

Chitosan and Chitosan Derivatives in Drug Delivery and Tissue Engineering

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Abstract Chitosan is a nontoxic, biodegradable, and biocompatible polysaccharide of $\beta(1-4)$ -linked D-glucosamine and N-acetyl-D-glucosamine. This derivative of natural chitin presents remarkable properties that have paved the way for the introduction of chitosan in the biomedical and pharmaceutical fields. Nevertheless, the properties of chitosan, such as its poor solubility in water or in organic solvents, can limit its utilization for a specific application. An elegant way to improve or to impart new properties to chitosan is the chemical modification of the chain, generally by grafting of functional groups, without modification of the initial skeleton in order to conserve the original properties. The functionalization is carried out on the primary amine group, generally by quaternization, or on the hydroxyl group. This review aims to provide an overview of chitosan and chitosan derivatives used for drug delivery, with a special emphasis on chemical modifications of chitosan to achieve specific biomedical purpose. The synthesis of the main chitosan derivatives will be reviewed. The applications of chitosan and these chitosan derivatives will be illustrated.

Keywords Chitosan · Chitosan derivatives · Drug delivery

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1 Introduction

Polymers are extensively used for the delivery of an active pharmaceutical ingredient. They can form a matrix or membrane that can control the release of a drug over a prolonged period, thus avoiding repetitive dosing. They can also be used to form (nano)carriers to deliver drugs, in particular poorly soluble drugs or biotechnology-based drugs. Both systems can protect the drug from degradation. Moreover, when the carrier is functionalized by a targeting agent, the encapsulated drug may be selectively released inside or near a specific tissue or organ. Polymeric delivery systems can modify the pharmacokinetics of a drug, leading to a higher therapeutic index by decreasing the side effects and/or increasing efficacy. Several polymeric drug delivery systems such as nanoparticles, micelles, hydrogels, or matrices are being studied worldwide. Generally, these systems are composed of a biocompatible polymer, degradable or not, and of an active pharmaceutical ingredient dispersed or covalently bound to the polymer. The release of the drug usually occurs by diffusion through the polymer, by degradation of the polymer, or by disorganization of the supramolecular structure of the carrier.

Among all the polymers available to be used for drug delivery systems, (bio) degradable polymers are highly recommended. Indeed, one of the key points of this kind of system is the removal of the carrier after the release of the active pharmaceutical ingredients. Moreover, to avoid side effects, in particular when the carrier is injected, the polymer must be biocompatible. For all of these reasons, natural polymers such as polysaccharides, polypeptides, or phospholipids are generally used as building blocks for the formulations [1].

This paper will focus on chitosan and chitosan derivatives developed for biomedical applications. In the first section, the remarkable properties of chitosan will be exposed. The main chemical modifications used to adapt this material for biomedical applications will be reviewed. Their applications in drug delivery systems and tissue engineering will then be discussed.

2 Production and Properties of Chitosan

Chitosan is a nontoxic, semicrystalline [2], biodegradable [3, 4], and biocompatible [5, 6] linear polysaccharide of randomly distributed *N*-acetyl glucosamine and glucosamine units (Fig. 1).

Chitosan is not widely present as such in nature and thus cannot be directly extracted from natural resources. Indeed, chitosan is a derivative of natural chitin, the second most abundant polysaccharide in nature after cellulose [2]. Typically, chitosan is obtained by deacetylation of the *N*-acetyl glucosamine units of chitin, generally by hydrolysis under alkali conditions at high temperature. The deacetylation of chitin is rarely complete. When the degree of acetylation falls below the value of 60 mol%, chitin becomes chitosan. In nature, chitin is present in life forms and more particularly in insects and crustaceans where it represents the major component of their exoskeleton. Chitin is also present in the cell wall of some mushrooms [7, 8]. Generally, chitosans produced from mushrooms present a narrow molecular weight distribution compared to chitosan produced from shrimps, and a non-animal source is considered to be safer for biomedical and healthcare uses.

Chitosan offers remarkable biological properties, which have paved the way for its application in the pharmaceutical and biomedical fields [9, 10] in new drug delivery systems [1, 11, 12] or as a scaffold for tissue engineering [13]. Indeed, chitosan has good mucoadhesive properties due to its positive charge [14], which increases the adhesion to mucosa and so the time of contact for drug penetration. Its haemostatic properties makes chitosan a good candidate for wound dressing [15, 16]. Moreover, the antibacterial property of chitosan also limits the risk of infection [17, 18].

Chitosan is a polycation whose charge density depends on the degree of acetylation and pH. So, chitosan chains are able to interact by electrostatic interactions with negatively charged molecules. It can form nanoparticles by ionic gelation with polyphosphates [19] and with nucleic acids [20–22].

However, chitosan suffers from a poor solubility in water, which is a major drawback for drug formulations. Indeed, chitosan is only soluble in acidic solutions of pH below 6.5, required to insure the protonation of the primary amine. In such cases, the presence of positive charges on the chitosan skeleton increases the repulsion between the different polymer chains, facilitating their solubilization.

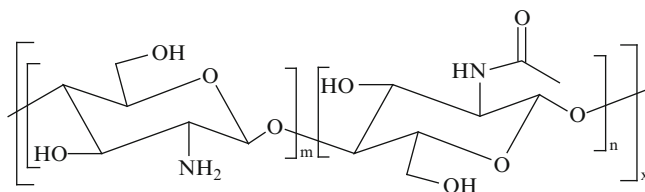


Fig. 1 Chemical structure of chitosan

As far as organic solvents are concerned, chitosan is slightly soluble in dimethyl sulfoxide (DMSO) and *p*-toluene sulfonic acid [23]. This poor solubility is a limitation for the processing of chitosan and is also a brake in its chemical modification. In order to tackle this drawback, chitosan oligomers are sometimes preferred. These oligomers (polymerization degree of around 20) are much more soluble into water compared to their polymer counterpart, even at physiological pH. Several methods for the synthesis of chitosan oligomers are reported that are mainly based on an acidic hydrolysis at high temperature. Nevertheless, final hydrolysis yields are often low and lead to a mixture of products (oligomers, glucosamine monomers) that must be purified [24, 25].

An important aspect for the application of chitosan in drug delivery systems is the fate of the chitosan in the body after absorption or injection. Generally, chitosan is eliminated by renal clearance but if the molecular weight is too large a degradation step by enzymes is required [26]. In the human body, three chitinases showed an activity leading to the formation of smaller chains [26]. Nevertheless, the rate of degradation depends on the molecular weight and the acetylation degree of the starting material [27].

3 Chemical Modifications of Chitosan

In order to improve or impart new properties to chitosan, chemical modification of the chitosan chains, generally by either grafting of small molecules or polymer chains onto the chitosan backbone or by quaternization of the amino groups, has been investigated. Chitosan chains possess three attractive reactive sites for chemical modification: two hydroxyl groups (primary or secondary) and one primary amine. The site of modification is dictated by the desired application of the final chitosan derivative. For example, the preservation of the primary amine is highly desirable for transfection application. Some chitosan derivatives having potential application in drug delivery and tissue regeneration are presented in Sects. 3.1–3.3.

3.1 *Quaternized Chitosan Derivatives*

Several chemical modifications of chitosan have been tested to make the solubility and/or positive charge of chitosan independent of pH.

The quaternization of the primary amine was investigated [28, 29]. This chemical modification increased the solubility of chitosan in water [30], keeping chitosan soluble over a wide pH range. In addition, the cationic character [23] can be controlled and kept pH-independent, which is desirable for improving the stability of ionic complexes [31, 32]. Typically, the reaction of chitosan with methyl iodide under basic conditions is the most straightforward route for quaternizing chitosan [33].

Among all the quaternized chitosans described into literature, *N,N,N*-trimethyl chitosan chloride (TMC) is the most widely applied for gene therapy applications [28, 33, 34]. The quaternization maintained and improved the muco-adhesive properties of chitosan, depending on the quaternization degree, which makes this chitosan derivative an ideal candidate for gene delivery [23]. Typically, TMC can be synthesized by reaction of chitosan with methyl iodide in the presence of sodium hydroxide into *N*-methyl-2-pyrrolidinone at 60 °C. In a second step, the iodide ion is substituted by chloride by an ion exchange process [34] (Fig. 2).

To enhance the delivery properties of TMC, Verheul and coworkers developed a synthetic route for the preparation of thiol-bearing TMC [35, 36]. Indeed, the presence of thiol increased the muco-adhesion of chitosan derivatives by formation of a disulfide bond with mucin proteins of the cell membrane [37, 38].

To increase the water solubility of chitosan, Toh et al. grafted succinic acid onto chitosan and demonstrated, by measurement of the cloud point, a higher solubility in water at pH 7.3 when 20 mol% of primary amine are converted into carboxylic acid [39]. Moreover, the grafting of carboxylic acids onto chitosan chains improved the transfection efficiency compared to pure chitosan but led to the formation of a weaker complex with DNA.

To improve the transfection efficiency of chitosan, the grafting of cationic polymer chains onto chitosan was also investigated [40–42]. Jere et al. successfully grafted low molecular weight poly(ethylene imine) (PEI) chains onto chitosan with formation of the corresponding chitosan-*g*-PEI copolymer [43]. The grafting of the PEI occurred in two steps. First, chitosan was reacted with potassium periodate in an acetate buffer leading to opening of the glucosamine ring with formation of two aldehyde groups. The reaction of the pendant primary amine of PEI oligomers with these aldehyde groups allowed the grafting with formation of an imine, which was

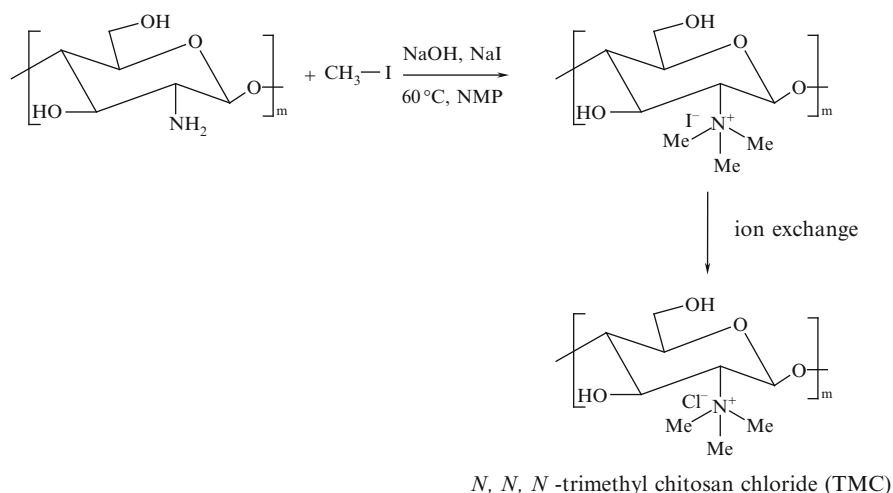


Fig. 2 General synthesis of *N,N,N*-trimethyl chitosan chloride

rapidly converted into amine by reduction with NaBH_4 during the second step. A one-step alternative strategy was proposed by Gao et al. based on the use of carbonyldiimidazole as coupling agent [44]. The grafting of poly(L-arginine) (PLR) chains was proposed by Noh et al. via formation of an amide bond between the primary amine of chitosan on the carboxyl acid of the PLR [45].

Another approach based on the introduction of amine groups onto chitosan was also proposed. Ghosn et al. investigated the introduction of secondary and tertiary amines to improve the transfection efficiency of chitosan [46]. This one-step synthesis was based on the grafting of a carboxylic acid-bearing imidazole onto chitosan by amide formation, mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and was simple and reproducible and improved the solubility and the buffering capacity of the chitosan derivatives.

3.2 Amphiphilic Chitosan Derivatives

To synthesize amphiphilic chitosan derivatives, the grafting of hydrophobic molecules was investigated. Initially, the grafting of hydrophobic alkyl chains onto chitosan is the most straightforward way to impart amphiphilic properties to chitosan. For this purpose, alkyl aldehydes or alkyl ketones were selectively grafted onto the primary amino groups of chitosan with formation of the corresponding Schiff base [47–49]. A reduction step mediated by sodium/potassium borohydride ($\text{NaBH}_4/\text{KBH}_4$) or sodium cyanoborohydride (NaBH_3CN) converted the imine group into the more stable amine with formation of the corresponding *N*-alkyl chitosan derivatives (Fig. 3).

For biomedical applications, the use of NaBH_3CN , able to generate toxic side products (e.g., HCN), is not acceptable. The synthesis and application in drug

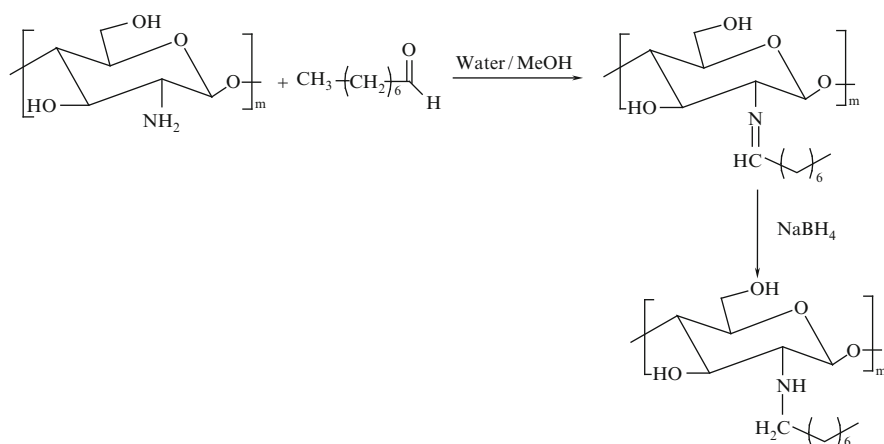


Fig. 3 Grafting of alkyl chain by reductive amination

delivery of *N*-alkyl chitosan derivatives with different chain lengths (C3, C5, C6, C8, C10, and C12) are reported in the literature [50–52]. Nevertheless, these *N*-alkyl-chitosan derivatives did not show optimum properties for the formulation of nanoparticles for drug delivery systems. In most cases, a double functionalization is proposed based on the grafting of both hydrophobic and hydrophilic moieties to improve the amphiphilic property of chitosan derivatives. As a representative example, Zhang et al. sequentially grafted octyl chains by reductive amination followed by the addition of sulfate groups onto chitosan chains [53, 54]. Typically, the reaction occurred in a water/methanol mixture in order to solubilize both hydrosoluble chitosan and liposoluble alkyl aldehyde. After reduction of the imine group, the primary hydroxyl groups of chitosan were selectively converted into sulfates by reaction of the *N*-octyl-chitosan with chlorosulfonic acid. Note the triple functionalization of chitosan by an octyl chain on some of the primary amines, by a poly(ethylene glycol) (PEG) chain on the remaining primary amines, and by a sulfate on some hydroxyl groups of chitosan chains. This provided both hydrophobic and hydrophilic character enhancement as proposed by Yao and coworkers [55, 56]. Another example was proposed by Zhang et al. who synthesized amphiphilic quaternized *N*-octyl-*N*-trimethyl chitosan chloride derivative by reaction of *N*-octyl-chitosan with iodomethane [49]. Huo et al. synthesized *N*-octyl-*O*-glycol-chitosan by successive reaction of chitosan chains with *N*-octyl-aldehyde followed by the ring opening of ethylene oxide under basic conditions [48]. Xiangyang successfully converted the remaining amino group of *N*-octyl-chitosan into carboxylic acid by reaction with succinic anhydride [57]. Li et al. proposed a similar strategy, but based on the use of phthalic anhydride [58]. An interesting approach was proposed by Liu et al. [59] whereby hexahydroxyphthalic acid was grafted onto *N*-octyl-chitosan by opening of acid anhydride by the remaining primary amine. Surprisingly, the hexahydroxyphthalic group was easily removed under acidic conditions leading to the precipitation of *N*-octyl-chitosan. The grafting of alkyl chains is not limited to reductive amination. Ercelen et al. grafted 2-(dodecen-1-yl)succinic anhydride onto oligo-chitosan chains by opening of the cyclic anhydride by the nucleophilic primary amine [47]. Lao et al. proposed the grafting of a C18 alkyl chain terminated by an epoxide followed by conversion of both remaining primary amines and hydroxyl groups into sulfate [60].

The grafting of aromatic groups as hydrophobic moieties was also investigated. Koutroumanis et al. grafted a 2-carboxybenzyl group by reductive amination [61]. Opnasasopit et al. reacted chitosan chains with phthalimic anhydride with formation of hydrophobic phthalimide-chitosan. The amphiphilic property was obtained by the grafting of poly(vinyl pyrrolidone) polymer chains terminated by a carboxylic acid group onto the primary amine, mediated by the crosslinker EDC [62]. Opnasasopit and coworkers extended this strategy by replacing poly(vinyl pyrrolidone) chains by PEG chains [63].

With the purpose of preparing chitosan-based amphiphilic copolymer with only natural and renewable compounds, the grafting of fatty acids was investigated by several research groups [64–67]. Generally, the grafting occurred by formation of an amide bond between the primary amine of chitosan and the terminal carboxylic

acid of the fatty acid, mediated by EDC in a water/alcohol mixture under vigorous stirring (Fig. 4).

Saturated stearic acid [65, 68–70] and unsaturated lineolic acid [66, 67] are two examples of fatty acids successfully grafted onto chitosan oligomers by this strategy. With this purpose, Zhang et al. preferred to first convert the carboxylic acid of oleic acid into acid chloride before it was reacted with chitosan in chloroform in the presence of pyridine [71]. Using a similar strategy, stearic, palmitic, or octanoic anhydride were grafted onto chitosan by Jiang et al. [64].

Steroid derivatives were also employed as natural compounds able to confer amphiphilic properties to chitosan. Several examples of grafting of 5 β -cholanic acid [72–75] and cholesterol [76] onto *O*-glycol-chitosan are reported in the literature (Fig. 5). For steroids, the strategy relies on the activation of the carboxylic acid by *N*-hydrosuccinimide in order to favor the grafting efficiency on the primary amine of glycol chitosan, mediated by EDC.

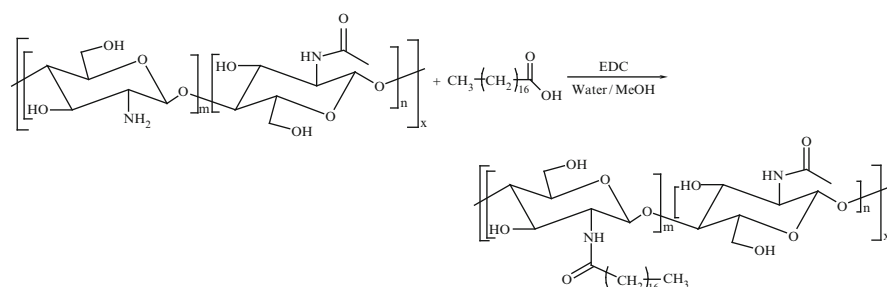


Fig. 4 Grafting of stearic acid onto chitosan

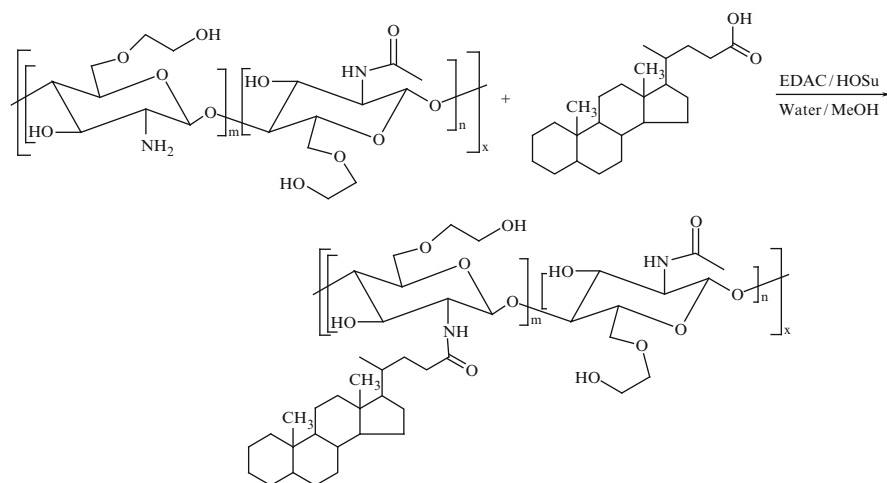


Fig. 5 Grafting of cholanic acid onto *O*-glycol chitosan

The grafting of hydrophobic biodegradable and biocompatible aliphatic polyester chains [77], and more particularly poly(ϵ -caprolactone) (PCL) chains [78–80], was investigated for the preparation of biocompatible and biodegradable chitosan-based amphiphilic grafted copolymers for nanoparticles formulation. For this purpose, two grafting strategies were investigated: the “grafting from” and the “grafting onto” techniques [81, 82] (Fig. 6).

In the “grafting from” technique, PCL chains are synthesized by the initiation of polymerization of ϵ -caprolactone directly by the primary amine, or the hydroxyl groups, present on the chitosan chain. A selective initiation of the polymerization exclusively by the hydroxyl groups can be reached if the primary amines are protected before polymerization and deprotected afterwards [80, 83, 84]. The grafting of PCL by “grafting from” initiated by the hydroxyl group was reported by Duan et al. [80]. Typically, the primary amines were protected by formation of a stable electrostatic complex with methylsulfonic acid, which is easily removed by precipitation in a phosphate buffer after polymerization. In the case of the “grafting onto” technique, polymer chains bearing an appropriate functional group at one chain-end were grafted onto the primary amine or hydroxyl groups of chitosan [85]. In the same way as for the “grafting from” technique, no selectivity on the grafting site was possible if the primary amines were not protected, e.g., by reaction with phthalic anhydride with formation of phthalimide-chitosan [78]. Moreover, the protection of the primary amine was also very helpful to solubilize chitosan in organic media. Ester or urethane links are two examples of organic functions used for the grafting of PCL terminated by a carboxylic acid [86] or an isocyanate group [78], respectively, onto hydroxyl groups of phthalimide-chitosan. Compared to the “grafting from”, the “grafting onto” technique allowed a better control of the number and molecular weight of the PCL grafts onto chitosan. The grafting of polymer chain onto chitosan is not limited to PCL. The grafting of PEG chains onto chitosan is widely described in the literature [87–91]. Recently, Casettari et al. grafted carboxylic acid-terminated PEG chains onto the primary amines of chitosan and compared the toxicity of the resulting grafted copolymer to chitosan [85]. However, the grafting of hydrophilic PEG chains was not enough to confer amphiphilic properties but can be coupled with the grafting of PCL chains. So, a heterografted chitosan bearing PCL and PEG chains was synthesized by Liu et al. by simultaneous grafting of carboxylic acid-terminated PEG and PCL onto the hydroxyl group of phthalimide-chitosan [92].

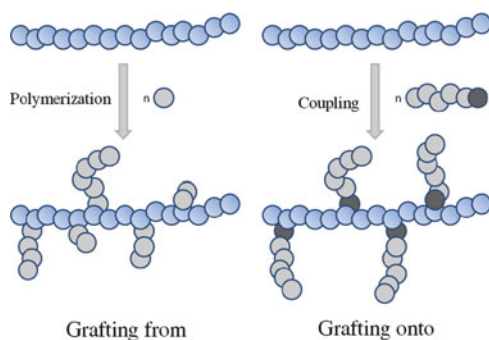


Fig. 6 The “grafting from” and the “grafting onto” techniques

3.3 Chitosan-Based Hydrogels

Due to its remarkable properties, chitosan has been applied to the synthesis of scaffolds or hydrogels dedicated to tissue engineering. A hydrogel is defined as a polymer network able to absorb an important quantity of water. These hydrogels are called physical gels if the origin of the network is due to physical phenomena (phase separation, sol–gel transition, etc.) and called chemical gels if the network is due to the formation of covalent links between the polymers chains. By its hydrophilic nature, chitosan can be applied as starting material for the elaboration of biodegradable and biocompatible hydrogels. Nevertheless, the properties of these chitosan-based hydrogels are not satisfying enough for specific applications [93]. In order to increase the mechanical properties of chitosan, Madhumathi prepared hydroxyapatite/chitosan composite [94]. Chitosan derivatives were then used for the synthesis of improved chitosan-based hydrogels. So, chitosan-g-PEG graft copolymer, synthesized by nucleophilic attack of the primary amine of chitosan onto chloride-terminated PEG, was able to form a stable physical hydrogel [95]. Chemical chitosan-based hydrogel was successfully prepared by Michael addition of a thiol-terminated six-armed star-shaped PEG onto acrylate-bearing chitosan [96]. Photopolymerizable chitosan-g-PEG was synthesized by Poon et al. for layer-by-layer cell encapsulation [97]. *N*-[(2-Hydroxy-3-trimethylammonium)propyl] chitosan chloride (HTCC) was chemically modified using glycidyltrimethylammonium chloride (GTMAC) by Shi et al. [98].

Another approach to improve the properties of chitosan hydrogels is via the preparation of polymer composites. Porous hydrogels of *N*-carboxymethyl chitosan/polyvinyl alcohol were prepared by Lee et al. [99]. Hydroxypropyl chitosan was combined with sodium alginate for the formation of biodegradable hydrogels [100]. Chitosan–hyaluronic acid composite was prepared by Tan et al. [101].

With the purpose of conferring thermosensitivity to chitosan-based hydrogels, Park et al. proposed the grafting of carboxylic acid-terminated poly(ethylene oxide-*b*-propylene oxide) block copolymer (Pluronic) onto the primary amine of chitosan, mediated by EDC coupling agent [102]. With the same purpose, Wang et al. grafted poly(*N*-isopropyl acrylamide) (NiPAM) chains onto chitosan by the copolymerization of acrylic acid-derivatized chitosan and *N*-isopropylacrylamide (NIPAAm) in aqueous solution [103].

4 Biomedical Applications of Chitosan and Chitosan Derivatives

Chitosan and chitosan derivatives have been extensively studied for drug delivery and other biomedical applications due to (1) their biocompatibility and low toxicity, (2) their possible formulation in nanoparticles or in gels, and (3) their cationic properties. An overview of their use in biomedical applications will be given for

gene delivery, solubilization of poorly soluble drugs, tissue engineering, and protein/vaccine delivery. These examples are not exhaustive but clearly demonstrate the benefit of chitosan derivatives in drug delivery.

4.1 Gene Delivery by Chitosan and Chitosan Derivatives

4.1.1 Chitosan as Carrier for Gene Delivery

Over the past few decades, many studies have reported the potential of gene therapy for purposes such as (1) silencing a gene (siRNA, shRNA), (2) compensating for defective genes, and (3) producing beneficial proteins or vaccines (DNA). However, the delivery of nucleic acids is confronted by many hurdles, like degradation by nuclease or lack of efficiency because their negative charges impair crossing over cellular membranes. For this purpose, viral vectors have been widely used for encapsulation of the genes. Nevertheless, these vectors present some immunogenic, cytotoxic, and oncogenic side effects [104, 105]. The substitution of these viral vectors by synthetic vectors made of polycationic polymers is an alternative way to protect the nucleic acids and to allow them to reach their therapeutic targets: the cytoplasm for siRNA or the nucleus for DNA.

Chitosan is one of the most commonly studied polymers in nonviral gene delivery [106]. Indeed, its positive charges under slightly acidic conditions allow its interaction with nucleic acids such as DNA or siRNA and the condensation of the nucleic acids into nanoparticles. In addition, the biocompatibility and low toxicity of chitosan enable its *in vivo* use [107]. However, the poor buffering capacity and poor solubility in water [46] make chitosan less efficient than other cationic synthetic polymers, such as PEI or PLL.

Two major formulation methods for nucleic acid-loaded nanoparticles are described in the literature. The simple complexation method consists in mixing chitosan and nucleic acids without any purification of the nanoparticles [108]. Using this process, Howard et al. obtained 78% of gene silencing on EGFP-H1299 cells [108]. However, as the obtained product might contain nucleic acid/chitosan complexes coexisting with free chitosan and free nucleic acids, this method could result in lower efficiency after intravenous administration. An alternative method, which ensures the entrapment of the nucleic acid into the nanoparticles, is ionic gelation involving the addition of a crosslinking agent. Therefore, in this method, formation of nanoparticles is based not only on electrostatic interactions but also on physical entrapment, resulting in stronger connections between the components. The most commonly used crosslinking agent is tripolyphosphate [109, 110]. Some authors used also polymers like alginate and polyguluronate as crosslinking agents [99].

The ability of chitosan to form complexes with nucleic acids is highly dependent on its structural characteristics. Indeed, the deacetylation degree (i.e., the percentage of deacetylated primary amine groups along the macromolecular chain)

determines the positive charge density of the polymer and, consequently, influences the electrostatic interactions with nucleic acids. To ensure a good complexation, the deacetylation degree must be higher than 65% [32]. Thibault et al. also reported that complexes with a greater degree of deacetylation showed a higher level of binding to cells, resulting in enhanced uptake [111].

Another physical characteristic to be considered is the molecular weight of chitosan, which influences the size and the stability of the nanoparticles. High molecular weight chitosan allows a better stability of the nanoparticles, which is advantageous for the protection of the nucleic acids but can also be an obstacle to the intracellular dissociation of complexes and therefore for nucleic acid release. Thus, the most efficient chitosans showed an intermediate stability and a kinetics of dissociation that occurred in synchrony with lysosomal escape [111]. For optimum DNA delivery and to ensure high transfection level, chitosan should have a molecular weight of between 10 and 50 kDa [106]. In contrast, for siRNA (13.4 kDa), higher molecular weight chitosan (65–170 kDa) can form stable nanocomplexes and induce a higher silencing efficacy [112]. Indeed, longer DNA strands may be able to compensate for the shorter chitosan chains in the assembly process.

The formation of nanoparticles also depends on the N:P ratio, defined as the molar ratio of chitosan amino groups to nucleic acid phosphate groups. The N:P ratio is different depending on the type of nucleic acid used, but in both cases an excess of chitosan is required to ensure good complexation of nucleic acids. For DNA, a ratio of between 3:1 and 5:1 is optimal [106], whereas higher ratios are necessary for siRNA. Indeed, Liu et al. showed that 50% and 80% EGFP (enhanced green fluorescent protein) silencing was obtained in H1299 cells at siRNA N:P ratios of 50 and 150, respectively, whereas only 10% knockdown occurred at N:P ratios of 2 and 10 [112].

4.1.2 Gene Delivery with Chitosan Derivatives

Although a few authors have reported good gene delivery efficiency using native chitosan, several publications demonstrate the limitations of this polymer. Indeed, one of the first restrictions is the poor solubility of the chitosan at physiological pH because of the partial protonation of the amino groups. This pH-dependence influences nucleic acid binding capacity and therefore the transfection effectiveness. Indeed, Zhao et al. obtained the highest transfection level on chondrocytes using chitosan/pEGFP complexes at pH 6.8 and 7 whereas a remarkable decrease was observed at pH 7.4, which may be due to the decondensation of the complex [113]. Therefore, decreasing the pH sensitivity of chitosan could be advantageous. Hence, among the chitosan derivatives used in gene delivery, TMC (Fig. 2) has been the most studied [114].

Moreover, in comparison to other cationic polymers like PEI, chitosan showed restricted transfection efficiency. This might be caused by the insufficient endosomal release of the complexes, due to the weak buffering capacity of chitosan [115]. Based on these findings, new strategies were developed like the elaboration

of innovative multicomponent formulations or the synthesis of new chitosan derivatives, e.g., a chitosan-g-PEI carrier efficiently and safely delivered siRNA to lung cancer cells [43].

Targeting function can also be grafted onto chitosan to achieve specific targeting. For example, RGD (arginine–glycine–aspartic acid) peptide has been conjugated with chitosan using a thiolation reaction. RGD enhances selective intratumoral delivery of siRNA loaded in RGD-chitosan nanoparticles and induces significant antitumoral activity [107]. Mannosylated chitosan-g-PEI has also been designed as a targeted gene carrier [40].

4.2 Chitosan Derivatives for the Delivery of Poorly Soluble Drugs

Amphiphilic copolymers present a double affinity for both hydrophilic and hydrophobic environments and are able to self-organize in water to form, in most cases, specific architectures such as micelles or vesicles, which can be used as carrier in drug delivery systems. The supramolecular organization in water generates small hydrophobic domains well-dispersed inside the solution. The self-assembled nanosized colloidal particles display a hydrophobic core surrounded by a hydrophilic outer shell in aqueous conditions, which allows the solubilization of hydrophobic drugs. Indeed, the inner core can serve as a nanocontainer for poorly soluble drugs. Micelles as drug carriers provide a set of advantages, i.e., increase water-solubility of sparingly soluble drug, improvement of bioavailability, reduction of toxicity, enhancement of permeability across the physiological barriers, and changes in drug biodistribution [116]. Because intravenous injection of a micellar solution induces extreme dilution by blood, polymer micelles could disassemble and release the loaded drug. However, their critical micellar concentration and kinetic stability was usually higher than those of surfactant micelles [68].

Hydrophobically modified chitosan derivatives have been designed to increase the solubility of poorly soluble drugs. However, chitosan is not optimal as the hydrophilic part of an amphiphilic self-assembling polymer because it is only soluble in acidic aqueous solutions with pH values lower than its pK_a value (6.5). Hence, glycol chitosan has been used to synthesize new amphiphilic chitosan-based polymers. These amphiphilic glycol chitosan derivatives are expected to self-aggregate and to ensure the solubility of poorly soluble drugs with a better stability in physiological conditions than chitosan derivatives.

Four major groups of hydrophobically modified chitosan have been used as potential drug delivery carrier for poorly soluble drugs: (1) steroid derivatives, (2) fatty acids derivatives, (3) aryl and alkyl derivatives, and (4) carboxymethyl derivatives of chitosan (Figs. 3–6). Others types of modified chitosan have also been synthesized [117–119].

Many of the poorly soluble drugs included in amphiphilic chitosan-based nanocarriers are anticancer drugs, e.g., paclitaxel, doxorubicine, camptothecin, and Mytomycin C. Besides increasing their solubility, the polymeric micelles

allow passive targeting in the tumor by the enhanced permeability and retention (EPR) effect. This is a form of selective delivery termed as “passive targeting” [116]. In addition to the size, the stability of the nanoparticles is an important parameter for a successful passive targeting. If the particles circulate in the bloodstream for longer periods, they can reach tumor sites more effectively. Some other therapeutics agents were also studied, e.g., anti-HIV, antifungal, and nonsteroidal anti-inflammatory agents, as well as corticosteroids and proteins.

Drug loading in the polymeric nanocarriers is generally achieved by a method requiring the use of organic solvent to dissolve the drug, dialysis, oil-in-water emulsion solvent evaporation, and solid dispersion. Drug incorporation can modify various physicochemical parameters of the carrier like size or surface charge.

4.2.1 Steroid Derivatives of Chitosan

The main steroids used to hydrophobically modify chitosan are 5 β -cholanolic acid [72, 120], cholic acid [121], and cholesterol [76, 122] (Fig. 5).

Hydrophobically modified glycol chitosan (HGC) with 5 β cholanolic acid has been extensively studied both in vitro and in vivo. This polymer was developed as a new Cremophor EL-free alternative carrier systems for docetaxel [74] and paclitaxel. Physical characteristics of the nanoparticules such as size, hydrophobic core, and stability depend on the degree of 5- β cholanolic acid substitution. The maximum loading content of paclitaxel into HGC nanoparticles was 10 wt% and the loading efficiency was above 90% [120]. Cytotoxicity studies on MCF7 breast cancer cells showed that HGC nanoparticles were less toxic than Cremophor EL, and allowed a higher dose of paclitaxel administration. The survival rate of mice that received 50 mg/kg paclitaxel in HGC nanoparticles increased substantially compared to 20 mg/kg PTX in Cremophor EL–ethanol solutions [120].

4.2.2 Fatty Acid Derivatives of Chitosan

Different fatty acids were used to generate amphiphilic chitosan derivatives: linoleic acid [66, 67], stearic acid [68, 70], and oleic acid [71] (Fig. 4).

The stability of the micellar structure can be controlled by adjusting the balance between hydrophobic acyl groups and hydrophilic chitosan in an *N*-acyl chitosan. The critical micellar concentration of chitosan modified with the smaller acyl chain length like octanoyl was weaker than that using a longer chain length like stearoyl because the hydrophobicity of the chitosan derivative was poorer [64]. Hence, the most studied fatty acid grafted on chitosan is stearic acid, especially stearic acid-grafted chitosan oligosaccharides (CSO-SAs). CSO-SA has been studied for the solubilization of several molecules, including lamivudine stearate [69], 10-hydroxycamptothecin [123], mytomycin [70], doxorubicine [71], and DNA [65]. As CSO-SA can rapidly release the drug by dilution, stearic acid was solubilized into the core of CSO-SA micelles and was shown to significantly

reduced doxorubicine release. This was because the enhanced hydrophobic interaction between stearic acid and stearic acid segments in CSO-SA forms a tightly packed hydrophobic core, and because of the ionic interaction between stearic acid and doxorubicine [68]. A second way to reduce the initial burst of drug release from CSO-SA micelles is to crosslink the shell of CSO-SA micelles using glutaraldehyde. The drug release could be highly controlled by the shell crosslinking of the micelles without affecting the cellular uptake and drug encapsulation efficiency of CSO-SA micelles [70]. PEGylation of CSO-SA did not affect the cellular uptake of the micelles by cancer cells, and significantly reduced the internalization of the CSO-SA micelles into macrophages [124].

4.2.3 Aryl and Alkyl Derivatives of Chitosan

N-mPEG-*N*-octyl-*O*-sulfate chitosan (mPEGOSC) was synthesized with various PEG chain lengths and various degrees of substitution. One of the derivatives was able to increase the concentration of entrapped paclitaxel by three orders of magnitude. Solubilization performance was influenced by crystallinity: the lower the degree of crystallinity, the higher the entrapment efficiency [55]. Micelle dissociation in plasma proceeded very rapidly for the first 5 min and then slowed down. The micelles based on PEGylated chitosan greatly decreased the accumulation in the liver and the spleen and slowed down the elimination of paclitaxel in the later stage of intravenous injection [56]. Paclitaxel-loaded *N*-octyl-*O*-glycol chitosan micelles showed lower toxicity and higher maximum tolerated dose than Taxol [48]. Other alkyl chitosans like *N*-succinyl-*N*-octyl chitosan [57] and *N*-octyl-*N*-trimethyl chitosan have been studied [49].

N-Phthaloylchitosan (PLC) is a typical aryl-modified chitosan developed to improve the solubility of poorly soluble drugs like camptothecin [63], retinoic acid [62], or prednisone acetate [125]. PLC showed concentration-dependent cytotoxicity in Hela cells, whereas none of the PLC-grafted poly(ethylene glycol) methyl ether (PLC-*g*-mPEG) micelles were cytotoxic in vitro [63]. PLC-*g*-mPEG improved the stability of a light-sensitive drug, all-*trans* retinoic acid from photodegradation [62].

4.2.4 Polycaprolactone Derivatives of Chitosan

Functionalization of chitosan with polycaprolactone (PCL) led to the synthesis of chitosan-PCL and a ternary derivative, chitosan-*g*-PCL-mPEG (CPP) [79, 80, 126]. Spherical micelles were formed through self-assembly of CPP in aqueous media. Encapsulation efficiency higher than 5% could be achieved. The micelles can be subjected to glutaraldehyde treatment to prolong the release of the incorporated drugs [126]. The importance of substituent grafting was again highlighted as an important factor for the morphology and the behavior of the nanoparticles [127, 128].

4.3 Chitosan and Chitosan Derivatives in Tissue Engineering

Tissue engineering aims to develop functional substitutes for damaged or diseased tissues through complex constructs of living cells, bioactive molecules, and 3D porous scaffolds that support cell attachment, proliferation, and differentiation. Such constructs can be formed either by seeding cells within a preformed scaffold or through injection of a mixture of living cells and solidifiable precursor to the defective tissue. As cell and bioactive molecule carriers, injectable scaffolds are appealing, particularly from the clinical point of view, because they offer the possibility of homogeneously distributing cells and molecular signals throughout the scaffold and can be injected directly into cavities with minimally invasive surgery. After injection and solidification, an in situ scaffold provides a temporary 3D matrix on which the cells can adhere, proliferate, and differentiate to form new, functional tissue [129].

Due to its properties, the natural biopolymer chitosan is an excellent candidate for the preparation of wound dressings and hydrogel scaffolds for tissue engineering. There are different ways to form hydrogels from chitosan. Chitosan could be used alone but this is rarely the case because pure chitosan hydrogel is fragile and has low mechanical strength, which limits its application in tissue engineering [130]. Chitosan has therefore been combined with other compounds or chemically modified to improve its properties for tissue engineering applications, in particular to create thermosensitive hydrogels that will gel in situ.

4.3.1 Chitosan Hydrogels for Tissue Engineering

Chitosan has been mainly combined with β -glycerophosphate [131–134] to make thermosensitive chitosan solutions. If highly deacetylated chitosan is mixed with β -glycerophosphate [135], it gels at 37 °C. The major function of β -glycerophosphate is to lower the surface electrostatic charge of chitosan and thus elevate the pH of the system [130]. By increasing the ratio of β -glycerophosphate in the hydrogel, the pH of the resulting solution was higher and the gelation time decreased [136]. The underlying mechanism is that the poly-alcohol group of β -glycerophosphate cuts off the chitosan chain, accelerating the formation of a hydrophilic shell around the chitosan molecule, and thus improving the chitosan chain protective hydration, which prevents the associative effects at low temperatures and neutral pH. However, with an increase in temperature, hydrophilic interactions and hydrogen bonding start playing an important role and trigger physical crosslinking throughout the whole solution, starting the gelation process [136].

However, the hydrogels obtained are weak and some toxicity has been reported, mainly due to the high osmolarity (781 mOsm for 0.8 w/v% chitosan/glycerophosphate [137]) induced by the addition of glycerophosphate [138, 139]. This is the reason why Kim et al. [134] dialyzed the chitosan solution to reduce the glycerophosphate concentration required to make chitosan gels at 37 °C. Others combined chitosan/glycerophosphate solutions with other compounds to increase the hydrogel modulus.

Collagen displays low immunogenicity, biocompatibility, and biological degradability. However, collagen has a unique molecular identifying signal system that can improve cellular adhesion, proliferation, and differentiation, hence providing a suitable scaffold bed for cellular expansion and differentiation. Collagen gels at body temperature, although with the disadvantages of having a fast degradation rate and a relatively low mechanical strength [136]. Chitosan/glycerophosphate/collagen hydrogels possess an excellent cellular compatibility. Chitosan/glycerophosphate has also been complemented with hydroxyethyl-cellulose [129] for cartilage reconstruction or for improving the myocardial performance in infarcted heart [140, 141], or with ethylcellulose [142] for neural repair. For each study, the hydrogels were biocompatible and improved significantly the function for which they were developed. Starch has also been added to chitosan/glycerophosphate [143]. The physical properties, including flexibility, of crosslinked chitosan hydrogels can be improved by blending chitosan with pregelatinized starch. The presence of starch in the system increased the water absorption of the hydrogel when compared to the system without starch.

Chitosan was also complemented with polyvinyl alcohol (PVA) for wound-healing [144] and bone regeneration [145]. The chitosan/PVA wound dressing was more swellable, flexible, and elastic because of its crosslinking interaction with PVA. The hydrogel significantly improved the wound healing effect compared with a gauze control and the conventional product. For bone regeneration, the chitosan/PVA blend was supplemented with hydroxyapatite, which significantly enhanced the gel strength. The authors reported that the weak chitosan chain association with phosphate resulted in an increase in gelation speed and an enhancement of gel strength. The early burst of drug release was minimized or avoided in comparison with the pure chitosan/PVA gel.

4.3.2 Chitosan Derivatives for Tissue Engineering

Chitosan has three types of reactive functional groups that allow modifications of chitosan to produce various useful hydrogels for tissue engineering applications [146].

Poon et al. [97] developed a chitosan-*g*-PEG-*g*-methacrylate copolymer that was both thermoresponsive and UV-curable. Cells remained mostly viable when they were encapsulated inside this gel and suffered little damage from the single brief UV exposure.

N-[(2-Hydroxy-3-trimethylammonium)propyl] chitosan chloride (HTCC) shows better water solubility, moisture retentiveness, antimicrobial activity, absorptive property, and cell proliferative capacity than chitosan [98]. To form a gel, HTCC was mixed with glycerophosphate. The mechanical and swelling properties of the hydrogel were readily controlled by pH, the content of HTCC, and the content of glycerophosphate. The gel could easily incorporate drug in the solution state, which was stable below or at room temperature and became transparent at 37 °C. In addition, these hydrogels possessed good biocompatibility, and the cells could adhere and migrate inside the hydrogel networks.

Park et al. [102] designed an injectable cell delivery chitosan–Pluronic hydrogel for articular cartilage regeneration. The chitosan–Pluronic solution underwent a sol–gel transition at around 25 °C. The chitosan–Pluronic hydrogel showed effective chondrocyte proliferation and promoted extracellular matrix expression compared with alginate hydrogel.

Tan et al. [101] proposed a new class of biocompatible and biodegradable composite hydrogels derived from water-soluble chitosan and oxidized hyaluronic acid upon mixing, without the addition of a chemical crosslinking agent. The gelation is attributed to the Schiff base reaction between amino and aldehyde groups of polysaccharide derivatives. *N*-Succinyl-chitosan and aldehyde hyaluronic acid were synthesized for preparation of the composite hydrogels. The results demonstrated that the composite hydrogel supported cell survival and that the cells retained chondrocytic morphology.

Thermosensitive chitosan hydrogels were also obtained through grafting with well-known thermosensitive synthetic polymers like poly(NIPAAm) [103]. Hydrogels were synthesized by the copolymerization of acrylic acid-derivatized chitosan (CSA) and NIPAAm in aqueous solution. Cell adhesion and spreading was higher on the surface of poly(NIPAAm-*co*-CSA) hydrogels than that of PNIPAAm hydrogel. These hydrogels showed more rapid detachment of cell sheets. When the temperature decreased, the poly(NIPAAm-*co*-CSA) hydrogel showed hydrophilicity and the cells spontaneously detached along with their deposited extracellular matrix.

As chemical crosslinking can cause toxicity, chitosan was chemically modified using *N*-acetyl-L-cysteine (NAC), with the degree of substitution of thiol groups kept below 50% to minimize interference with biological function, and then crosslinked by disulfide bond formation in air [147]. Disulfide-crosslinked chitosan hydrogels were rapidly formed, their mechanical and swelling properties being controlled by the content of free thiol, concentration of thiol, and the molecular weight of chitosan. In vitro release of insulin and bovine serum albumin (BSA) was dependent on loading efficiency, composition of thiolated chitosan, and the drug entrapped, but the drug bioactivity was not affected during formation of the hydrogels. These hydrogels exhibited good compatibility and cells could adhere and migrate inside the hydrogel networks.

For corneal regeneration, Liang et al. [148] describe an in situ-formed hydrogel based on a water-soluble derivative of chitosan, hydroxypropyl chitosan, and sodium alginate dialdehyde. The composite hydrogel was both nontoxic and biodegradable and showed that corneal endothelial cells transplanted using the composite hydrogel could survive and retain normal morphology.

5 Conclusion

Chitosan has received considerable attention as a functional biopolymer for diverse pharmaceutical and biomedical applications. It is a nontoxic, biocompatible, and biodegradable polymer. Chitosans can be formulated as nanocarriers mainly by

ionic interactions, leading to drug-loaded colloidal systems with mucoadhesive and controversial permeation-enhancer properties. They can also be formulated as hydrogels.

However, for most applications, practical use of chitosan has been limited by its physicochemical properties, in particular its low solubility above pH 6.5 and the pH-dependence of the ionic interactions in the formulations. Hence, chitosan derivatives have been recently developed to widen and improve the potential biomedical applications of chitosan.

Due to its cationic properties, chitosan has been extensively used for gene delivery. However, due to its low buffering capacity and the low stability of nucleic acid-loaded chitosan nanoparticles, several chemical modifications have been introduced to improve transfection efficiency *in vivo*: (1) quaternization to improve solubility and stability of the nanoparticles, (2) grafting of polymer chains such as PEI to improve endosomal escape, or (3) grafting of ligands for specific cell targeting.

When a hydrophobic moiety is conjugated to chitosan, the resulting polymer can self-assemble and encapsulate poorly soluble drugs. Grafting a steroid, fatty acid, or PCL onto chitosan or glycol chitosan leads to nanocarriers that are useful for drug delivery, in particular passive or active targeting of anticancer drugs to tumors.

Chitosan can form 3D scaffold that are too weak to be useful in tissue engineering. Hence, inclusion in the chitosan matrix and/or grafting onto chitosan of other substances such as collagen, other biopolymers, or hydroxyapatite has been achieved to improve the mechanical properties of the scaffold and to mimic the nanostructure of the tissue for a better cell adhesion/infiltration and/or to provide thermosensitivity for *in situ* gelation.

The chemical modifications of chitosan described are not exhaustive but clearly demonstrate their benefit for drug delivery. Similarly, besides the described improvements in delivery of genes or poorly soluble drugs and in scaffolds for tissue engineering, other improvements in the delivery of therapeutic peptide and proteins or vaccines using chitosan-based systems have also been reported.

However, before the translation can be made to a marketed product containing modified chitosan as an excipient, extensive preclinical studies, including toxicological studies, as well as clinical studies are required. Moreover, scaling up of the chemical synthesis and precise physicochemical characterization of the novel polymers will be needed.

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