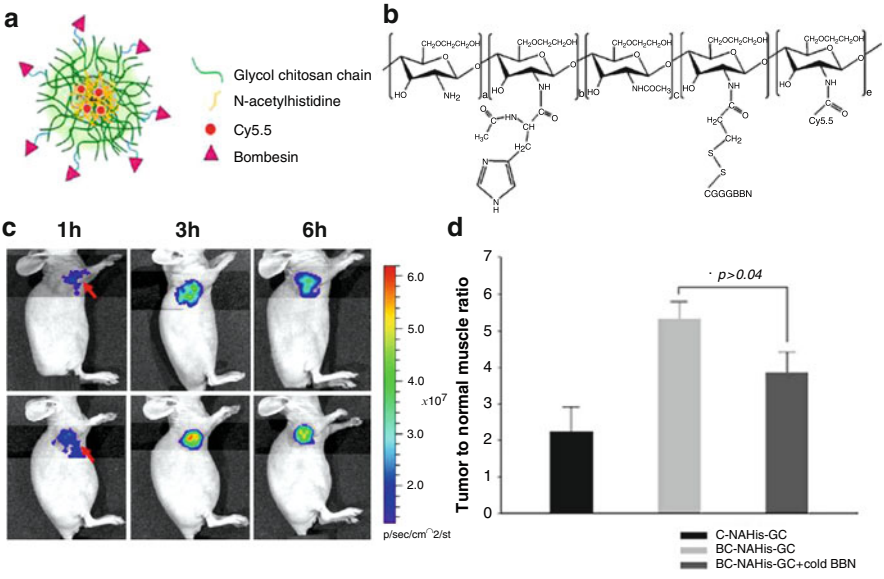

Fig. 3 Fluorescent optical imaging of tumor with bombesin–Cy 5.5-*N*-acetylhistidine–glycol chitosan nanoparticles (BC-NAHIS-GC). (**a**) BC-NAHIS-GC nanoparticle. (**b**) Chemical structure of BC-NAHIS-GC. (**c**) In vivo noninvasive fluorescent imaging of mice with PC3 tumors: C-NAHIS-GC (*upper*) and BC-NAHIS-GC (*lower*). (**d**) Ex vivo quantification of fluorescence in tumor tissue (tumor to normal ratio). From Lee et al. [34]

nanoparticles doped with QDs for delivery of HER-2-specific siRNA, and the results suggested that they are easily internalized into cells [38]. In another study, cadmium–selenium hybrid QDs were crosslinked to chitosan–poly(methacrylic acid) nanogels [39]. The covalently crosslinked hybrid nanogels had good structural stability and changed physical property in response to a pH change to release the anticancer drug temozolomide. In in vitro cytotoxicity tests, there were no signs of morphological damage to B16F10 cells, and free hybrid nanogels were low-cytotoxic in concentrations up to 520 mg/mL for 2 h. These studies only contain in vitro data, but we expect that chitosan nanoparticles have potential for efficient in vivo optical imaging using QDs.

3.2 MR Imaging

MRI has been one of the most powerful noninvasive diagnostic imaging modalities and provides detailed anatomical information for tumor diagnosis [40]. In clinical diagnosis, contrast agents such as iron oxide nanoparticles and gadolinium (Gd)-based molecules are necessary to improve enhancement of signals in the disease tissue surrounding the normal tissue. There are two major subgroups of MR contrast agents that are currently being developed. The first type are positive contrast agents like Gd-based chelates, which cause a reduction in the T1 relaxation time and appear extremely bright on the T1-weighted images [40, 41]. The second type are negative contrast agents such as superparamagnetic iron oxide nanoparticles (SPIONs), which produce a predominantly spin–spin relaxation effect, resulting in shorter T1 and T2 relaxation times [40]. This enables the enhancement of T2-weighted images and gives dark MR images. The functional modification of these MR contrast agents with nanoparticles for efficient delivery, tumor targeting, and molecular imaging has been attempted by many researchers [40, 42, 43].

Among contrast agents, SPIONs have attracted the interest of many researchers over the last 10 years in an effort to achieve an efficient MRI for more accurate tumor diagnosis [40, 44]. Although considerable efforts have been made to develop magnetic nanostructures, further modification is required to improve the physiological character of SPIONs. SPIONs can be easily prepared in nanosizes and show promising magnetic properties. However, it has been shown that bare SPIONs tend to aggregate in suspension, resulting in limitations for clinical applications [40, 42]. Furthermore, for in vivo application when injected intravenously, SPIONs need to be suspended in aqueous solution with good stability. They can be easily functionalized with biological substrate for efficient tumor targeting without rapid elimination of them by the reticulo-endothelial system (RES). Therefore, for in vivo application of SPIONs as MRI contrast agents, stabilization of SPIONs is highly desirable. Surface modification of SPIONs with biocompatible polymers (mostly coated with dextran) such as PEG, polylactic acid, albumin, or hyaluronic acid has been explored to achieve enhanced colloidal stability, prolonged blood circulation,

and improved specificity to target site [42, 45–47]. Surface-modified SPIONs showed enhanced blood circulation and sufficient stability for in vivo applications, such as diagnosis of disease, MR-guided drug delivery, and imaging of cell trafficking and migration [40, 48–50]. There are also several studies on the modification of SPIONs with chitosan-derived polymers, which can be used to coat and stabilize the SPIONS due to their biocompatibility. Shi et al. prepared SPIONs coated with carboxymethyl chitosan (CMCS) for imaging human mesenchymal stem cells (hMSCs) with MRI [51]. In this preparation, CMCS was used for increasing the stability of SPION, resulting in good dispersion in aqueous media and facilitating the uptake of the CMCS-coated SPIONs by stem cells.

Nanoparticles have been introduced into a multidisciplinary research field for various biomedical applications such as diagnosis and therapy. Particularly, the use of polymeric nanoparticles is attractive for the development of multimodality imaging platforms due to their biodegradable and biocompatible character [52–54]. Polymeric nanoparticles can encapsulate SPIONs, providing enhanced MR relaxivity and tumor targeting. Cheong et al. have recently developed SPION-loaded chitosan-based nanoparticles for hepatocyte-targeted gene delivery and imaging [55]. The encapsulation of SPION has been performed using water-soluble chitosan (WSC)–linoleic acid (LA), which formed self-assembled nanoparticles (SCLNs) through conjugation of the hydrophobic moiety, LA. In this study, encapsulated SPIONs in SCLNs with pEGFP (plasmid DNA encoding enhanced green fluorescent protein) are useful for monitoring gene delivery into hepatocytes by MR imaging. Oleic acid-coated SPIONs were prepared with a diameter of 11.7 nm and loaded into WSC-LA nanoparticles by a solvent-evaporation method, resulting in SPION clusters inside the hydrophobic core, with a content of 37.68% (w/w).

As described above, Gd-based chelates were considered as a T1 MRI contrast enhancement agent. Saha et al. reported chitosan-based Gd-DTPA microspheres for Gd neutron-capture therapy and as an MRI agent [56]. However, the size distribution of 3–12 μm makes it difficult to apply in vivo. As an optical/T1 dual imaging agent, Tan et al. prepared chitosan nanoparticles that encapsulated both QDs and Gd-DTPA [57]. They demonstrated that Gd-DTPA within the chitosan nanobeads had a high MR relaxivity, which may be useful for a MR contrast agent, but they did not carry out in vivo experiments. In a similar fashion, we recently reported that Cy5.5-conjugated self-assembled chitosan nanoparticles (CNPs) grafting Gd-DOTA [Cy5.5-CNP-Gd(III)] have been used as an efficient MR and optical imaging probe (Fig. 4) [58]. The highest weight ratio of Gd(III) in the Cy5.5-CNP-Gd (III) nanoparticles was 6.28 wt%, with a diameter of 350 nm. CNPs have proved their potential for in vivo molecular imaging and drug delivery systems due to their efficient accumulation in solid tumor tissues through the EPR effect and their ideal in vivo characteristics, such as biocompatibility and biodegradability, and prolonged blood circulation time. Therefore, Cy5.5-CNP-Gd(III), in which MR and NIRF dual modalities are introduced to the chitosan nanoparticles, can be successfully used for in vivo tumor imaging by optical and MR imaging systems.

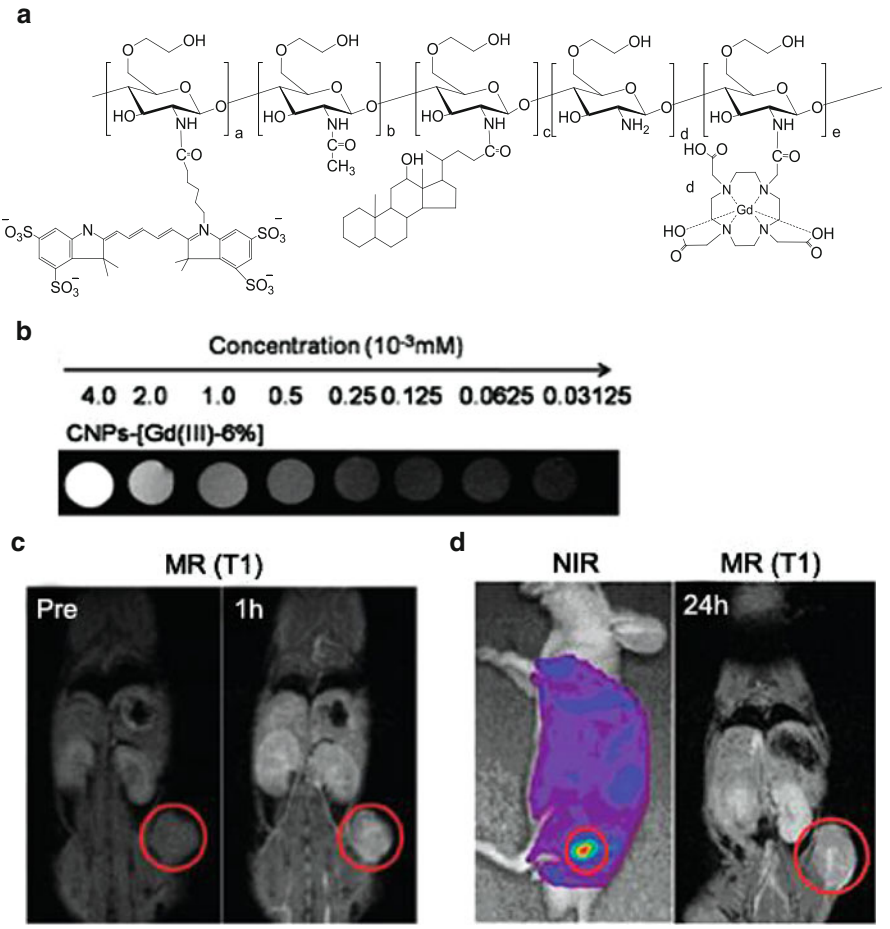

Fig. 4 MR/optical dual imaging with Gd(III) encapsulated in Cy5.5-conjugated glycol chitosan nanoparticles [Cy5.5-CNP-Gd(III)]. (**a**) Chemical structure of Cy5.5-CNP-Gd(III). (**b**) T1-weighted spin-echo MR image (1.5T) of Cy5.5-CNP-Gd(III) in phosphate-buffered saline, showing bright MR signals. (**c**) Coronal slices of T1-weighted MR images of Cy5.5-CNP-Gd(III) in SCC7 tumor-bearing mice. *Circles* indicate SCC-7 tumor tissue. (**d**) NIRF and T1-weighted MR dual images at 1 day post-injection of Cy5.5-CNP-Gd(III) (5 mg/kg). From Nam et al. [58]

4 Application for Tumor Therapy

Various chemical or biological drugs for clinical uses have been incorporated into chitosan-based nanoparticles. Many studies have described the characteristics, efficiency, and safety of these chitosan-based nanoparticle systems containing chemical drug or therapeutic agents, such as antitumor genes or siRNA. Many amphiphilic chitosan derivatives constitute a hydrophobic core of self-assembled

nanoparticles, and these nanoparticles can load hydrophobic drugs inside themselves because of the affinity of drug and core-forming molecules. Therapeutic genes usually require different methods to build up nanoparticles. The cationic chitosan provides electrostatic interaction with negatively charged therapeutic genes, and this charge–charge interaction can be utilized for producing nanosized complexes. These therapeutic nanoparticles can reach tumor sites through the EPR effect and targeting moieties, and they need to overcome intracellular barriers and release the therapeutics into the cytosol for achievement of desired goals [59].

4.1 Anticancer Drug Delivery

Doxorubicin (DOX) is the one of the most widely used anticancer drugs and inhibits the nucleic acid synthesis of cancer cells. DOX has many advantages as an anticancer drug, but it shows a number of side-effects on heart and bone marrow, resulting in a narrow therapeutic index [60]. Since targeted DOX delivery to cancer cells can reduce the side effects, researchers have attempted to load DOX to tumor-homing nanoparticles. DOX has a primary amino group and becomes positively charged at pH 7.4. DOX in its natural form is hydrophobic, and it is commonly used in a hydrochloric acid salt formulation [61]. In a previous study, DOX-GC conjugates containing up to 5 wt% DOX formed self-assembled nanoparticles in aqueous solution [62]. The final DOX loading content in the nanoparticles was 38.9%, and the DOX-GC conjugates precipitate at DOX loadings above 5.5 wt% because of increased hydrophobicity. The physicochemical properties and the cytotoxicity of DOX-modified and loaded GC nanoparticles was evaluated [7]. DOX-GC nanoparticle showed lower cytotoxicity than the free form of DOX. In another study, DOX and dextran conjugates were encapsulated in 100-nm chitosan nanoparticles [63]. When encapsulated in the chitosan nanoparticles, DOX-dextran enabled faster in vivo tumor regression. In addition, several active targeting chitosan nanoparticles using transferrin or folate could also deliver DOX to tumor cells with high efficiency [26, 30].

As a mitotic inhibitor, paclitaxel (PTX) is another drug actively used in cancer chemotherapy. PTX is soluble in diverse organic solvents, but poorly soluble in aqueous solutions. Originally, it was dissolved in Cremophor EL (polyethoxylated castor oil) and ethanol, but it bound to albumin and showed unintended toxicity. Previous studies using chitosan-based nanoparticles loaded with PTX presented relatively low cytotoxicity compared to PTX in Cremophor EL. Hydrophobically modified GC nanoparticles, 200–400 nm in size, had a similar antitumor effect to PTX formulated in Cremophor EL without severe toxicity [8]. Qu et al. demonstrated that PEGylated chitosan micelles also showed decreased plasma protein adhesion and increased circulation time, thus evading the elimination of PTX in the late stage of intravenous injection [10]. Docetaxel is a semisynthetic analog of PTX, and was loaded into hydrophobically modified GC nanoparticles in an attempt to reduce the side effects of free docetaxel by using tumor targeting [64].

Camptothecin (CPT) is a hydrophobic quinoline alkaloid that inhibits the enzyme topoisomerase I. FDA-approved CPT-based anticancer drugs are used in first therapy of cancer with 5-fluorouracil [60], but insolubility and rapid hydrolysis have prevented wide clinical applications of CPT in cancer therapy. CPT has an anticancer active lactone form in acidic conditions (pH < 5), which becomes an inactive carboxylate form at basic pH [65]. CPT was incorporated into polymeric micelles using *N*-phthaloylchitosan-grafted polyethylene glycol methyl ether (mPEG), and *N*-trimethyl chitosan (TMC) or hydrophobically modified GC nanoparticles [11, 66, 67]. Stability tests investigating the preservation of CPT within the nanoparticles under physiological condition (pH 7.4, 37°C) and optimal controlled release of the active lactone form supported the idea that modified chitosan-derived nanoparticles have considerable potential as CPT carriers. The biodistribution, tumor targeting ability, time-dependent excretion profile, and anticancer efficacy of CPT-GC nanoparticles were also confirmed in vivo by noninvasive animal imaging systems [67]. Liu et al. evaluated the high therapeutic effects of TMC nanoparticles with the recorded tumor growth and histopathological changes (Fig. 5) [66]. In addition, quantitative analysis of expression of a specific protein like proliferating cell nuclear antigen (PCNA) supported the possible therapeutic value of the nanoparticles. The tumor volume in the group treated with CPT-loaded nanoparticles was significantly smaller, and PCNA expression was also reduced compared with other groups. As shown, chitosan-based nanoparticle systems can be practically applied for delivery of hydrophobic anticancer drugs.

4.2 Photodynamic Therapy

Photodynamic therapy (PDT) is one of the therapeutic methods especially used for the treatment of various cancers [68]. In this method, a photosensitive drug called the photosensitizer is injected locally, intravenously or intraperitoneally, and should accumulate in target tissue with high concentration. Because of the fluorescence of photosensitizers and their high concentration in the target tumor site compared with surrounding normal tissue, the visualization and imaging of tumors are enabled by fluorescence [69]. To achieve efficient tumor therapy, the tumor should be exposed to laser light, and the laser-excited photosensitizer produces reactive oxygen species like singlet oxygen ($^1O_2$). These can kill tumor cells by phototoxic damage to major cellular organelles such as mitochondria [70]. Especially for in vivo therapy, laser of the red or near-infrared wavelength is more widely used than blue wavelengths, because of the differences in absorbance by photosensitizers. This optimal absorption wavelength of the photosensitizer is a crucial factor in PDT because the light of a longer wavelength can penetrate through the tissue deeply and enable maximum therapeutic results for large tumors. PDT has been applied clinically for the treatment of a variety of cancer types. In the last decade, many promising results have been obtained for lung cancer, head and neck cancer, skin cancer, locoregional breast cancer recurrences, and prostate

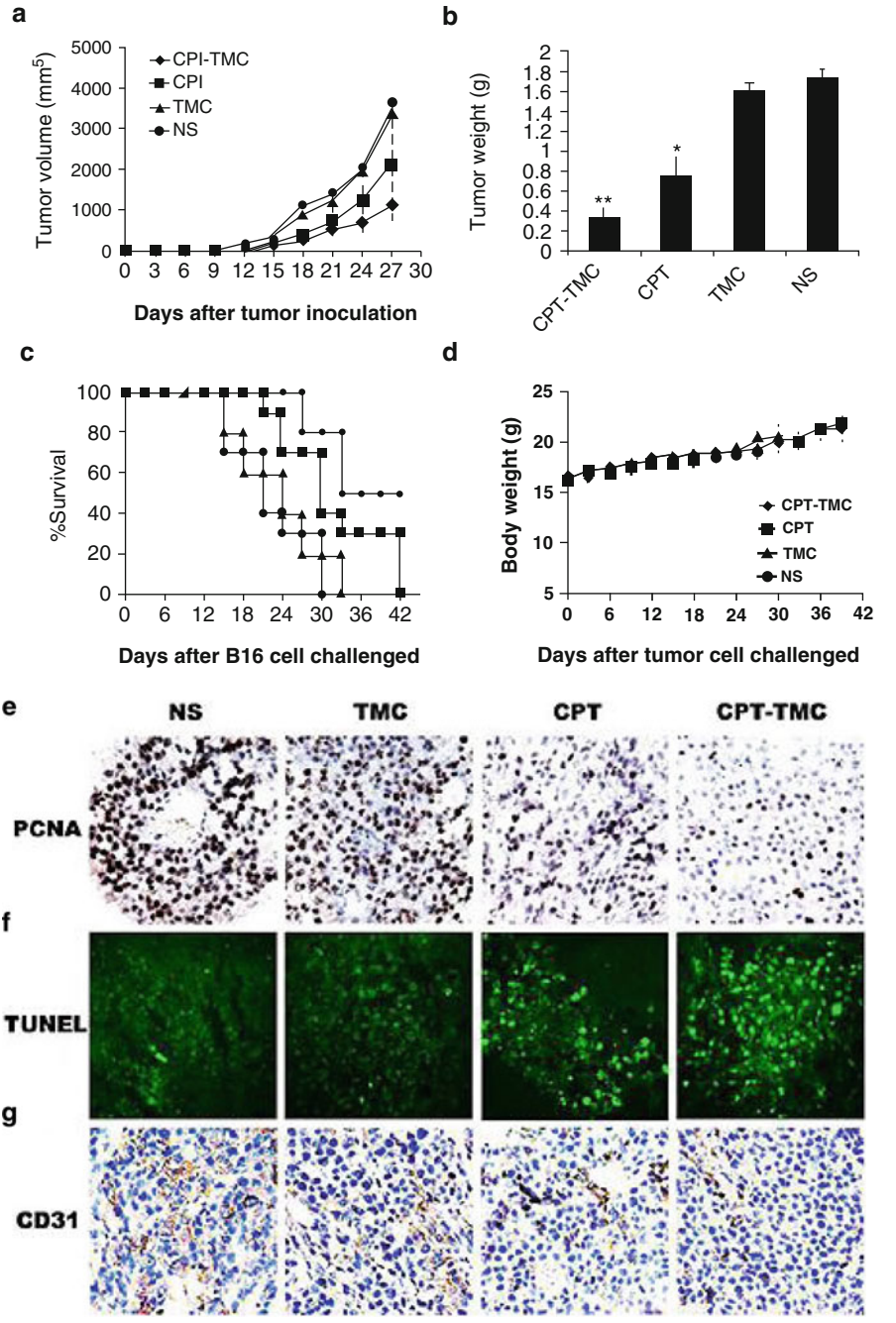

Fig. 5 Tumor therapy with camptothecin encapsulated in *N*-trimethyl chitosan nanoparticles (CPT-TMC). (**a**) Tumor volume growth curve of B16-F10 tumor-bearing mice with CPT-TMC. Comparison of (**b**) tumor weight, (**c**) survival curves, and (**d**) body weight changes in mice treated

cancer [71, 72]. PDT is also an attractive clinical modality for several other therapeutic applications like inflammations [73].

In spite of these promising results, there are some problems still to be solved in current PDT. The major limitation is the nonselective accumulation of the photosensitizers, which could cause severe damage to normal tissue after laser irradiation. Moreover, patients should stay in the absence of sunlight for a long time after the administration of the photosensitizer, because photosensitizers may result in skin phototoxicity. Various delivery methods such as dendrimers, liposomes, micelles, and nanoparticles have been developed to overcome these problems [74, 75].

Chitosans and chitosan derivatives are also attractive materials in PDT. Chen et al. used GC as immunoadjuvant in photodynamic therapy with two photosensitizers, photofrin and *m*-tetrahydroxyphenylchlorin [73]. The treatment with GC could enhance antitumor immune response and the effects were more highly effective than other immunoadjuvants like complete Freud adjuvant or *Corynebacterium parvum*. The combination of immunological stimulation by GC and active tumor destruction by photosensitizers could significantly enhance the cure rate of tumor therapy.

Hu et al. encapsulated chlorine e6 (Ce6) in stearic acid-grafted chitosan micelles [76]. The size of micelles was 270–300 nm and they could contain 5–20% Ce6. They were stable spherical nanostructure in aqueous condition and enabled sustained release of Ce6 over 12 h. Importantly, they could enhance the uptake of Ce6 to A549 (human lung cancer) and HeLa (human cervix carcinoma) cells, and showed high phototoxicity, proving their potential for cancer therapy.

In vivo PDT with chitosan was performed by Schmitt et al. [77]. They used chitosan nanogel prepared by crosslinking with tripolyphosphonate and Ce6, as photosensitizer. This chitosan nanogel was about 40–140 nm and could more efficiently deliver Ce6 to macrophage cells than fibroblast cells. Macrophages play a crucial role in inflammatory disease, and they are major targets of clinical treatments at the inflammation site. In vivo evaluation of serum amyloid A (SAA) levels showed the clinical results of a rheumatoid arthritis model. SAA levels after PDT with Ce6 encapsulated in chitosan nanogel were comparable with those obtained after intra-articular injection of methylprednisolone, the currently used corticoid for rheumatoid arthritis patients.

Recently, Lee et al. showed highly efficient PDT with an in vivo tumor model using chitosan-based nanoparticles (Fig. 6) [78]. They prepared self-assembled amphiphilic glycol chitosan–5β-cholanic acid conjugates and encapsulated the hydrophobic photosensitizer, PpIX, with a high drug-loading efficiency of over

Fig. 5 (Continued) with CPT-TMC and in mice treated with CPT or TMC alone. NS indicates treatment with normal saline. (**e**) Tumor sections immunostained with an antibody against PCNA showing the differences in positive nuclei in tumor tissues. (**f**) Apoptosis of tumor tissues examined by TUNEL assays. (**g**) Tumor sections immunostained with anti-CD31 antibody (*brown*) for angiogenesis assay. From Liu et al. [66]

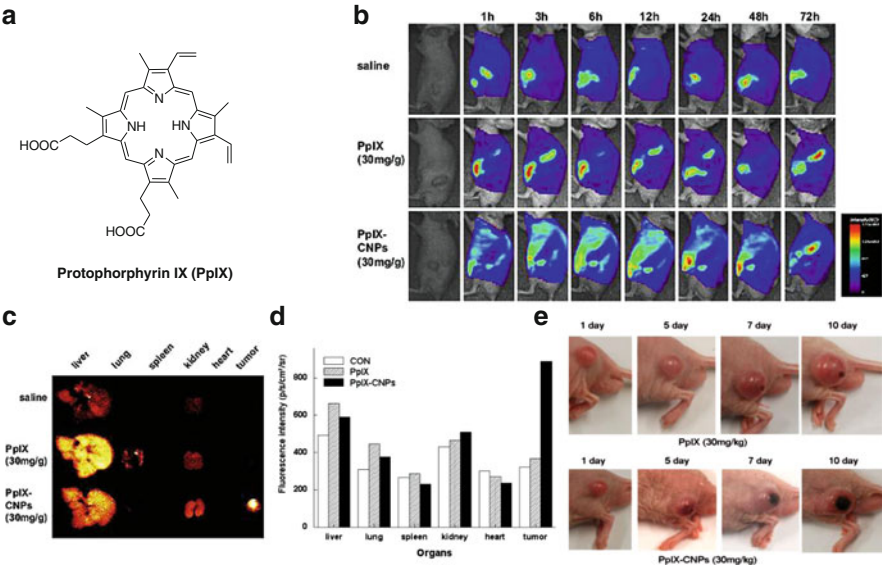

Fig. 6 Photodynamic therapy with protophorphyrin IX encapsulated in glycol chitosan nanoparticles (PpIX-CNP). (**a**) Chemical structure of the photosensitizer PpIX. (**b**) In vivo time-dependant whole-body imaging of athymic nude mice bearing SCC7 tumors after i.v. injection of PpIX-CNPs. (**c**) Ex vivo images of normal organs (liver, lung, spleen, kidney and heart) and tumors excised at 3 days post-injection of PpIX-CNP (**d**) Quantification of in vivo biodistribution of free PpIX and PpIX-CNPs recorded as total photon counts per second per centimeter squared per steradian (p/s/cm^2/sr) for each excised organ. (**e**) Photodynamic therapeutic efficacy of free PpIX (30 mg PpIX/kg body weight) and PpIX-CNPs (30 mg/kg of PpIX) in SCC7 tumor-bearing mice. From Lee et al. [78]

90%. These PpIX-loaded chitosan-based nanoparticles (PpIX-CNPs) showed a sustained release profile of PpIX and were biocompatible to tumor cells without irradiation. With SCC7 murine cell carcinoma cells, we observed fast cellular uptake of the PpIX-CNPs and the released PpIX exhibited high phototoxicity after laser irradiation. In vivo imaging and therapy with SCC7 tumor-bearing mice showed enhanced tumor specificity and increased therapeutic efficacy of PpIX-CNPs compared to free PpIX. These results indicated that these chitosan-based nanoparticle systems have great potential as fine delivery system for photodynamic therapy.

4.3 Gene Delivery

Research on gene delivery has expanded during the past 20 years because gene therapy is a potential candidate for therapies to overcome many obstinate diseases. Gene materials include plasmid DNA (pDNA), oligonucleotides, and small RNA

molecules. Recently, the use of siRNA for gene silencing has been paid more attention because of its highly specific silencing ability. The siRNAs targeting overexpressed oncogenes or mutated tumor suppressor genes are being vigorously investigated in cancer studies. In spite of the specific gene suppression effect, therapeutic genes are not yet widely used in clinical trials because naked therapeutic genes are easily degraded by nucleases in plasma and are rapidly cleared through the kidneys. Moreover, they show poor cellular uptake and low transfection efficiency in their original form due to the strong negative charge of phosphate backbone [79, 80]. Therefore, many kinds of viral vectors or nonviral vectors are being developed for gene therapy. The transfection efficiency of nonviral vectors is still lower than that of viral vectors, but they have advantages of safety and cost.

Chitosan-based carriers are being used as one of the typical nonviral vectors. The primary amine groups in the chitosan backbone present a positive charge at acidic pH, and this cationic property is utilized for application as a gene delivery carrier. The cationic chitosan backbone can interact with anionic pDNA or siRNA to form stable nanosized particles in an aqueous condition. At this moment, it is crucial to consider the proper ratio of chitosan derivatives to therapeutic genes for obtaining particles with desirable physicochemical properties. The physicochemical properties such as size and electrical ζ-potential are important because the stability of the particles and the ability for gene delivery are entirely dependent on them. The particle size of the chitosan–gene complex is also dependent on the molecular weight of the chitosan polymer. The degree of chitosan deacetylation can affect DNA binding, release, and transfection efficiency [81, 82]. Previous studies reported that chitosan–siRNA nanoparticles formed using chitosan of high molecular weight (114 and 170 kDa) and high degree of deacetylation (84%) formed the most stable structure and exhibited the highest gene knockdown in vitro [81, 83].

Numerous researchers have studied chitosan nanoparticles as carriers of pDNA, and showed that PEGylation or thiolation of chitosan prolongs the plasma circulation time and enhances the transfection efficiency of pDNA [84, 85]. In a previous study, combination of PEGylated chitosan and low molecular weight polyethylenimine (PEI, 1.2 kDa) demonstrated effective gene delivery and transfection in mice bearing C6 xenograft tumors [47]. Another study implied the use of chlorotoxin–chitosan conjugates to enhance uptake into glioma cells in vivo with the same animal model and gene (pEGFP-CS2) [86]. Nanocarrier uptake into the cancer cells and the biodistribution including tumor accumulation were confirmed in the paper. In another paper, gene therapy using the p53 gene and urocanic acid-modified chitosan was described [87]. Urocanic acid-modified chitosan nanoparticles mediated p53 transfection in HepG2 cells and induced high levels of p53 mRNA and protein. Consequently, apoptosis and growth inhibition of HepG2 cells were observed in vitro and in vivo (Fig. 7).

siRNA is the small RNA molecule that is typically used for the technique of RNA interference. The siRNA, when introduced into cells, forms RNA-induced silencing complexes and induces degradation of homologous mRNA and knockdown of the relevant protein [88]. Previous studies introduced a chitosan-based siRNA nanoparticle delivery system, which demonstrated 77.9 and 89.3%

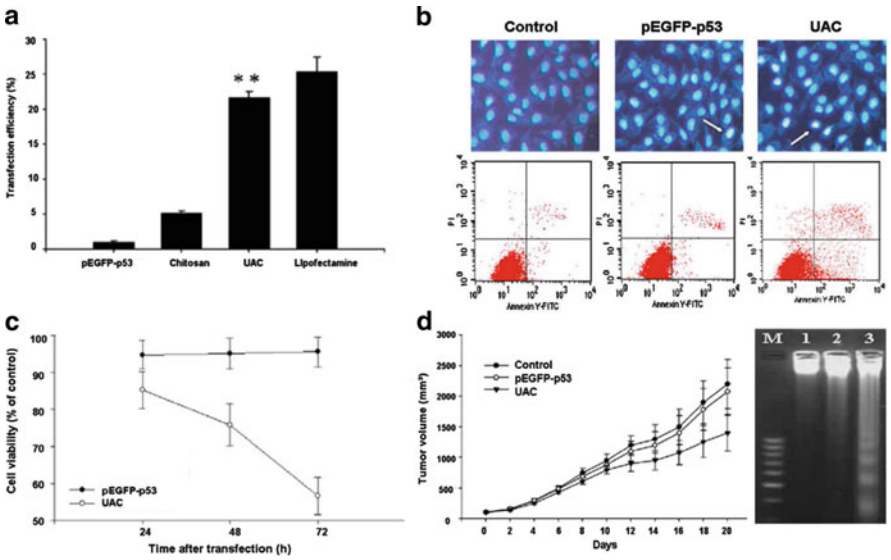

Fig. 7 Gene therapy with p53 gene (PEGFP-p53) and urocanic acid-modified chitosan nanoparticle (UAC). (**a**) In vitro transfection efficiency examined with pEGFP gene. (**P < 0.01 compared with untreated control). (**b**) DAPI staining for apoptosis imaging and FACS analysis for Annexin V. *Arrows* indicate typical apoptotic cells. (**c**) Inhibitory effects of UAC-mediated p53 transfection on the proliferation of HepG2 cells. (**d**) Tumor suppression of HepG2 tumor-bearing mice after intratumoral injection of naked DNA and UAC/pEGFPp53 complexes (*left*). DNA fragmentation assay on HepG2 nude mice xenografts after treatments (*right*). *Lane M* marker, *Lane 1* control, *Lane 2* pEGFP-p53 alone, *Lane 3* UAC/pEGFP-p53 complexes. From Wang et al. [87]

reduction of endogenous EGFP in H1299 human lung carcinoma cells and murine peritoneal macrophages in vitro [89]. Subsequently, siRNA delivery with tumor-homing chitosan-based nanoparticles was evaluated in vivo. Huh et al. introduced strongly positively charged PEI polymer to GC polymer–siRNA complexes [90]. GC-PEI nanoparticles carried red fluorescent protein (RFP)-matched siRNA to the tumor site of RFP-expressing B16F10 tumor-bearing mice, and RFP suppression was confirmed in vivo. Although low transfection efficiency of the polymer carriers has not been fully overcome, chitosan-based nanoparticles are still strong candidates for gene delivery systems in cancer treatment.

5 Conclusion

In this review, recent applications of chitosan nanoparticles for tumor imaging and therapy have been discussed. Recently, chitosan has been applied to molecular imaging based on many imaging agents, which enables in vivo live imaging. It also has profitable chemical and biological characteristics as a drug carrier, and hence

chitosan-based nanoparticles have been widely investigated for tumor therapy. Different groups of researchers have studied various sizes and characteristics of chitosan nanoparticles using chitosan polymers of different molecular weight and deacetylation degree [91]. In addition, diverse chitosan nanoparticles can be further modified by chemical conjugation with amine groups in the chitosan polymers. Hydrophobically modified, PEGylated, glycolated, or ligand-conjugated chitosan nanoparticles can improve their solubility, stability, passive targeting abilities, and cellular uptake efficiency.

Use of chitosan-based nanoparticles with diverse probes and imaging modalities could be used to trace biological and biochemical processes in living subjects. In previous studies, real-time in vivo imaging and treatment monitoring were carried out effectively. In addition, active targeting and controlled drug release enables enhanced tumor targeting and therapy. Although some chitosan-based nanoparticles that include drugs or other therapeutic agents displayed insufficient drug delivery efficiency, others showed large tumor accumulation and outstanding therapeutic effects with rational design and optimization. With the advantages of chitosan and its derivatives, such as biocompatibility or the convenience of availability, this kind of review on tumor targeting chitosan-based nanoparticles should be helpful to many biomedical researchers.

The current chitosan-based nanoparticles can be further modified to have better properties for an ideal carrier of imaging agents or drugs. Therefore, we expect that these chitosan nanoparticles will continue to provide attractive and advanced tools as nanocarriers, especially for cancer research and treatment.

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Chitosan-Coated Iron Oxide Nanoparticles for Molecular Imaging and Drug Delivery

Hamed Arami, Zachary Stephen, Omid Veiseh, and Miqin Zhang

Abstract Iron oxide nanoparticles (IONPs) are a new class of nanomaterials which have attracted extensive interest for application in in vivo magnetic resonance imaging (MRI) due to their intrinsic superparamagnetic and biodegradable properties. Performance of the IONPs is largely dependent upon the properties of their surface coatings, which serve to prevent nanoparticle agglomeration, reduce the risk of immunogenicity, and limit nonspecific cellular uptake. Among the coating materials studied to date, chitosan has drawn considerable attention. Commonly derived from crustacean shells, chitosan is a natural linear polysaccharide and has ample reactive functional groups that can serve as anchors for conjugation of therapeutics, targeting ligands, and imaging agents. Because of these unique attributes, chitosan-coated IONPs are becoming more desirable for cancer imaging and therapy applications. This chapter discusses the current advances and challenges in synthesis of chitosan-coated IONPs, and their subsequent surface modifications for applications in cancer diagnosis and therapy.

Keywords Cancer · Chitosan · Drug delivery · Gene therapy · Imaging · Iron oxide · Magnetic nanoparticles · MRI · Nanomedicine

Contents

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1 Introduction

Recent advances in nanotechnology are providing new opportunities for the development of advanced nanoparticle formulations for biomedical applications. One of the greatest benefactors of this research is in the area of oncology, where these nanoparticles have the potential to drastically improve the diagnosis and treatment of many forms of cancer [1, 2]. A highly promising avenue of this research is the development of iron oxide nanoparticle (IONP) formulations [3, 4]. IONPs uniquely possess intrinsic superparamagnetic properties that enable noninvasive tracking through magnetic resonance imaging (MRI). IONPs are biodegradable and amendable for surface modifications. Because of these unique traits, IONPs have become an attractive platform for the development of new cancer diagnostic and therapeutic tools for a broad spectrum of applications, such as in vivo molecular imaging, drug delivery, and gene therapy [5–10].

Critical to the engineering of successful IONP formulations is the development of suitable nanoparticle surface coatings. Uncoated IONPs aggregate in biological solutions due their large surface area to volume ratio, forming large clusters and rending them unsuitable for biomedical applications. Therefore, an appropriate coating is needed to reduce the surface free energy in order to protect the nanoparticles against agglomeration and to serve additional functions. In the past decades, a number of materials have been evaluated as IONP coatings, including polyethylene glycol (PEG) [11], dextran [12], polyethyleneimine (PEI) [13], phospholipids [14], and more recently chitosan [5].

Chitosan is a naturally found polysaccharide that is derived from chitin, the major component of the exoskeleton of crustaceans. It is nontoxic, biocompatible, and biodegradable. Chitosan differs from other commonly available polysaccharides in that it is readily amendable to further modification due to the presence of amino and hydroxyl functional groups in its molecular structure. Furthermore, it is cationic, and has the ability to form polyelectrolyte complexes with metals and anionic biomolecules [15]. Combined, these biological and chemical traits render chitosan a highly desirable biomaterial for drug delivery applications [16, 17]. For decades, chitosan and its derivatives have been used as polymeric nanoparticle formulations for the delivery of therapeutics [15, 16, 18]. However, considerable interest has recently grown in evaluating chitosan-based polymers as coating materials for IONPs [19].

In the last 5 years, the unique combination of chitosan and IONPs has paved the way to several emerging breakthroughs in oncology-directed nanomedicine applications, such as development of IONPs capable of permeating across the

blood–brain barrier (BBB) for brain tumor imaging [5] and IONPs as targeting and magnetic nanoparticles for cancer cell-specific chemotherapeutic and gene delivery [20]. Herein, we review the current synthesis and coating strategies for development of chitosan-coated IONPs and their potential applications for drug delivery and gene therapy.

2 Synthesis of IONP Cores

The first step towards the development of chitosan-coated IONP nanoparticles is the synthesis of uniformly shaped and sized IONP nanoparticle cores. Over the years, many approaches have been evaluated for the synthesis of IONP cores [21]. These approaches can be generally divided into two groups: top-down (mechanical attrition) and bottom-up (chemical synthesis) approaches [22]. Of the two routes, the chemical synthesis approach is generally favored as it is better suited to production of nanoparticles with uniform size and shape (typical deviation is less than 10% of the nanoparticle batch) [23]. The chemical synthesis approach can be further subcategorized into two major chemical processes: coprecipitation and thermal decomposition. Both chemical processes have been exploited for the development of chitosan-coated IONPs; however, each process has its own advantages and limitations, as discussed below.

The coprecipitation of iron chlorides is the most commonly used approach [24]. This synthesis scheme is based on the coprecipitation of Fe^{2+}/Fe^{3+} salt solutions through the addition of base to an aqueous mixture under inert conditions. The products of this reaction are mixtures of γ-Fe_2O_3 (maghemite), and Fe_3O_4 (magnetite) nanocrystals [21, 24]. Of the two crystal structures, magnetite is preferred because it produces superior magnetic properties [3]. To favor more magnetite formation the reaction conditions need to be optimized through adjusting the molar ratios of iron chloride species, strength of base added, and reaction temperatures [24].

The size and morphology of the nanocrystals formed by this method can be controlled by regulating the kinetics of the reaction, specifically by separating the nucleation and growth phases of nanocrystal formation. This can be accomplished by controlling the rate at which the pH of the reaction solution is increased during the coprecipitation reaction. Nucleation occurs rapidly when the pH of the solution is sufficiently high (more than pH 9) to cause the reactants to reach a supersaturation limit and begin to precipitate from the solution. As the pH of the solution is further increased, the IONP nuclei formed begin to grow in size through what is referred to as the growth phase of nanocrystal formation. The kinetics of the growth phase is much slower and relies upon further diffusion of the reactants from solution to nuclei surfaces. In order to ensure that uniformly sized nanoparticles are produced, it is crucial to control the rate of pH change during coprecipitation of the IONP [25].

The major advantage of the coprecipitation chemical scheme for synthesis of IONP cores is that the produced nanocrystals are hydrophilic and do not need additional processing steps or modifications to ensure their stability in aqueous solutions. Water dispersion is a key requirement for the preparation of nanoparticles for biomedical use, and thus this approach has been most prevalently applied for preparation of chitosan-coated IONPs. Other advantages of this strategy are its potential for large-scale manufacturing, cost efficacy, and ease of production. Conversely, the major limitation of coprecipitation is the difficulty in maintaining consistency in nanoparticle size and morphology between batches. This can be circumvented with some degree of tolerance through the accurate control of synthesis parameters such as reactant type and concentration, rate of pH change, temperature, and ionic strength of the reaction solution [21, 26–29].

Thermal decomposition of iron organometallic compounds such as ferrous acetylacetone [$Fe(acac)_3$] and $Fe(CO)_5$ and subsequent seed growth is another method for the synthesis of IONPs of magnetite and maghemite nanocrystals [30]. Surfactants are needed in these reactions to provide a matrix that restricts nanoparticle growth after nucleation. A commonly used surfactant is a combination of 1,2-hexadecanediol, oleic acid, and oleylamine. The IONPs produced through this method of synthesis are coated with ocleic acid and are only dispersible in organic solvents post-synthesis. To control the size and morphology of nanoparticles prepared using this method, reaction parameters such as time, temperature, precursor type, precursor ratios, and addition of external seeds can be modulated to produce IONPs with desired specifications [21].

The IONPs prepared through thermal decomposition are superior in terms of materials properties to those prepared through coprecipitation reactions. This is mainly attributed to the fact that thermal decomposition reactions occur at significantly higher temperatures than coprecipitation and thus allow for higher rate of diffusion of reactants during the growth phase. Consequently, the IONP cores produced are highly uniformly sized, shaped, and monodispersed. They also form perfect crystals of homogeneous composition. This higher order crystallinity produces superior magnetic properties such as saturation magnetization, which corresponds to enhanced detectability by MRI.

However, there are several major drawbacks in using IONPs prepared through thermal decomposition for biomedical applications. The most notable limitation is that the surfactants used in these reactions are considered toxic. Further, these molecules are difficult to remove from IONP formulations after synthesis and pose long-term safety concerns, which have precluded their use in clinical settings. Another challenge is that the produced nanoparticles are hydrophobic in character and require additional postpreparative processing to either remove the hydrophobic surfactants, or covalently modify the surface with hydrophilic polymers to render the nanoparticles water-soluble [31].

3 Synthesis of Chitosan-Coated IONPs

3.1 Chitosan

Chitosan is a biocompatible natural linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units. Chitosan is produced commercially by deacetylation of chitin, the structural element in the exoskeleton of crustaceans and the cell walls of fungi. After cellulose, chitosan is the most abundant natural polymer. In recent years, increasing attention has been paid to chitosan as an underutilized biomaterial in various applications, including water treatment, membranes, hydrogels, adhesives, antioxidants, biosensors, food packaging, and drug delivery [32, 33].

Chitosan possesses several unique biological and physicochemical properties that render it specifically suitable for biomedical applications. One of the most desirable traits is its biodegradability [34]. Chitosan can be broken down in the body by specialized enzymes (chitonases) produced by bacteria found in the colon of humans. Chitonases can degrade polymers of chitosan to small fragments that can then be readily eliminated by the body through renal clearance. Biodegradation is crucial to development of pharmaceutical reagents because it ensures a desirable metabolic fate in the body, which eliminates safety concerns [35]. Numerous studies have confirmed that chitosan is nontoxic to humans and other mammals, and currently the US Federal Drug Administration is considering listing chitosan as a "generally regarded as safe" (GRAS) material for pharmaceutical and food applications [36].

A key physiochemical trait of chitosan is its unique molecular structure, which contains abundant reactive hydroxyl and amino functional groups. These functional groups provide anchor sites for further conjugation of functional molecules to chitosan. The abundant amino functional groups present in the molecular structure of chitosan also ensure a net cationic character, which enables electrostatic complexation with anionic drugs and biotherapeutics. Combined, these functionalities render chitosan highly amendable to further modification, and a desirable polymer platform for a broad range of biomedical applications.

Chitosan's initial use as a biomaterial was prominently for tissue engineering applications, where chitosan has been used as scaffolding material for tissue regeneration [33]. For these applications, chitosan's unique bioadhesive properties proved to be highly beneficial in promoting cell adhesion growth [37, 38]. More recently, chitosan has been evaluated as a biomaterial for the development of carriers for drug and gene delivery applications [15]. Here chitosan's flexibility for covalent modification and complexation with anionic biotherapeutic molecules has enabled the development of many promising carrier formulations [15, 18]. These carrier formulations have proven be highly effective in improving the therapeutic index of therapies delivered to target cells by improving the cell permeation of drug compounds. For gene therapy applications, the anionic character of chitosan enables nucleic acid loading, and protects this therapeutic cargo against degradation

by systemically present nucleases [39, 40]. These nucleic acid-loaded chitosan-based carriers can then readily permeate cell membranes to deliver gene therapeutics. It is believed that these carriers may circumvent many limitations associated with the conventional viral approach because they are more biocompatible, and nonimmunogenic [16].

Modified versions of chitosan, known as chitosan derivatives, are also being developed to improve the physicochemical properties of chitosan for drug delivery applications. For example, water solubility of the chitosan is a very important characteristic and can be improved by addition of new hydrophilic functional groups or polymers to its molecular structure [41–51]. Grafting of PEG to chitosan (PEGylation) has been reported as one of the most widely applied modifications for this material. Such a process can increase the physical stability of the chitosan in biological media and its circulation time in blood [51]. Application of some other types of biocompatible polymers, such as alginate, hyaluronic acid, and pluronic acid, can also enhance the stability and pharmacological performance of the chitosan [18, 52, 53].

3.2 Synthesis of Chitosan-Coated IONPs

The application of chitosan as coating material for IONPs is a relatively new and emerging field of research, but is believed to provide new opportunities for the development of IONP-based imaging and therapeutic delivery tools or devices. To accomplish coating of IONPs, numerous methods have been reported in the literature. A list of the various strategies used to develop chitosan-coated IONPs is given in Table 1. These methods can be generally categorized as ex situ or in situ methods. Through the ex situ methods, the IONPs are first produced using methods described in Sect. 2, and then stabilized with a chitosan-based coating after initial purification of the IONPs. By contrast, using the in situ method, chitosan-coated IONPs are synthesized in the presence of the chitosan-based polymers and thus the IONPs are simultaneously produced and coated with the polymer.

3.2.1 Ex Situ Coating Approaches

Ex situ coatings of chitosan can be applied to IONP cores produced through either coprecipitation or thermal decomposition methods. IONPs synthesized through thermal decomposition are hydrophobic in character and can only be dispersed in nonpolar organic solvents [54]. Conversely, IONPs prepared through coprecipitation are hydrophilic and can readily be disperse in polar aqueous solvents [55].

Physical interaction between the nanoparticles and chitosan or its derivatives is the most commonly used method for stabilization of the IONPs. For example, Zhu et al. [56] used different functional polysaccharides such as chitosan, *O*-carboxymethyl chitosan (OCMCS) and *N*-succinyl-*O*-carboxymethyl chitosan

Table 1 Reported methods for coating of IONPs with chitosan and their applications in biomedical fields

Coating method	Polymer structure	IONP type	CS/HS (nm)	Application	References
Ex situ	CS	Hydrophilic	10/40–60	Drug delivery	[56]
			60/100		[57]
			Microspheres	MRI	[58]
			16/20	Drug delivery/	[59]
			8/107	imaging	[60]
			–	Hyperthermia	[61]
			10.3/–		[62]
			15/20	–	[63]
			Microsphere		[64]
			14–20/42		[65]
			15/–		[66]
			5–10/200		[67]
			10/–		[68]
		Hydrophobic	10–30/500		[69]
	N-Hexanoyl CS	Hydrophilic	7–13/50–150	Gene delivery	[70]
	Carboxymethyl CS		8/11	–	[71]
			6–10/55.4	MRI	[55]
	N-Succinyl-*O*-carboxymethyl CS		14/17	–	[72]
			10/40–60	Drug delivery	[56]
	O-Carboxymethyl CS		14–20/38	–	[65]
			10/40–60	Drug delivery	[56]
	CS-DMSA		17/–	–	[73]
	CS-*g*-Pluronic acid		40/1200		[74]
	CS-*g*-poly(NIPAAm-*co*-DMAAm)		5/8	Drug delivery	[75]
	Glycol CS		10/81	–	[76]
		Hydrophobic	10/273	MRI	[54]
	CS–linoleic acid		12/67	Gene delivery	[77]
			12/67	MRI/targeting	[78]
In situ	CS	Hydrophilic	–/87.2	MRI	[79]
			10–20/–	–	[80]
			16/hydrogel		[81]
	HTCC		–/274	MRI	[82]
	CS-*g*-PEG		7.5/112	Gene delivery/	[6]
			7.5/43	imaging	[8]
			7/62		[20]
			7/–	MRI/optical	[83]
			7/33	imaging	[5]
			7/120	Targeting	[84]

CS/HS core size and hydrodynamic size, *CS* chitosan, *DMSA meso*-2,3-dimercaptosuccinic acid, *NIPAAm N*-isopropylacrylamide, *DMAAm N*,*N*-dimethylacrylamide, *HTCC N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride, *PEG* polyethelene glycol

(NSOCMCS) to coat hydrophilic IONPs. The polymer to nanoparticles ratios, as well as the mixing of base play important roles in determining the size distribution and uniformity of the coating [65, 72]. Lee et al. mixed amphiphatic chitosan–linoleic acid copolymer with hydrophobic nanoparticles [78]. In their method, the

hydrophobic end of the polymer was self-assembled on the surface of the nanoparticles, while the hydrophilic end dispersed the nanoparticles in the aqueous media. Therefore, the water dispersibility of the nanoparticles was improved considerably after coating with the copolymer.

Since simple mixing approaches may not have the highest coating efficiency, crosslinking of the chitosan coating can be used to increase the stability of the nanoparticles. Yuan et al. [75], encapsulated the IONPs with a thermosensitive smart copolymer, chitosan-*g*-poly(*N*-isopropylacrylamide-*co*-*N*,*N*-dimethylacrylamide) and stabilized the coating using the crosslinking process. However, when these crosslinkers are used, additional investigations are needed to evaluate their toxicity and appropriate ratios [85].

The emulsion–diffusion–evaporation method was recently used to improve the uniformity of chitosan coatings. Lee et al. [54], used this method to make biodegradable chitosan-coated iron oxide/poly(D,L-lactic-*co*-glycolic acid) (PLGA) nanoparticles. In this method, hydrophobic PLGA and IONPs were mixed in dichloromethane and the organic solvent was evaporated after being emulsified in a solution mixture of polyvinyl alcohol and positively charged glycol chitosan. While a very good coating was formed around the nanoparticles, the size of the nanoparticles was reported to be about 275 nm.

3.2.2 In Situ Coating Approaches

The in situ method for the preparation of chitosan-coated IONPs involves the coprecipitation of IONPs in the presence of chitosan-based polymers. In this scheme, hydrophilic IONP cores precipitate from solution, forming nuclei with high surface energies, and rapidly adsorb chitosan polymers through physical binding. Under these conditions, uniform layers of chitosan-based polymers can be assembled on IONPs to stabilize each discrete nanoparticle. The chitosan-coated IONPs prepared through this in situ method are monodispersed, and surface modified with a uniform coat of chitosan polymers [86].

The major limitation associated with this in situ method is lack of flexibility in the formulation of chitosan polymers which can be used during synthesis. For example, modified versions of chitosan polymers may not appropriately assemble onto IONP surfaces during nanoparticle formation, causing incomplete coating. Another limitation is that the molecular weight of the chitosan polymer being applied as a coating will need to be sufficiently low to ensure solubility under the basic conditions needed for IONP coprecipitation [79].

One promising strategy that may enable higher molecular weighted chitosan polymers to form applicable coatings, which can be used in conjunction with the in situ synthesis method, is the development of chitosan copolymers soluble at high pH. For example PEG can be grafted to chitosan polymers to improve the solubility of chitosan under high pH conditions [87]. Veiseh et al. demonstrated that such copolymers can be used to coat IONPs prepared through the in situ method [5, 6]. The produced IONPs were reported to be of uniform size and relatively small

hydrodynamic size (32 nm). The same approach was successfully used by Kievit et al. [8, 20] and Gunn et al. [84].

4 Applications of Chitosan-Coated IONPs for Molecular Imaging and Therapy

4.1 Chitosan-Coated IONP Cancer Targeting Strategies

The ability of an imaging agent to specifically target diseased tissue increases the signal to noise ratio (SNR), potentially allowing for earlier detection of small solid tumors and metastatic cells. In addition, targeting of diseased tissue by therapeutic agents limits the adverse side effects of chemotherapeutic treatment on healthy tissue. Early IONPs relied on passive targeting, which refers to the accumulation of a carrier at a diseased tissue site by exploiting physiochemical and pharmacological characteristics [88]. Examples of passive targeting include uptake of nanoparticles by kupffer cells in the liver and the enhanced permeation and retention (EPR) effect, which is the primary route for passive targeting in solid tumors. The EPR effect aids in nanoparticle uptake by way of leaky vasculature, which allows particles generally less than 150 nm in diameter to cross from vasculature into the interstitium. Poor lymphatic drainage then aids in the entrapment of the particles in the solid tumor.

Active targeting strategies provide mechanisms for specific accumulation of imaging agents within tumors. These strategies are based on targeting of the unique molecular signatures of cancer cells, such as overexpressed growth factors and nutrient receptors (Fig. 1) [84, 89]. Many types of ligands have been investigated for targeting of these markers, including antibodies, peptides, small molecules, and aptamers [1]. Active targeting is not only important for improving the diagnosis of cancer, but plays a crucial role in nanoparticle therapeutics, as discussed in Sect. 4.3.

Gunn et al. recently developed an IONP coated with chitosan/PEG for tumor targeting that employs a novel active targeting strategy based on pretreatment with a recombinant fusion protein (FP) [84]. In this two-step method, a cell-targeting FP composed of a single-chain antibody and streptavidin is used to specifically pre-label cancer cells. In a subsequent step, biotinylated IONPs are applied that bind to the streptavidin of the FP on the cancer cells. In this study, cancer cell lines, Ramos and Jurkat were utilized along with two FPs, anti-CD20 and anti-TAG-72 CC49, which specifically target Ramos ($CD20^+/TAG72^-$) and Jurkat ($CD20^-/TAG72^+$) cells, respectively. Ramos and Jurkat cells were each treated with either anti-CD20 or anti-TAG-72 CC49, followed by treatment with chitosan/PEG-coated IONPs labeled with fluorophore and biotin (NPB-AF647). Figure 1 shows the results of flow cytometry experiments that demonstrate specific targeting by NPB-AF647 of Ramos cells pretreated with anti-CD20 and Jurkat cells pretreated with anti-TAG-72 CC49. This approach would allow for the use of a wide range of

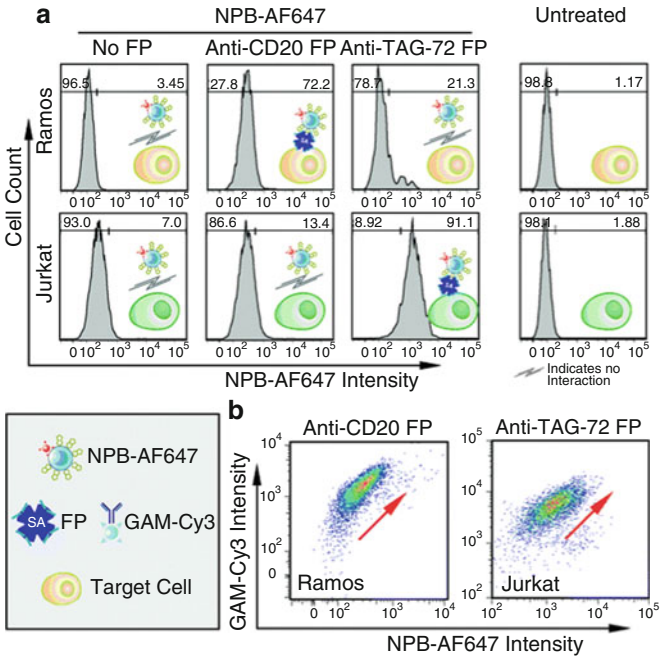

Fig. 1 Cell targeting with NPB-AF647. (**a**) Flow cytometry analysis of Ramos and Jurkat cells treated with IONPs coated with chitosan/PEG and labeled with fluorophore and biotin (*NPB-AF647*) after pretreatment with different fusion proteins (*FP*) or receiving no FP pretreatment. Ramos and Jurkat cells receiving neither FP nor nanoparticle treatment are given as reference (*right column*). (**b**) Dependence of NPB-AF647 cell-binding on FP prelabeling, demonstrated by flow cytometry, where the secondary antibody, goat-antimouse-Cy3 (*GAM-Cy3*), indicates FP labeling. *SA* streptavidin. Reprinted from [84] by permission from RSC, Molecular Biosystems. Copyright 2011

streptavidin-containing FPs for targeting a vast array of diseased tissue with a single nanoparticle system.

4.2 Applications of Chitosan-Coated IONPs in Molecular Imaging

Nanoparticle probes can improve molecular imaging techniques by providing enhanced SNRs and better spatial resolution, giving greater information at the molecular and cellular levels of biological systems [90]. Each imaging modality has its advantages and limitations and no one modality can give a comprehensive snapshot of a biological system. Although IONPs were developed specifically as MR imaging contrast agents, advances in coating technologies have allowed for the addition of other functional imaging moieties, including those for optical and

nuclear imaging. These multifunctional IONPs can be designed to incorporate complementary imaging modalities that synergistically provide more accurate information in vivo. The incorporation of polymers such as chitosan on the IONP surface has facilitated these advances by providing a great number of reactive sites for the addition of imaging moieties through simple amine reactive chemistries.

4.2.1 MR Imaging

MRI is one of the most powerful and versatile imaging techniques clinically available today [3, 19]. Unlike X-ray and computed tomography (CT), MRI does not rely on ionizing radiation, and gives higher spatial resolution in soft tissue, which is crucial to the imaging of cancer. MRI is primarily used to produce anatomical images of organs and tissue and can also provide information on the physiochemical state and vascularization of tissue [91]. This technique employs a strong magnetic field that aligns the magnetic moments of hydrogen protons. A radio frequency pulse is then applied, changing the direction of the magnetic moments of these protons. Upon removal of the radio frequency, protons relax to their original state at varying rates. There are two relaxation times of interest, longitudinal relaxation time (T_1) and transverse relaxation time (T_2). Variation in relaxation, which leads to image contrast, is due to the differences in proton density as well as the chemical and physical nature of tissues. Due to their excellent biocompatibility and magnetic properties, IONPs are currently the most promising of T_2 agents being investigated [3].

Current in vivo MRI research with chitosan-coated IONPs includes stem cell tracking and tumor imaging. Stem cell therapy holds promise for prevention and therapeutic care of many devastating diseases. The success of this therapy depends on the survival, homing and engraftment of transplanted stem cells in target tissue. Current biochemical and immunohistochemical analysis methods to determine the successful targeting of diseased tissue only give snapshot information as to the fate of stem cells [92]. Several studies have shown successful MRI stem cell tracking with use of chitosan-coated IONPs [3, 89, 93]. This method relies on the labeling of stem cell preinjection and allows for noninvasive, real-time imaging of the fate of injected stem cells.

Recently, Reddy et al. demonstrated that labeling of mesenchymal stem cells (MSCs) with superparamagnetic iron oxide nanoparticles (SPIONs) coated with chitosan did not alter the proliferation, surface marker expression or differentiation capacity of MSCs [92]. For comparison, Resovist, a commercially available MR contrast agent coated with carboxydextran, showed alteration to the chondrogenesis capacity of MSC. In addition, the chitosan-coated SPION-loaded MSCs did not show any toxic effects in viability studies over a 3-week period. Further, in vivo MR imaging of the chitosan-coated SPION-loaded MSCs injected into the brain of an ischemic brain rabbit model enabled the tracking of the stem cells for up to 2 weeks (Fig. 2).

IONPs have been investigated extensively as contrast agents in the detection and diagnosis of tumors [3]. Recently, Veiseh et al. [5] have shown successful penetration

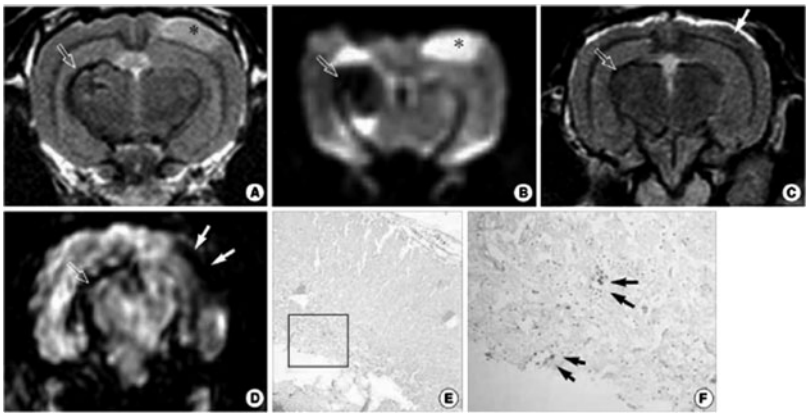

Fig. 2 In vivo MRI of rabbit ischemic brain that received mesenchymal stem cells labeled with chitosan-SPION. *Asterisk* indicates ischemic area and *open arrows* indicate chitosan-SPION-labeled mesenchymal stem cells at the injection site. (**a**, **b**) T2WI and diffusion weighted image (DWI) of brain immediately after injection of mesenchymal stem cell at contralateral side of ischemic area (*open arrows*) on day 4 of ischemia. (**c**, **d**) T2WI and susceptibility weighted image (SWI) 16 days after stem cell transplantation. At the ischemic site, dark signal (*white arrows*) on SWI matches mesenchymal stem cells (*black arrows*) on Prussian staining (**e**, **f**). (**e**, **f**) Prussian blue staining detected iron-labeled stem cells at the liquefied infarct area in a section of 4% paraformaldehyde-fixed brain tissue on day 16. Reprinted from [92] by permission from The Korean Academy of Medical Sciences, Journal of Korean Medical Science. Copyright 2010

of the BBB with an actively targeted chitosan-coated SPION to image brain tumors in a genetically engineered mouse model (Fig. 3). Chlorotoxin (CTX), which is derived from the venom of Israeli scorpions, was used as a targeting agent. In this study, the authors were able to show a significant increase in R_2 values ($1/T_2$) in the cerebellum (tumor region) in comparison to the frontal lobe (healthy region). Not only were these IONPs able to traverse the BBB, they were able to do so without compromising the integrity of the BBB and showed no toxicity effects on the liver. The ability of these chitosan-coated IONPs to cross biological barriers shows potential for a wide range of use as an MRI contrast agent in a variety of tumor types.

4.2.2 Fluorescent Imaging

Fluorescence imaging uses a sensitive low-light camera to detect fluorescence emission from specific tissue of in vitro samples or whole-body living animals labeled with fluorophores. In general, fluorophores with emission at the near-infrared (NIR) region are preferred due to their ability to overcome photon attenuation in living tissue [94]. Traditionally, small-molecule NIR dyes have been used, but development of fluorescent organic, biological and inorganic nanoparticles for fluorescence imaging offer new, powerful tools for in vivo applications [95].

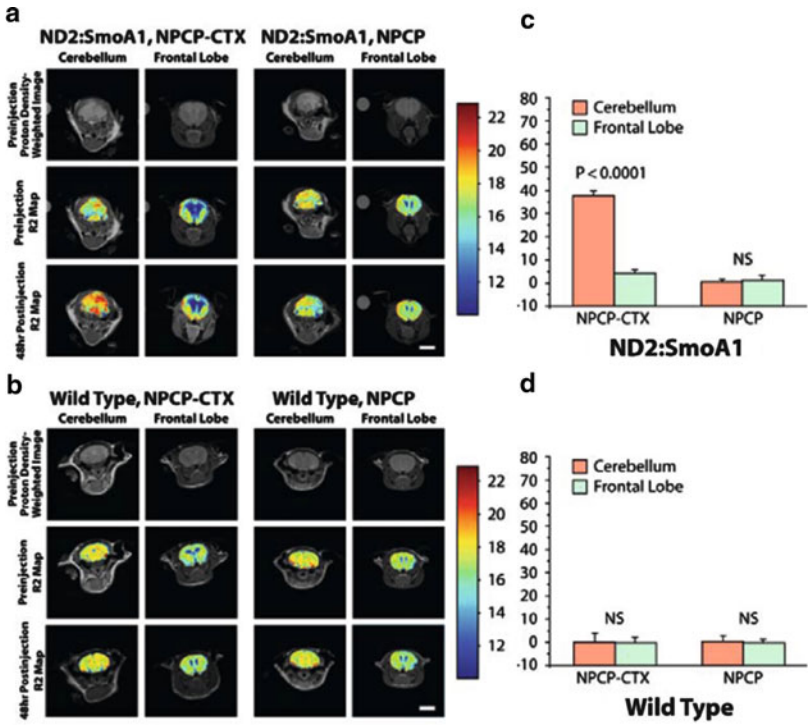

Fig. 3 In vivo MRI of brain tumors and signal quantification. In vivo MR images of ND2:SmoA1 (**a**) and wild-type mice (**b**) acquired before and 48 h after administration of nanoprobes comprising IONPs coated with chitosan/PEG and either labeled with CTX (*NPCP-CTX*) or unmodified (*NPCP*). Coronal cross-sections of the frontal lobe (no tumor present) of the cerebral hemisphere and cerebellum (containing tumor tissues) were analyzed. Colorized R_2 maps of the brain region were superimposed onto proton density-weighted images. Varying R_2 values (s^{-1}) from low to high were visually represented in colors generated from the gradient (*right*). Change in R_2 was quantified by dividing the change in R_2 before and after nanoprobe injection, and by the preinjection R_2 response for ND2:SmoA1 (**c**) and wild-type mice (**d**). Both targeting (NPCP-CTX) and nontargeting (NPCP) nanoprobe systems were evaluated in ND2:SmoA1 and wild-type mice. Reprinted from [5] by permission from AACR, Cancer Research. Copyright 2009

Fluorescently labeled nanoparticles offer several advantages over small-molecule NIR dyes. Small-molecule dyes are cleared quickly from the body, limiting the number of fluorescent molecules that can reach the tissue of interest, reducing SNRs. Nanoparticles demonstrate increased blood circulation times and allow for the loading of multiple dye molecules to improve the SNR. Tight, specific binding of imaging probes to the tissue of interest is crucial to successful imaging. Small-molecule NIR dyes have limited capacity for ligand conjugations, whereas the large surface area of nanoparticles allows for the addition of numerous targeting ligands. The binding affinity of targeted fluorescent nanoparticles is improved due to the principals of multivalency [94, 96].

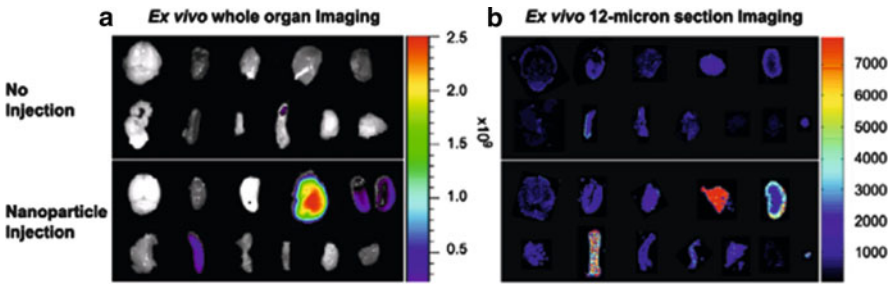

Fig. 4 Ex vivo NIR fluorophore imaging of each organ. Fluorescence image of whole organs in noninjected animals (*top*) and in animals injected with CTX-labeled chitosan-based nanoparticles incorporating the NIF dye Cy5.5 (NP-CTX-chitosan-Cy5.5) (*bottom*). Images were acquired 6 h postinjection. (**a**) Mice were injected with 200 mL of 1 mg/mL. Six hours after injection, the animals were euthanized and the organs were collected. Ex vivo fluorescence images of whole organ was obtained using the Xenogen imaging system. The spectrum gradient bar corresponds to the fluorescence intensity unit p/s/cm^2/sr. (**b**) Fluorescence image of 12-μm sections obtained using the Odyssey imaging system. The spectrum gradient bar corresponds to relative fluorescence level. (*Top row*: brain, heart, lung, liver, and kidney. *Bottom row*: pancreas, spleen, small intestine, colon, gonad, muscle, and bone marrow). Bone marrow is only shown with the Odyssey scanner. Reprinted from [83] by permission from Public Library of Science, PLoS One. Copyright 2010

Fluorescently labeled IONPs may serve as a platform for the next generation of multifunctional probes for multimodality imaging. These nanoparticles provide fluorescence by conjugation of numerous small NIR molecules on the polymeric surface of the particle. Recently, Lee et al. demonstrated the usefulness of fluorescent imaging in the pharmacokinetic and biodistribution evaluation of nanoparticles using chitosan-based IONPs [83]. The aim of the study was to develop a rapid, radioactive-free method to determine nanoparticle biodistribution profiles to help inform decisions determining particle size, coatings, etc., early in nanoparticle development. The results showed a serum half-life of 7–8 h and prolonged stability of the nanoparticles in physiological fluids and in vivo. The IONPs were distributed primarily in the spleen and liver of healthy mice (Fig. 4). Other organs showed very low levels of nanoparticles. The results indicate the potential of chitosan-coated IONPs for imaging of cancer and other diseases. In addition, the magnetic and fluorescent properties of these probes could allow for the coregistration of MRI and fluorescent images to improve diagnostic capabilities.

4.3 Drug Delivery

4.3.1 Drug Loading and Release

Modification to the surface coating of IONPs is often needed to facilitate effective loading and controlled release of therapeutic agents. Therapeutic agents can be

incorporated on the surface of IONPs in a number of ways, including chemical linkage, incorporation of drug in a degradable outer shell, or through electrostatic interactions. Currently the most common organic linkers for covalent bonding of therapeutics to IONPs include amine, carboxylic acid, aldehyde and thiol-containing molecules [97]. Amines are positively charged and can form amide bonds with negatively charged carboxylic acids. Aldehydes are able to form imide bonds with amine groups, while thiols are able to form disulfide bridges with other thiol-containing molecules, such as cysteine residues present on proteins and peptides [97, 98].

Although covalent linkages provide a convenient and stable method for therapeutic conjugation, this method is not always possible or preferred. A promising option for incorporation of DNA on the surface of IONPs for gene therapy is to use electrostatic interactions between the negatively charged phosphate backbone of DNA and the cationic polymers on the surface of IONPs. PEI was among the first cationic polymers reported to bind and condense DNA. In addition to their binding capacity, the positive charge of cationic polymers can also serve as a release mechanism from lysozomal entrapment [97]. Cationic polymers buffer the intralysozomal pH, causing an influx of protons and subsequent rupture of the lysozome, freeing DNA. Although PEI has shown promise as a gene transfection agent, its cytotoxicity has led researchers to investigate other cationic polymers such as chitosan for application in gene therapy [99].

4.3.2 Chitosan-Based Magnetic Nanoparticles for Chemotherapeutic Delivery

Chitosan-based systems have been investigated extensively for their drug delivery capabilities and have shown promise in an array of applications, including vaccine delivery, chemotherapeutic treatment, and gene therapy [33, 78, 92, 94, 100, 101]. Due to their surface chemistry and morphology, chitosan-based magnetic nanoparticles may also serve as a drug delivery platform with inherent advantages over polymer–drug conjugates [24]. These advantages include high surface area-to-volume ratio and numerous reactive amine groups, allowing for the loading of a large amount of chemotherapeutic agents and specific targeting of diseased tissue. In addition, magnetic nanoparticles allow real-time MR imaging of the therapeutic treatment.

Chitosan-coated IONPs have been investigated as drug carriers for a variety of chemotherapeutic agents. Recently, Wang et al. demonstrated the successful loading of the chemotherapeutic agent 5-fluorouracil onto IONPs with chitosan as an enveloping agent and glutaraldehyde as a crosslinking agent [102]. In this study, the drug-releasing properties in physiological conditions of the chitosan-enveloped IONPs were evaluated by UV–vis spectrum analysis. The results showed that at 30 h only 68% of the drug had been released, indicating the potential of these IONPs for controlled drug release applications.

Zhu et al. investigated the loading and release of the chemotherapeutic drug camptothecin (CPT) with three variations of chitosan surface-modified IONPs [56]. The IONPs were stabilized with chitosan, OCMCS, or NSOCMCS. After physical absorption of CPT on the polymer surface of the IONPs, the positively charged chitosan-modified SPION was not stable, whereas the ampholytic OCMCS-modified SPION and the negatively charged NSMCMS-modified SPION showed good stability. Drug release studies of OCMCS-SPION showed continued release over a 10-day period, while the NSOCMCS-SPION demonstrated a burst of drug release in the first 2 days with very little release thereafter. Cell viability studies showed that both the OCMCS- and NSOCMCS-modified IONPs without drug where not toxic to 7721 cancer cells, whereas the CPT-loaded version demonstrated greater cell death than free CPT at comparable concentrations.

Recently, Yuan et al. developed a stimulus-responsive chitosan smart polymer-coated SPION as a drug carrier [75]. In this study, doxorubicin (DOX) was conjugated to the SPION surface via acid-labile hydrazone bonds and encapsulated by the smart polymer chitosan-*g*-poly(*N*-isopropylacrylamide-*co*-*N*,*N*-dimethylacrylamide) [chitosan-*g*-poly(NIPAAm-*co*-DMAAm)]. This thermosensitive polymer exhibits a low critical solution temperature (LCST) of 38°C. At or above the LCST, the polymer collapses on itself, releasing the bound DOX. The system exhibits low drug release at temperatures below the LCST and higher drug release above the LCST. Whereas increased drug release was demonstrated above the LCST at physiological pH, a decrease in pH further increased the rate of release of DOX due to the acid-labile bonding of DOX on the SPION surface.

4.3.3 Chitosan-Based Magnetic Nanoparticles for DNA and siRNA Delivery

Beyond drug and targeting ligand conjugation, the amine groups of chitosan along with those of other polycationic polymers can complex with negatively charged DNA and siRNA for use in gene therapy [101, 103, 104]. Gene therapy holds potential for treatment of disease through modification of specific cellular functions. DNA-based gene therapy is used to introduce genetic material into cells in order to alleviate symptoms or prevent occurrence of disease by expression of the delivered genetic material. Short interfering ribonucleic acid (siRNA)-based gene therapy, on the other hand, relies on the binding of siRNA to complementary messenger RNA (mRNA). In this approach, siRNA-bound mRNA inhibits translation of mRNA to protein, effectively silencing the gene of interest. Both DNA and siRNA therapies are hampered by inefficient and nonspecific delivery of the therapeutic to targeted cells. In addition, current viral packaging methods raise serious safety concerns [101, 103]. While polycationic polymers may not have the safety implications associated with viral vectors, toxicity is still an issue due to cell membrane disruption by positively charged moieties on the polymer. Chitosan-coated IONPs have shown promise in alleviating these limitations.

In one approach, Bhattarai et al. developed IONPs stabilized with a hexanoyl chloride-modified chitosan (Nac-6-IONPs) for enhancement of adenoviral gene

delivery [70]. K562 cells were targeted by Nac-6-IONPs in vitro using an external magnetic field (magnetonfection) and treated with adenovirus (Ad) containing reporter gene *LacZ* (Ad/*LacZ*). Results showed that Nac-6-IONPs/Ad/*LacZ* is able to transduce K562 cells more efficiently than Ad/*LacZ* alone. Furthermore, in vivo results of transduction of lung and intestine by magnetonfection with Nac-6-IONPs/Ad/*LacZ* demonstrated that Nac-6-IONPs could be applied to other cell lines. The authors hypothesize that the IONPs stabilized with modified chitosan increase transduction efficiency either by interactions with the cell membrane or by destabilization of the endosomal environment. These results suggest that chitosan-stabilized IONPs could drastically lower effective viral doses, reduce incubation times, and prevent delivery to nonspecific sites.

Nonviral, in vitro gene delivery studies by Kievit et al. have shown that IONPs coated with chitosan, PEG, and PEI have significantly reduced toxicity compared to nanoparticles coated with PEI alone, while showing transfection efficiencies of a green fluorescent protein (GFP) reporter gene on par with the commercially available transfection agents Polymag and Lipofec [20]. Not only were the chitosan/PEG/PEI-coated IONPs less toxic to cells than PEI-coated IONPs but transfection efficiency was significantly greater for the chitosan-coated IONPs at low ratios of DNA to IONP. A more recent study by Kievit et al. further demonstrated that this engineered system is able to function in vivo [8]. Mice bearing C6 xenograft flank tumors were injected via the tail vein with either targeted CTX-labeled chitosan/PEG/PEI-coated IONPs or with a nontargeted vector. MR imaging indicated that both the targeted and nontargeted nanovectors were taken up by tumors equally, but GFP expression was shown to be greater in the tumor regions of mice treated with the targeted nanovector (Fig. 5). These results suggest that the targeting moiety did not enhance accumulation of the DNA-loaded nanovectors in the tumor region, but did promote specific uptake in C6 cells.

Veiseh et al. recently developed a cancer cell-specific IONP/siRNA nanovector for efficient and specific delivery of siRNA [6]. Similar to the work by Kievit et al., the IONP was coated with a PEG-modified chitosan polymer and was subsequently grafted with PEI. The nanovector was further modified with CTX and fluorphore-labeled GFP siRNA. In vitro studies with GFP-positive glioma C6 cells showed increased uptake of the CTX-labeled nanovector over the nontargeted nanovector, as determined by flow cytometry and in vitro MRI. In addition, GFP expression was shown to be greatly reduced for cells treated with the siRNA-loaded, CTX-targeted nanovector (Fig. 6).

5 Conclusions and Outlook

IONPs have been extensively studied for applications in nanomedicine due to their biocompatibility, biodegradability, and superparamagnetism that enables their use as a contrast agent for MRI and as a therapeutics carrier for in vivo applications. Of the many approaches for the synthesis of iron oxide cores,

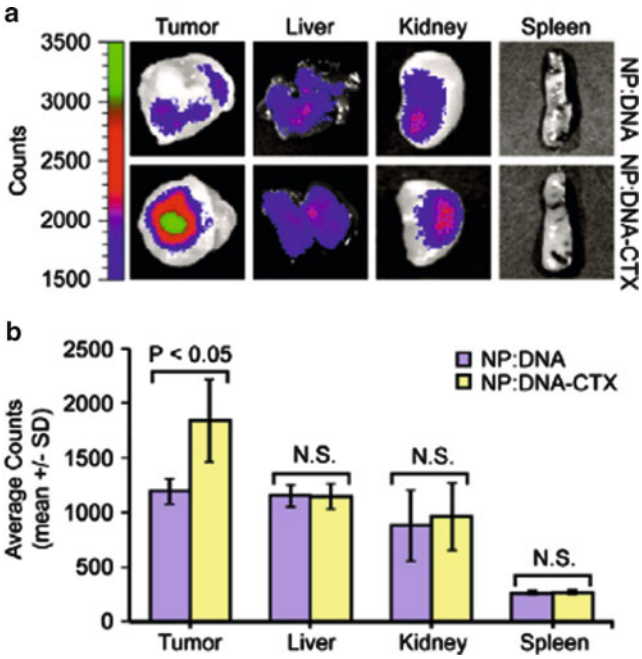

Fig. 5 Enhanced delivery of GFP encoding DNA to C6 glioma cells in vivo using CTX-labeled chitosan/PEG/PEI-coated IONPs (*NP*). (**a**) Xenogen images of tumors, livers, kidneys, and spleens from C6 xenograft tumor-bearing mice, harvested 48 h after treatment, indicating GFP fluorescence levels. (**b**) Average counts over the tumors and clearance organs, quantified using the IVIS Xenogen software ($n = 3$). Reprinted from [8] by permission from ACS, Journal of ACS Nano. Copyright 2010

coprecipitation and thermal decomposition have shown to be the most effective approaches in the production of nanoparticles with uniform size, composition, and shape. Although thermal decomposition tends to yield nanoparticles with superior materials properties to those synthesized by coprecipitation, the resulting hydrophobic surfaces require additional exhaustive steps to produce water-soluble nanoparticles. On the other hand, the coprecipitation approach generates nanoparticles with hydrophilic surfaces that do not need additional steps to become stable in aqueous solutions. In addition, the coprecipitation synthesis can be performed in the presence of a polymer that, through physical absorption, further stabilizes the surface of the nanoparticle. Chitosan and its derivatives have emerged as some of the more promising of the many polymer systems that have been investigated for both ex situ and in situ coating approaches for IONPs. Chitosan's attractiveness as a coating material for IONPs arises from its biodegradability, cellular compatibility, and ample functional groups that can serve as anchors for the addition of functional molecules. The unique combination of chitosan and IONPs has great potential in an array of nanomedicine applications.

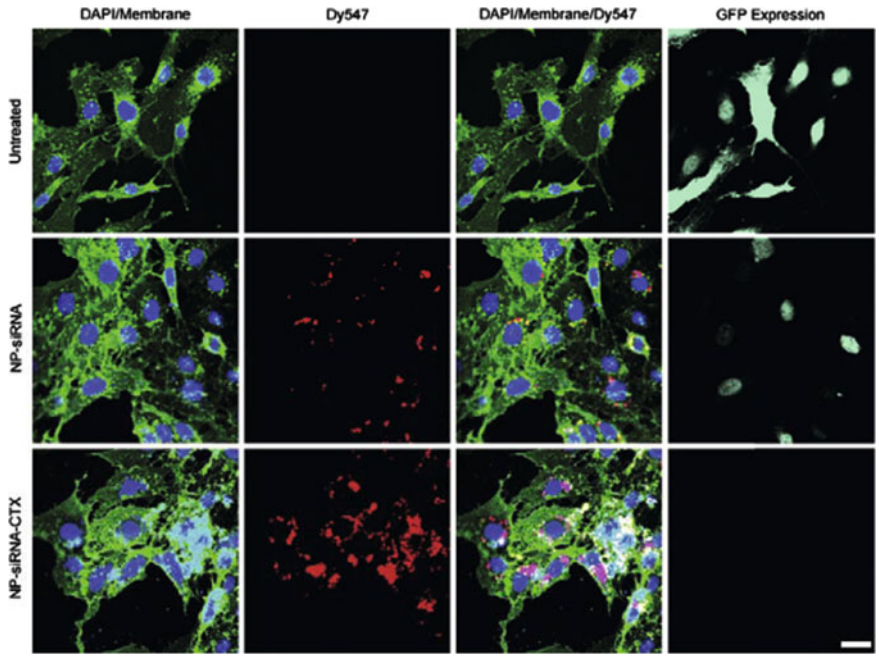

Fig. 6 Confocal fluorescence images of C6 GFP-positive cells illustrating targeted siRNA delivery and enhanced knockdown of gene expression. Cells were imaged 48 h post-treatment with IONPs coated with chitosan/PEG/PEI and then loaded with siRNA (*NP-siRNA*; *second row*) or with the nanoparticles further modified with CTX (*NP-siRNA-CTX*; *third row*), with untreated cells (*first row*) as a reference. Cell nuclei are shown in *blue*, cell membranes in *green*, siRNA in *red*, and GFP expression levels in *light green* (*fourth column*). *Scale bar*: 20 mm. Reprinted from [6] by permission from Elsevier, Biomaterials. Copyright 2010

As a first step toward clinical application, chitosan-coated IONPs have demonstrated successful tracking of stem cells as well as targeting and imaging of cancer by MR and fluorescent imaging in vivo. In addition, this novel nanoparticle is emerging as one of the top candidates for the targeted delivery of traditional chemotherapeutic agents as well as for packaging and delivery of DNA and siRNA for gene therapy.

Although there are still questions to be answered, chitosan-coated IONPs show great promise in the successful diagnosis and treatment of the most devastating diseases, including cancer. Once critical design parameters have been optimized, the nontoxic, cost-effective, and efficient chitosan-based multifunctional IONPs can move forward to clinical biomedical applications.

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Chitosan: Its Applications in Drug-Eluting Devices

Mei-Chin Chen, Fwu-Long Mi, Zi-Xian Liao, and Hsing-Wen Sung

Abstract Chitosan, a naturally occurring polysaccharide derived from chitin, has been widely applied in drug delivery, tissue regeneration, wound healing, blood coagulation, and immunostimulation due to its well-known biocompatibility and biodegradability. Additionally, because of its unique cationic nature and the gel/film/matrix-forming capabilities, chitosan has been considered as a promising material for the development of medical devices. The current concept for developing medical devices often comprises the functionality of controlled release of bioactive agents such as drugs, proteins, or growth factors in order to fulfill their clinical applications. However, in biological fluids, the hydrophilic chitosan matrices may swell and deform dramatically through hydration, thus resulting in a rapid loss of the encapsulated drugs from the delivery device. Considerable efforts have therefore been made in chemically modifying chitosan to improve its physical properties and functionality. This review article focuses on the versatile modifications of chitosan matrices (ionic or chemical crosslinking) and the most recent research activities in drug-eluting devices, including vascular stents, artificial skin, bone grafts, and nerve guidance conduits.

Keywords Biodegradable materials · Controlled release · Crosslinking agent · Drug-eluting stent · Tissue regeneration

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Contents

1 Introduction

Chitin [poly(*N*-acetyl-1,4-β-D-glucopyranosamine)] is an abundant mucopolysaccharide that is found in the exoskeleton of crustaceans, the cuticles of insects, and the cell walls of fungi [1, 2]. It is structurally similar to cellulose, consisting of 2-acetamido-2-deoxy-β-D-glucose with a β-(1→4) linkage. Chitosan is obtained by the deacetylation of chitin, which is a copolymer of 2-acetamido-2-deoxy-D-galactopyranose (GlcNAc) and β-(1→4)-2-amino-2-deoxy-D-glucopyranose (GlcN), with a GlcN content of more than 50%. Applications of chitin are fewer than those of chitosan because chitin is chemically inert and soluble in neither water nor acid, whereas chitosan is relatively reactive and can be processed in various forms [3]. The solubility of chitosan depends on the distribution of free amino and N-acetyl groups. In dilute acids (pH < 6.5), the free amino groups are protonated and the molecule thus becomes soluble [4]. Accordingly, chitosan can be easily molded in various forms, such as powder, paste, film, fiber, porous scaffold and others, for various applications.

Owing to its well-known antibacterial nature, minimal foreign body reactions, biocompatibility, and biodegradability, chitosan is regarded as a good candidate for tissue engineering [4–6], wound healing [7–9], drug delivery [10–13], and gene delivery applications [14–17]. Functional modifications of chitosan by graft copolymerization or crosslinking are sometimes made to introduce desired properties and enlarge the range of the potential applications. In recent years, chitosan-based medical devices have shown great potential for drug delivery, because of their controlled and sustained release properties. Many studies reported that tissue

regeneration can be accelerated by the association of proteins or growth factors with controlled drug delivery devices. This review summarizes the most recent investigations of versatile modifications of chitosan, and of chitosan-based drug-eluting devices and their formulations to fulfill various clinical requirements.

2 Modification and/or Crosslinking of Chitosan

2.1 Chitosan Chemistry

The presence of both reactive amino and hydroxyl groups in chitosan provides opportunities for chemical modification to impart useful physicochemical properties and distinctive biological functions [18–21]. Some of the potential chemical modification reactions have been reported [22–24]. Specific reactions that involves the $–NH_2$ group at the C-2 position and nonspecific reactions of –OH groups at the C-3 and C-6 positions (especially esterification and etherification) give rise to such chemical reactions as acylation, quaternization, carboxymethylation, galactosylation, sulfation, and thiolation, which provide a variety of products with, for example, antibacterial, antifungal, antiviral, anticoagulation, and transmucosal properties [25, 26]. The aforementioned chemically modified chitosan derivatives and their applications are discussed in the rest of this section.

2.1.1 Acylation

N-Acyl chitosan derivatives are typically synthesized using anhydrides (linear or cyclic), acyl chlorides, and lactones [27–32]. The degree of acetylation (DA) of chitosan by a controlled N-acetylation reaction can be adjusted by mixing chitosan with acetic anhydride in a molar ratio of 0.1–2.0. Modifying the DA of chitosan significantly affects the material properties that are critical to tissue engineering applications, by increasing degradation times and enhancing cell adhesion [33]. Moreover, chitosan has been selectively N-acylated with linear aliphatic anhydrides of different chain length, including acetic, propionic, butyric, and hexanoic anhydrides, to prepare *N*-acetyl chitosan, *N*-propionyl chitosan, *N*-butyryl and *N*-hexanoyl chitosan, respectively [29]. The hydrophobic interaction of N-acylated chitosan that has been substituted with longer acyl chains is stronger than with shorter acyl chains. In contrast, the drug release rates of *N*-acylchitosan microspheres with long acyl chains are less susceptible to enzymatic degradation than are their *N*-acetylchitosan counterparts [34].

Since the amino group is more active than the hydroxyl groups, a new method for the selective O-acylation of chitosan in methanesulfonic acid (MSA) has been developed [35]. The N,O-acylation of chitosan has also been conducted by dissolving the *O*-acyl chitosans that are synthesized via an MSA protection method in *N*,

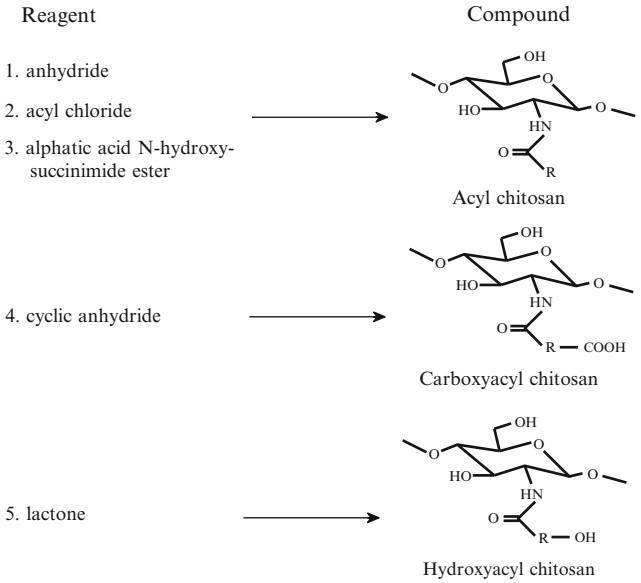

Fig. 1 Reaction scheme for acylation of chitosan

N-dimethylacetamide and then performing N-acylations on the *O*-acyl chitosans [36, 37].

Chitosan with aliphatic side chains such as stearoyl, palmitoyl, and octanoyl groups has been hydrophobically modified by reacting carboxylic anhydride with chitosan through N-fatty acylation reactions [27, 28]. *N*-Palmitoyl chitosan (NPCS) has also been synthesized by reacting palmitic acid *N*-hydroxysuccinimide ester with chitosan. This NPCS was developed as a pH-triggered, in situ gelling system, which underwent a rapid nanostructural transformation within a narrow pH range (pH 6.5–7.0) [38, 39]. In dilute aqueous media, NPCS can self-assemble into nanoparticles, owing to the hydrophobic interaction between their conjugated palmitoyl groups [40]. The extent of cellular uptake of NPCS nanoparticles increased with the degree of substitution (DS) of palmitoyl groups in NPCS. The internalization of NPCS nanoparticles was clearly related to the lipid raft-mediated routes. Figure 1 shows the reaction scheme for the aforementioned acylation of chitosan.

2.1.2 Quaternization

N-Trimethyl chitosan chloride (TMC) with introduced quaternary amino groups on the chitosan chain has been synthesized using iodomethane in an alkaline solution of *N*-methyl pyrrolidinone (NMP). Quaternization is based on the nucleophilic alkylation of the primary amino group at the C-2 position of chitosan [41]. TMC

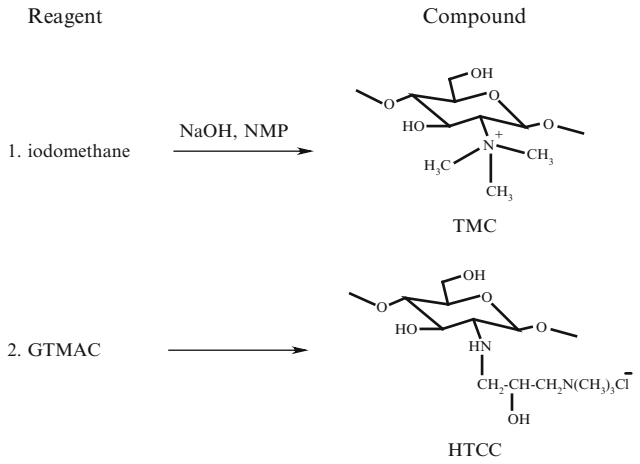

Fig. 2 Reaction scheme for quaternization of chitosan

is the most important quaternized chitosan derivative, and has been proven to effectively increase the paracellular permeation of hydrophilic macromolecular drugs across the mucosal epithelia by opening the tight junctions [42]. The degree of substitution of TMC crucially influences its ability to open up the tight junctions of intestinal mucosa. A degree of quaternization of approximately 40–50% is reportedly optimal for transepithelial delivery of both low molecular weight compounds [43, 44] and proteins [45–48].

Chitosan has been modified using glycidyl trimethyl ammonium chloride (GTMAC) to produce another quaternized chitosan derivative, *N*-(2-hydroxyl) propyl-3-trimethylammonium chitosan chloride (HTCC). When a primary amino group at the C-2 position of chitosan reacted with GTMAC, the chain of the quaternary ammonium group thus obtained was longer than that of the TMC. The preliminary protein release test from HTCC or modified HTCC nanoparticles in vitro reveals that they are functional carriers for protein delivery [49]. Figure 2 shows the reaction scheme for the aforementioned quaternization of chitosan.

2.1.3 Sugar-Modification

Carbohydrates such as lactose can be grafted onto the chitosan backbone at the C-2 position by reductive alkylation, or can be introduced at the C-6 position without opening of the ring [50]. These sugar-modified chitosan derivatives can be used for drug targeting because the specific recognition of cells, viruses, and bacteria by sugars has been proven. D- and L-fucos-bound chitosan derivatives have been synthesized to interact specifically with lectin and cells [51].

Asialoglycoprotein receptors are known to be present on hepatocytes at a high density of 500,000 receptors per cell, and they are retained on several human

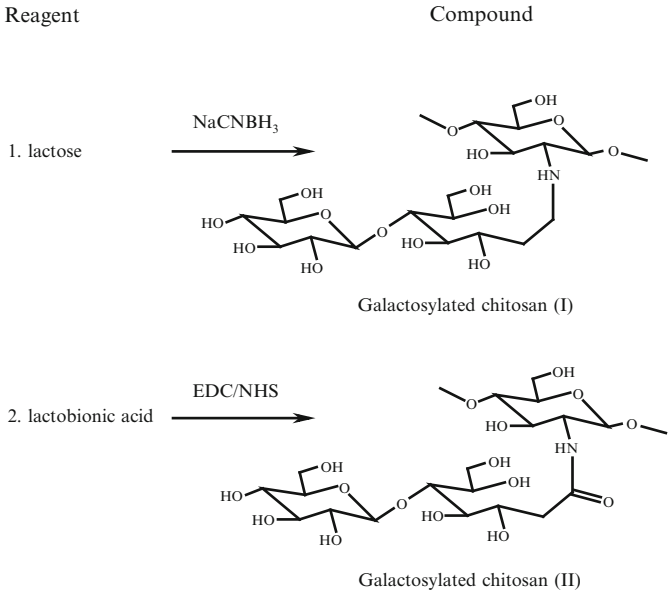

Fig. 3 Reaction scheme for sugar-modification of chitosan

hepatoma cell lines [52, 53]. A lactosaminated *N*-succinyl-chitosan derivative has been synthesized as a liver-specific drug carrier in mice to target asialoglycoprotein receptors [54]. Galactosylated chitosan (GC) can be synthesized by coupling chitosan with lactobionic acid via an active ester intermediate using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). GC has potential as a synthetic extracellular matrix (ECM) for hepatocyte attachment [55]. Chitosan bearing single or antennary galactose residues has also been investigated to determine its specific targeting to HepG2 cells after it is formed into nanoparticles or complexes with DNA [56–58]. The GC/DNA complex has a much higher transfection efficiency than the chitosan/DNA complex on HepG2 [58, 59], indicating that a galactose ligand that is attached to GC not only helps the GC/DNA complex bind to asialoglycoprotein receptors, but also promotes the internalization of GC/DNA complex within membrane-bound vesicles or endosomes [15]. Figure 3 shows the reaction scheme for the aforementioned sugar-modification of chitosan.

2.1.4 Carboxymethylation

Both, *N*-carboxymethyl and *O*-carboxymethyl chitosan derivatives have been prepared under different reaction conditions using monochloroacetic acid in the presence of NaOH to achieve the N-versus-O selectivity [60, 61]. The selective route for synthesizing N-carboxymethylation utilizes glyoxylic acid in a reductive amination sequence [62]. *N*-Carboxymethyl and 6-*O*-carboxymethyl chitosan

(*N*-CC and 6-*O*-CC) are both polyampholytic chitosan derivatives, whose molecules contain –COOH groups and $–NH_2$ groups. These carboxymethyl chitosans are not only soluble in water, but also have numerous outstanding chemical, physical, and biological properties, including high viscosity, film formation, gel formation, nontoxicity, biodegradability, biocompatibility, and antibacterial and antifungal activities, all of which make them attractive for used in biomedical, pharmaceutical, and cosmetic applications [63, 64].

N,*O*-carboxymethyl chitosan (*N*,*O*-CC) is a chitosan derivative that has carboxymethyl substituents at some of both the amine and the 6-hydroxyl sites of its glucosamine units. It can be easily prepared using chitosan, sodium hydroxide, and isopropanol with chloroacetic acid [65]. *N*,*O*-CC as a wound dressing can stimulate the extracellular lysozyme activity of fibroblasts and considerably promote the proliferation of skin fibroblasts. *N*,*O*-CC is also used in the development of various functional hydrogels, including superporous or pH-sensitive hydrogels, which are used for protein drug delivery [66–69].

Another carboxymethyl chitosan derivative called *N*-succinyl chitosan is obtained by introducing succinyl groups at the N-terminal of the glucosamine units of chitosan [70]. Various macromolecule–antitumor drug conjugates could be prepared from the synthesis process of *N*-succinyl chitosan and drug conjugation [71]. For example, *N*-succinyl chitosan can directly react with an activated ester of glutaric mitomycin [54, 72] or with mitomycin C using carbodiimide as a coupling reagent [73]. Figure 4 shows the reaction scheme for the aforementioned carboxymethylation of chitosan.

2.1.5 Sulfation

N-, *O*- or *N*, *O*-sulfate derivatives of chitosan have been synthesized using numerous methods in the preparation of bioactive sulfated polysaccharides with anticoagulant activity, immunomodulating effects, and anti-virus activity [74]. Various reagents have been used in the sulfation of chitosan, including concentrated sulfuric acid, oleum, sulfurtrioxide, sulfurtrioxide/pyridine, sulfurtrioxide/trimethylamine, sulfur trioxide/sulfur dioxide, chlorosulfonic acid–sulfuric acid, and chlorosulfonic acid, which is the most commonly used [75–77]. These syntheses have been performed under both homogeneous and heterogeneous conditions in such media as dimethylformamide (DMF), DMF–dichloroacetic acid, tetrahydrofuran, and formic acid [78]. Sulfated derivatives of chitosan exhibit different blood anticoagulant activities depending on the degree and sites of substitution [79–81]. Increasing the sulfur content in chitosan increased its anticoagulant activity. 6-*O*-carboxymethyl chitosan *N*-sulfate exhibited 23% of the activity of heparin [79], and the O-sulfated form of 6-*O*-carboxymethyl chitosan exhibited 45% of the activity of heparin in vitro [80]. *N*-Carboxymethyl chitosan 3,6-disulfate exhibited anticoagulant activity similar to that of heparin [81].

N-Sulfate, 6-O-sulfated, and 2-N,6-O-sulfated chitosans were synthesized and their effects on the bioactivity of bone morphogenetic protein-2 (BMP-2) were

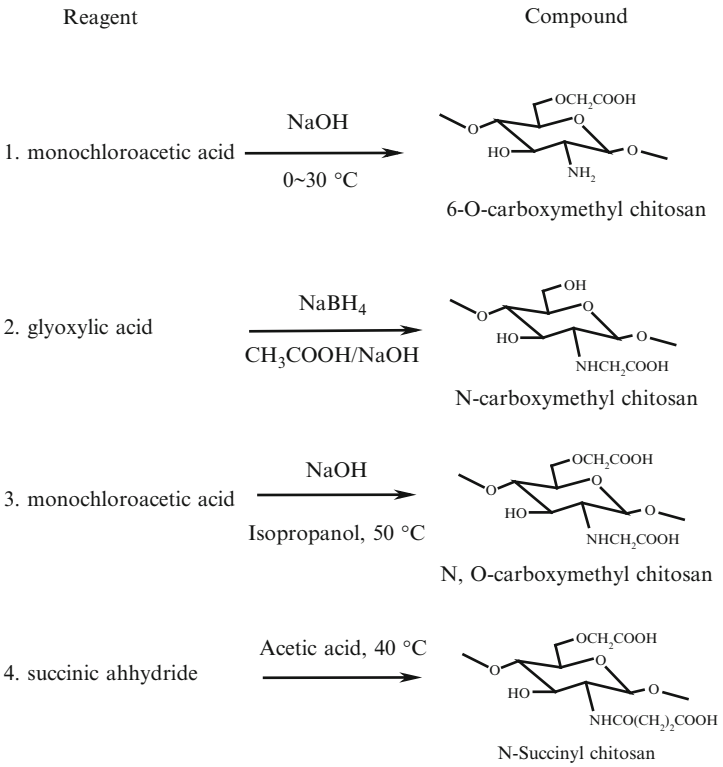

Fig. 4 Reaction scheme for carboxymethylation of chitosan

compared. The 6-O-sulfated substitution primarily increased the bioactivity of BMP-2, while 2-*N*-sulfate acted as a subsidiary group, which provided less activity. A low dose of 2-N, 6-O-sulfated chitosan markedly enhanced the activity of alkaline phosphatase (ALP) and the mineralization that was induced by BMP-2 [82]. N,O-Sulfated carboxymethyl chitosan that is substituted with 6-*O*-carboxymethyl groups and 2-*N*, 3-*O*, and 6-*O* sulfate groups may have well-balanced steric and electrostatic structures that can stably interact with basic fibroblast growth factor (bFGF) to protect the growth factor against proteolytic inactivation. The sulfate groups appeared to bind predominantly to basic amino acid residues, while the carboxymethyl groups complemented the sulfate groups, stabilizing the binding structure [83]. Figure 5 shows the reaction scheme for the aforementioned sulfation of chitosan.

2.1.6 Thiolation

Thiolated chitosans are synthesized by covalently coupling thiol groups to chitosan backbones. They exhibit in situ gel-forming properties owing to the formation of

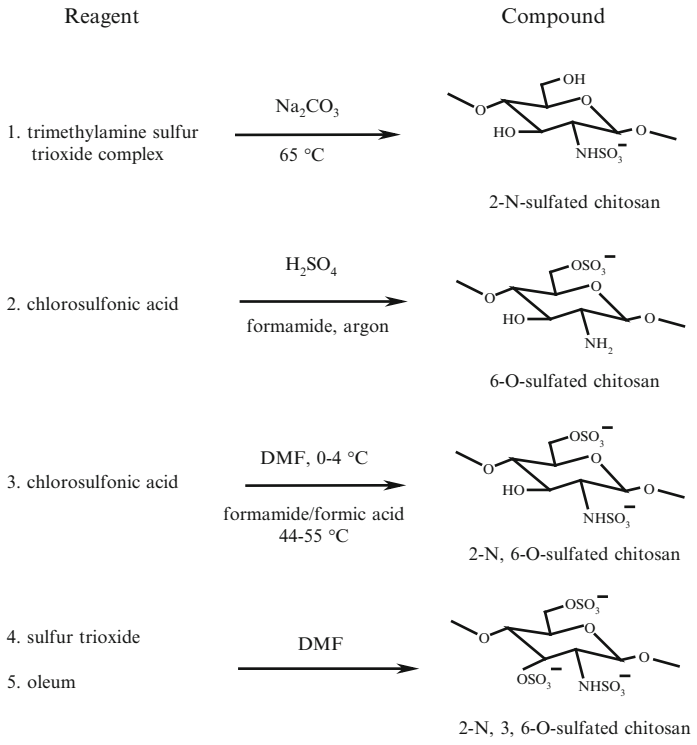

Fig. 5 Reaction scheme for sulfation of chitosan

inter- and/or intramolecular disulfide bonds at pH values above 5 [84]. Sulfhydryl-bearing agents such as cysteine or thioglycolic acid can be covalently bound to chitosan. The thiolation reaction of chitosan involves amide bond formation between the carboxylic acid group of the thiol reagent and the primary amino group of chitosan, mediated by a water-soluble carbodiimide [84, 85]. Thiolated chitosans can also be prepared by modifying chitosan with 2-iminothiolane or isopropyl-*S*-acetylthioacetimidate, which is crosslinkable in situ. As well as a sulfhydryl group, a cationic moiety is also introduced in the form of an amidine substructure, resulting in a chitosan–4-thio-butylamidine conjugate (chitosan–TBA) or chitosan–thioethylamidine conjugate (chitosan–TEA) [86, 87]. A new system of thiolated chitosan that is based on chitosan–thioglycolic acid (chitosan–TGA) in the presence of oxidizing agents such as hydrogen peroxide (H_2O_2), sodium periodate ($NaIO_4$), ammonium persulfate [$(NH_4)_2S_2O_8$] and sodium hypochlorite (NaOCl) has been examined to increase the dynamic viscosity and reduce the reverse volume phase time [88].

Chitosan has mucoadhesive properties [89]; however, the mucoadhesive properties of chitosan can be significantly improved by the immobilization of thiol groups on the polymer. The mucoadhesion is enhanced by the formation of disulfide bonds with cysteine-rich subdomains of mucus glycoproteins, and these bonds are

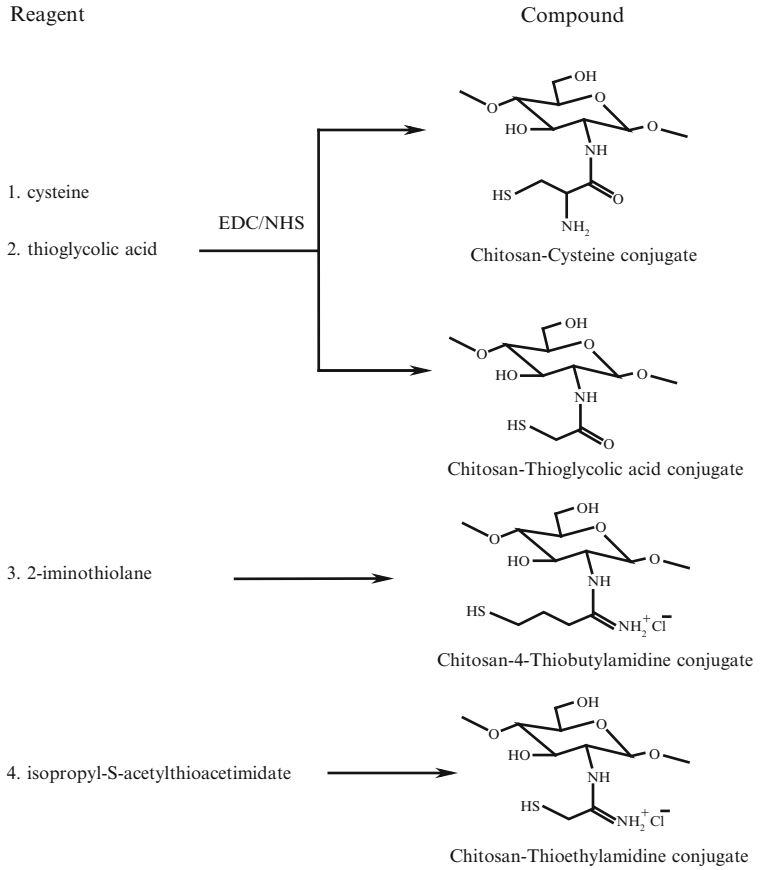

Fig. 6 Reaction scheme for thiolation of chitosan

stronger than non-covalent bonds [89, 90]. Chitosan–TBA conjugates provide 140-fold stronger mucoadhesion than unmodified polymer [84, 86]. These favorable results can be explained by the fact that chitosan–TBA combines the formation of disulfide bonds with improved ionic interactions between the additional cationic amidine groups of the modified chitosan and the anionic moieties that are provided by sialic acid and sulfonic acid substructures in the mucus layer. Figure 6 shows the reaction scheme for the aforementioned thiolation of chitosan.

Thiolation of chitosan strongly improved the permeation-enhancing capabilities of chitosan, facilitating the paracellular transport of hydrophilic compounds. The immobilization of thiol groups can strongly improve the permeation-enhancing effect of chitosan. Uptake of the fluorescent dye rhodamine-123 in the presence of chitosan–TBA was three times that of unmodified chitosans [91]. Permeation studies were also carried out using rhodamine-123 as a permeability glycoprotein substrate, indicating that chitosan–TBA potentially inhibits the ATPase activity of permeability glycoprotein in the intestine [92]. Furthermore, chitosan–TBA seems

to inhibit protein tyrosine phosphatase, opening tight junctions to increase the permeation of hydrophilic compounds [93].

2.2 Ionic Modification

Chitosan is acid-soluble because its crystal structure can be broken down under acidic conditions. To preserve the stability of chitosan gel under gastrointestinal tract delivery or enzymatic degradation, the amine groups on its polymeric chains must be fixed by chemical reagents. The ionic interactions between the positively charged amino groups on chitosan and either small anionic molecules, such as sulfates, citrates, and phosphates, or some metal ions, have been successfully used in the preparation of chitosan beads or hydrogels [94, 95].

Small anionic molecules bind to the protonated amino groups on chitosan via electrostatic attractions, but metal ions form coordinate-covalent bonds with chitosan rather than electrostatic interactions [96]. Tripolyphosphate (TPP), which can interact with chitosan by electrostatic attractions, has been recognized as an acceptable food additive by the US Food and Drug Administration (FDA) [95]. In the ionotropic gelation method, chitosan dissolved in aqueous acidic solution is added dropwise with constant stirring to TPP solution to form spherical particles. Since the pK_a of chitosan is in the range of 6.3–6.7, chitosan gel beads that are cured at pH values of less than 6 were really ionically crosslinked. However, chitosan gel beads that are cured at pH values of greater than 7 are coacervation-phase inversion-controlled, and depend slightly on ionic-crosslinking [97]. The ionic-crosslinking density of chitosan beads can be improved by changing the pH value of the curing agent, TPP, from basic to acidic [98].

The process by which chitosan is ionically crosslinked is simple and frequently performed under mild conditions without the use of organic solvents. Hence, several researchers have explored its potential pharmaceutical use [99, 100]. Bodmeier et al. have developed a new approach for preparing chitosan nanoparticles based on the ionotropic gelation method [101], forming positively charged, stable chitosan nanoparticles with sizes in the range of 250–400 nm. Subsequently, ionically crosslinked chitosan nanoparticles have been extensively used to deliver various small molecular drugs and bioactive macromolecules. Anticancer or protein drugs such as doxorubicin or insulin can be effectively entrapped into the chitosan nanoparticles during ionotropic gelation process [102, 103].

2.3 Modification by Aldehydes

Chitosan and its derivatives have been extensively developed for biomedical applications because of their biocompatibility and enzymatic degradability. Glutaraldehyde, a bifunctional aldehyde, is traditionally utilized to crosslink chitosan.

The crosslinking process involves formation of imine bonds between the amino groups on chitosan chains and bifunctional glutaraldehyde crosslinker [104]. However, glutaraldehyde is generally considered to be relatively toxic and the fate of this crosslinking reagent in human body has not been established. The glutaraldehyde crosslinked chitosan gels can be restructured into tablets, membranes, beads, and nano- or microspheres. Recent studies have demonstrated that crosslinking is one of the main factors that affect the physical and swelling properties of chitosan-based gels [105].

The degree of crosslinking of chitosan depends on the degree of deacetylation but is found to be independent of the molecular weight of chitosan [105]. The concentration of glutaraldehyde and the reaction temperature are also used to control the degree of crosslinking in chitosan-based microspheres [105–107]. Crosslinked chitosan microspheres has been investigated for potential use in a drug delivery system for delivering anticancer drugs, such as 5-fluorouracil, cisplatin, oxantrazole and others [108–111]. Additionally, chitosan can be crosslinked by glutaraldehyde to form a network to promote the sustained release of bioactive reagents, including centchroman and progesterone [112, 113]. Crosslinked chitosan microspheres reportedly release hydroquinone faster than do their uncrosslinked counterparts [114]. The rate of release depends on the degree of crosslinking of chitosan microspheres. Gupta and Jabrail also reported an extended, constant release of centchroman, a selective estrogen receptor modulator, from crosslinked microspheres for 60–70 h, whereas this process continued for only 10 h from uncrosslinked microspheres [113]. These studies demonstrate that drug release rates may be manipulated by controlling the degree of crosslinking of the chitosan hydrogels or microspheres.

2.4 Modification by Genipin

Genipin, a naturally occurring heterocyclic compound, can be obtained from its parent compound, geniposide, which is isolated from the fruits of *Gardenia jasminoides* ELLIS. Genipin and its related iridoid glucosides have been extensively used as antiphlogistics and cholagogues in herbal medicine [115]. Comparing the cytotoxicity of genipin to that of glutaraldehyde in vitro using 3T3 fibroblasts via the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay reveals that genipin is about 5,000–10,000 times less cytotoxic than glutaraldehyde [116]. In an in vitro 3T3 cell culture or an in vivo rat model, a chitosan membrane or injectable chitosan microspheres that are crosslinked with genipin histologically exhibited less cytotoxicity, better biocompatibility, and slower degradation rate than their glutaraldehyde crosslinked counterparts [117, 118]. Relevant studies strongly suggest that the compatibility of genipin is superior to that of glutaraldehyde.

The above results indicate that the crosslinking reactions were pH-dependent. Under neutral and acidic conditions, genipin reacted with chitosan via a nucleophilic

attack by the primary amino group, on the olefinic carbon atom at the C-3 position of deoxyloganin aglycone, followed by opening of the dihydropyran ring and attack by the secondary amine group on the intermediate aldehyde group, forming heterocyclic amines [119]. The heterocyclic amines were further associated to form crosslinked networks with short chains that comprised dimmer, trimer, and tetramer bridges. Under strongly basic conditions, genipin underwent a ring-opening polymerization prior to crosslinking with chitosan [120]. The crosslinking bridges consisted of polymerized genipin macromers or oligomers. This ring-opening polymerization of genipin was initiated by extraction of a proton from the hydroxyl groups at the C-1 position of deoxyloganin aglycone, followed by opening of the dihydropyran ring to enable an aldol condensation [120].

Genipin has been adopted to crosslink chitosan in the preparation of hydrogels, wound dressings, scaffolds, films, beads, and nano- or microspheres for wound healing, tissue engineering, and drug delivery [68, 117, 118, 121–123]. Novel chitosan gel beads have been synthesized by a coupled ionic and chemical co-crosslinking method using TPP and genipin as crosslinkers. The pH-dependent ionic/chemical co-crosslinking mechanism has an obvious effect on the swelling property and enzymatic degradation behavior of the prepared chitosan gel beads [124]. A pH-sensitive hydrogel of *N*,*O*-carboxymethyl chitosan (*N*,*O*-CC) and alginate, crosslinked by genipin, for protein drug delivery has been reported [68]. The amount of albumin (BSA) that is released at pH 1.2 is relatively low (20%), while that released at pH 7.4 is significantly greater (80%). These results clearly suggest that the genipin-crosslinked *N*,*O*-CC/alginate hydrogel may be a suitable polymeric carrier for site-specific delivery of protein drugs in the intestine.

2.5 Modification by Other Crosslinking Agents

In addition to glutaraldehyde and genipin, numerous bifunctional reagents have been used to crosslink chitosan covalently, such as epichlorohydrin, diisocyanate, or epoxy compounds 4-butanediol diglycidyl ether or ethylene glycol diglycidyl ether (EGDE) [125–129]. Figure 7 shows the reaction schemes for crosslinking chitosan with these bifunctional reagents, as well as with TPP, glutaraldehyde, and genipin. Among those bifunctional reagents, EGDE may be the most suitable crosslinker for reaction with chitosan to prepare flexible films. Observably, crosslinking with glutaraldehyde increases the tensile strength of 6-*O*-CC/water-soluble polyurethane (WPU) composite membranes, but reduces their elongation. In contrast, the elongation of 6-*O*-CC/WPU membrane increased upon reaction with EGDE [130]. Recently, a novel biodegradable stent, made of chitosan/poly(ethylene oxide) blend films that were crosslinked with EGDE and exhibit shape-memory, was developed for the sustained release of sirolimus [131]. Other studies, such as those involving the preparation of highly porous microspheres by crosslinking chitosan with EGDE to create an open porous surface structure have been performed with the goal of sustained release of the antigen of the Newcastle disease

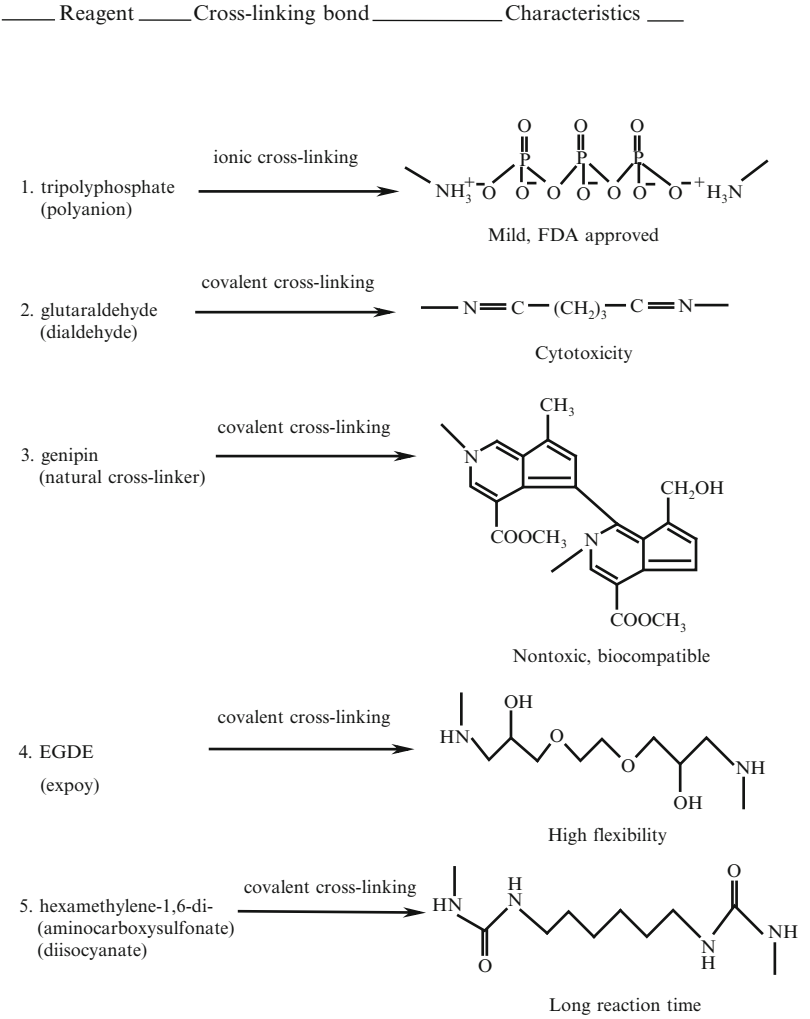

Fig. 7 Reaction schemes for crosslinking chitosan with TPP, glutaraldehyde, genipin, and other bifunctional reagents

vaccine [132]. Furthermore, quaternary ammonium, and aliphatic and aromatic acyl groups, can be introduced into the porous chitosan beads to adsorb the anti-inflammatory drug indomethacin via both electrostatic and hydrophobic interactions [133].

Chitosan solutions that are physically mixed with β-glycerophosphate can be injected into the body in liquid form, forming a gel in situ at the body temperature. The rate of gelation depends on the degree of chitosan deacetylation, the concentration of β-glycerophosphate, and the temperature and pH of the final solution [134]. The in situ gelation mechanism involves neutralization of the ammonium

groups in chitosan, allowing strengthened hydrophobic and hydrogen bonding between the chitosan chains at elevated temperatures. A new, mild approach to in situ hydrogel formation via a peroxidase-catalyzed crosslinking reaction that takes only seconds was recently developed. This approach has shown great potential for making injectable hydrogels for biomedical applications, such as tissue engineering and drug or protein delivery [135, 136].

3 Drug-Eluting Stents

Atherosclerosis is the primary cause of coronary heart disease, which is characterized by a narrowing (stenosis) of the arteries that supply blood to tissues of the heart [137–139]. Metallic coronary stents were originally developed to prevent abrupt artery closure and to reduce the likelihood of restenosis, which is associated with percutaneous transluminal coronary angioplasty. They are, however, limited by the frequent occurrence of restenosis, which is caused by smooth muscle proliferation, and associated neointimal hyperplasia and target lesion revascularization [140].

Drug-eluting stents (DES) are a revolutionary technology because of their unique ability to provide both mechanical and biological solutions simultaneously to the target artery [141]. Generally, each DES comprises three components: the backbone stent, the active pharmacologic compound, and a drug-carrier vehicle, which controls drug elution [142]. The biological effects of the pharmacological agents and the interaction of DES components with the arterial wall have been demonstrated to attenuate greatly in-stent restenosis, relative to that associated with a bare-metal stent (BMS) [140].

Using polymers as drug delivery vehicles typically enables the best controlled and sustained drug release [143]. Current commercially available DES (such as Cypher, Taxus, Xience V, Promus and Endeavor) comprise a metallic stent with a coating of a non-erodable synthetic polymer that contains antiproliferative agents [140]. The drugs can inhibit the proliferation and migration of vascular smooth muscle cells (VSMC), which are important factors in the development of neointima formation, preventing restenosis [144]. After the drug elutes from the polymer coatings, the residual synthetic polymer coatings remain in place. Eventually, the permanent presence of the nonresorbable polymer may lead to complications such as an exaggerated inflammatory response and neointimal hyperplasia at the implant site [145, 146]. A principal target of current research is to develop the next generation of DES with a biodegradable and biocompatible coating to minimize these unfavorable effects.

Chitosan is a biodegradable, nontoxic and tissue-compatible polymer; it is regarded as an excellent candidate for drug delivery applications. Many studies have been conducted to explore the feasibility of using chitosan as a drug reservoir for DES, as described in the following sections.

3.1 *Eluting Coating for Metallic Stents*

Although DES have dramatically reduced in-stent restenosis, histopathological assessment in autopsy cases has demonstrated delayed healing and incomplete endothelialization. Additionally, evidence exists of hypersensitivity reactions in patients with stent thrombosis who had received DES [147]. A hypersensitivity reaction is caused by the non-erodable polymer. The mechanisms by which DES cause a prolongation of arterial healing and endothelialization, which may be susceptible to late thrombosis, are poorly understood [147]. Recent investigations have found that antiproliferative drugs (sirolimus and paclitaxel) reduce neointimal formation by impeding VSMC proliferation and migration, and retard the normal healing of the injured arterial wall [147].

Bioactive surfaces and compounds that promote vascular healing are emerging to improve stent performance and patient outcomes [140]. A biomacromolecular layer-by-layer (LbL) coating of chitosan/heparin (CS/HP) onto a coronary stent has been designed to accelerate re-endothelialization and healing after coronary stent deployment [148]. Chitosan has promise as a bioactive material for implant devices because of its capacity to enhance wound healing and cell attachment [7]. Heparin is the most often used anticoagulant reagent in clinical use. It incontrovertibly suppresses subacute in-stent thrombus [149]. The results of in vitro culturing of porcine iliac artery endothelial cells, as well as a hemocompatibility evaluation, support the claim that the combination of chitosan and heparin can make the stent surface compatible with endothelial cells and hemocompatible.

A porcine coronary injury model and an arteriovenous shunt model were used to evaluate the application of such a surface-modified stainless steel stent in vivo. In the second week, neointimal layers were present on the surfaces of some BMS samples, whereas the intimal tissues seemed to be more integrated on the surfaces of all of the CS/HP-coated stents. After 4 weeks of implantation, the healing on all BMS and CS/HP-coated stents was completed. No obvious inflammation response to any of the CS/HP-coated stents was observed, suggesting the good biocompatibility of the CS/HP coating and the safety of its in vivo application (Fig. 8) [148].

Endothelialization of the inner wall of the stented arteries was further examined by SEM (Fig. 9) [148]. In the second week of development, cell attachment occurred on the BMS surface with a relatively low density. These cells appeared to be infantile and undeveloped, while on the CS/HP-coated stents, the luminal surface of the vessel wall and the stent struts were covered with confluent shuttle-like endothelial cells. The endothelialization of the BMS surfaces was statistically estimated to be approximately 30% as observed by SEM, whereas that of the surfaces of the CS/HP-coated stents was almost 100%. After 4 weeks, the attached and grown shuttle-shaped endothelial cells completely covered the BMS and the CS/HP-coated stents. Therefore, the evidence conclusively established that the natural self-assembly coating accelerated the re-endothelialization process in the coronary stent system. This finding may provide a solution to the delayed healing problem of DES, and thus the natural self-assembly coating may have great potential in future use as a drug reservoir.

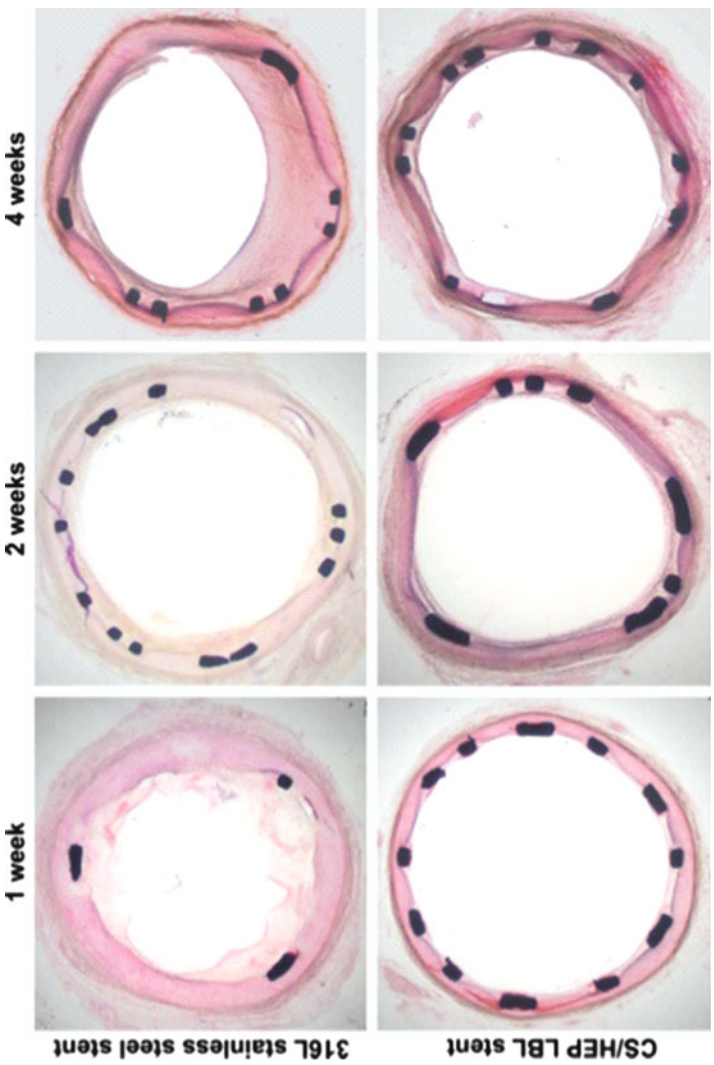

Fig. 8 Typical optical photographs (25×) of the cross-section slices of porcine arteries implanted with the BMS- and CS/HP coated stents after implantation for 1, 2, and 4 weeks. From [148]; reproduced by permission of Elsevier

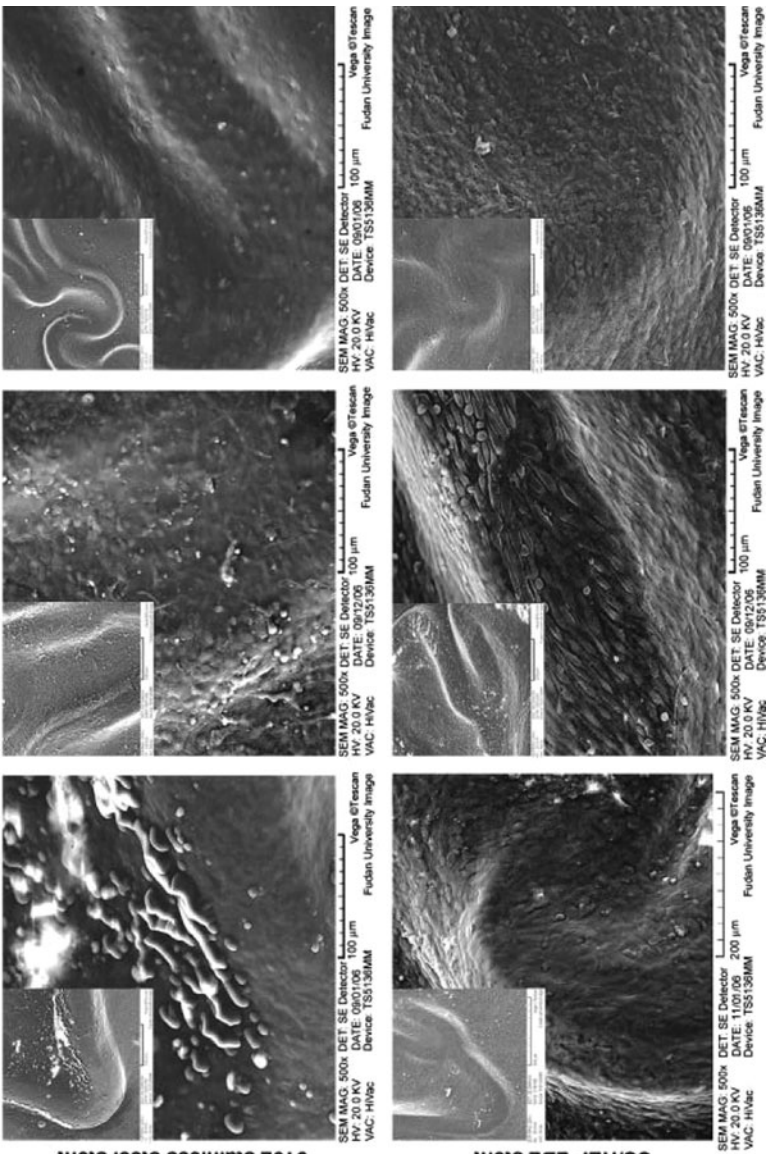

Fig. 9 Typical SEM images (500×; *inset* 200×) of the inner wall of stented arteries after stent implantation for 1, 2, and 4 weeks. From [148]; reproduced by permission of Elsevier

A separate attempt has also been made to use the LbL coating method to form self-assembling chitosan/hyaluronan (CS/HA) coatings for endovascular stents [150]. HA, a naturally occurring polyanion, plays an important structural and mechanical role in various tissues. The inhibitive effects of HA with respect to hyperplasia, observed after either systemic or local delivery, suggest that the antiproliferative effects of HA may be related to its anti-inflammatory properties [151]. Related results have demonstrated that CS/HA multilayer-coated NiTi disks have better antifouling properties than unmodified NiTi disks, as demonstrated by a decrease in the adhesion of platelets in an in vitro assay (38% reduction). This result may be attributable to the hydration layer that surrounds HA molecules on the surface of the disks [152]. The hydrogel-like surface that is formed by the multilayers exhibited good hemocompatibility.

To determine whether the CS/HA multilayers may be exploited as in situ drug delivery system, sodium nitroprusside (SNP) was incorporated in the multilayers. SNP, a nitrous oxide donor that spontaneously decomposes in biological environments [153], is extensively used clinically to reduce blood pressure and has emerged as a promising modality in the treatment of restenosis [154]. SNP-loaded multilayers have been shown to reduce platelet adhesion below that associated with multilayers devoid of the drug. The enhanced thromboresistance of the self-assembled multilayer, together with the anti-inflammatory and wound healing properties of HA and chitosan, are expected to reduce neointimal hyperplasia that is associated with stent implantation.

The LbL self-assembly of chitosan and polyanions into multilayers has emerged as an efficient and versatile approach for forming biologically active surfaces [150]. CS/HP LbL modification is simpler and less expensive than other methods of facilitating vascular healing (CD34 antibody-coated or RGD peptide-coated stents) [155, 156]. An LbL coating increases stability during sterilization and storage. Additionally, bioactive molecules, such as DNA or proteins, may be incorporated into the multilayer during the polyelectrolyte deposition process, forming drug-releasing interfaces on various substrates [148].

3.2 Eluting Membrane for Metallic Stents

Membrane-covered stents have also been studied with a view to their use in the treatment of coronary diseases [157–159]. A stent that is covered with chitosan/polyethylene oxide (CS/PEO) membrane has been shown to have suitable properties for endoscopic implantation [160]. The results of mechanical tests indicate that a blend of chitosan with PEO of high molecular weight can greatly improve the elasticity and strength of chitosan. CS/PEO membrane-coated stents have been demonstrated to sustain mechanical deformation during endoscopic expansion and to resist physiological blood pressure. Additionally, the blended membrane has good hemocompatibility because it has a lower adsorption than a non-blended CS membrane. To study further the capacity of the CS/PEO membrane to act as a drug

reservoir, SNP was used as a model drug and loaded in the membrane via an ionic interaction between the anionic drug and CS. The SNP-loaded membrane further reduced platelet adhesion. These results suggest that the CS/PEO membrane could also be easily loaded with other bioactive drugs for the treatment of various pathologies.

3.3 Polymeric Stent Crosslinked by an Epoxy Compound

Even though polymer-coated metallic DES have revolutionized the treatment of obstructive coronary diseases, they do not provide an optimal solution [161]. FDA reports and autopsy findings suggest that metallic DES may be a cause of systemic and intrastent hypersensitivity reactions that, in some cases, have been associated with late thrombosis and death [162]. Other reports have further suggested that the most likely cause of the hypersensitivity reaction is the non-erodable polymer coating of the DES [145, 146, 163]. Additionally, in-stent restenosis may be associated with allergic reactions to the nickel (stent component) and molybdenum (stent impurity) in metallic stents [146, 164].

Stents are superior to angioplasty because they provide scaffolding of the vessel and prevent elastic recoil and detrimental remodeling following revascularization [140]. However, whether the presence of a permanent stent is favorable, or whether it would be more advantageous that the stent was degraded and absorbed by the body once its task was done, is unclear [140]. Recently, biodegradable polymeric stents have attracted much interest as an alternative to metallic stents [131, 165, 166].

A self-expandable polymeric stent, made of chitosan-based films crosslinked with an epoxy compound (EGDE), and exhibiting shape memory has been developed [131, 165, 166]. The flexibility of chitosan films can be significantly improved by blending with glycerol and PEO. Since covalent crosslinks form in the stent matrix, a chitosan-based stent has shape memory (Fig. 10) [165]. It can expand (~150 s) from its crimped (temporary) to its fully expanded (permanent) states upon hydration (Fig. 11) [165], markedly faster than can polymeric stents that are made of poly-L-lactic acid (PLLA) (~20 min at 37°C for the Igaki-Tamai stent) [167] or PLLA/poly-D-L-lactide-glycolide (~8 min at 37°C for the stent that was developed by Venkatraman et al.) [168]. Rapid self-expandability of the stent is advantageous, as it helps prevent migration of the stent during its in vivo deployment. In a preliminary animal study, an implanted chitosan-based stent was found to be intact and no thrombus was formed in the stent-implanted vessel.

Sustained delivery of antiproliferative drugs to prevent in-stent restenosis is crucial for an ideal biodegradable polymeric stent. However, the drugs (sirolimus and paclitaxel) that are most commonly used in currently available DES systems are both lipophilic. The hydrophilic nature of chitosan matrices makes them unable to entrap poorly soluble therapeutic agents and greatly limits their range of applications as drug delivery systems. A consistent limitation of

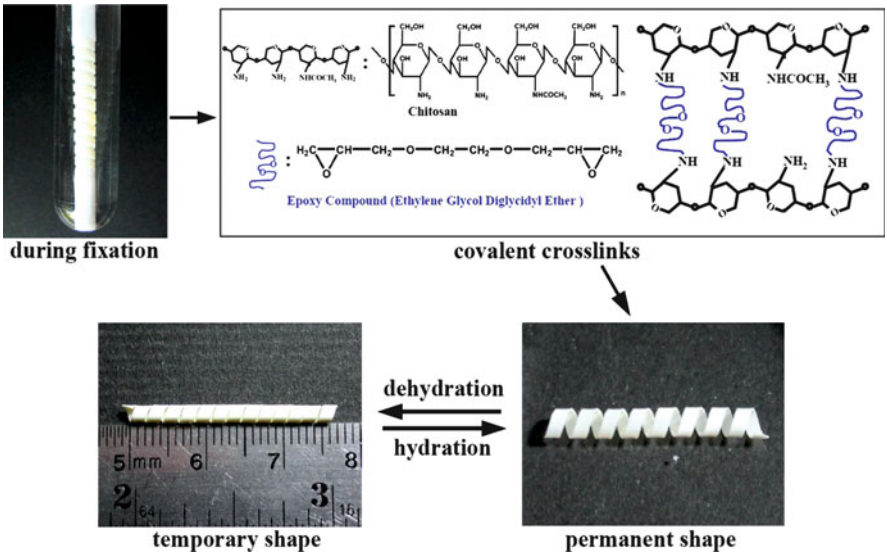

Fig. 10 Crosslinking structures and photographs of the permanent and temporary shapes of the polymeric (CS/glycerol/PEO400) stent. The shape-switching process is reversible and controlled by hydration or dehydration of the crosslinked stent. From [165]; reproduced by permission of the American Chemical Society

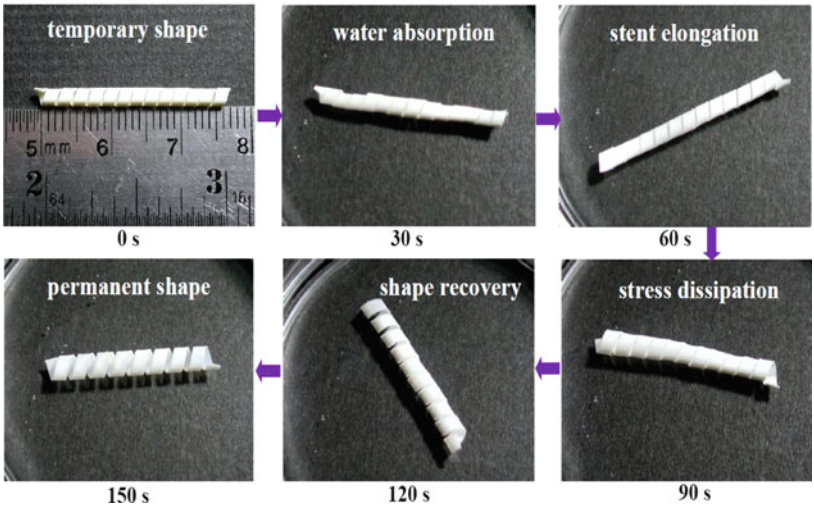

Fig. 11 Photographs of the time courses of self-expansion of the polymeric (CS/glycerol/PEO400) stent, immersed in phosphate-buffered saline, stimulated by hydration. From [165]; reproduced by permission of the American Chemical Society

these hydrophilic systems appears to be the rapid loss of therapeutic agents because of the lack of sufficiently strong interactions between the lipophilic drug and the hydrophilic polymer. Additionally, large aggregates may be formed by the interaction among lipophilic pharmaceuticals in the drug-loading process, resulting in a high local concentration and, thus, toxicity at the sites of the aggregates [169].

A nanoscale drug entrapment strategy has been proposed for the controlled and sustained release of sirolimus in chitosan-based stents [166]. Sirolimus was first entrapped in the core of self-assembled Pluronic block copolymer L121 (PEO_5-PPO_{68}-PEO_5) micelles; the hydrophilic outer shell of L121 micelles then maintained their uniform dispersion and stability in the hydrophilic chitosan matrix (Fig. 12) [166]. The SEM results indicated that large aggregates of sirolimus were present at random locations in the control group (sirolimus/chitosan-blended stents, arrow in Fig. 13a, left) [166], whereas spherical micelles were uniformly dispersed in the experimental stents with sirolimus-loaded micelles (Fig. 13b) [166].

In an in vitro drug release study, an initial burst release of more than 40% of sirolimus from the control stents was observed on the first day of the experiment, whereas no apparent burst release from the experimental stents occurred (Fig. 14) [166]. After 10 days, the cumulative percentages of drug that were released from the

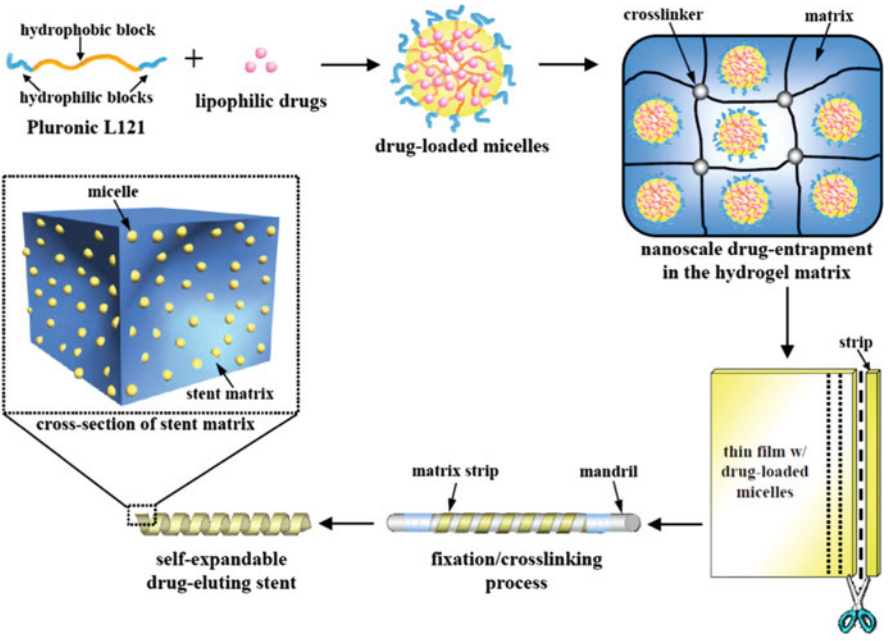

Fig. 12 Illustration of the concept of nanoscale drug entrapment strategy and the fabrication process of the self-expandable drug-eluting stent. From [166]; reproduced by permission of Elsevier

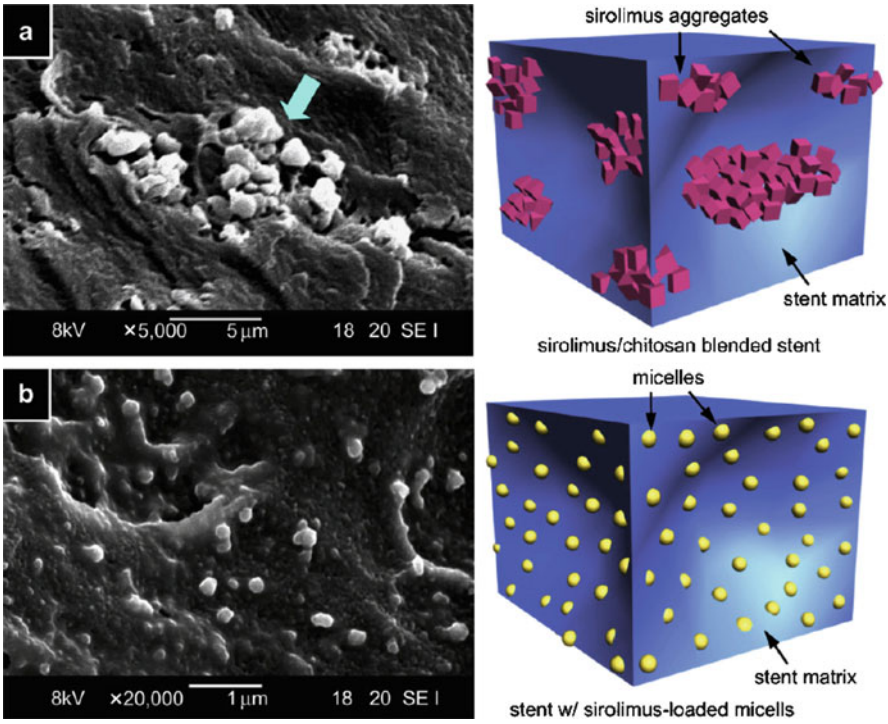

Fig. 13 Dispersibility of drug or drug-loaded micelles in test stents. SEM images and illustrations showing (**a**) the cross-sectional views of the sirolimus/chitosan-blended stent (*arrow* in SEM image indicates the aggregates of sirolimus), and (**b**) the stent with sirolimus-loaded micelles. From [166]; reproduced by permission of Elsevier

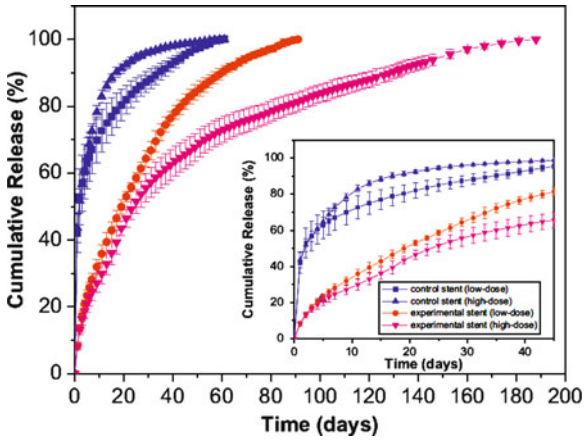

Fig. 14 In vitro cumulative release profiles of the control and experimental stents. The *inset* shows the initial release profiles within the first 45 days. From [166]; reproduced by permission of Elsevier

low-dose and high-dose control stents were approximately 70% and 80%, respectively. These were followed by continuous release for about 2 months. In contrast, the cumulative percentage of the drug that was released in 10 days from the experimental group was much lower, being 35% (low-dose stent) or 28% (high-dose stent); this was followed by a prolonged and sustained drug release for up to 3 months (low-dose stent) or 6 months (high-dose stent). These results indicate that a nanoscale drug entrapment strategy can prevent the drug from aggregating and beneficially reduce its initial burst release, greatly extending the duration of drug release.

Abnormal VSMC proliferation is involved in restenosis following percutaneous transluminal angioplasty [170, 171]. Previous studies have demonstrated that the macrolide antibiotic sirolimus can inhibit VSMC proliferation by blocking cell cycle progression at the G_1–S transition [172]. To evaluate the activity of the released sirolimus, rat thoracic aorta smooth muscle cells (RASMC) were co-incubated with test stents for 3 days. The cell viability of RASMC was significantly reduced when they were co-incubated with either the control or experimental stent, because of the released sirolimus. The antiproliferative activities of sirolimus that was released from these two stents on RASMC were similar to each other (Fig. 15) [166]. Cell cycle analysis revealed that the sirolimus that was released from the stent retained its original activity in inhibiting RASMC proliferation during the G_1 phase, which was consistent with the proven effects of the agent on cell cycle signaling and proliferation.

To investigate the efficacy of the prepared DES in inhibiting restenosis, test stents were individually deployed into the infrarenal abdominal aorta of rabbits. Six weeks after stenting, stent-released sirolimus had greatly altered the intimal response to stent implantation. In the group treated with an empty stent, a marked neointima was observed (inset in Fig. 16a) [166] and many RAM11-positive macrophages (shown in green in Fig. 16d) were present around the stent struts [166]. In contrast, the neointimal area was markedly reduced (insets in Fig. 16b, c) [166], and less macrophage infiltration was observed (Fig. 16e, f) [166] following implantation of the control or experimental stent, revealing the antiproliferative and anti-immune effects of the released sirolimus.

Re-endothelialization occurs after vascular injury and, similarly, following stent placement [144]. Endothelial cells are conventionally believed to proliferate and migrate from intact neighboring artery segments, eventually leading to the re-endothelialization of the injured segment [144]. The degree of CD31-positive cellular coverage (blue arrows in Fig. 16a−c), an indication of re-endothelialization [166], was notably higher in arteries that were implanted with an empty stent than in those that received sirolimus-loaded stents (control or experimental stents).

Recent studies have reported that sirolimus reduces neointimal formation by inhibiting the proliferation and migration of VSMC and delays the normal healing processes of the injured arterial wall [147]. In the control group, poor endothelial cell junction formation (Fig. 16b) [166] and thrombus-like substance deposition around the stent struts (indicated by the arrow in Fig. 16b, inset) were observed at 6 weeks after implantation, possibly owing to the adverse side effects of the initial

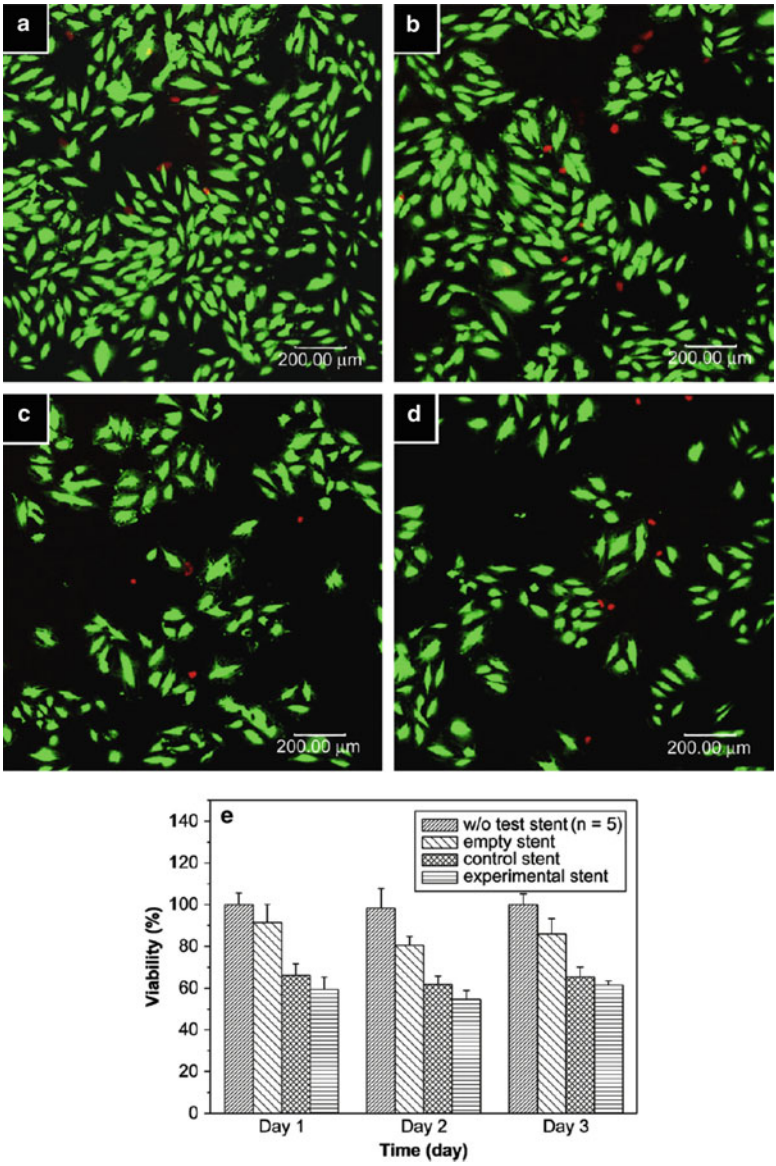

Fig. 15 Results of the activity assay of the sirolimus released from various test stents. Confocal laser scanning microscope (CLSM) images showing the viability of RASMC following treatment with test stents for 3 days: (**a**) no treatment; (**b**) treated with the empty stent; (**c**) treated with the control stent; and (**d**) treated with the experimental stent. (**e**) The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay of RASMC cultured in a polystyrene dish alone (without test stent) and those co-incubated with the empty stent, control stent, or experimental stent for distinct time periods. The cell viability was expressed as a fraction of viable cells and normalized to that of cells cultured in a dish alone. From [166]; reproduced by permission of Elsevier

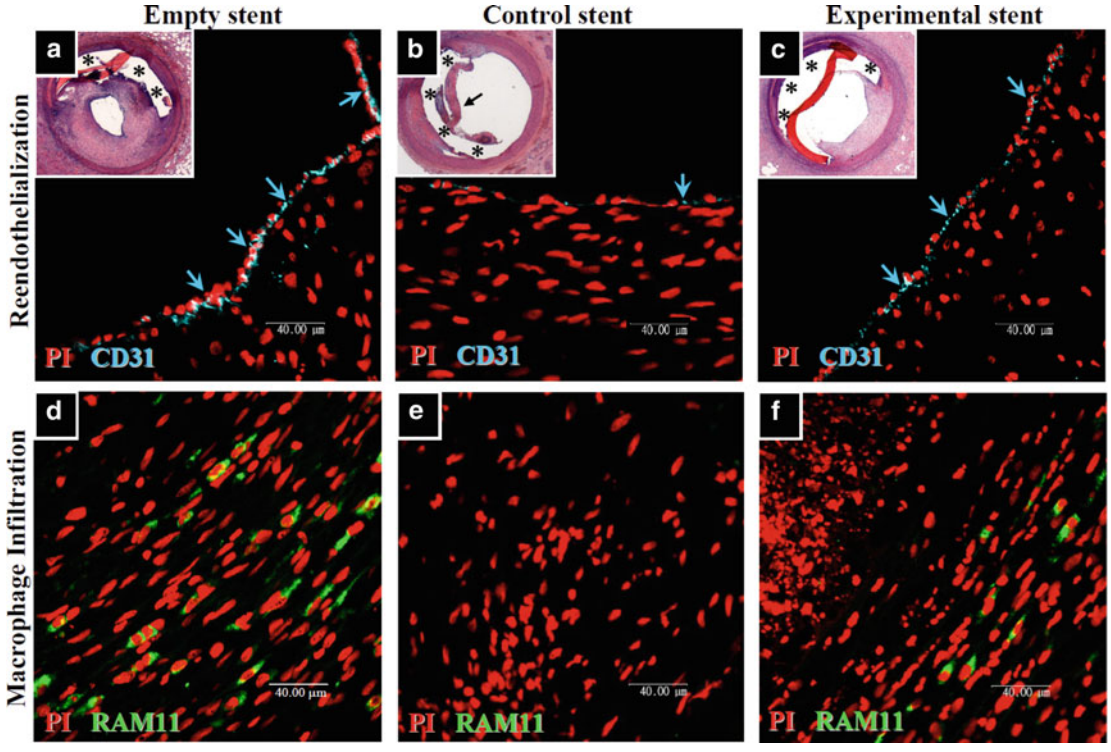

Fig. 16 Results of the vascular response to stent implantation at 6 weeks postoperatively. CLSM images show (**a**–**c**) re-endothelialization and (**d**–**f**) macrophage infiltration after stent implantation: (**a**,**d**) the empty stent; (**b**,**e**) the control stent; and (**c**,**f**) the experimental stent. The immunofluorescence was performed with mouse anti-human CD31 (PECAM-1, *blue*; *arrows* indicate sites of re-endothelialization), mouse anti-rabbit macrophages (RAM11, *green*) and propidium iodide (PI, *red*). The *insets* of (**a**–**c**) are the same sections stained with hematoxylin–eosin. *Sites of stent struts. Because of histological preparation, stent struts have been lost or migrated slightly. *Arrow* in *inset* to (**b**) indicates site of thrombus-like substance deposition. From [166]; reproduced by permission of Elsevier

burst release of sirolimus. In contrast, the experimental stent with nanoscale sirolimus-entrapment within L121 micelles provides sustained release without any apparent burst (Fig. 14) [166], preventing undesirable side effects such as delayed endothelial healing that would be caused by the overdose of sirolimus (Fig. 16c) [166]. The patient must receive a safe and effective dosage of the drug.

3.4 Polymeric Stent Crosslinked by Genipin

Most polymeric stents are flexible along their longitudinal axes, facilitating the delivery of stents through a tortuous vessel. However, one of the limitations of the use of polymers as stent matrices is their inherent mechanical weakness. Covalent crosslinking has been extensively utilized for improving structural stability and the mechanical properties of many engineering materials [173, 174]. The fixation of materials using various crosslinking agents reportedly may form distinct crosslinking structures, which affect their mechanical properties and crosslinking characteristics [175]. A naturally occurring crosslinking agent, genipin, which has a cyclic molecular structure, has been utilized to crosslink chitosan-based stents (genipin stents) as polymeric stents with enhanced mechanical strength [176].

The results of mechanical tests indicate that the genipin stent has a significantly higher ultimate compression load (1,123 $\pm$ 77 mN) and collapse pressure (2.5 $\pm$ 0.1 bar) than the epoxy stent (856 $\pm$ 148 mN, 1.9 $\pm$ 0.1 bar). These results are attributable to the various crosslinking structures that are formed in stent matrices (Fig. 17) [176]. The two epoxide functional groups in the epoxy compound that was used in the study crosslinked the amine groups of chitosan in the stent matrix [177]. In this way, a linearly crosslinked structure between the adjacent chitosan molecules may be formed intermolecularly.

In contrast, genipin reacts spontaneously with the amine groups of chitosan to form a nitrogen-iridoid, which undergoes dehydration to an aromatic monomer. Dimerization occurs in the second stage, perhaps via a radical reaction [178]. Hence, genipin can form a heterocyclic intermolecular crosslinking structure in the stent matrix. The heterocyclic crosslinking structure that is formed in the genipin stent matrix is denser than the linear crosslinking structure in the epoxy stent matrix, because of it is bulky and cyclic nature (Fig. 17) [176]. Therefore, the mechanical strength of the genipin stent greatly exceeds that of its epoxy counterpart. These results suggest that the cyclic crosslinked structures formed within the genipin stent matrix improved its mechanical properties.

The in vivo vascular responses of the genipin stent were investigated in rabbit infrarenal abdominal aortas. At 3 months postoperatively, the retrieved arteries remained patent, and no thrombosis was observed. An almost intact layer of endothelial cells was observed on the stent-implanted vessel wall. Endothelialization is one of the most promising mechanisms for reducing the thrombogenicity of any cardiovascular prostheses [179]. To evaluate its potential use in developing a

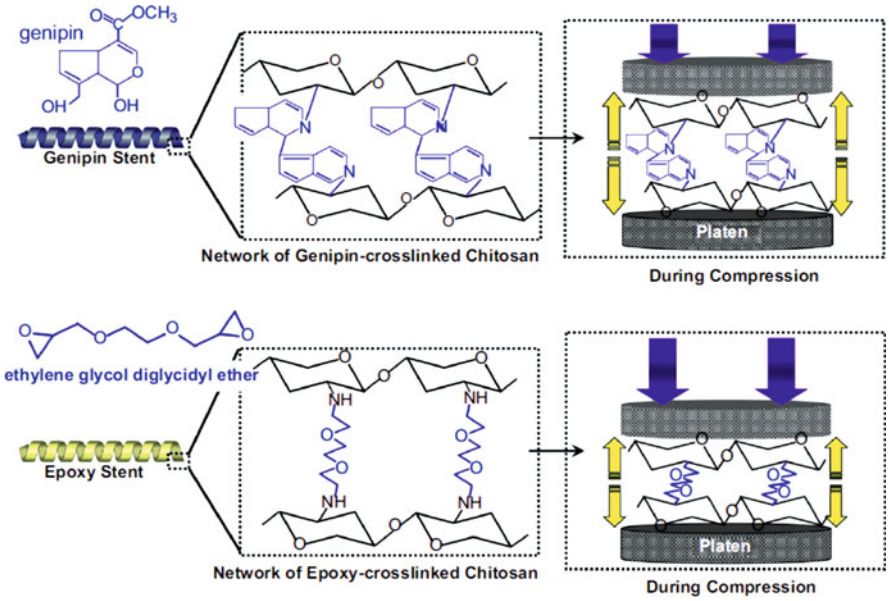

Fig. 17 Illustration of the crosslinking structures formed in the genipin stent and the epoxy stent, and effects of the crosslinking structures on their mechanical strength. From [176]; reproduced by permission of Elsevier

drug delivery vehicle, the genipin stent was loaded with sirolimus. The genipin stent that was coated with hydrophobic heparin (Duraflo, Edwards Life Science) had a linear sustained release profile (Fig. 18) [176], and the released sirolimus maintained its original activity in inhibiting smooth muscle cell proliferation. These findings suggest that the genipin stent with improved mechanical strength can be used for effective local drug delivery.

4 Drug-Eluting Medical Devices

Chitosan-based materials have a wide range of applications, including wound dressings, cartilage and bone grafts, and nerve guidance conduits. In recent years, much attention has been paid to chitosan-based devices owing to their minimal foreign body reactions, intrinsic antibacterial nature, biocompatibility, biodegradability, and ability to be molded into various geometries and forms (such as porous or tubular structures) enabling cell in-growth and nerve conduction [180]. Degradable polymeric implants eliminate the need for a second surgical operation and can prevent some of the problems that are associated with stress shielding during post-healing. Furthermore, they can be used simultaneously to deliver therapeutic

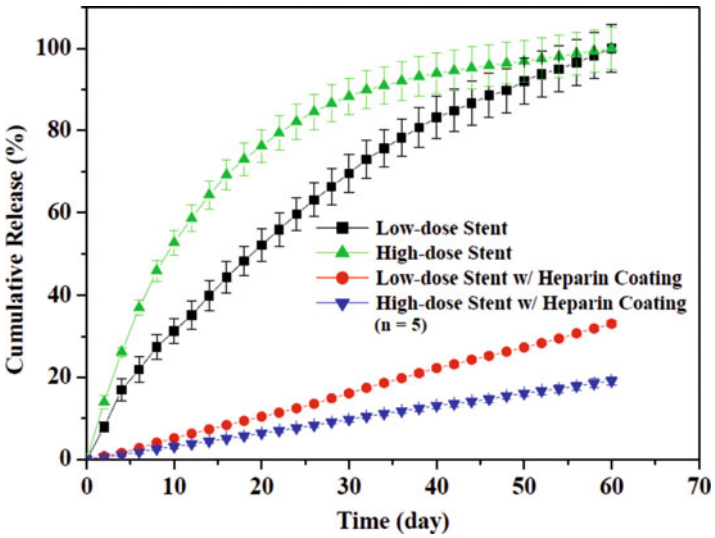

Fig. 18 Cumulative release profiles of sirolimus from the genipin stents with different formulations. From [176]; reproduced by permission of Elsevier

drugs for treating infections or to deliver growth factors to accelerate new tissue growth [181].

Many investigations have demonstrated that tissue regeneration is optimized by the association of proteins or growth factors with a sustained release carrier. Chitosan-based devices have been shown to deliver such agents in a controlled fashion. Sections 4.1–4.4 discuss chitosan-based drug-eluting medical devices for use in tissue engineering and their promotion of the in-growth and biosynthetic ability of tissues.

4.1 Wound Dressings and Artificial Skin

Wound healing is a complex process that involves inflammation, granulation tissue formation, ECM deposition, angiogenesis, and remodeling [182]. Chitosan oligosaccharides are known to have a stimulatory effect on macrophages and polymorphonuclear cells, and chitosan is a chemo-attractant for neutrophils. Accordingly, chitosan promotes the formation and re-epithelization of granulation tissue. Therefore, it is useful for healing open wounds [183]. Many studies have shown that chitosan-based materials in the form of non-wovens, nanofibers, composites, films, and sponges can accelerate wound healing and dermal regeneration [184]. Various wound dressings that are made from chitosan are commercially available. These include Chitosan Skin, HemCon, TraumaStat and others.

To increase healing efficacy, chitosan can be used in combination with many pharmacological agents, such as growth factors, antibiotics, and antibacterial agents. The application of growth factors that can induce fibroblast and/or endothelial cell proliferation for healing-impaired wounds may increase the rate and extent of formation of granulation tissue, and thereby stimulate wound repair. Many growth factors have been incorporated into chitosan-based devices to accelerate healing; these include fibroblast growth factors bFGF and FGF-2 [185–188], platelet-derived growth factor (PDGF) [189], and recombinant human epidermal growth factor (rhEGF) [190].

However, owing to the high diffusibility and the very short half-life of growth factors, their use in treating healing-impaired wounds is not always successful. A photo-crosslinkable chitosan hydrogel can control the release of fibroblast growth factors, thus serving as a drug carrier and inducing neovascularization in vivo [185, 191]. Chitosan hydrogel gradually releases incorporated FGF-2 molecules upon its biodegradation, and chitosan hydrogels that incorporate FGF-2 greatly improve wound healing in healing-impaired obese diabetic (db/db) mice [185].

DNA-incorporating chitosan matrices have been developed as platforms for gene delivery and as reservoirs for the localized and sustained expression of growth factors [192]. Plasmid DNA (pDNA), encoding vascular endothelial growth factor-165 (VEGF-165)/TMC complexes were loaded into a bilayer porous collagen-chitosan/silicone membrane dermal equivalents, which were used in the treatment of full-thickness burn wounds [193]. TMC/pDNA complexes can be released in a sustained manner from collagen-chitosan scaffold for up to 28 days. The super-coiled structure of the released DNA remains, but its content decays with incubation time. Relevant results indicate that the TMC/pDNA-VEGF group had more newly formed and mature blood vessels than other groups, and faster regeneration of the dermis.

Wound infection is one of the most important factors that delay healing. As well as facilitating normal physiological wound repair, a dressing can importantly prevent infection. Adding antimicrobial agents to wound dressings is an effective method. The direct delivery of such agents to the wound site is favorable, especially when systemic delivery can cause organ damage because of toxicological issues that are associated with the preferred agents. Many groups have successfully used chitosan-based wound dressings for the controlled release of antimicrobial agents to prevent infection.

As noted above, polyelectrolyte multilayers that are assembled from cationic chitosan and anionic polymer are a useful platform for loading drugs inside multilayers. Since LbL structures are assembled by electrostatic interaction or hydrogen bonding, charged or polar substances can be sandwiched within the multilayers. This approach has also been used to fabricate drug-loaded wound dressing. Tetracycline (TC), the most effective antibiotic, was sandwiched between a poly(vinylacetate) (PVAc) layer and a chitosan/alginate nanosheet (PVAc-TC-nanosheet) [194]. Under physiological conditions, TC was released from the nanosheet for 6 h. The potential efficacy of the PVAc-TC-nanosheet as an antimicrobial material was studied using a murine cecal puncture model. An in vivo study showed that

treatment with a PVAc-TC-nanosheet increases survival rates and reduces the incidence of bacterial peritonitis.

Silver has been used as an antimicrobial agent for a long time in the form of metallic silver, silver sulfadiazine ointment, or silver nanoparticles. A chitosan-based wound dressing with improved hemostatic and antimicrobial properties was prepared by incorporating a procoagulant (polyphosphate) and an antimicrobial agent (silver) [195]. The silver-loaded wound dressing has been found to exhibit a significantly greater bactericidal activity than an unloaded control, completely killing *Pseudomonas aeruginosa* and consistently killing >99.99% of *Staphylococcus aureus*. A bilayer chitosan wound dressing, consisting of a dense upper layer (skin layer) and a sponge-like lower layer (sublayer), has been designed for the controlled delivery of silver sulfadiazine to treat infected wounds [196]. An in vitro drug release study reveals that the silver that is released from a bilayer chitosan dressing has a slow release profile. In vivo antibacterial tests have confirmed that such a wound dressing effectively inhibits the growth of *P. aeruginosa* and *S. aureus* in an infected wound over the long term.

4.2 Cartilage Graft

Chitosan is regarded as a potential material for use in cartilage tissue engineering to modulate chondrocyte morphology, in differentiation, and in the stimulation of chondrogenesis [197]. Chitosan-based scaffolds not only efficiently support chondrogenic activity [198, 199], but also allow the ability of chondrocytes to synthesize cartilage ECM proteins [199]. Chondrocytes that are cultured in chitosan scaffolds may maintain their round shape, a normal phenotypic characteristic [200].

Although much attention has been paid to chitosan scaffolds, their use is restricted because of limited cell adhesion [201, 202]. Efforts have been made to solve this problem by incorporating ECM molecules into chitosan matrices. Cartilage ECM is composed of mainly type II collagen and glycosaminoglycans (GAGs). To mimic the natural environment of cartilage ECM, chitosan is commonly combined with collagen or/and hyaluronan as a fundamental material for regenerating cartilage. A recent investigation demonstrated that chitosan fibrous scaffolds that are coated with type II collagen promote the attachment and distribution of mesenchymal stem cells and promote chondrogenic differentiation. Additionally, the number of cells, the matrix production, and the expression of genes that are specific to chondrogenesis were improved [201].

Many studies [203, 204] have addressed the regeneration of osteochondral defects by promoting the repair of articular cartilage, which can be further enhanced using controlled release approaches. An ideal scaffold for tissue engineering not only provides a temporary three-dimensional support for the formation of tissues, but also acts as a carrier for important signal molecules [205]. The cationic nature of chitosan allows it to electrostatically interact with anionic GAGs and proteoglycans that are distributed widely throughout the body and with other negatively charged

species [206]. This property is crucial because many cytokines and growth factors are known to be modulated by GAGs [207].

Insulin-loaded chitosan particle-aggregated scaffolds were developed as a controlled release system [205]. Insulin was used as a potent bioactive substance to induce chondrogenic differentiation. In vitro release studies have shown that the concentrations of insulin that are released to the medium were correlated closely with initial loading. Prechondrogenic cells (ATDC5) that are seeded in 5% insulin-loaded scaffolds typically exhibit a chondrocytic round morphology with visible cell condensation. Insulin-loaded scaffolds upregulated the Sox-9 and aggrecan expression of ATDC5 cells after 4 weeks of culture to levels above those of unloaded scaffolds.

To promote the in-growth and biosynthetic ability of chondrocytes, chitosan-based scaffolds were adopted to deliver growth factors in a controlled manner. Transforming growth factor beta1 (TGF-β1) is an important regulator of the proliferation and differentiation of chondrocytes and can promote the synthesis of specific ECMs [208]. Porous collagen/chitosan/GAG scaffolds that are loaded with TGF-β1 reportedly promote cartilage regeneration for cartilage defects [206, 209]. A porous freeze-dried chitosan scaffold that incorporates TGF-β1-loaded microspheres has also been used to treat cartilage defects. TGF-β1 is released in a sustained fashion, promoting the proliferation of chondrocytes and matrix synthesis [209].

Like the direct delivery of recombinant proteins, gene therapy can be used to introduce growth factors. Gene therapy favors local expression more than does the continuous injection of recombinant growth factors, because DNA is more stable and flexible than proteins, and is therefore likely to be compatible with established sustained delivery systems. The feasibility of the use of gene-activated chitosan-gelatin matrices for primary chondrocytes culture and the expression of the TGF-β1 gene in vitro has been established [210]. The incorporation of pDNA into the scaffolds is associated with burst release in the first week and sustained release for the following 2 weeks. A gene that is the transfected into chondrocytes expresses TGF-β1 protein stably for 3 weeks. The histological and immunohistochemical results confirm that the primary chondrocytes that are cultured into the chitosan–gelatin scaffold maintained their round shape and their own characteristic high secretion of specific ECM.

4.3 Bone Graft

Ideal bone substitutes should exhibit osteogenic, osteoinductive, and osteoconductive properties. They should be resorbed and gradually replaced by newly formed bone [211]. Chitosan has been suggested as a potential material for promoting bone regeneration because of its apparent osteoconductive [212] and biodegradable properties. It has been shown to promote the growth of osteoblasts and promote mineral-rich matrix deposition in culture [212]. Additionally, chitosan promotes

the recruitment and attachment of osteogenic progenitor cells, thus facilitating bone formation [213].

However, after grafting chitosan matrices, bone forms over a long period (of many months or years) [214]. The chitosan matrix serves physically in bone defects as a bone substitute, and seems not to suffice to induce rapid bone regeneration in the initial stage of bone regeneration [214]. Chitosan matrices need the additional functions of shortening the bone-forming period and improving the bone-forming efficacy. Chitosan has been demonstrated to be effective in regulating the release of bioactive agents. Incorporating bioactive materials such as growth factors may be very advantageous for improving bone-forming efficacy [214, 215].

Platelet-derived growth factor-BB (PDGF-BB) is an osteoblast mitogen and chemotaxin that has been shown in many preclinical studies to accelerate bone healing [216]. A PDGF-BB-loaded chondroitin-4-sulfate-chitosan sponge is reportedly useful in controlling PDGF-BB release and physically serves as a scaffold to facilitate osteoblast proliferation. Owing to the interaction between positively charged PDGF-BB and negatively charged chondroitin sulfate, incorporated chondroitin sulfate effectively controls the release of PDGF-BB from the sponge and increases the porosity of the sponge. Relevant results indicate that PDGF-BB that is released from the sponge retains its biological activity and enhances the migration and proliferation of osteoblasts [214].

The delivery of multiple growth factors involved in tissue regeneration could mimic the conditions of natural tissue formation. In the bone-forming process, the optimal release kinetics of growth factors must be established, not only with respect to local concentrations but also to the duration of action of the growth factors in the damaged tissue. A brushite–chitosan system has been developed to realize physiologically relevant VEGF/PDGF profiles for bone repair [211]. PDGF acts in the first stage of bone repair, and so this growth factor is dispersed in the brushite for fast release [217]. VEGF acts after PDGF. Consequently, VEGF is pre-encapsulated in alginate microspheres that are present in the chitosan sponge, reducing the release rate and thereby prolonging the presence of the growth factor at the damage site.

In vivo studies have shown that 80% of the PDGF was released from the brushite–chitosan composite scaffold within 2 weeks, while only 70% of VEGF was delivered in 3 weeks. Both growth factors that were released from the constructs remained near the implantation site (5 cm) with negligible systemic exposure. The results indicate that the brushite–chitosan system can control the release rate and localization of both growth factors in a bone defect. PDGF/VEGF-loaded brushite–chitosan scaffolds greatly promoted bone formation [211].

Another investigation also demonstrated the synergistic enhancement of bone formation by both growth factors in combination [218]. Bone morphogenetic proteins (BMPs) can improve bone formation by inducing the chondroblastic and osteoblastic differentiation of mesenchymal stem cells (MSCs) [219]. BMP-2 and BMP-7 have been shown to be the most effective BMPs for stimulating complete bone morphogenesis [220], and have been approved by the FDA for clinical use [221, 222]. A chitosan-based scaffold that contains two populations of nanocapsules for delivering BMPs sequentially has been developed [218]. Poly(lactic acid-*co*-

glycolic acid) nanocapsules that are loaded with BMP-2 and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) nanocapsules that are loaded with BMP-7 enable the early release of BMP-2 and the longer term release of BMP-7. The results indicate that sequential growth factor delivery is more effective than the use of an individual growth factor in tissue engineering because it mimics the natural process of healing.

Chitosan-based composite scaffolds are also used as drug delivery systems in the antibiotic treatment of osteomyelitis, which is a common bone disease caused by a bacterial infection of the bone modular cavity, cortex, and/or periosteum following implantation [223, 224]. Macroporous chitosan scaffolds that are reinforced by β-tricalcium phosphate (β-TCP) and calcium phosphate invert glasses were originally designed as both drug carriers for controlled drug release and scaffolds for bone regeneration [223]. Related results indicate that incorporating β-TCP and glass into the chitosan matrix effectively reduces the initial burst release of the antibiotic gentamicin sulfate from the composite chitosan scaffolds, perhaps because calcium phosphates are more stable than chitosan in physiological media and have a much lower dissolution rate therein. Therefore, controlled biodegradation and drug release can be achieved by the hybridization of chitosan and calcium phosphates. MG63 osteoblast-like cells that are seeded on the composite scaffolds grow and migrate into the scaffolds, suggesting good cell biocompatibility of the composite scaffolds. Calcium phosphate/chitosan composite scaffolds were originally fabricated for potential use as a bone substitutes and a synergistic drug carrier, and they can be utilized to deliver biological active agents to promote bone regeneration.

4.4 Nerve Guidance Conduit

Large-gap nerve damage that cannot be directly repaired using sutures has usually been treated using nerve autografts, but this approach suffers from donor site morbidity, inadequate return of function, aberrant regeneration, and shortage of donor tissue [225, 226]. An alternative method is to use a nerve guide conduit. Nerve conduits are tubular structures that are used to bridge the gap of a severed nerve, and thereby acting as guides for the regenerating axons, and as barriers to the in-growth of scar-forming tissue [227].

Chitosan-based composites have also been regarded as promising materials for nerve repair [225, 228–231]. In particular, nerve guide conduits that are made from chitosan and poly(caprolactone) nanofibrous matrix have excellent mechanical and biological properties in vitro [225]. Additionally, a pilot in vivo study demonstrated the regeneration of nerve fibers after implantation of the chitosan/poly(caprolactone) fibrous matrices in a sciatic nerve defect in rats for 1 month. Some groups have also attempted to use a nerve graft that comprises an outer microporous conduit of chitosan and interior oriented filaments of polyglycolic acid for bridging a 30-mm defect in sciatic nerves in six Beagle dogs [231]. Six months postoperatively, in the chitosan/polyglycolic acid graft group, the dog sciatic nerve trunk had been reconstructed with restored nerve continuity and functional recovery, and its

target skeletal muscle had been re-innervated, improving the locomotion of the limb that had been operated upon. These investigations establish the feasibility of a chitosan-based nerve graft for regenerating peripheral nerves.

However, physical nerve guidance by a nerve conduit may not suffice to optimize recovery [227]. The transience of the increase in growth factor expression following nerve injury, mainly by Schwann cells [232], is thought to compromise the ability of cut axons to maintain their regenerative state [233]. The prolonged delivery of exogenous growth factors to the injured nerve may thus sustain the drive to regenerate injured axons when endogenous growth factor expression has been downregulated [234].

Attempts to improve the regeneration process by including growth factors in the nerve conduit have had partial success [235, 236]. One method of loading conduits with growth factors involves blending of the factor with the scaffold polymer, making the factor an intrinsic part of the conduit, which is released as the conduit is degraded. This method has been used to integrate glial cell line-derived neurotrophic factor (GDNF) into chitosan conduits before experimental sciatic nerve injuries are inflicted. The GDNF-loaded conduit can promote axon regeneration in the early stages of recovery at around 6 weeks post-implantation, but after 9 and 12 weeks, no differences between the GDNF that contained the conduit and the empty chitosan controls can be observed [235].

Like growth factors, laminin peptides facilitate nerve regeneration. One study utilized GDNF–laminin-blended chitosan (GLC) nerve guides in rat peripheral nerve injury models to promote functional nerve recovery, as determined by gait analysis and measurements of muscle mass [236]. The results indicated a greater functional restoration in the group that was treated with GLC than was achieved using the unblended chitosan nerve guides. Muscle weights of the GLC group revealed less atrophy and greater restoration of functional strength than in the unblended control groups. Additionally, according to behavioral testing, the GLC group regained sensation while the control groups exhibited no restoration. The study that involved those tests verified that adding GDNF and laminin to chitosan nerve guides accelerated both functional and sensory recovery.

Although modification of conduits by the inclusion of growth factors has been shown to promote nerve regeneration under some conditions, little is known about the effect of their delivery kinetics on peripheral nerve regeneration [227]. The timing of growth factor delivery is probably important in determining the degree of axon regeneration [234]. Clearly, further development and refinement of the drug delivery technique are required.

One group has begun to develop nerve conduits with an adjustable rate of release of nerve growth factor (NGF) [227]. The nerve conduit, which comprises a polyelectrolyte alginate/chitosan complex, is coated with layers of poly(lactide-*co*-glycolide) to control the release of embedded NGF. The related study revealed that the release kinetics could be efficiently adjusted by incorporating NGF at various radial locations within the nerve conduit. The release of bioactive NGF in the low nanogram per day range was sustained for at least 15 days. The designed

nerve conduits are thus promising candidates for exploring the effect of release kinetics on nerve regeneration in the future.

5 Conclusions

Controlled drug-eluting devices, which can be easily tailored and conveniently prepared to release drugs at specified controlled rates, are beneficial to the healing process. This review reports the utility of chitosan as a potential material for developing drug delivery devices and their sustained release ability for various pharmacological agents, such as growth factors, proteins, antibiotics, or antibacterial drugs. However, the effect of prolonged delivery of therapeutic agents to lesion sites may not be as straightforward as expected. It is likely that the delivery timing plays an important role in affecting the efficacy of tissue regeneration. Therefore, further development and refinement of the delivery technique is required for current systems.

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