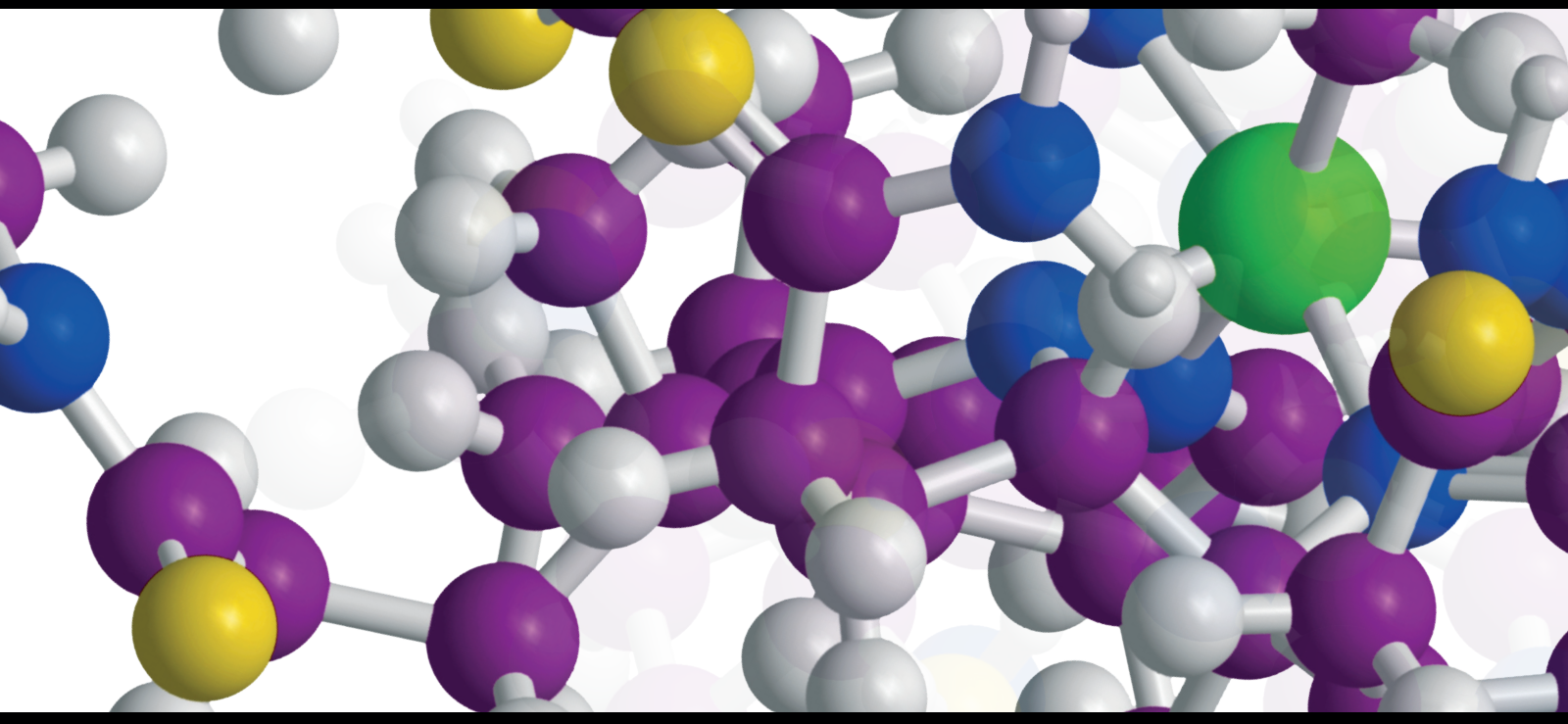
International Journal of Carbohydrate Chemistry

Chitosan and Chitosan Derivatives for Biological Applications: Chemistry and Functionalization

Guest Editors: Bruno Sarmento, Francisco M. Goycoolea, Alejandro Sosnik, and José das Neves



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Editorial Board

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Editorial

Chitosan and Chitosan Derivatives for Biological Applications: Chemistry and Functionalization

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It is with great satisfaction that we present this special issue in the International Journal of Carbohydrate Chemistry. Chitosan chemical derivatives have received increasing interest over the past decades due to their advocated chemical, biological, and functional advantages over unmodified chitosan. These include, but are not limited to, better solubility in aqueous solutions over a wider range of pH, gelling properties, possibility to revert the net charge from polycationic to polyanionic, design of hydrophobic derivatives with amphiphilic character and capacity to harness self-assembling nanostructures and chemical conjugates with an assortment of bioactive and therapeutic molecules; improved biocompatibility (e.g., hemocompatibility); enhanced properties for complexing pDNA or siRNA.

Undoubtedly, life sciences and technologies is the realm where chitosan and chitosan derivatives have raised greater scientific interest. A recent search in the Web of Knowledge^{SM} reveals that more than half out of the total number of published papers and patents including the terms "chitosan" or "chitosan derivatives" (>54 000) published over the past two decades are directly related to the life sciences. The number of papers published in this field has kept a sustained increasing trend over the past years, and only in 2010 a total of ca. 1800 papers have appeared in this field. This analysis clearly illustrates that the interest in chitosan in the biological sciences continues to increase and to receive funding by research agencies worldwide.

In this special issue of the International Journal of Carbohydrate Chemistry, we have put together a collection of six papers (five reviews and one research article). The review papers provide an overview of the state of the art of a large repertoire of chitosan derivatives and materials derived from them in various fields of life sciences, spanning pulmonary drug delivery (F. Andrade et al.), DNA condensation (K. E. Kador and A. Subramanian), biomaterials for tissue regeneration (G. D. Guerra et al.), food microbiology (D. de Britto et al.), and crop protection (M. E. I. Badawy and E. I. Rabea). In turn, the research article (S. E. Noriega and A. Subramanian) focuses on chitosan films for chondrocytes culture. The areas encompassed by these papers are among the ones that currently represent the most prominent opportunities for the utilization of chitosan and chitosan derivatives; hence, it is foreseeable that the interest in the recent advances in the field will continue but to increase in the coming years.

We are confident that this special issue will attract the attention of a wide audience worldwide that will also benefit from the many advantages associated to open access journals like the International Journal of Carbohydrate Chemistry.

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Review Article

Quaternary Salts of Chitosan: History, Antimicrobial Features, and Prospects

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Recently, increasing attention has been paid to water-soluble derivatives of chitosan at its applications. The chemical characteristics and the antimicrobial properties of these salts can play significant role in pharmacological and food areas mainly as carriers for drug delivery systems and as antimicrobial packaging materials. In the current paper, a historical sequence of the main preparative methods, physical chemistry aspects, and antimicrobial activity of chitosan quaternized derivatives are presented and briefly discussed. In general, the results indicated that the quaternary derivatives had better inhibitory effects than the unmodified chitosan.

1. Background

One of the first reports about methylation process of chitosan was presented by Wolfrom et al. in their study on carboxyl-reduced heparin published in 1963 [1]. Despite employing a very methylant agent (dimethylsulfate), only a 3,6-O-methylated product was claimed by the authors. In the seventies, Nudga et al. studied a series of N-alkylated chitosans [2–4] and described the preparation of a derivative with a 78% of quaternary amino sites. Then, Muzzarelli and Tanfani, in 1985 [5], prepared N-trimethyl chitosan iodide in a controlled three-step reaction: (a) started by a preparation of an N-alkyl chitosan via reductive alkylation, in which the chitosan amino groups react with appropriated aldehyde, generating a Schiff's base; (b) this compound is treated with a reducing agent, to yield the corresponding N-alkyl chitosan (c) then an overmethylation with an alkyl halide is carried out, generating, finally, the quaternary salt. With these sequences Muzzarelli and Tanfani obtained N,N,N-trimethyl chitosan (TMC) by reacting N-permethylated chitosan, which was previously prepared by treating chitosan with formaldehyde followed by reduction with sodium borohydrade and trimethylation with methyl iodide. Although, according to the authors, the product 60% trimethylated was not water soluble and characterized only with ^{13}C NMR and elemental analysis. This effort was made, aiming its application as antibiotic and ion exchange material.

In the following year, Domard et al. [6], also reacting chitosan with methyl iodide, gave an important contribution mainly on the role of the reagents in the quaternization. They established that the combination of N-methyl-2-pyrrolidone (NMP) and NaOH favors the quaternization better than sole addition of organic bases, for example, triethylamine. In order to understand the chemical structure of the TMC, several papers were published, emphasizing the features and signal changes as observed by nuclear magnetic resonance spectroscopy [7, 8]. At this time, three important branches of application for TMC have emerged, to be known: (i) as gene delivery tool; (ii) as absorption enhancer for hydrophilic drugs transport across epithelium (iii) for cosmetics applications.

The first topic started back in 1996-97 by the publications from Murata et al. [9, 10] who measured the cytotoxic activity and the formation of polyelectrolyte complex with DNA. Those works were based on the derivative synthesis

as proposed by Domard et al. [6]. After a long period of latency regarding this specific application, in 2005 Kean et al. [11] returned to this area, publishing results of a study about the toxicity and transfection efficiency of TMC derivatives with respect to the degree of trimethylation. After that, several other papers have been published in the field, highlighting the polyelectrolyte complex (PEC) formation [12]; aspects of nanoparticles cytotoxicity [13]; structure-activity relationship determination [14, 15]; reviews of use of chitosan derivatives as functional polymers [16–18].

The second issue began with Kotze et al. [19] in 1997 by reporting that TMC, as drug excipient, had a superior efficiency as absorption enhancer for hydrophilic drugs across intestinal epithelia than the chitosan itself. This potential for pharmacological applications increased considerably the interest for this polyelectrolyte. Aiming at better efficiency, the synthetic path was deeply studied, allowing, consequently, a better comprehension of its chemical structure [20–30]. The absorption enhancer property of the TMC did not confine only to intestinal epithelia but for other epithelial tissues. It showed also to be efficient, as documented for buccal mucosa [31, 32], mammalian airway [33], rabbit cornea [34], and derma [35]. Despite the huge amount of publications and data on chitosan quaternization, the synthetic route had remained almost the same, generally with a reaction involving a suspension of NMP and methyl iodide. Furthermore, these studies also demonstrate (see, e.g. [25, 29]) that strong inorganic bases, such as NaOH, are more efficient for quaternization than organic ones. As TMC gained attention for use in oral drug delivery, some reviews on the subject have appeared [16, 28, 36–38].

The last application can be related to the publication of Lang et al. [39] regarding the synthesis of quaternary salts for use as additive in cosmetic products. The authors proposed alternative synthetic routes as well as new applications for the derivative. One of the suggested methodologies was the covalent addition of a substituent containing a quaternary ammonium group. It could be achieved by reacting chitosan with a quaternary ammonium epoxide, such as glycidyl-trimethylammonium chloride. The main focus was the cosmetic market, as evidenced by several patents refereed in that paper.

More recently, an important branch of application for chitosan quaternary salt related to antimicrobial action has gained attention. It started by Kim et al. [40] testing several chitosan quaternary salt against *Staphylococcus aureus*. The synthetic path was based on that proposed by Muzzarelli and Tanfani [5]. In this way, the authors described the reaction of chitosan with formaldehyde, butyraldehyde, n-octylaldehyde, and n-dodecylaldehyde, treated the resulting Schiff's bases with sodium borohydrade, obtaining the quaternary salts via methyl iodide synthetic route. In fact, the antibacterial activity of the prepared salts was higher than that found for chitosan itself and increased with increasing chain length of the alkyl substituent. Following this line, in 2001, Jia et al. [41], also prepared several quaternary chitosan salts and tested against gram-negative bacteria *Escherichia coli*. It was seen that these salts exhibited higher *in vitro* activity against *E. coli* than chitosan, mainly in acid medium. Particularly, other combinations including alkyl, aryl moieties and carboxymethylation in chitosan quaternary salt also showed to be efficient against gram-negative and gram-positive bacteria [42–45].

With respect to its physical and chemical properties, works have reported the chemoselectivity with methyl iodide [46], the thermal degradation [47], the viscosity behavior [48], new synthetic route via dimethylsulfate [49, 50], the degree of quaternization via solid state ^{13}C NMR [51], and procedures for preventing the occurrence of O-methylation [52, 53].

Finally, the utmost research is the synthesis and application of chitosan quaternary salts as nanoparticles. It started from the work of Xu et al. [54], who reported the preparation of quaternary salt via reaction between glycidyl-trimethyl-ammonium chloride and chitosan and the formation of nanoparticles based on ionic gelation process with sodium tripolyphosphate (TPP). They described nanoparticles in the size range of 110–180 nm with drug entrapment efficiency up to 90%, for evaluation of bovine serum albumin as a model drug. Several other important applications have emerged for TMC nanoparticles obtained by ionic gelation with TPP, such as nasal [55–57] and oral [58] vaccine delivery system; protein carrier [59–61], and insulin releaser [62]. Then, several methods for the preparation and applications of TMC were reported, as: (i) by emulsion-diffusion-evaporation technique for gene transfer process [63]; (ii) by electrostatic self-assembling between TMC and insulin [64, 65] and cysteine [66], for controlled release applications; (iii) synthesis and characterization of gold, silver, and platinum TMC nanoparticles [67]; (iv) via ionotropic gelation with alginate for drug delivery [61, 68–70]; (v) alkyl chitosan salts micelles (or nanomicelles) formed via ultrasonication in aqueous media for nutraceuticals and functional foods [71, 72]; (vi) via ionotropic gelation with poly(γ-glutamic acid) for insulin controlled transport [73].

In spite of this vast literature on production and applications of TMC nanoparticles for delivery system, it is reported that TMC nanoparticles loaded with insulin were found to be less effective in permeating epithelial cells than the corresponding TMC in its free soluble form (not as nanoparticles) [74]. The reduced available amount of positive charge at the surface of the nanoparticles was claimed as responsible for this lower performance. Moreover, an important finding in that research was the strongest ability of TMC in opening the tight junction in comparison with the parent chitosan and other quaternary salts, for example, dimethylethyl chitosan and diethylmethyl chitosan.

2. Physical-Chemical Aspects

Chitin and chitosan are $\beta(1 \rightarrow 4)$ glycans whose chains are formed, respectively, by 2-acetamide-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. Chitosan is generally prepared by the deacetylation of chitin, and, in fact, none of them can be considered a homopolymer. Chitin, regularly isolated from the carapaces of marine animals such as crabs and shrimps, contains some

fraction (approximately 5–10%) of 2-amino-2-deoxy-D-glucopyranose units. Completely deacetylated chitosan is, however, rarely prepared due to the simultaneous occurrence of extensive depolymerization.

The chitosan's amino group behaves as nucleophile that bonds to and yields products with a variety of electrophiles, so the methylation reaction proceeds via electrophilic substitution of nitrogen. The most common electrophiles already reported on chitosan methylation are alkyl halides (R–$\mathbf{C}^{\delta+}H_2$–X) and dialkyl sulfate (R–$\mathbf{C}^{\delta+}H_2$–SO_4–$\mathbf{C}^{\delta+}H_2$–R). In both cases the reaction proceeds presumably by a S_N2 mechanism and liberates H^+ as a by-product. However, low pH values affect the forward reaction in two aspects: first, decreasing the substitution degree and, second, promoting the glycoside bond cleavage. The first event occurs when H^+ is captured by the unshared electron pair of the nitrogen, stopping the reaction at the amino site. The last event causes chain depolymerization, yielding low molecular weight derivatives [48]. These problems can be overcome by a proper addition of strong bases [6, 7, 25]. On the other hand, the added OH^- base is also a nucleophile that can react with the electrophile and produces alcohols. It seems that the slow addition of the strong base during the course of the reaction is the best way to avoid this problem.

The main parameters that characterize a chitosan's derivative are its average degree of quaternization, $\overline{DQ}$, and molecular weight. The $\overline{DQ}$ is usually determined by ^{1}H NMR spectroscopy [22, 24, 46], although potentiometric and conductimetric methods have also been proposed [41, 46, 48]. As the reaction is conducted in a heterogeneous medium, monomethylated, dimethylated, and trimethylated species are generated randomly. This has complexed and even leaded to misinterpretation of the ^{1}H NMR spectrum. Although, solid state CP-MAS ^{13}C NMR technique has been emerged as an alternative method to calculate TMC's $\overline{DQ}$ [51]. Despite the usefulness of FTIR spectroscopy in determining the degree of acetylation of chitosan [75] on TMC characterization, it has been limited to qualitative analysis [40, 41, 44, 47, 49]. Regarding the molecular weight, comparative viscosimetric studies have been adopted [25], but, as increasing the applicability of such derivatives, absolute method based on Gel Permeation Chromatograph (GPC) has given precise data [15, 27, 42, 76–82]. In the following, the main features of these techniques in TMC characterization are discussed.

Sample preparation for FTIR spectroscopy can be accomplished by KBr pellets or casting thin film on acrylic dish. The last method, however, makes use of the excellent film ability of such polymer and gives good spectra with low noise. Two main differences between the parent chitosan and TMC FTIR occur in the interval of 1700–1200 cm^{-1} (see Figure 1). First, the TMC's spectra show a band near 1475 cm^{-1}, "*A*", correspondent to asymmetrical stretching of C–H in the methyl groups. This band is absent in the spectrum of the starting chitosan. Second, the band assigned to angular deformation of N–H in amino groups (near 1590 cm^{-1}, "*B*") is reduced for the derivatives, and, generally, is overlaid by the signal at 1630 cm^{-1} (carbonyl stretching of acetamide moiety, "*C*"). The major problem in using FTIR in

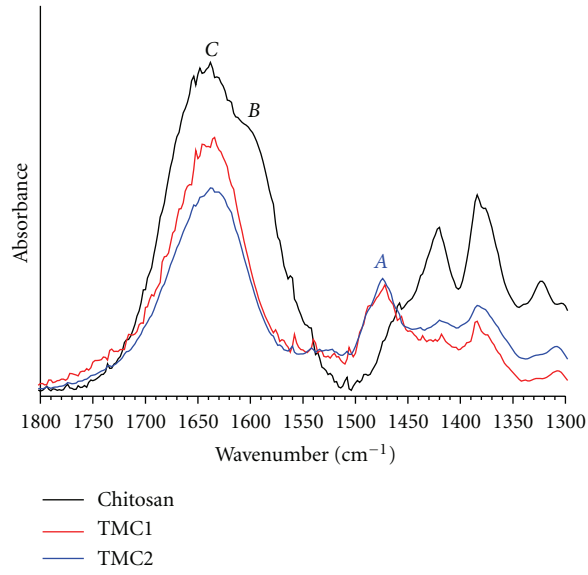

Figure 1: Typical FTIR spectra of chitosan and TMC.

quantitative analysis may be related to lack of proportionality between the signal "*A*" and the $\overline{DQ}$. As seen in Figure 1, the two TMC derivatives have different $\overline{DQ}$ but the intensity of the signal "*A*" is equal in two samples [49].

The ^{1}H NMR spectroscopy is also a useful tool in characterization and has been largely used in $\overline{DQ}$ calculation. Some misinterpretation regarding peaks assignments was raised before [8] but corrected in a subsequent paper [22]. Currently, the peaks assignments shown in Figure 2 are accepted as the correct one. Although, for certain applications, not only the $\overline{DQ}$ is important, but also the degree of dimethylation and O-methylation as well. For this knowledge, ^{1}H NMR spectroscopy has been used [15, 77]. ^{1}H NMR spectroscopy was also used as quantitative technique in the characterization of O-methyl free TMC derivatives [46, 52, 78, 79]. The strategy for such attempt was based on the method described by Muzzarelli and Tanfani [5] with some modification. According to the authors, the two-step reaction (dimethylation with formic acid-formaldehyde followed by a mild methylation with iodomethane without addition of strong OH^- base) not only avoided the O-methylation reaction but also prevented the chain scission. However, some limitations of this technique in substitution calculation were discussed recently [51] and are related mainly to the low intensity of the proton linked to C1 of the glycoside ring, generally took as reference signal [52]. This can induce a super estimation of such parameters.

The solid-state CP-MAS ^{13}C NMR spectroscopy, which combines the techniques of proton dipolar decoupling (DD), magic angle spinning (MAS), and crosspolarization (CP), has emerged as an alternative tool in TMC characterization and $\overline{DQ}$ calculation [49–51]. A methylated chitosan may show the following evidences (Figure 3): (a) 106.1 ppm, attributed to C1 signal of the glycoside ring when there is no substitution on the near amino group; (b) 100.4 ppm, attributed to C1 signal when there is substitution on the near

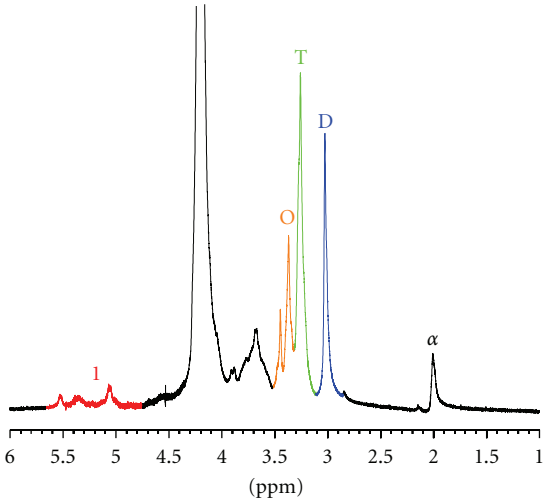

Figure 2: Typical ^{1}H NMR spectrum of TMC. For chemical structure and legends, see Figure 3.

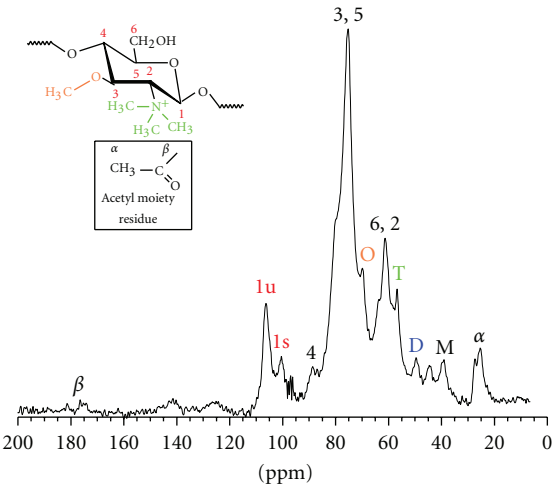

Figure 3: Typical solid state CP-MAS ^{13}C NMR spectrum of TMC. The labels stand for 1u: C1 (amino unsubstituted); 1s : C1 (amino substituted); O : O-methylated; T : N,N,N-trimethylated; D : N,N-dimethylated M : N-monomethylated signals.

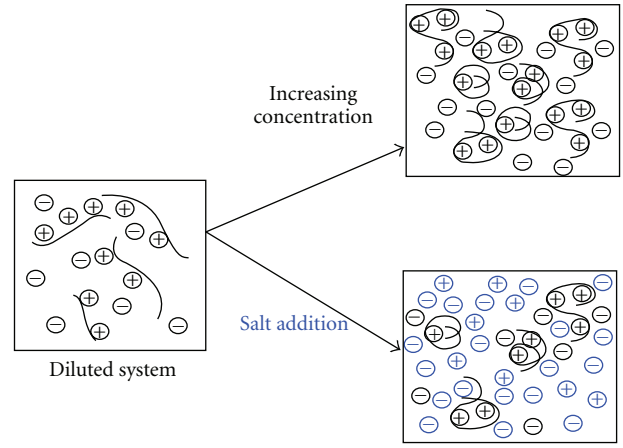

Figure 4: Illustration of the different chain conformations of a polyelectrolyte in function of the concentration and ionic strength.

amino group; (c) 37.9 ppm, attributed to carbon of methyl groups in N-monomethylated site; (d) 48.1 ppm, correspondent to carbon of methyl groups in N,N-dimethylated site; (e) 56.4 ppm, related to the carbon of methyl groups in N,N,N-trimethylated site (f) 68.5 ppm, correspondent to the carbon of methyl groups in O-methylated site. In fact, the signals labeled as "1u", "1s", and "T" showed reasonable proportionality according to degree of substitution [82]. Such technique was also useful to characterize other chitosan quaternary salts, for example, N-butyl-N,N-dimethylchitosan, N-octyl-N,N-dimethylchitosan, and N-dodecyl-N,N-dimethylchitosan [50].

Degradations which occur during the reaction can be assessed by measuring the intrinsic viscosity, $[\eta]$, since it is related to the average molecular weight by the Mark-Houwink equation, $[\eta] = K\overline{M}_v^a$. The drop in $[\eta]$ can be of 90% in comparison with parent chitosan, depending on the reaction condition [25], until of only 5% [8]. But, in most of the cases, intermediate values within this range are common [27, 48, 49, 76, 77, 82]. Absolute molecular weight technique (GPC) also shows such depolymerization, situating M_w near 1.0–3.0 g/mol $\times 10^5$ [27, 76, 77, 82]. In fact, a plot of $\log[\eta]$ versus $\log M_w$ showed values of 0.39 and 2.14 $\times 10^{-4}$ for the empirics Mark-Houwink's parameters a and K, respectively [77]. However, instead of a drop, Verheul et al. [52] found a slight increase in M_w, rising of 42 kDa up to 84 kDa, which was attributed to limited or even absence of chain scission during reaction by the method of O-methylation free.

The usefulness of viscosity data is not limited to molecular weight estimation, but it spread light on chain conformation as well. Chain conformation, in its turn, can help to understand the antimicrobial activity of the polymer. Particularly in the case of cationic polyelectrolyte, as chitosan and its quaternary salts, the viscosity, in aqueous solution free of added electrolytes, shows an apparent paradoxical behavior called polyelectrolytic effect in which the viscosity increase, as the dilution increase, (Figure 4). The charged groups repeal each other, causing chain expansion, increasing the hydrodynamic volume and, consequently, increasing the viscosity. In the presence of added electrolytes, the charges will be screened, and, consequently, the polyelectrolyte chain will adopt a coiled conformation.

TMC itself shows the polyelectrolyte effect (Figure 5) and the viscosimetric study of the interaction of TMC with other charged species like surfactant (Figure 5), anionic polyelectrolyte, and other complexant polymers can explain the mode of action between the quaternary salt and the cell wall of a microorganism. Further studies must be developed in this area.

The hydrophilic character is another important property of chitosan quaternary salt that shows the net force presented in the surface of a smooth solid film. This property is useful in protecting coating experiments in which the interactions

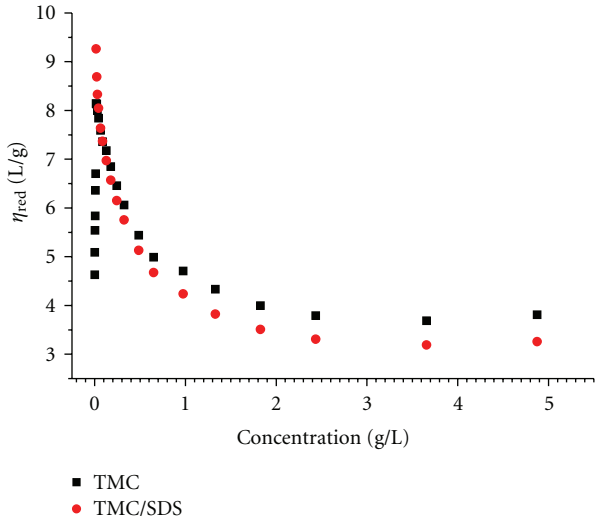

Figure 5: Polyelectrolytic effect of TMC and TMC/sodium dodecyl sulfate (SDS) complex in aqueous solution (adapted from [83]).

existent in the interfaces peel/coating film/microorganism spores play an important role. It is generally measured by contact angle technique and expressed in terms of wettability [49, 84]. For TMC, a sharp decrease in the contact angle values was found when compared with the parent sample due the presence of permanent positive charges which increased the hydrophilic character of the quaternary salts. On the other hand, the methyl groups, having apolar character, also contribute to hydrophilic character decreasing, as seen for highly substituted samples [84].

3. Antibacterial Action of Chitosan Quaternary Salts

The mode of action of antimicrobial activity of chitosan derivatives is strictly linked with that described for chitosan itself [85–87]. In fact, the main objective of the derivatives synthesis is to enhance such intrinsic property of chitosan. Particularly for quaternary salts, the advantage over the parent chitosan is attributed to its permanent positive charge and the synergetic effect of the pendant alkyl moiety. Further, the solubility of chitosan at physiological pH is low, while for the quaternary salts the solubility is high both in acid and basic conditions.

In fact, the better antibacterial activity of quaternary salt cannot rely only on charge density because at acid medium the chitosan chain is almost completely protonated. As pointed out by Xu et al. [44], it is necessary also to consider the degree of ionization and the chain conformation. For example, at moderated acid conditions, the repulsive forces among quaternary groups ($-N^+(CH_3)_3$) are weaker than that among protonated chitosan amine groups, which increases the chain flexibility and facilitates the interaction with bacteria cell envelope. Despite this, the increase of the $\overline{DQ}$ is not a guarantee of better antibacterial activity. It depends on the pH. Some investigations showed that the antibacterial efficiency decreased as the $\overline{DQ}$ increased at acid conditions, but increased as the $\overline{DQ}$ increased at neutral conditions [44, 88]. Here, the knowledge of chain conformation is fundamental for a better comprehension of this behavior.

The cell wall of bacteria is a complex structure made mainly of lipopolysaccharide (gram-negative) and peptidoglycan associated with teichoic acid (gram-positive), both having anionic groups (phosphate, carboxyl, N-acetylglucosamine, N-acetylmuramic acid, etc.) that can interact (hydrogen bond, electrostatic, etc) with the polycationic structure of the quaternary salt. The chelant effect of quaternary salts on divalent cations present on cell wall also contributes to disrupt the integrity of the membrane [85].

The presence and length of alkyl moiety on amine group is another relevant question on such derivatives. The hydrophobic affinity between the alkyl chain and phospholipids of the bacterial membrane has assumed to enhance the antibacterial activity [40, 41, 43, 89]. In this attempt, several chitosan derivatives were synthesized, involving several paths as alkylation, covalent addition of a substituent containing a quaternary ammonium group and extent methylation. Such derivatives have a very complex structure with variable antibacterial activity [90, 91]. However, a series of N-(2-quaternary ammoniumyl) acyl derivatives with different chain moiety did not exhibit better antibacterial activity than the TMC [45]. In another study, the methylated N-aryl chitosan did not improve so much the antibacterial activity, with similar results that are found for pure TMC [42].

E. coli and *S. aureus* have been adopted as model of gram-negative and gram-positive bacteria, respectively, in most of the studies, with MIC (Minimum Inhibitory Concentration) as lower as 0.125 μg/mL for *E. coli* and 0.0625 μg/mL for *S. aureus* [44]. However, other bacteria as *Enterococcus facialis* and *Pseudomonas aeruginosa* have shown susceptible to TMC, with MIC of 128 and 256 μg/mL respectively [45]. *Streptococcus mutans*, responsible for dental caries in humans, was also susceptible to chitooligosaccharide quaternized via covalent addition of glycidyl trimethylammonium chloride [92]. In the presence of a 0.1% solution, the quaternary derivative inhibited near 90% of the colonies growth, while the unmodified counterpart only 10% after 5 hours of exposure. Belalia et al. [93] also gave an important contribution, studding the action of quaternary salt against *Listeria monocytogenes* and *Salmonella typhimurium* in their attempt to improve the properties of biocomposite for packaging purposes. The derivative was based on Muzzarelli and Tanfani [5] method and showed inhibition of 96% of *L. monocytogenes* and 100% of *S. typhimurium* development.

The most important issue from now is to establish the effectiveness of such derivatives at *in situ* experiments. The antibacterial activity of chitosan has been proved in several *in situ* experiments as food preservative, packaging material, edible film and coating, wound dressing materials, cotton fabrics, and many others [86]. In this line, experiments with TMC were reported, showing the effectiveness of electrospun mats of TMC/poly(vinyl alcohol) [94] and mats of TMC/poly(vinyl pyrrolidone) [95] against *E. coli* and *S. aureus*. The potentiality of such mats was regarding wound-dressing applications. Antibacterial activities of finished

cotton fabrics impregnated with chitosan derivative were also evaluated, although the synthesis path was similar to that complex one cited above, based on alkylation, covalent addition of a quaternary substituent, and further methylation [96]. Related to food area, the work of Belalia et al. [93] contributed to knowledge in elaboration of effective antibacterial biopackagings with improved mechanical property.

In this way, the antibacterial activity of cast TMC film helps to understand the behavior of *in situ* conditions. For this, a qualitative test can be performed by agar diffusion method [97]. Film cuts were placed on the medium, and the inhibitory activity was measured based on the average diameter of the clear inhibition zone. Figure 6 illustrates the measurable zone.

The diameters of inhibitory zones surrounding film discs as well as the contact areas of edible films with agar surface were reported with significant results. A wide clear zone on solid media has been observed for S. *aureus* growth inhibition, whereas inhibition for *E. coli* is not effective, in some way confirming better antibacterial activity against gram-positive bacteria. The film concentration also appears to be important, mainly for the TMC, whose inhibition zone increases exponentially with the concentration (Figure 7). For commercial chitosan, the bacterial activity is inferior and not dependent on the concentration.

4. Chitosan Quaternary Salt and Antifungal Activity

Similar to antibacterial properties, the derivative synthesis has the main objective to enhance the antifungal property of chitosan largely reported in the literature [87, 98–100]. Particularly for fungi, such derivative study is more important once chitosan generally has a stronger antimicrobial activity against bacteria rather than against fungi [101]. Common plant pathogenic fungi such as *Botrytis cinerea*, *Penicillium expansum, Rhizopus stolonifer*, *Penicillium digitatum*, and *Penicillium italicum*, among many others, have been tested with chitosan, owing to its impact in the agricultural business.

In order to enhance the chitosan antifungal activity some methodology has been proposed. For example, the synergistic effect of microbial antagonists, for example, *Candida saitoana Cryptococcus laurentii*, enhanced significantly the antifungal activity of chitosan [101]. Accordingly, chitosan was effective against *P. expansu* spore germination *in vitro* and *in vivo* when introduced on the wound of Fuji apple (*Malus domestica* Borkh.). They found a complete inhibition of *P. expansum* spore germinations at concentrations of 0.3% or above of chitosan for *in vitro* test. On the other hand, after addition of *C. laurentii* antagonist the inhibitory concentration dropped to 0.1%. Addition of essential oil [102] or changing the organic acid, for example, acetic, sorbic, propionic, lactic, and glutamic acids [103], were also proposed to enhance the chitosan antifungal activity, representing an actual alternative to synthetic fungicide such as thiabendazole.

Finally, the chemical modification of chitosan as potential antifungal has emerged, however, with few publications. Some chitosan derivatives studied as antifungal were N,O-carboxymethyl chitosan [104], glycolchitosan [105], and thiourea derivative [106]. It was found that the hydrosoluble derivative N,O-carboxymethyl chitosan was less effective than chitosan in reducing the radial growth of the fungi *B. cinerea* and *R. stolonifer,* while the acid soluble derivative glycolchitosan was more effective than chitosan against the fungi *B. cinerea* and *P. expansum*. Once again, it looks that fungicidal effect depends on the charge density of the chitosan chain. Also, Rabea and colleagues [107–110] have synthesized a series of N-alkyl and N-aryl chitosan derivatives with superior fungicidal and insecticidal functions that founded for chitosan, although soluble only in acid conditions.

Recently, Badawy [111] has shown that the quaternary salts of alkyl chitosan derivatives also exhibit antifungal property against plant fungi *Pythium debaryanum*, *B. cinerea* and *Fusarium oxysporum*. Accordingly, *in vitro* results showed that the best antifungal quaternary salts were N-octyl-N,N-dimethylchitosan against *B. cinerea* (383 mg/L), *F. oxysporum* (812 mg/L), and *P. debaryanum* (440 mg/L), on the basis of the effective concentration that caused 50% inhibition of mycelial growth (EC_{50}). TCM also showed a good activity with EC_{50} = 1467, 875, and 907 mg/L respectively, for *B. cinerea*, *F. oxysporum,* and *P. debaryanum*. This is a good achievement when compared with chitosan that showed EC_{50} > 3000 mg/L in all the cases. Despite the solubility of those salts in aqueous media, the author used a mild acetic acid solution to enhance salt solubility. The action of those quaternary salts was also effective against spore germination, generally increasing with an increase in the chain length of alkyl substituent.

Other hydrosoluble chitosan derivatives, bearing salicylaldehyde and pyridinium ion [112], were tested against some plant-threatening pathogenic fungi *Cladosporium cucumerinum*, *Monilinia fructicola*, *Colletotrichum lagenarium*, and *F. oxysporum*. The best performance of those salts was near 500–1000 μg/mL.

Chitosan quaternary salt has shown good activity against fungal deterioration of *in situ* experiments with apple [113]. The TMC showed important antifungal activity against *P. expansum* which was strongly dependent on the characteristics of the polymer (Figure 8). By comparing the samples TMC1 ($\overline{DQ}$ = 18%, low solubility and low viscosity) and TMC2 ($\overline{DQ}$ 45%, high solubility and high viscosity), it was found that the latter presents superior antifungal activity. The chitosan derivatives N-butyl-N,N-dimethylchitosan, N-octyl-N,N-dimethylchitosan, and N-dodecyl-N,N-dimethylchitosan also showed good antifungal activity (Figure 8). The introduction of alkyl substituents on the chitosan chain strongly decreases the polymer solubility, a property which seems to play a very important role in the antifungal activity. The chitosan derivative TMC dissolved in water solution exhibited moderate antifungal activity against *P. digitatum* and *B. cinerea* with fungal spread 28.6% and 45.5%, respectively, as compared to the respective controls 34.9% and 51.5%. As observed for *P. expansum*, the acetic

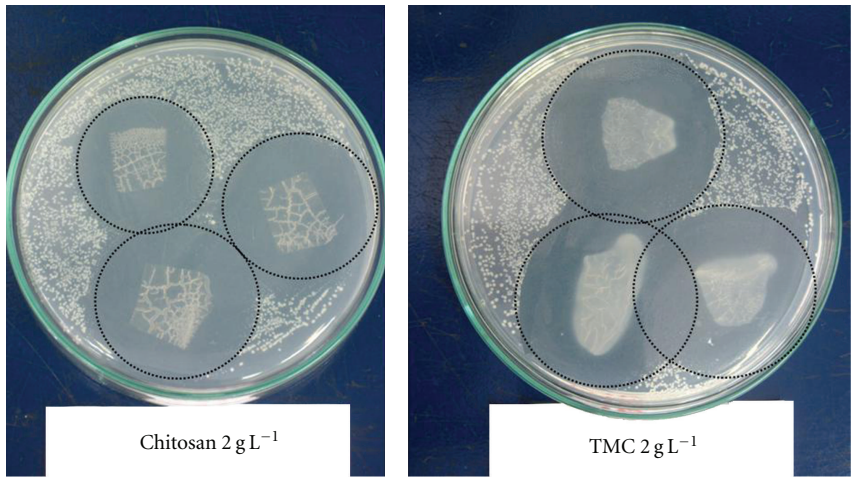

Figure 6: Examples of inhibitory effect of chitosan and TMC film against gram-positive bacteria *S. aureus*.

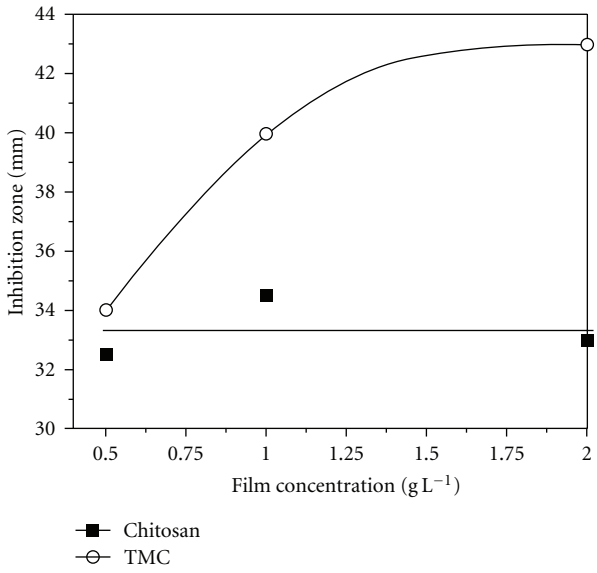

Figure 7: Inhibition zone in function of the film concentration as measured against the bacteria *S. aureus*.

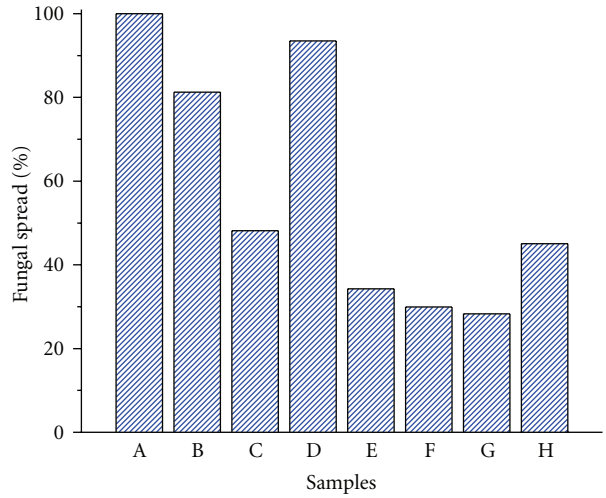

Figure 8: *P. expansum* colonies development on cut apple surface coated with different coating solutions after 7 days of storage in a greenhouse at 28°C and 80% humidity. (A) acetic acid control; (B) water control; (C) chitosan; (D) TMC1; (E) TMC2; (F) N-butyl-N,N-dimethylchitosan; (G) N-octyl-N,N-dimethylchitosan; (H) N-dodecyl-N,N-dimethylchitosan.

acid medium was favorable to fungal spread on cut apple surface, reaching values of 100% and 87.1% for *P. digitatum* and *B. cinerea*, respectively, while both control samples were 100%.

The mechanism of action of quaternary salts against fungi still debated but generally is attributed to formation of polyelectrolyte complex between different charged species, in a similar way that took for antibacterial action [111]. On the other hand, microscopic observation has shown that chitosan oligomers can also diffuse inside hyphae interfering on the enzymes activity responsible for the fungus growth [106].

5. Conclusion

Quaternary salts of chitosan can be prepared with different degree of substitution mainly via methyl iodide or dimethylsulfate synthetic route. The last one shows the advantage of better control and reproducibility of the reaction steps. New and more security synthetic routes, free of hazardous methylant agent, can be developed to attenuate the purification costs for grade suitable for pharmaceutical and food areas. The water solubility is attained by introduction of CH_3 moiety in the amino group, generating a permanent cationic polyelectrolyte. This feature enhances the chitosan

applicability in a large pH range opening up a broad range of possibilities. Ordinary, by increasing the reaction time and reaction steps, a higher degree of N-quaternization is achieved. However, the overmethylation can yield products with low degrees of substitution and polymerization. The antifungal activity represents a potential application as edible coating and film for this hydrosoluble derivative once the acid medium used for preparation of chitosan solution is very aggressive to cut fruit tissue. Regarding the antibacterial activity, it revealed higher inhibition against gram-positive than gram-negative bacteria. Finally, nanoparticles-based quaternary salts promise to improve these already cited properties. TMC and other chitosan quaternary salts have effectively antimicrobial activity; however, experiments *in situ* must help the applications in real conditions.

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Review Article

A Biopolymer Chitosan and Its Derivatives as Promising Antimicrobial Agents against Plant Pathogens and Their Applications in Crop Protection

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Recently, much attention has been paid to chitosan as a potential polysaccharide resource. Although several efforts have been reported to prepare functional derivatives of chitosan by chemical modifications, few attained their antimicrobial activity against plant pathogens. The present paper aims to present an overview of the antimicrobial effects, mechanisms, and applications of a biopolymer chitosan and its derivatives in crop protection. In addition, this paper takes a closer look at the physiochemical properties and chemical modifications of chitosan molecule. The recent growth in this field and the latest research papers published will be introduced and discussed.

1. Introduction

Plant pathogens are considered economically important agricultural micro-organisms around the world. They induce decay on a large number of agricultural crops during the growing season and postharvest. Chemical pesticides provide the primary means for controlling the plant pathogens. However, continuous use of such compounds has faced two major obstacles: increasing public concern regarding contamination of perishable agricultural products with pesticide residues and proliferation of resistance in pest populations [1–3]. Hence, there is growing emphasis on environmentally friendly technologies in pest control, and evaluation of various alternatives to reduce dependency on harmful synthetic pesticides [4–6]. Consequently, several nonchemical treatments have been proposed for pest control. Although these approaches have been shown to reduce pests, each has limitations that can affect their commercial applicability. When used as stand-alone treatments, none of the nonchemical control methods has been clearly shown to offer a consistently economic level of disease control that warrants acceptance as an alternative to synthetic pesticides. Among these strategies, some satisfactory results have been reported using natural compounds such as chitosan as safe alternative to hazardous pesticides with negligible risk to human health and the environment [7]. Chitosan, as the most abundant naturally occurring amino-polysaccharide, possesses many of these attributes and has attracted attention because of its unique physiochemical characteristics and biological activities [6, 8, 9]. From a biological standpoint, chitosan and its derivatives are very attractive for agriculture applications, which are closely related to human safety and fitness. For example, these compounds can function as seed soaking, root applying, and spray agents; all of these play an important role on plant disease control and stress resistance [6, 10].

The origin of chitosan (pronounced *Kite-O-San*) can be traced back to 1811 when "chitin", from which it is derived, was first discovered by Henri Braconnot, a professor of the natural history in France. According to some researches, while Braconnot was conducting research on mushrooms, he isolated what was later to be called chitin [11, 12]. Chitin was the first polysaccharide identified by man, preceding cellulose by about 30 years. In the 1830s, there was a man

who authored an article on insects in which he noted that similar substance was present in the structure of insects as well as the structure of plants. He then called this amazing substance as "chitin". Basically, the name chitin is derived from Greek, meaning "tunic" or "envelope". The concept was further known in 1843 when Lassaigne demonstrated the presence of nitrogen in chitin. In 1859, Professor C. Rouget subjected chitin to alkali treatment, which resulted in a substance that could, unlike chitin itself, be dissolved in acids. The term "chitosan" was given to deacetylated chitin by Hoppe-Seiler [13]. While chitin remained an unused natural resource for a long time, interest in this polymer and its derivatives such as chitosan and chitooligosaccharides has increased in recent years due to their unique properties. Intense interest applications grew in the 1930s; however, the lack of adequate manufacturing facilities and competition from synthetic polymers hampered the commercial development in this period. Renewed interest in the 1970s was encouraged by the need to better utilize shellfish shells and the scientists worldwide began to chronicle the more distinct properties of chitin and chitosan to understand the potential of these natural polymers. In the early 1960s, chitosan was investigated for its ability to bind with the red blood cells. That time also, it was considered as a hemostatic agent. Then, for the past three decades, chitosan has been used in water purification. Since then, numerous research studies have been undertaken to find ways to use these materials. Today, it is known as a dietary supplement that is good for weight loss. In fact, it has been marketed for such purpose for about 20 years in Japan as well as in Europe. Many people even call it as the "fat blocker" [14–18].

Chitosan is a linear aminopolysaccharide of glucosamine and *N*-acetylglucosamine units and is obtained by alkaline deacetylation of chitin extracted from the exoskeleton of crustaceans such as shrimps and crabs, as well from the cell walls of some fungi [19]. The following major characteristics of chitosan make this polymer advantageous for numerous applications: (1) it has a defined chemical structure; (2) it can be chemically and enzymatically modified; (3) it is physically and biologically functional; (4) it is biodegradable and biocompatible with many organs, tissues, and cells; (5) it can be processed into several products including flakes, fine powders, beads, membranes, sponges, cottons, fibers, and gels. Consequently, chitosan has found considerable application in various industrial areas [6, 20–22].

Owing to its high biodegradability, nontoxicity, and antimicrobial properties, chitosan is widely-used as an antimicrobial agent either alone or blended with other natural polymers. To broaden chitosan's antimicrobial applicability, comprehensive knowledge of its activity is necessary. The paper reviews the current trend of investigation on antimicrobial activities of chitosan and its derivatives against plant pathogens. The antimicrobial activity depends on several factors such as molecular weight, degree of deacetylation, solubility, positive charge density, chemical modification, pH, concentration, hydrophilic/hydrophobic characteristic, chelating capacity, and type of microorganism. Mode of antimicrobial action is discussed in parts of the active compound and the target microorganisms collectively and independently in same complex. It has immense structural possibilities for chemical modifications to generate novel properties, functions, and applications especially in agricultural area. Therefore, different physiochemical properties and chemical modifications of chitosan molecule are also comparatively discussed. Finally, the general antimicrobial applications of chitosan and perspectives about future studies in this field are considered.

2. Chitosan Structure and Natural Origin

Chitin, occurring as a structural polysaccharide in the outer skeleton of animals belonging to the phylum Arthropoda (animals with an outer skeleton) and a component of the cell walls of certain fungi and algae, is quite abundant. It is also produced by a number of other living organisms in the lower plant and animal kingdoms, serving in many functions where reinforcement and strength are required. In contrast, chitosan is much less abundant in nature than chitin and has so far been found only in the cell walls of certain fungi [23]. Chitin is the raw material for all commercial production of chitosan and glucosamine, with estimated annual production of 2000 and 4000 tons, respectively [24]. Most commonly, chitin forms the skeletal structure of invertebrates. At least 10 Gtons (1×10^{13} Kg) of chitin are constantly present in the biosphere [25]. Chitin is a linear polymer of $(1 \rightarrow 4)$-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit), which is insoluble in aqueous solvents. It also has many structural similarities with cellulose such as conformation of the monomers and diequatorial glycosidic linkages. Chitosan may be considered as a family of linear binary copolymers of $(1 \rightarrow 4)$-linked A-units and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit). The term chitosan does not refer to a uniquely defined compound; it merely refers to polysaccharides having different composition of A and D units, which is a white, hard, inelastic, and nitrogenous polysaccharide. It has been proposed to define chitin and chitosan based on their solubility in aqueous acetic acid, that is, chitosan as soluble and chitin as insoluble [26].

3. Production of Chitosan

A variety of procedures have been developed and proposed over the years for preparation of pure chitosan. Several of these form the basis of chemical processes for industrial production of chitosan from crustacean shell waste. For chitin production, the raw materials most abundantly available are the shells of crab, shrimp, and prawn (69-70%) [16, 19, 27]. Because chitin is associated with other constituents, harsh treatments are required to remove them from chitinous material to prepare chitin and then chitosan on a large scale. Proteins are removed from ground shells by treating them with either sodium hydroxide or by digestion with proteolytic enzymes such as papain, pepsin, trypsin, and pronase [28]. Minerals such as calcium carbonate and calcium phosphate are extracted with hydrochloric acid. Pigments such as melanin and carotenoids are eliminated with 0.02% potassium permanganate at 60°C or hydrogen

peroxide or sodium hypochlorite. Conversion of chitin to chitosan generally is achieved by hydrolysis of acetamide groups of chitin. This is normally conducted by severe alkaline hydrolysis treatment due to the resistance of such groups imposed by the *trans*-arrangement of the C2-C3 substituents in the sugar ring [29]. Thermal treatments of chitin under strong aqueous alkali are usually needed to give partially deacetylated chitin (degree of acetylation, DA < 30%), regarded as chitosan. Usually, this process is achieved by treatment with concentrated sodium or potassium hydroxide solution (40–50%) at 100°C or higher to remove some or all the acetyl groups from the polymer [19, 30, 31]. This process, called deacetylation, releases amine groups (NH_2) 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. The deacetylation process is carried out either at room temperature (homogeneous deacetylation) or at elevated temperature (heterogeneous deacetylation), depending on the nature of the final product desired. However, the latter is preferred for industrial purposes. In some cases, the deacetylation reaction is carried out in the presence of thiophenol as a scavenger of oxygen or under N_2 atmosphere to prevent chain degradation that invariably occurs due to peeling reaction under strong alkaline conditions [32]. One other method of preparing chitosan of improved purity is to dissolve the materials in an acid (e.g., acetic acid) and filter to remove extraneous materials. The clarified product is then lyophilized to give a water-soluble chitosonium acid salt or precipitated with NaOH, washed, and dried to give a product in the free amine form [19].

Recent advances in fermentation technologies suggest that the cultivation of selected fungi can provide an alternative source of chitin and chitosan. The amount of these polysaccharides depends on the fungi species and culture conditions. Fungal mycelia are relatively consistent in composition and are not associated with inorganic materials; therefore, no demineralization treatment is required to recover fungal chitosan. Usually, the Zygomycetes class has higher amounts of chitin and chitosan in their cell walls when compared to other classes of fungi [33–42]. The use of biomass from fungi has demonstrated great advantages, such as: independence of seasonal factor, wide-scale production, simultaneous extraction of chitin and chitosan, extraction process is simple and cheap resulting in reduction in time and cost required for production, and also absence of proteins contamination, mainly the proteins that could cause allergy reactions in individuals with shellfish allergies [33, 43–47]. However, to optimize the production of chitin and chitosan from fungi, complex or synthetics cultures media, which are expensive are usually used. It has become necessary to obtain economic culture media that promote the growth of fungi and stimulate the production of the polymers. Recently, microbiological processes were used for chitin and chitosan production by *Cunninghamella elegans* grown by submerse fermentation in economic culture medium, yam bean (*Pachyrhizus erosus* L. Urban), as substrate [48]. The main characteristic of yam bean is the simple manipulation and low nutrition requirements when compared with other similar cultures, and tuberous roots yields are up to 60 ton/ha. The extraction of chitin and chitosan from different species of mushrooms (i.e., *Agaricus bisporus*, *Auricularia auriculajudae*, *Lentinula edodes*, *Trametes versicolor*, *Armillaria mellea*, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, and *Pleurotus eryngii*) has been also illustrated [39, 49]. The mushroom, *P. sajor-caju,* showed highest yield of biomass and *L. edodes* was the lowest when compared with others under submerged fermentation. The processes and conditions for the extraction of chitin and chitosan from mushroom were nearly same in the methods of Crestini et al. [50] and Pochanavanich and Suntornsuk [39], and were different in Mario et al. [49]. Moreover, the chitin composition and structure have been studied in insects, terrestrial crustaceans, and nematodes. However, their demineralization studies were carried out using HCl (1-2 N) for 0.3–96 h at 25–100°C, which is stronger than the demineralization process of aquatic crustacean materials [38, 51–55].

4. Physiochemical Properties of Chitosan

4.1. Crystalline Structure. Since chitosan is a heterogeneous polymer consisting of GlcN and GlcNAc units, its properties depend on the structure and composition. Ogawa and Yui [56] studied the crystalline structure of different chitin/chitosan samples prepared by two different procedures: (a) the partial deacetylation of chitin, and (b) the partial reacetylation of a fully deacetylated chitin (pure chitosan). It was observed that the partially reacetylated material was less crystalline than pure chitosan. They also showed that, for the preparation of a less crystalline chitosan sample, it is preferable to proceed via reacetylation of fully deacetylated chitosan rather than direct solid-state deacetylation of chitin. This preferred treatment also resulted in less anhydrous crystals. Generally, the step of dissolving the polymer results in a decrease in the crystallinity of the material. However, it also depends on the secondary treatment (reprecipitation, drying, and freeze-drying). In addition, the origin may affect the residual crystallinity of chitosan, which in turn controls the accessibility to internal sorption sites and the diffusion properties (rehydration and solute transport) and also deacetylation procedure may affect the solid state structure of chitosan [57–59].

4.2. Degree of N-Acetylation. An important parameter to examine closely is the DA in chitin, that is, the ratio of GlcNAc to GlcN structural units. In chitin, the acetylated units prevail (DA ≥ 90%), where as chitosan is fully or partially *N*-deacetylated derivative with a DA of less than 30%. This ratio has a striking effect on chitin and chitosan solubility and solution properties. To define this ratio, attempts have been made with several analytical methods [18, 60–65], which include IR, UV, and NMR spectroscopies, pyrolysis gas, gel permeation chromatography (GPC), thermal analysis, various titration schemes, and acid hydrolysis. Furthermore, in case of chitin, the DA determines the suitable conditions of deacetylation, a complex process still requiring investigation in order to assess the feasibility of quicker preparation of

chitosan. Thus, the search for quick, user-friendly, low cost, and accurate method to determine the DA has been one of the major concerns over many decades. However, the inherent complexity of this particular system turns this apparently simple analytical problem into a very difficult one, which is well illustrated by the extensive number of different techniques, either destructive or nondestructive, yielding direct or nondirect and frequently non reproducible values [26, 66–69]. It is worth noting that nondestructive methods offer the advantage of avoiding manipulations of the polymers such as hydrolysis, pyrolysis, or derivatization, the consequences of which are not always well known. Especially, FTIR spectroscopy is considered to be a very attractive technique, as it is nondestructive, fast, sensitive, user-friendly, low priced, and suitable for both soluble and nonsoluble samples [62]. Proton NMR spectroscopy is a convenient and accurate method for determining the chemical structure of chitosan and its derivatives [61, 70–73]. NMR measurements of chitosan compounds are, however, limited to samples that are soluble in the solvent, which limits the analysis of chitosan with DA values lower than 0.3 in aqueous solutions. A typical proton NMR spectrum of chitosan is shown in Figure 1. The signal at δ 3.20 ppm was attributed to H-2 of GlcN residue. The intense band at 4.8–5.30 ppm is related to OH groups and HDO (solvent). In this region, as observed more clearly from an extended spectrum, some different anomeric protons (H-1 of GlcN and GlcNAc units) are appeared at 4.88–5.00 ppm. The DA is calculated from the integral ratio between protons of acetyl group and the GlcN protons. The degree of deacetylation (DDA) is calculated from the integral ratio between the proton on C-2 and the glucose unit protons [71, 74, 75]. The ^{1}H-NMR spectra also allowed us to propose a method to determine the degree of substituent (DS) value and the determination is based on the ratio between the protons of the substituent and the protons of the pyranose unit [71, 76–79].

The role of the DA on the chain stiffness has essentially been assessed by viscometry considering the Mark-Houwink-Kuhn-Sakurada (MHKS) relationship [80, 81], $[\eta] = KM^a$, and by static light scattering through the relation $R_G = KM^\nu$, where $[\eta]$ and R_G correspond to the intrinsic viscosity and the radius of gyration, respectively. Experiments have been performed either with parent samples having different degrees of polymerization [82] or with samples fractionated by size exclusion chromatography [83]. Many authors found an increase of the a coefficient with DA and concluded that acetyl groups induce some stiffness to the chains [82, 84, 85], whereas the dependence of ν on DA is less significant [83, 85, 86].

4.3. Molecular Weight. As polysaccharides in general, chitosans are polydisperse with respect to molecular weight (MW). The MW difficulty encountered in this respect concerns the solubility of the samples and dissociation of aggregates often present in polysaccharide solution. As to choosing a solvent for chitosan characterization, various systems have been proposed, including an acid at a given concentration for protonation together with a salt to screen the electrostatic interaction because of the MW chitosan is an average over the whole distribution of Mws. The MW heterogeneity of polysaccharides can be described by several types of average MW. The two most common methods in use for averaging are the number-average, Mn (which weighs the polymer molecules according to the number of molecules having a specific MW) and the weight-average, Mw (which weighs the polymer molecules according to the weight of molecules having a specific MW). The MW of chitosan depends on its source and deacetylation conditions (time, temperature, and concentration of alkali). Chitosan obtained from deacetylation of crustacean chitin may have a MW over 100,000 Da. Consequently, it is necessary to reduce the MW by chemical or enzymatic methods to much lower MW for easy application and high biological activity.

In order to evaluate the MW of polymeric chain, various methods can be used extensively. Viscometric [87] and GPC [88] techniques are easy to perform and low time consuming. On the other hand, they are empirically related to the MW, because the measurement depends upon the hydrodynamic volume of the macromolecule, which is a function of the MW conformational properties, and polymer-solvent interaction. As a consequence, a calibration curve is required. The viscosity of chitosan solutions is measured by using Ubbelohde Viscometer. The running times of the solution and solvent are used to calculate the relative viscosity (η_{rel}), specific viscosity (η_{sp}), and reduced viscosity (η_{red}) as follows: $\eta_{rel} = t_{ch}/t_{sol}$; $\eta_{rel} = t_{ch}/t_{sol}$;$\eta_{red} = \eta_{sp}/c$, where t_{ch} and t_{sol} are the running times of the chitosan solution and solvent, respectively and c is the chitosan concentration in g/dL. The intrinsic viscosity, defined as $[\eta] = C(\eta_{red})_{c=0}$, is obtained by extrapolating the η_{red} versus concentration data to zero concentration and the intercept on the ordinate is the intrinsic viscosity [82, 89–91]. Finaly the average molecular weight (M) is calculated based on the MHKS equation ($[\eta] = KM^a$), where K and a are viscometric parameters depending on the solvent [92, 93]. For example, a chitosan dissolved in 0.5 M CH_3COOH/0.2 M CH_3COONa, K and a were found to be 3.5×10^{-4}) and 0.76, respectively according to Terbojevich et al. [94] and Wang et al. [82]. On the contrary, the light scattering (LS) method [95–97] gives absolute values for MW, but the technique is more difficult and sometimes the data are not easy to interpret.

4.4. Solubility and Charge Density. Chitin and chitosan degrade before melting, which are typical for polysaccharides with extensive hydrogen bonding. This makes it necessary to dissolve them in an appropriate solvent system to impart functionality. For each solvent system, polymer concentration, pH, counter ion concentration, and temperature effects on the solution viscosity must be known. When the DDA of chitin reaches about 50% (depending on the origin of the polymer), it becomes soluble in aqueous acidic media and is called chitosan. Comparative data from solvent to solvent are not available. As a rule, the maximum amount of polymer is dissolved in a given solvent towards a homogeneous solution. Subsequently, the polymer is regenerated in the required form. A coagulant is required for polymer regeneration or solidification. The nature of the coagulant is also highly dependent on the solvent and

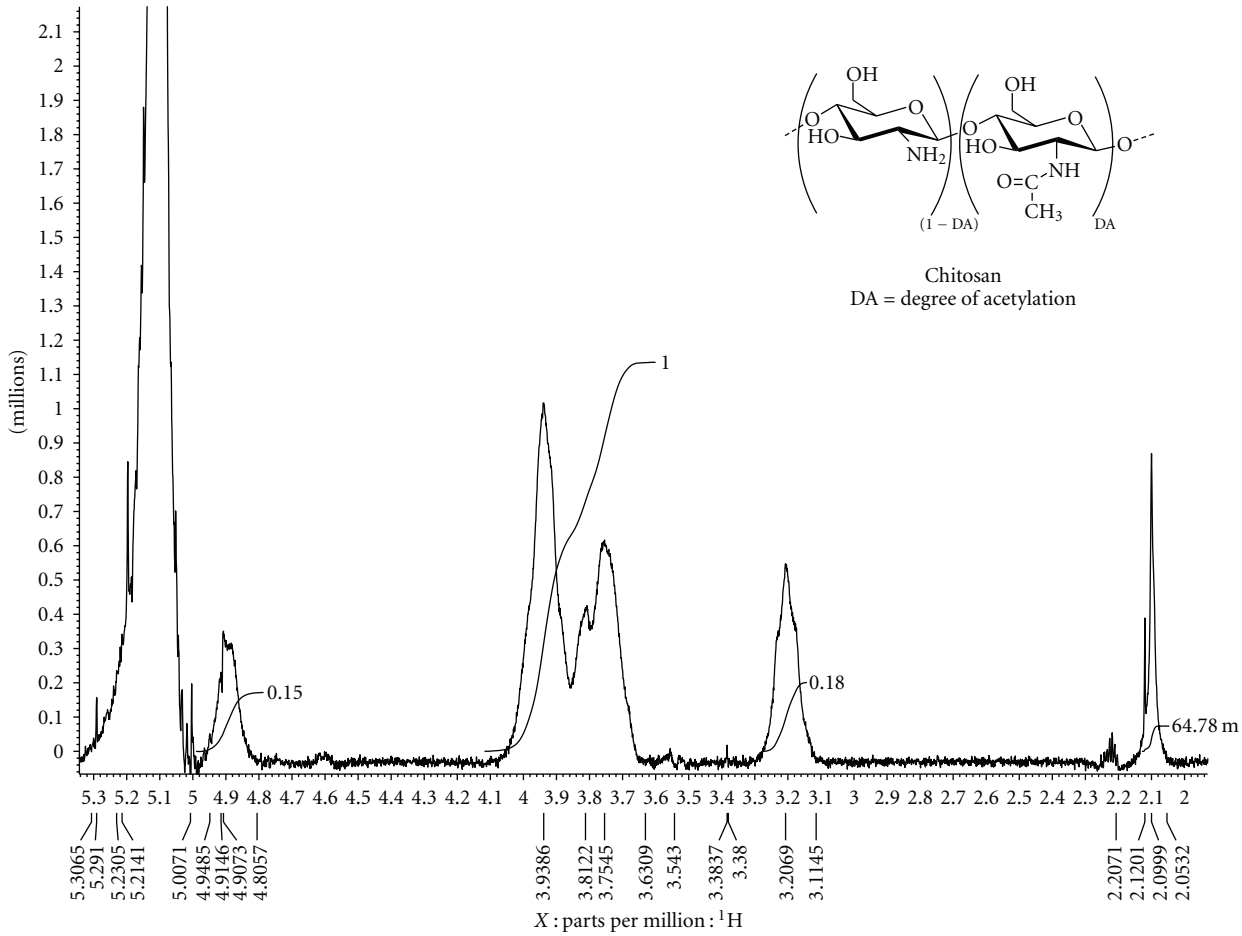

Figure 1: ^{1}H-NMR spectrum (300 MHz) of chitosan in 0.5% CD_3COOD/D_2O at 25°C.

solution properties as well as the polymer used [63, 98]. Chitosan, being a cationic polysaccharide in neutral or basic pH conditions, contains free amino groups on C-2 of GlcN unit and hence, is insoluble in water. In acidic pH, amino groups can undergo protonation thus, making it soluble in water. Therefore, solubility of chitosan depends upon the distribution of free amino and *N*-acetyl groups [99]. Usually 1–3% aqueous acetic acid solutions are used to solubilize chitosan [100]. The macromolecule chains are stretched caused by electrostatic repulsion of the NH_3^+ groups. The stretched chains will tend to be coiled with addition of salt because of the charge screening effect of added salt. The extent of solubility depends on the concentration and type of acid, where as the solubility decreases with increasing concentration of acid and aqueous solutions of some acids such as phosphoric, sulfuric, and citric acids are not good solvents [101]. The charge density of chitosan, that is, the degree of protonation of amino groups, is determined by the chemical composition, MW, and external variables such as pH and ionic strength. Dissociation constants (pKa) for chitosan range from 6.2 to 7, depending on the type of chitosan and conditions of measurement [61, 91, 102, 103]. A number of solvents for chitin and chitosan can be found in the literature. Generally, the solubility decreases with an increase in MW [63, 104]. Moreover, few attempts have been made to enhance chitosan's solubility in organic solvents [105–108]. However, many other attempts have been made to enhance its solubility in water. One major reason is because most biological applications for chemical substances require the material to be processible and functional at neutral pH. Thus, obtaining a water soluble derivative of chitosan is an important step towards the further application as a biofunctional material [70, 109, 110].

4.5. Viscosity. Viscosity is an important factor in the conventional determination of chitosan MW and in determining its commercial applications. Higher MW chitosan often render highly viscous solutions, which may not be desirable for industrial handling. However, lower viscosity chitosans may facilitate easy handling. The solution viscosity of chitosan depends on its molecular size, cationic character, and concentration as well as the pH and ionic strength of the solvent [111]. The determination of the intrinsic viscosity of polyelectrolyte is an effective method to study the sensibility of polyelectrolyte to the addition of salt. Therefore, the dilute solution viscosity of three chitosan samples (MW = 2.6×10^5, 5.6×10^5, and 1.06×10^6 Da) was measured both in solutions of NaCl with different ionic strengths

(0.01, 0.03, 0.06, and 0.10 mol/L) and in those of NaCl, KCl, $CaCl_2$, and $BaCl_2$ with the same ionic strength (0.06 mol/L), respectively [112]. It was found that the response of the intrinsic viscosity of chitosan to the added salts proceeds in the order of anion $Na^+ > K^+ > Ba^{2+} > Ca^{2+}$. According to the comparison of the ionic radius and hydrate number among the four anions, it is clear that the influence of intrinsic viscosity of chitosan on the added salt was due to both the anion radius and anion solvent power. Meanwhile the parameter of the MHKS equation was estimated to be 0.78, 0.70, 0.67, and 0.62 in corresponding added salt ionic strength (0.01, 0.03, 0.06, and 0.10 mol/L) [113].

4.6. Chemical Reactivity. Chitosan has three reactive groups, that is, primary (C-6) and secondary (C-3) hydroxyl groups on each repeat unit and the amino (C-2) group on each deacetylated unit. These reactive groups are readily subject to chemical modifications to alter mechanical and physical properties of chitosan. The typical reactions involving the hydroxyl groups are etherification and esterification. Selective *O*-substitution can be achieved by protecting the amino groups during the reaction [71]. The presence of a nucleophilic amino group allows for selective *N*-substitution, such as *N*-alkylation and *N*-acylation by reacting chitosan with alkyl halides and acid chlorides, respectively [71, 75, 114–117]. The alternative method for the *N*-alkylation is a reductive alkylation, where the amino group is converted to an imine with an aldehyde or ketone, and subsequently reduced to an *N*-alkylated derivative [26, 70, 76, 118–120].

4.7. Film-Forming Properties. In recent years, increasing interest in edible films has developed mainly due to concern over the disposal of conventional synthetic plastic materials derived from petroleum. Degradation of plastics requires a long time and most of them end up overburdening on landfill. Conversely, edible films from renewable agriculture products not only are degraded readily after their disposal, but also can extend the food shelf life, thus improving the quality of food. Among various available edible film materials, considerable attention has been given to chitosan because of its unique properties. It has been extensively studied for applications as films or membranes. These films can be described as biofilms with a homogeneous matrix, stable structure, good water barrier, and mechanical properties [121–124]. The functional properties of chitosan films are improved when chitosan is combined with other film-forming materials. Hoagland and Parris [125] prepared chitosan-pectin laminated films by interaction between the cationic groups on chitosan with the anionic groups on pectin. Hosokawa et al. [126] reported that when biodegradable films were made from chitosan and homogenized cellulose oxidized with ozone the number of carbonyl and carboxyl groups on the cellulose interacting with the amino groups on the chitosan increased. The water resistance of chitosan film was ameliorated by the incorporation of hydrophobic materials such as fatty acids to enhance the film's hydrophobicity [127]. Starch has been used to produce biodegradable films to partially or entirely replace plastic polymers because of its low cost and renewability. However, a wide application of starch film is limited by its water solubility and brittleness [128, 129].

4.8. Gelling Properties. Hydrogels are three-dimensional networks that swell in water and aqueous solutions. These materials, based on both natural and synthetic polymers, are currently attracting a great deal of interest as bioactive molecules and in tissue engineering. Among natural biopolymers of interest, chitosan stands out due to its unique combination of favorable properties such as hydrogel forming. Chitosan hydrogels can be divided into two classes: physical and chemical. Chemical hydrogels are formed by irreversible covalent links, whereas physical hydrogels are formed by various reversible links. For various reasons, physically cross-linked hydrogels have attracted increasing attention as bioactive compounds.

The preparation and characterization of a few hydrogels of chitosan have been reported, such as thermoreversible chitosan-oxalate, chitosan-aldehyde gels [130–132], and chitosan-alginate [133]. So far, no simple ionic and nontoxic cross-linking agent has been found that gives reproducible chitosan gels at low concentrations, such as calcium ions for gelling of alginates. However, aqueous chitosan gels crosslinked with molybdate polyoxy-anions have been reported, resulting in transparent, thermoirreversible gels that are able to swell several times their original size in aqueous solutions, depending on the ionic strength [134]. Different chitosan gels made with covalent cross-linking have been reported, with cross-linking with glutaraldehyde being the most widely applied [135, 136]. In addition, an enzymatic gelling system with chitosan has been reported [137–139].

4.9. Ion Binding. Chitosan is proved to have the best chelating properties among other natural polymers [140]. Responsible for complex formation are amino groups of chitosan, in which nitrogen is a donor of electron pairs, although hydroxyl groups may also participate in sorption. The mechanism of combining these reactive groups with ions of metals is much differentiated and can depend on the ion type, pH, and also on the main components of the solution. The complexes formation could be also described based on Lewis acid-base theory: metal ion (acting as the acid) is the acceptor of a pair of electrons given by chitosan (acting as the base).

In relation to food applications of chitosan, its application as a cholesterol-lowering agent [141, 142] and more controversial use as a weight-reducing agent, knowledge on the selective binding of essential metal ions to chitosan is important. Most studies of ion binding to chitosan have been aimed at determining whether chitosan binds to a given ion, whereas only a few studies have involved determining the selectivity of binding of different ions to chitosan. Rhazi et al. [143] determined the selectivity of mixtures of the ions $Cu^{2+} > Hg^{2+} > Zn^{2+} > Cd^{2+} > Ni^{2+} > Co^{2+} = Ca^{2+}$, using potentiometric and spectrometric methods. Vold et al. [144] reported the selectivity of different chitosans in binary mixtures of Cu^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+}, showing that chitosan could bind Cu^{2+} in large excess of the other metal ions. Recent years, chitosan-metal complexes attracted

great interests for their potential use in agriculture, medical industry, and food industry [145–148]. It is well known that both chitosan and metals such as Ag+, Cu^{2+}, Ni^{2+}, and Zn^{2+} have the properties of disinfection and bactericide [147, 149]. After chitosan binds to metal ions through nitrogen and or oxygen, the bindings are likely to leave some potential donor atoms free and these free donor atoms enhance the antimicrobial activity [148]. So it stands a good chance that chitosan-metal complexes exhibit enhanced ability of the antimicrobial activity of chitosan molecule, which will be very favorable to their applications in agriculture, medical industry, and food industry [147, 148].

4.10. Emulsification. Even though chitosan alone does not produce emulsions, Cho et al. [150] reported that emulsifying capacity of egg yolk increased with the addition of chitosan. At a concentration of 0.5%, better emulsifying capacity was observed compared with at 0.1 or 0.3% chitosan. In general, chitosan emulsions tend to be very stable under temperature changes and aging. With viscosity, the DA is reported to be a determining factor in the emulsification properties of chitosan. The protein solution containing chitosan with intermediate DDA produces less effective emulsion compared with that containing chitosan with higher DDA. The optimum chitosan DDA for sunflower oil emulsification is 81 and 89 as reported by Del Blanco et al. [151] and Rout [152], respectively.

5. Modification of Chitosan Structure and Properties

Chemical modifications of chitosan are increasingly studied as it has the potential of providing new applications. With regard to its unique properties such as biocompatibility, biodegradability, and no toxicity to mammals, it is widely used in fields like biotechnology, pharmaceutics, cosmetics and agriculture. In particular the antimicrobial activities of chitosan and its derivatives have aroused considerable recent interest. Unfortunately, in spite of the chitosan advantages, the poor solubility, low surface area, and porosity of chitosan are the major limiting factors in its utilization. Its solubility is limited at a pH higher than 6.5 where chitosan starts to lose its cationic nature. This problem is probably the major limiting factor for chitosan utilization, that is, its application in biology, since many enzyme assays are performed at neutral pH. If water-soluble chitosan would be easily accessible, it is expected that the biological and physiological potential would increase dramatically.

Chitosan can be modified by physical or chemical processes in order to improve the mechanical and chemical properties. Chitosan is a multinucleophilic polymer due to the presence of the amino group at C-2 and hydroxyl groups at C-3 and C-6 in the GlcN residue. Chitosan membrane is swollen in water; the amino groups may be protonated and leave the hydroxide ions free in water, which may contribute to the ionic conduction in the membrane. The initial sites where substitution occurs are the more nucleophilic amino groups. However, the experimental conditions and protection of the amino groups reduce the intermolecular hydrogen bonding and creates space for water molecules to fill in and solvate the hydrophilic groups of the polymer backbone [75]. For introducing alkyl or substituted alkyl groups selectively at the amino groups, reductive alkylation is the most reliable procedure. Chitosan is treated with an aldehyde to give an imine (Schiff base), which is easily converted into an *N*-alkyl derivative by reduction with sodium borohydride or sodium cyanoborohydride [76, 78, 119, 120, 153, 154]. These reactions are facile; the DSs are generally high and the products are soluble in water or dilute acids. The chitosan derivatives mentioned in the literatures showed that one can differentiate specific reactions involving the $–NH_2$ group at the C-2 position or nonspecific reactions of –OH groups at the C-3 and C-6 positions (especially esterification and etherification) [26, 155–158]. The positive charges on chitosan can also participate in ionic interactions, particularly with polyanions such as alginate and pectin. The complexes formed by electrostatic interaction between COO^- or SO_4^{--} and NH_4^+ [159] have been proposed for the recovery of suspended solids from aqueous food processing streams [160, 161] that can be used for animal feed.

Hydroxyalkyl chitosans are usually obtained in reactions of chitosan with epoxides. Depending on the reaction conditions (pH, solvent, and temperature); the reaction may take place predominantly at the amino or hydroxy groups giving *N*-hydroxyalkyl- or *O*-hydroxyalkyl chitosans or a mixture of both types. Under neutral and acidic conditions, *N*-hydroxyalkyl chitosan is preferred, leading to DS value <2. However, under alkaline conditions, the strongly nucleophilic oxygen ions will react much faster, resulting in *O*-hydroxyalkyl chitosan with DS values > 2 [26, 162, 163].

Acylation of chitosan was the usual method involving reacting chitosan under homogeneous reaction conditions with either an acid chloride or acid anhydride [164]. Acylation was shown to proceed smoothly at the free amino groups preferentially and then more slowly at the hydroxyl groups [164]. Complete *N*-acylation has been achieved by treating chitosan with cyclic acid anhydrides in aqueous homogeneous media at pH 4 to 8. Some of the resulting *N*-carboxyacyl chitosans were successfully converted into the corresponding imido forms by thermal dehydration [165]. *N*-acetylation of chitosan can be controlled when carried out in aqueous acetic acid solutions or in a highly swollen gel state in pyridine. With this gel, 50% *N*-acetylation was achieved, and the product was found to be soluble in neutral water [166, 167]. In case the swelling of chitosan is not sufficient, even the product with a similar DA does not give a homogeneous solution in water. Furthermore, no appreciable degradation is expected during the acetylation, and hence water-soluble chitosans with desired MWs can be prepared. Partial acetylation is also possible in homogeneous solutions in aqueous acetic acid/methanol [144] or in aqueous acetic acid [168] to give water-soluble products. The highest water solubility was again observed for a DA of 0.5. Under appropriate conditions similar to those for the benzoylation of chitin [169], chitosan was benzoylated (DS up to 2.5) with benzoyl chloride in methanesulfonic acid [170, 171].

N-saturated fatty acyl chitosan derivatives soluble in water, aqueous alkaline and acid solutions were prepared [117, 172, 173]. Acyl substitution was reported to take place on both *O*- and *N*-positions under a large excess of acid chloride. The successful preparation of *N*,*O*-acyl chitosans in $MeSO_3H$ as solvent was performed by Sashiwa et al. [79, 174] and also in our laboratory [71]. In this method, chitosan was dissolved in $MeSO_3H$ and the acid chlorides were added dropwise. The homogenous mixture was neutralized with $NaHCO_3$, then dialyzed and lyophilized to obtain the (*N*,*O*-acyl) chitosan derivatives with *O*-substitution as a major product. A noteworthy point is that both moderate substitution of *N*,*O*-acyl groups and moderate MW are important factors in obtaining highly biologically active compounds. Although the selective *O*-acylation of chitosan in $MeSO_3H$ (owing to the salt formation of the primary amino group with $MeSO_3H$) was reported [175], the detailed chemical structure and the protecting effect of $MeSO_3H$ on the amino group are not clear yet. The preparation of *O*,*O*-didecanoyl chitosan was also reported through a protected *N*-phthaloyl chitosan as intermediate [176]. However, this method needs several steps for the protection and deprotection of the *N*-phthaloyl groups [177]. Some *N*-carboxyacyl chitosans were also prepared by reaction of chitosan with intramolecular carboxylic anhydrides including maleic, glutaric, phthalic, and succinic [115, 165, 178]. As related compounds, some cyclic phthalimido derivatives of chitosan were reported [179]. In addition, *N*-carboxyacyl chitosans filaments were synthesized by suspended chitosan in methanol and carboxylic anhydrides were added [116, 180]. These compounds are usable as new functional materials in many fields because of their hydrophilic and acidic properties.

Grafting of chitosan allows the formation of functional derivatives by covalent binding of a molecule, the graft, onto the chitosan backbone [181]. The properties of the resulting graft copolymers are controlled by the characteristics of the side chains, including molecular structure, length, and number [182]. The cross-linking agents can be of varying length and contain other functional groups than those involved in cross-linking [183]. Partial cross-linking by di/polyfunctional reagents enables the use of chitosan for metal adsorption in acidic medium. Several bi- or polyfunctional cross-linking agents such as glutaraldehyde [184–187], ethylene glycol diglycidyl ether [188, 189], glyoxal [190], epichlorohydrin [191, 192], benzoquinone [193], and cyclodextrin [194–196] have been used. The fact that the cross-linking agents cited before are neither safe nor environment friendly has led to the use of water-soluble cross-linking agents such as sodium trimetaphosphate, sodium tripolyphosphate, or carboxylic acids [197].

One of the important strategies to increase both the solubility and positive charge density of chitosan is based on the introduction of quaternary ammonium groups into chitosan. This modification has got the commonly accepted term "quaternization of chitosan". Thus, derivatives soluble in water and in both acidic and basic physiologic circumstances may be good candidates for the polycationic biocides [198, 199]. Many efforts to synthesize quaternized chitosan derivatives have been reported. For example, Muzzarelli and Tanfani [157] reported the formation of *N*,*N*-dimethyl chitosan and the preparation of *N*,*N*,*N*-trimethyl chitosan iodide with formaldehyde and sodium borohydride. Trimethyl chitosan ammonium iodide was also obtained by reaction of a low acetyl content chitosan with methyl iodide and sodium hydroxide under controlled conditions [200, 201]. Water-soluble quaternary ammonium salts of *N*,*N*,*N*-trimethyl, *N*-*N*-propyl-*N*,*N*-dimethyl, and *N*-furfuryl-*N*,*N*-dimethyl chitosans were also prepared by reacting of *N*-alkyl chitosan derivatives with methyl iodide [109, 202]. Stepnova et al. [203] discovered a new, smooth, and one-step method for preparation of quaternized chitosans by means of reaction with betaine in the presence of the coupling reagent 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline in aqueous media at pH 5.5 ± 0.5. This reaction results in preparation of *N*-((trimethylammonio)acetyl) chitosan chloride and its amphiphilic derivatives. Other derivatives of quaternary chitosans as water-soluble and antimicrobial agents were recently prepared in our laboratory based on three-step process [70]. Schiff bases were firstly synthesized by the reaction of chitosan with aliphatic aldehydes followed by a reduction with sodium borohydride to form *N*-alkyl chitosans. *N*,*N*,*N*-dimethyl alkyl chitosans were then obtained by a reaction of chitosan containing *N*-butyl, (-pentyl), -hexyl, -heptyl, and -octyl substituents with methyl iodide.

The –OH and $–NH_2$ groups on the skeleton of chitosan are good ligands to coordinate with transition metal ions to get chitosan-metal complexes [146–148]. Moreover, the amine group of chitosan is modified using many chemical methods including chitosan 6-*O*-sulfate [204, 205], *N*-sulfated chitosan [206], and *N*-methylene phosphonic chitosans [207, 208]. So the functional groups of chitosan are easily modified by many organic reactions: tosylation [209], alkylation [210], carboxylation [211], sulfonation [205], Schiff base [212], and quaternary salt [157].

6. Application of Chitosan in Crop Protection

6.1. Chitosan and Its Derivatives as Antimicrobial Agents against Plant Pathogens (In Vitro Studies). The enormous increase in the number of relevant research papers and patents revealed a surprisingly high level of chitosan research activity from both academic and industrial scientists. Many literatures reported that chitosan and its derivatives have antimicrobial and plant-defense elicit function [6, 213–218]; therefore, these compounds are considered as useful pesticides in the control of plant diseases. The ideal antimicrobial polymer should possess the following characteristics: (1) easily and inexpensively synthesized, (2) stable in long-term usage and storage at the temperature of its intended application, (3) soluble in water or neutral media, (4) does not decomposed to and/or emit toxic products, (5) should not be toxic or irritating to those who are handling it, (6) can be regenerated upon loss of activity, and (7) biocidal to a broad spectrum of pathogenic microorganisms in brief times of contact [219].

Numerous studies on the antimicrobial activity of chitosan and its derivatives against most economic plant pathogens have been investigated [26, 70, 71, 76–78, 145, 220–227] and reviewed [6, 8, 10, 100, 218, 228–231]. Their antimicrobial activity has received considerable interest due to the problems associated with harmful synthetic antimicrobial agents [221, 232]. Chitosan's inhibition was observed on different development stages such as mycelial growth, sporulation, spore viability and germination, and the production of fungal virulence factors. It has been commonly recognized that the antimicrobial activity depends on the MW, DA, pH of chitosan solution and, of course, the target organism [202, 226, 232–244]. The natural antimicrobial characteristics of chitosan and its derivatives have resulted in their use in commercial disinfectants. Chitosan has several advantages over other types of disinfectants in that it possesses a high-antimicrobial activity, a broad spectrum of activity, and a low toxicity for mammalian cells [245]. Generally yeasts and moulds are the most sensitive group, followed by gram-positive bacteria and finally gram-negative bacteria [223]. The inhibitory activity was higher at pH 6.0 (pK_a value of chitosan = 6.2) than pH 7.5, when most amino groups are in the free base form.

Variation in sensitivity between closely related micoorganisms was illustrated in an experiment in which plant pathogenic fungi were screened for sensitivity to chitosan in liquid media [246]. One *Cytosporina sp.* isolate was completely inhibited by 75 mg/L chitosan, while a second isolate of the same genus was unaffected by 1000 mg/L. Chien and Chou [247] noted that the antifungal activity of chitosan depends on the type, concentration and test organism. For example, at 0.1%, chitosan of 92.1 kDa showed a higher growth inhibition of 76.2% on *P. italicum* than did chitosan of 357.3 kDa (71.4%), while at 0.2%, the antifungal activity exerted by chitosan of 357.3 kDa was higher than chitosan of 92.1 kDa against *P. italicum*.

A report by Benhamou et al. [248] indicated that chitosan derived from crab-shell at concentrations of 500 and 1000 mg/L was effective in reducing disease incidence caused by *F. oxysporum* f. sp. *radicis-lycopersici*. At the same time El Ghaouth et al. [249] revealed that chitosan was effective in inhibiting mycelial growth of *P. aphanidermatum* completely at a concentration of 400 mg/L. In our laboratory, we found that chitosan concentration increased (750–6000 mg/L), the radial growth of *A. alternata*, *B. cinerea*, *Colletrotichum gloeosporioides,* and *Rhizopus stolonifer* was decreased [250]. The same effect was reported on *Sclerotinia sclerotiorum* when chitosan concentrations increased from 1% to 4% [251]. Other studies showed a linear decrease of growth of *R. solani* as the chitosan concentration gradually increased from 0.5 to 6.0 mg/mL [252]. However, a complete growth inhibition was recorded against *F. oxysporum*, *R. stolonifer*, *P. digitatum,* and *C. gloeosporioides* at concentrations of 3% [253, 254].

The fungicidal activity of three commercial chitosan samples (3.60×10^5, 6.11×10^5, and 9.53×10^5 Da) was tested in our laboratory against plant pathogenic bacteria of *Agrobacterium tumefaciens, Corynebacterium fascians, Erwinia amylovora, E. carotovora, Pseudomonas solanacearum,* and *Sarcina lutea* [250]. The results indicated that chitosans of 6.11×10^5 and 9.53×10^5 Da were more potent in bactericidal activity than 3.60×10^5 Da chitosan and a chitosan of 9.53×10^5 Da exhibited a good antibacterial potency especially against *C. fascians* with MIC 500 mg/L. Moreover, three different MWs (0.5×10^4, 3.7×10^4, and 5.7×10^4 Da) chitosan were prepared in our laboratory from a commercial sample of chitosan (2.9×10^5 Da) and evaluated against bacteria of *A. tumefaciens* and *E. carotovora* and fungi of *A. alternata*, *B. fabae*, *F. oxysporum,* and *R. stolonifer* [255]. Chitosan of 0.5×10^4 Da exhibited a good antibacterial potency against *A. tumefaciens* with MIC 2600 mg/L, while chitosan of 3.7×10^4Da was the most active against *E. carotovora* with MIC 950 mg/L. The antifungal activity was increased with decrease of the MW and chitosan of 0.5×10^4 Da exhibited a high antifungal potency against *B. fabae*, *F. oxysporum*, and *R. stolonifer* while the fungus of *A. alternata* was more sensitive to a chitosan of 3.7×104 Da. This fact is in agreement with studies of Kim and Rajapakse, [256] and Zhang et al. [257] who reported that oligochitosans, obtained by hydrolysis or degradation of chitosan, was not only water-soluble but also have shown to be more effective than chitosan. Interestingly, oligochitosan (hexamer unit) that elicited maximal pisatin formation also exhibited higher antifungal activity against *F. solani* than the lower with degree of polymerization [223]. Xu et al. [258] added that the oligochitosans prepared by enzymatic depolymerization were more effective than the original chitosan in inhibiting mycelial growth of nine phytopathogens *F. graminearum*, *Phytophthora capsici*, *Verticillium dahliae*, *A. solani*, *B. cinerea*, *C. orbiculare*, *Exserohilum turcicum*, *F. oxysporum,* and *Pyricularia oryzae* and their inhibition on different stages in life cycle was observed. Hirano and Nagao [259] testing high- and low-molecular-weight chitosan on different fungal species and they found that the best fungicidal activity on mycelia occurred in media supplemented with low-molecular-weight chitosan. However, Bautista-Baños et al. [260] indicated that no differences in the fungicidal pattern among the three different types of chitosan, whereas there was a higher fungicidal effect as chitosan concentration increased (0.5–2.0%). Meng et al. [261] reported that both of chitosan (350 kDa) and oligochitosan (6 kDa) strongly inhibited spore germination and mycelial growth of two phytopathogenic fungi *A. kikuchiana* Tanaka and *Physalospora piricola* Nose. El Ghaouth et al. [215] found that a chitosan at concentrations ranged from 750 to 6000 mg/L was very effective in inhibiting spore germination and germ tube elongation of *B. cinerea* and *R. stolonifer*. Furthermore, this biopolymer at a concentration greater than 1500 mg/L induced morphological changes in *R. stolonifer*. Hernández-Lauzardo et al. [262] confirmed that the spore morphology of *R. stolonifer* presented variations in area, form, and optical density in chitosan solutions.

Control of two sapstain fungi *Leptographium procerum* and *Sphaeropsis sapinea* by a combination of chitosan or chitosan oligomer and an albino strain of *Trichoderma harzianum* was tested by Chittenden and Singh [263]. There was no mycelial growth of the fungi regardless of chitosan concentrations used when either *L. procerum* or *S. sapinea* were simultaneously inoculated with *T. harzianum*.

However, the dose response of chitosan or chitosan oligomer was apparent when *T. harzianum* was not simultaneously inoculated with test fungi but introduced later. There was a greater growth reduction at higher concentrations (0.075–0.1%) of chitosan, and overall chitosan oligomer was more effective than chitosan aqueous solution. Chitosan alone was able to restrict or delay the germination of spores but the combination of chitosan and *T. harzianum* inhibited spore germination and hence colony formation of test fungi regardless of time delay.

In addition, chitosan shows an antiviral activity against plant viruses. It was shown that chitosan inhibited the productive infection caused by the bacteriophage, the efficiency of inhibition of bacteriophage depending directly on the final concentration in the medium [264]. Major factors of suppressing phage infections by chitosan are phage particle inactivation and inhibition of bacteriophage reproduction at the cellular level. Evidently, chitosan may be used for induction of phagoresistance in industrial microorganism cultures to prevent undesirable phagolysis caused by inoculum contamination by virulent bacteriophages or by spontaneous prophage induction in lysogenic culture.

According to the data published on the antifungal and antibacterial activities of chitosan which indicated low activity observed against plant pathogens, several research groups have started to modify a chitosan molecule to produce high-antimicrobial active compounds. For examples, *N*-sulfonated and *N*-sulfobenzoyl chitosans [234], *N,N,N*-trimethyl chitosan [109], *N,O*-acyl chitosans [71, 79], *O*-acyl chitosans [77], hydroxyethyl acryl chitosan [265], dimethylpiperazine and trimethylpiperazine chitosans [266], carboxymethyl chitosans [267, 268], acyl thiourea chitosans [269], chitosan *N*-betainates [270], *N*-succinoyl chitosans [225], and *N*-heterocyclic chitosans [76]. We have prepared in our laboratory some of chitosan derivatives through the reductive amination reaction as described by Borch et al. [271] with various aldehydes. We noted that *N*-alkylation or -arylation of chitosan with aliphatic or aromatic aldehydes, respectively, effectively enhanced the antifungal activity of chitosan [77, 78, 119, 120]. For example, *N*-(*o,p*-diethoxybenzyl)chitosan was the most active one with EC_{50} of 400 and 468 mg/L for *F. oxysporum* and *P. debaryanum,* respectively. With the same methods and techniques, but different kinds of aldehydes, we have synthesized a series of *N*-benzyl chitosan derivatives and the fungicidal assessment has been investigated against *B. cinerea* and *P. grisea* [119]. The data revealed that *N*-(*o,o*-dichlorobenzyl) chitosan was the most active compound against *B. cinerea* with an EC_{50} of 520 mg/L. However, *N*-(benzo[d][1, 3]dioxol-5-ylmethyl) chitosan, and *N*-(methyl-4H-chromen-4-one) chitosan as new *N*-(heterocyclic) chitosan derivatives were the most active against *P. debaryanum* and *F. oxysporum* [76]. Guo and coworkers [222] added that the Schiff bases of chitosan and the *N*-substituted chitosan derivatives had a slight activity against *B. cinerea* Pers., and the inhibitory indices were 26.8%, 33.5%, 39.3%, and 32.3% at 1000 ppm, respectively, compared with 45.4% at chitosan. Previously, we synthesized derivatives of *N,O*-acyl chitosans [71] and the data indicated that *N,O*-(*p*-chlorobutyryl) chitosan, *N,O*-decanoyl chitosan, *N,O*-cinnamoyl chitosan and *N,O*-(*p*-methoxybenzoyl) chitosan were the most active compounds against *B. cinerea* (EC_{50}= 430, 440, 450 and 500 mg/L, respect.) and were 12- to 13-fold more active than the native chitosan (EC_{50} > 3000 mg/L).

Previously Muzzarelli et al. [272] prepared five chemically modified chitosans and tested their antifungal activities against *Saprolegnia parasitica*. Results indicated that, as for the chitosan-bearing broth assay, *S. parasitica* did not grow normally; on the first day for methylpyrrolidinone chitosan and *N*-phosphonomethyl chitosan and on the second day for *N*-carboxymethyl chitosan, a tightly packed precipitate was present at the bottom of the test tubes instead of the fluffy fungal material as in the control. In contrast, *N*-dicarboxymethyl chitosan seemed to favor fungal growth, while dimethylaminopropyl chitosan did not significantly differ from the control.

N,N,N-dimethylalkyl chitosans as quaternary and water-soluble chitosan compounds were recently prepared in our laboratory to test their antimicrobial activities against the most economic plant pathogenic bacteria *A. tumefaciens* and *E. carotovora* and fungi *B. cinerea, F. oxysporum,* and *P. debaryanum* [70]. Quaternary chitosans enhanced the antibacterial activity and *N,N,N*-dimethylpentyl chitosan was the most active with MIC 750 and 1225 mg/L against *A. tumefaciens* and *E. carotovora,* respectively. However, both of *N,N,N*-dimethylpentyl chitosan and *N,N,N*-dimethyloctyl chitosan were significantly the highest in fungal mycelial growth inhibition of *B. cinerea*, *F. oxysporum* and *P. debaryanum*. In addition, spore germination of *B. cinerea* and *F. oxysporum* was significantly affected with the compounds at the tested concentrations and the inhibition activity was increased with an increase in the chain length of the alkyl substituent. Previously Hernández-Lauzardo et al. [236] reported that spore germination of *R. stolonifer* was affected by different MWs chitosan (1.74×10^4, $2.38 \times 10^4$and 3.07×10^4 Da). They found that chitosan of 1.74×10^4 and 2.38×10^4 Da markedly reduced spore germination, but no significant effects were found among the tested concentrations (1.0, 1.5, and 2.0 mg/mL). However, they observed a complete inhibition of spore germination with a chitosan of 3.07×10^4 Da. Recently, the effect of *N*-(benzyl) chitosan derivatives on spore germination of *F. oxysporum* was evaluated in our laboratory at 250, 500, and 1000 mg/L [78]. All the derivatives had better inhibition of spore germination, about threefold compared with chitosan. *N*-(*p*-dimethylaminobenzyl) chitosan, *N*-(*p*-ethylbenzyl) chitosan, *N*-(*o*-methoxybenzyl) chitosan, and *N*-(*o,p*-diethoxybenzyl) chitosan significantly exhibited high inhibition percentage (>90%) of spore germination at 1000 mg/L.

6.2. Chitosan and Its Derivatives in Plant Disease Control (In Vivo Studies). The plant protection activity of chitosan compounds have been well documented in many different plant systems [10]. The control diseases of chitosan include bacteria, fungi, and viral diseases. In this section, the effects of these compounds on several plants will be discussed.

In 1980, Professor Hadwiger at Washington State University reported that oligochitosan can induce soybean against *F. solani* [273]. Oligochitosan from the *F. solani* f. sp. *phaseoli* cell walls could elicit defense reaction in pea pod tissue. Concentrations of oligochitosan as low as 0.9 and 3 mg/mL elicited phytoalexin induction and inhibited the germination of *F. solani*, respectively. This was the first publication of chitosan-induced plant resistance. From then, a series of excellent work was conducted in his laboratory. These creative researches led to an increased tide on the study of chitosan-induced plant defense.

The soilborne phytopathogenic fungi *F. solani* and *C. lindemuthianum* were inhibited by chitosan and *N*-(carboxymethyl) chitosan [274–276]. Benhamou et al. [248] added that chitosan concentrations of 0.5 and 1 mg/mL showed high plant protection from *F. solani,* when seed coating and soil amendment were performed. Although chitosan at 0.1 mg/mL induced a delay in disease development (root lesions visible by 4 days after inoculation), emergence of wilting symptoms occurred between 7 and 10 days after-inoculation, while death of about 80% of the plants was recorded 1 week later. *F. acuminatum*, *Cylindrocladium floridanum,* and other plant pathogens of interest in forest nurseries were inhibited by chitosan [277]. Similarly, *Aspergillus flavus* was completely inhibited in field-growing corn and peanut [278]. Generally, chitosan has high-antifungal activity, but it is less effective against fungi with a chitin or chitosan component in their cell walls [246]. Part of the effect observed by chitosan on the reduction of soilborne pathogens comes from the fact that it enhances plant defense responses. The other part is linked to the fact that it is composed of polysaccharides that stimulate the activity of beneficial micro-organisms in the soil such as *Bacillus,* fluorescent, *Pseudomonas*, actinomycetes, mycorrhiza, and rhizobacteria. This alters the microbial equilibrium in the rhizosphere disadvantaging plant pathogens. Beneficial organisms, on the other hand, are able to compete them through mechanisms such as parasitism, antibiosis, and induced resistance [279–284].

Great advancement of chitosan on rice disease control has been achieved in recent years. In 2002, Agrawal and coworkers reported the effect of chitosan in initiating defense response in the leaves of rice for the first time [285]. After treatment with 0.1% chitosan, necrotic streaking was clearly observed on the upper side of rice leaves. Enhanced defense against rice blast pathogen, *Magnaporthe grisea* 97-23-2*D*1, was observed in *H7S* rice seedlings treated with oligochitosan. In this experiment, 5 mg/L oligochitosan solution showed the best effect and the disease control was more than 50% [286]. Chitosan was tested in rice production by Boonlertnirun et al. [287] and its application by seed soaking and soil application four times throughout cropping season significantly increased rice yield over the other treatments. However, application by seed soaking and spraying the foliar four times tended to show ability on disease control. Zeng and Shi [288] developed a new type of organic rice seed coating agent using liquid-based polymeric adhesives. By using chitosan as the main raw material, modified with sodium hydroxide and polymerised with plant growth regulators and other additives, the novel seed coating agent is a safer, cheaper, and more environmentally friendly alternative. Results of antifungal tests showed that the antifungal efficiency of the seed-coating agent was increased with increasing amount of dosage; a 1 : 20 concentration was the best for inhibiting growth of the two phytopathogens *R. solani* and *F. moniliforme*.

Wheat is another important source of staple food, especially in cold countries; therefore, Russian scientists conduct more research in this area. Studies conducted on wheat infection with *Bipolaris sorokiniana* indicated that oligochitin with a MW of 5–10 kDa and the DA of 65% has good effect on controlling wheat disease [289, 290]. The ability of oligochitosan to promote wheat resistance to pathogenic toxin was also validated [291]. Chitosan treatment (2–8 mg/mL) of wheat seeds (two cultivars of spring wheat (Norseman and Max)) significantly improved seed germination to recommended seed certification standards (>85%) and vigour at concentrations >4 mg/mL by controlling seed-borne *F. graminearum* infection. The germination was <80% in the control and >85% in chitosan-treated seeds. The reduction of seed-borne *F. graminearum* was >50% at higher chitosan treatments compared with control [292].

Tobacco is an important economic crop and a model plant for research. Many reports reveal that chitosan can induce tobacco's resistance to tobacco mosaic virus, tobacco necrosis virus, and *Phytophthora parasitica*. For example, Falcon studied the effect of different sizes and DA of chitosan derivatives on tobacco protection against *P. parasitica* [293]. The results of their experiment showed that different chitosans have distinct effects on this disease control, though less acetylated chitosan were better for inhibition of *P. parasitica* growth, partially acetylated chitosan were more effective in protecting tobacco against this pathogen by systemic induction of plant immunity.

Sclerotinia rot is the most harmful disease on oilseed rape production. The inducing resistance of oligochitosan to *Sclerotinia sclerotiorum* on *Brassica napus* was studied [294]. However, oligochitosan did not affect the radial growth of *S. sclerotiorum* colonies; it reduced the frequency and size of rot compared with controls when applied to oilseed rape before inoculation. The best pretreated time was 3 days before inoculation, and the best inducing resistance concentration of oligochitosan was 50 μg/mL. Oligochitosan can be modulated into steady colloid solution, so it can be used as a seed-coating agent. It does not influence the seed sprout and emerge, but can obviously suppress the emergence of *S. sclerotiorum*; the control rate of three species of rape was 34.19–44.10% [295].

Chitosan was shown to inhibit the systemic propagation of viruses and viroids throughout the plant and to enhance the host's hypersensitive response to infection. Potato is a tuberous crop from the perennial *Solanum tuberosum* of the *Solanaceae* family. It is an essential crop in the world. The effect of the chitosan-induced resistance to viral infection was investigated in potato plants. The plants were sprayed with different molecular weights of chitosan solution (1 mg/mL) and the greatest antiviral activity was shown by chitosan of 120 kDa. In another experiment, potatoes were

infected with potato virus X after chitosan pretreatment. It was found that chitosan treatment significantly decreased the number of systemically infected plants compared to control, and the treated leaves also accumulated less amount of virus than the control leaves [296]. The antiviral activity of chitosan depends on the average degree of polymerization, the degree of *N*-deacetylation, the positive charge value, and the character of the chemical modifications of the molecule. Possible mechanisms of suppressing viral infections by chitosan are also discussed [264, 296–301]. Chitosan applied by spraying or inoculating leaves protected various plant species against local and systemic infection caused by alfalfa mosaic virus, tobacco necrosis virus, tobacco mosaic virus, peanut stunt virus, cucumber mosaic virus, and potato virus X [300, 301]. The ability of chitosan to suppress viral plant infections does not depend on the virus type because chitosan affects the plant itself by inducing resistance to the viral infection. Imitating the contact of the plant with a phytopathogen, chitosan induces a wide spectrum of protective reactions in the plant, which limit a systemic spread of the viruses and viroids over the plant and lead to the development of systemic acquired resistance [296–301].

The effect of oligochitosan and oligochitin on gray mould caused by *B. cinerea* in cucumber plants were evaluated by Ben-Shalom et al. [302]. It was shown that oligochitosan and oligochitin had different effects on this cucumber-pathogen interaction. Although complete inhibition of *Botrytis* conidia germination was found at 50 ppm chitosan solution *in vitro*, chitosan also controlled the gray mould in treated plants compared with control plants. But there was no effect of oligochitin on both pathogen growth on PDA and leaves. Besides this fungicidal effect, spraying chitosan 1, 4, and 24 h before inoculation with *B. conidia* decreased gray mould by 65, 82, and 87%, respectively. However, spraying chitosan on the leaves decreased gray mould incidence only by 52% 1 h after inoculation. These results suggest that the antifungal and elicitor activity of chitosan are both necessarily for the control of gray mould in cucumber [302]. *P. aphanidermatum (Edson) Fitzp* is an aggressive and economically important pathogen in greenhouse-grown cucumbers. Especially in substrates like rockwool which exhibit high water retention capacity, it can flourish and spread rapidly by zoospores. The pathogen causes severe root and crown rot, which result in wilting and death of plants. A recent paper showed that chitosan cannot induce defense to this pathogen when used alone. But the application of chitosan in combination with *Lysobacter enzymogenes* 3.1*T*8 (a biocontrol bacterium) reduced the number of diseased plants by 50%–100% in four independent experiments relative to the *Pythium* control. [303, 304].

Vasyukova et al. [305] reported that low-molecular-weight water-soluble chitosan (5 kDa), obtained after enzymatic hydrolysis of native crab chitosan, was shown to display an elicitor activity by inducing the local and systemic resistance of *S. tuberosum* potato and *Lycopersicon esculentum* tomato to *P. infestans* and nematodes, respectively. Chitosan induced the accumulation of phytoalexins in tissues of host plants; decreased the total content; changed the composition of free sterols producing adverse effects on infesters; activated chitinases, $\beta-$glucanases, and lipoxygenases; stimulated the generation of reactive oxygen species. The activation of protective mechanisms in plant tissues inhibited the growth of taxonomically different pathogens (parasitic fungus *P. infestans* and root knot nematode *Meloidogyne incognita*). In addition, the potential of *B. pumilus* strain SE 34 in combination with chitosan, for inducing defense reactions in tomato plants inoculated with *F. oxysporum*, was studied [306]. A substantial increase in the extent and magnitude of the cellular changes induced by *B. pumilus* was observed when chitosan was supplied to bacterized tomato plants. These changes were characterized by a considerable enlargement of the callose-enriched wall appositions deposited onto the inner cell wall surface in the epidermis and the outer cortex.

Chitosan of 350 kDa was more effective at 25°C than oligochitosan (6 kDa) in controlling of the disease in pear fruit caused by two phytopathogenic fungi of *A. kikuchiana* Tanaka and *Physalospora piricola* Nose [261]. When treated with oligochitosan, pear fruit increased the activities of chitinase and b-1,3-glucanase. Differently, chitosan treatment significantly increased peroxidase activity of pear fruit. The results suggested that chitosan and oligochitosan triggered different mechanism for pathogenicity inhibition and disease control.

Chitosan compounds are used as biopesticides in many grape-producing countries. It was reported that oligochitosan (1500 Da and a DA of 20%) at 200 μg/mL dramatically reduced the infection of grapevine leaves by *Plasmopara viticola* and *B. cinerea*. Dose-response experiments showed that maximum defense reactions and control effect of *B. cinerea* were achieved with 75–150 μg/mL [307–309]. Similarly, on apple and watermelon, chitosans can induce plant defense against canker and anthracnose.

Guan et al. [310] examined the use of chitosan to prime maize seeds. Although chitosan had no significant effect on germination under low temperatures, it enhanced germination index, reduced the mean germination time, and increased shoot height, root length, and shoot and root dry weights in two tested maize lines. In both tested lines, chitosan induced a decline in malonyldialdehyde content, altered the relative permeability of the plasma membrane and increased the concentrations of soluble sugars, proline, peroxidase, and catalase activities. In other studies, seed priming with chitosan improved the vigor of maize and wheat seedlings. It was also reported that such treatment led to an increase of seed resistance to certain diseases and improve their quality and/or their ability to germinate [292, 311]. Similarly, peanut seeds soaked in chitosan were reported to exhibit an increased rate of germination and energy, lipase activity, and gibberellic acid and indole acetic acid levels [312]. Ruan and Xue [313] showed that rice seed coating with chitosan may accelerate their germination and improve their tolerance to stress conditions. In carrot, seed coating helps restrain further development of Sclerotinia rot [251]. It has also been reported that chitosans can activate plant defense to disease on several other plants such as barley [314], pearl millet [315], carrot [316], sunflower [317], and coconut [318].

6.3. Chitosan and Its Derivatives in Postharvest Application. Fruits and vegetables deteriorate rapidly after harvest and in some cases do not reach consumers at optimum quality after transport and marketing. The main causes of their deterioration are dehydration, with the subsequent weight loss, color changes, softening, surface pitting, browning, loss of acidity, and microbial spoilage, among others. One of the potential approaches to extend the storability of these perishable commodities is to apply edible coatings or films on the surface, followed by a cold storage [319]. Therefore, the use of bioactive substances such as chitosan to control postharvest microbial diseases has attracted much attention due to imminent problems associated with chemical agents [215, 216, 320]. It has become a promising alternative treatment for fruits and vegetables due to its natural character, antimicrobial activity, and elicitation of defense responses [321, 322]. Indeed, chitosan is an ideal preservative coating for fresh fruits and vegetables because of its film-forming and biochemical properties [323] and has led to prolonged storage life and controlled decay of several fruit crops [324]. Chitosan coating is likely to modify the internal atmosphere without causing anaerobic respiration, since chitosan films are more selectively permeable to O_2 than to CO_2 [325].

Strawberry is among the most perishable fruits and is vulnerable to physical injuries and microbial infection. El Ghaouth and coworkers [215, 326] investigated the effect of chitosan coating on decay and quality of strawberries. Fruits were inoculated with spore suspension of *B. cinerea* or *R. stolonifer* and subsequently dipped in chitosan solutions (1.0 and 1.5% in 0.25 N HCl). In both studies, chitosan coating significantly reduced the decay of strawberries compared with the control. Chitosan coating decreased the respiration rate of strawberries with a greater effect at higher concentration. The improved storability of fresh strawberries by chitosan-based coating also has been documented [319, 327–330].

Li and Yu [331] reported that chitosan significantly delayed the postharvest development of brown rot disease caused by *Monilinia fructicola* on peach fruit. Effects of chitosan coating on browning of litchi (*Litchi chinensis*) fruit were also investigated by several workers [332–334]. Chitosan coating, irrespective of concentration 1 and 2% dissolved in 2% glutamic acid delayed changes in contents of anthocyanins, flavonoids, and total phenolics. It also delayed the increase in polyphenol oxidase (PPO) activity, and partially inhibited the increase in peroxidase activity [332, 335]. Jiang et al. [333] also similarly observed that chitosan of 2% in 5% acetic acid coating delayed the decrease in anthocyanin content and the increase in PPO activity. Such effects of chitosan coating were also observed with peeled litchi fruit [336], longan fruit [337], and fresh-cut Chinese water chestnut vegetable [338]. Dependence of browning rate of chitosan-coated litchi fruit on the initial pericarp water content [332], pericarp pH, and dehydration rate during storage [334] has been reported.

Penicillium is the most harmful citrus fruit postharvest pathogen and infects the fruit through microinjuries generated in the flavedo, during harvesting and processing. However, nowadays, consumers around the world demand high-quality food, without chemical preservatives, leading to increased effort in discovering new natural antimicrobials. Accordingly, the fungistatic effects of chitosan have been investigated. For example, coating of citrus fruit with chitosan was effective in controlling fruit decay caused by *P. digitatum* and *P. expansum* and chitosan of 15 kDa at 0.2% was more effective in controlling the growth of fungi than chitosan of 357 kDa [339].

Moreover, edible coatings can be used as a vehicle for incorporating functional ingredients such as antioxidants, flavors, colors, antimicrobial agents, and nutraceuticals [327, 340–342]. Several workers have endeavored to incorporate calcium [327, 328], vitamin E [327, 343], potassium [319], or oleic acid [330] into chitosan film formulation to prolong the shelf life and to enhance the nutritional value of fruits.

Gray mold and blue mold rots caused by *B. cinerea* and *P. expansum*, respectively, in sweet cherry fruit were reduced by preharvest spraying or postharvest dipping of chitosan [344]. Liu et al. [345] added that the control effects of chitosan on both fungi significantly decreased in tomato fruit at 5000 and 10000 mg/L and the gray mold was better controlled than blue mold. Previous investigations on chitosan coating of tomatoes have shown that it delayed ripening by modifying the internal atmosphere that reduced the decay [215, 216, 320]. Recently we investigated the effectiveness of different molecular weights chitosan on the gray mold caused by *B. cinerea* as *in vivo* in tomato fruit (*Solanum lycopersicum* L. var. *lycopersicum*) stored at different temperatures [220]. The treatments significantly reduced fungal decay and all compounds at concentrations of 2000 and 4000 mg/L exhibited complete fungal control in wound-inoculated fruit. In addition, chitosan had potential for the elicitation of defense markers, including total soluble phenolics, PPO activity and total protein content. This finding suggests that the effects of chitosan may be associated with direct fungitoxic properties against the pathogen, and the elicitation of biochemical defense responses in fruits [220]. After treatment with chitosan, various defense responses have been induced, including the elicitation of phenylalanine ammonia lyase (PAL) activity in grape berries [324, 346], chitinase, and β-1,3-glucanase in oranges, strawberries, and raspberries [345, 347, 348].

7. Factors Affecting Antimicrobial Activity of Chitosan

The extent of the antimicrobial action of chitosan is influenced by intrinsic and extrinsic factors such as MW, DDA, pH, temperature, solubility, derivatization, type of organism and so on. It is necessary to understand these factors for the effective application of chitosan and its derivatives as antimicrobial agents against plant pathogens.

7.1. Molecular Weight. Effect of chitosan MW on the antimicrobial activities has been explored and most investigators used the uncertain term of low MW (LMW) chitosan for a partially depolymerized chitosan not indicating exactly its MW [349]. Only a few data on the bactericidal activity of LMW chitosan could be compared depending on bacteria

tested, conditions of biological test and chitosan MW, but even in this case the results did not correspond to each other. Thus, chitosan of 9300 Da restricted growth of *E. coli*, while chitosan of 2200 Da promoted growth of this bacterium [350]. Increase in the MW led to a decrease in chitosan activity against *E. coli* in some studies [351, 352], while in the others an increased activity for a high MW (HMW) in comparison with LMW chitosan have been found [353]. In contrast to the above mentioned results, no differences in HMW and LMW chitosan activities were found against *E. coli* [354, 355] and *Bacillus subtilis* [351, 354].

It has been demonstrated that LMW chitosans (of less than 10 kDa) have greater antimicrobial activity than native chitosans. However, a DP of at least seven is required; lower MW fractions have little or no activity [227]. Chitosan with a MW ranging from 10,000 to 100,000 Da would be helpful in restraining the growth of bacteria. In addition, chitosan with an average MW of 9300 Da was effective against *E. coli*, while that with a MW of 2200 Da accelerated growth of the same bacteria [356]. Tanigawa et al. [357] reported that D-glucosamine hydrochloride (chitosan monomer) did not show any growth inhibition against several bacteria, whereas chitosan was effective. This suggests that the antimicrobial activity of chitosan is related to not only its cationic nature but also to its chain length. Shimojoh et al. [358] also found that chitosan of 220,000 Da was most effective, whereas chitosan of 10,000 Da was the least effective in their bactericidal activities. However, the antimicrobial activity of chitosan of 70,000 Da was better than of 426,000 Da for some bacteria, but for the others, the effectiveness was reversed. Yalpani et al. [359] reported that medium MW chitosans showed higher antimicrobial activities against *B. circulans* than chitooligosaccharides (DP 2–30), whereas they were less effective against *E. coli* than chitooligosaccharides. From the results of Shimojoh et al. [358] and Yalpani et al. [359] one can notice that the relationship between MW of chitosan and the antimicrobial activity can be affected by the test organisms. Numerous researchers have reported that the antibacterial activity of chitosan is a MW dependant [291, 354, 357, 360, 361]. Hwang et al. [362] concluded that chitosan with MW bout 30,000 Da exhibited the highest bactericidal effect on *E. coli* from their investigation of MW range of 10,000–170,000 Da. Jeon et al. [354] suggested that the MW of chitosan is critical for the inhibition of microorganisms and suggested the required MW be higher than 10,000 Da for better antimicrobial activity.

The antimicrobial activity of different MWs chitosan and chitosan oligomers (DP 2–8) against several plant pathogens were examined by Hirano and Nagao [259]. It was observed that the increases in MW increased the number of inhibited fungi. The strongest growth inhibition was observed with LMW and the weakest was observed with HMW chitosan. Kendra and Hadwiger [223] examined the antifungal effect of chitosan oligomers on *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli*. The antifungal activity was found to increase as the polymer size increased. Monomer and dimer units did not show any antifungal activity at 1000 μg/mL. However, heptamer (DP = 7) showed maximal antifungal activity and the minimum concentrations were identical to those observed for both native chitosan and the acid-cleaved chitosan. We examined the antimicrobial activity of 3.60×10^5, 6.11×10^5 and 9.53×10^5 Da chitosans against plant pathogenic bacteria *A. tumefaciens, C. fascians, E. amylovora, E. carotovora, P. solanacearum* and *S. lutea* and fungi *A. alternata*, *B. fabae*, *F. oxysporum*, *P. digitatum*, *P. debaryanum* and *R. solani*. The results indicated that chitosans of 6.11×10^5 and 9.53×10^5 Da were more potent in bactericidal activity than chitosan of 3.60×10^5 Da and chitosan of 9.53×10^5 Da exhibited a good antibacterial potency especially against *C. fascians* with MIC 500 mg/L. The data demonstrated that the fungicidal activity was increased as MW increase and chitosan of 9.53×10^5 Da was the most potent one against all the tested fungi [250]. Recently we also investigated the antifungal activity of depolymerized chitosans (0.5×10^4, 3.7×10^4, 5.7×10^4, and 2.9×10^5 Da) on the gray mold caused by *B. cinerea* as *in vitro* and *in vivo* on tomato fruit [220]. In an *in vitro* experiment, the result demonstrated that the antifungal activity increased as the chitosan MW decreased. In an *in vivo* study, chitosan with MW of 5.7×10^4 Da was the most effective among those tested.

It is difficult to find a clear correlation between MW and antimicrobial activity, generally the antimicrobial activity increases as the MW of chitosan increases. However, the activity decreases over a certain high MW. The discrepancies between data may result from the different DDA and MW distributions of chitosan. The evaluation of only the MW dependence of the antimicrobial activity requires a wide MW range of chitosan samples with the same DDA. It is almost impossible to obtain this because chitosan is a natural polymer. From the existing data, it is difficult to determine what the most optimal MW for the maximal antimicrobial activity is. The selection of MW of chitosan could be thought to be more dependent on its application.

7.2. Degree of Deacetylation. The antimicrobial activity of chitosan is directly proportional to the DDA of chitosan [291, 357, 358, 363]. The increase in DDA means the increased number of amino groups on chitosan. As a result, chitosan has an increased number of protonated amino groups in an acidic condition and dissolves in water completely, which leads to an increased chance of interaction between chitosan and negatively charged cell walls of micro-organisms [224]. Variation of deacetylation process yielded chitosan with significant differences in DDA% as well as variation of the MW. Simpson et al. [364] reported that chitosan with a DDA of 92.5% was more effective than chitosan with a DDA of 85%. On the contrary, Ikinci et al. [365] reported that change in DDA (73, 84 and 95%) did not have any effect on the antimicrobial activity of chitosan against *Porphyromonas gingivalis*.

Hongpattarakere and Riyaphan [366] prepared chitosan from black tiger shrimp carapace by deacetylation process performed in 50% NaOH at 100°C under vacuum, nitrogen, and regular atmospheres. Each condition was maintained for 0.5, 1, and 2 h. MIC values of chitosans varied depending on conditions of deacetylation processes, reaction times, and test micro-organisms. In general, chitosan prepared

under atmosphere showed the lowest MIC value or highest inhibitory effect on test micro-organisms, whereas that deacetylated under nitrogen showed the least inhibitory effect. Chitosan obtained from 1 h of deacetylation under regular atmosphere showed the lowest MIC value (625 ppm) against *E. coli* and *S. aureus*, while *Candida albicans* was inhibited at MIC value of 312.5 ppm due to its higher DDA and lower MW compared to chitosan deacetylated under vacuum and nitrogen atmospheres.

7.3. The pH. The antimicrobial activity of chitosan is strongly affected by the pH [226, 243, 244, 291]. Lower pH increases the antimicrobial activity for much the same reasons, in addition to the "hurdle effect" of inflicting acid stress on the target organisms. Tsai and Su [226] examined the antimicrobial activity of chitosan (DDA 0.98) against *E. coli* at different pH values of 5.0, 6.0, 7.0, 8.0, and 9.0. The greatest activity was observed at pH 5.0. The activity decreased as the pH increased and chitosan had little antibacterial activity at pH 9.0. Other researchers [243, 291] reported that chitosan had no antimicrobial activity at pH 7.0 due to the deprotonation of amino groups and poor solubility in water. This suggests that the antimicrobial activity comes from the cationic nature of chitosan.

7.4. Temperature. Temperature also has an effect on the antimicrobial activity of chitosan. Higher temperature (37°C) has been shown to enhance its antimicrobial activity compared to refrigeration temperatures. However, the greatest single influence on antimicrobial activity is the surrounding matrix. Tsai and Su [226] examined the effect of temperature on the antibacterial activity of chitosan against *E. coli*. The cell suspensions in phosphate buffer (pH 6.0) containing 150 ppm chitosan were incubated at 4, 15, 25, and 37°C for various time intervals and the surviving cells were counted. The antibacterial activity was found to be directly proportional to the temperature. At the temperatures of 25 and 37°C, the *E. coli* cells were completely killed within 0.5 and 1 hr, respectively. However, at lower temperatures (4 and 15°C) the number of *E. coli* declined within the first 5 hrs and then stabilized. The authors concluded that the reduced antimicrobial activity resulted from the decreased rate of interaction between chitosan and cells at a lower temperature.

7.5. Cations and Polyanions. Young et al. [367] observed that chitosan-induced leakage of UV-absorbing material from Glycine max was strongly inhibited by divalent cations in the order of $Ba^{+2} > Ca^{+2} > Sr^{+2} > Mg^{+2} > Na^{+} > K^{+}$. It was assumed that the cations displaced Ca^{+2} released from the cell surface, formed complexes stabilizing the cell membrane, and consequently reduced the chitosan-induced leakage. Young and Kauss [368] reported that chitosan caused the release of Ca^{+2} present on *Glycine max* cell and/or plasma membrane, which destabilized the cell membrane and further induces leakage of intracellular electrolytes. They suggested that the cross-linking of chitosan (polycation) with phospholipids or protein components in the cell membrane affects the membrane permeability, which further causes leakage of intracellular substances and finally causes the death of cell. Tsai and Su [226] also reported that reducing bactericidal effect of chitosan against *E. coli* by the addition of salts containing alkaline earth metals such as $MgCl_2$, $BaCl_2$, and $CaCl_2$ was observed. The order of effectiveness was $Ba^{+2} > Ca^{+2} > Mg^{+2}$. The authors proposed that the cations form complexes with chitosan and consequently the reduced available amino groups of chitosan led to the reduced bactericidal effect unlike Young's assumption [367]. In addition to the reduced chitosan-induced leakage by cations, the leakage was also reduced by the addition of polyanions such as sodium polygalacturonate and sodium poly-Laspartate. The complete prevention of electrolyte leakage was observed when the number of carboxyl groups in the polyanions was equal to that of the amino groups of chitosan. It was attributed to the formation of polycation (chitosan)-polyanion complexes, which were observed by formation of precipitate. However, monomeric galacturonate and aspartate did not show any effect on the leakage and no precipitation of chitosan was observed. The explanation of this provided by the authors was that individual ionic bonds between anionic monomers and polycations could dissociate, but the multiple bonds between polyanion and polycation would not dissociate at the same time.

8. Mode of Antimicrobial Action of Chitosan

Chitosan is a natural polymer and has no antigenic properties, and thus is perfectly compatible with living tissue. Its antithrombogenic and hemostatic properties make it very suitable for use in all fields of biology. The exact mechanisms of the antimicrobial activities of chitosan and its derivatives are still unknown. It is known that chitosan antimicrobial activity is influenced by a number of factors that act in an orderly and independent fashion. Because of the positive charge on the C-2 of the glucosamine monomer below pH 6.0, chitosan is more soluble and has a better antimicrobial activity [6, 234]. The polycationic structure forms unnecessarily in acidic conditions because the grafted groups of specific derivatives may change the *pKa* of chitosan and cause protonation at higher pH value. When the positive charge density of chitosan strengthens, the antibacterial property will increase consequently, as is the case with quaternized chitosans [70, 109, 369, 370] and chitosan metal complexes [145, 148, 371]. On the contrary, if the polycationic property of chitosan is deprived or reversed, the corresponding antimicrobial capacity will be weakened or lost. Therefore, large amounts of amino groups are able to enhance the antimicrobial activity. Accordingly, native chitosan with higher DDA shows a stronger inhibitory effect than that a molecule with a lower DDA. Moreover, it has been reported that asparagine *N*-conjugated chitosan oligosaccharide that possesses two positively charged sites provides strong interaction with carboxyl-negative charges on the bacteria cell wall [354, 372].

Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular

constituents [6, 234, 241, 373–376]. Papineau et al. [239] and Sudarshan et al. [243] reported that chitosan acts mainly on the outer surface of the bacteria. At a lower concentration (<200 mg/L), the polycationic chitosan does probably bind to the negatively charged bacterial surface to cause agglutination, while at higher concentrations the larger number of positive charges may have imparted a net positive charge to the bacterial surfaces to keep them in suspension. Chitosan interacts with the membrane of the cell to alter cell permeability. For example, fermentation with baker's yeast is inhibited by certain cations, which act at the yeast cell surface to prevent the entry of glucose. UV-absorption studies indicated that chitosan caused considerable leakage of proteinaceous material from *P. oaroecandrum* at pH 5.8 [377]. Chitosan also acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth [274, 275]. It also activates several defense processes in the host tissue [215], acts as a water-binding agent, and inhibits various enzymes.

Another mechanism is that the positively charged chitosan interacts with cellular DNA of some fungi and bacteria, which consequently inhibits the RNA and protein synthesis [243, 378]. In this mechanism, chitosan must be hydrolyzed to low MW to penetrate into the cell of micro-organisms however, this mechanism is still controversial. Tokura et al. [350] examined the antimicrobial action of chitosan with average MW of 2200 and 9300 Da having DDA of 0.54 and 0.51, respectively. It was observed that chitosan of 9300 Da was stacked on the cell wall and inhibited the growth of *E. coli*. However, the chitosan of 2200 Da, which permeated into the cell wall, accelerated the growth of *E. coli*. They suggested that the antimicrobial action is related to the suppression of the metabolic activity of the bacteria by blocking nutrient permeation through the cell wall rather than the inhibition of the transcription from DNA. Chitosan also inhibits toxin production by *A. alternata* and macerating enzyme production by *Erwinia* in addition to eliciting phytoalexin production [379, 380]. The effects of chitosan on growth inhibition of plant pathogenic fungi in fruits and vegetables were correlated with the reduction of aflatoxin, elicitation of phytoalexin and phenolic precursors, enhanced production of chitinases, and other factors relevant to the plant defenses [220, 381, 382]. El Ghaouth et al. [215, 216, 320] added that the fungistatic properties of chitosan against *R. stolonifer* were related to its ability to induce morphological changes in the cell wall. In addition to the direct effect of chitosan on different plant pathogens, it also activates several defense processes in treated plants or their products at postharvest stage. These defense mechanisms include accumulation of chitinases, synthesis of proteinase inhibitors, and lignification and induction of callous synthesis [320, 325]. For example, when applied on wounded wheat leaves, chitosan-induced lignifications, and consequently restricted the growth of nonpathogenic fungi in wheat. Chitosan inhibited the growth of *A. flavus* and aflatoxin production in liquid culture, preharvest maize, and groundnut, and it also enhanced phytoalexin production in germinating peanut [274, 275].

Unfortunately, in spite of the chitosan advantages, it is only soluble in acidic aqueous solutions with pH values lower than 6.5. At higher pH values, amino groups of chitosan macromolecules become unprotonated and chitosan forms insoluble. This problem is probably the major limiting factor for its utilization, that is, its application in biology, since many enzyme assays are performed at neutral pH. If water-soluble chitosan would be easily accessible, it is expected that the biological and physiological potential of chitosan would increase dramatically. One of the strategies to increase both the solubility and positive charge density of chitosan macromolecule is based on the introduction of quaternary ammonium groups in chitosan. This modification has got the commonly accepted term "quaternization of chitosan". Thus, water-soluble chitosan derivatives soluble to both acidic and basic physiologic circumstances may be good candidates for the polycationic biocides [70, 383, 384]. The antimicrobial action of such compounds is believed to occur when the compounds are absorbed onto the bacterial cell surface, increasing the permeability of the lipid cell membrane and causing death through the loss of essential cell materials. In addition, these derivatives of chitosan are generally more active against gram-positive bacteria than their corresponding monomers. Antimicrobial activity generally increases as the content of the quaternary ammonium moiety increases. The antimicrobial activities of quaternary chitosan derivatives were evaluated against some of gram-positive, gram-negative bacteria, and fungi. It was found that the activity increased with increasing chain length of the alkyl substituent, and this was attributed to the contribution of the increased hydrophobic properties of the derivatives. These results clearly demonstrated that hydrophobicity and cationic charge of the introduced substituent strongly affect the antibacterial activity of quaternary chitosan derivatives [70, 110].

It can be concluded that the exact mechanism of the antimicrobial action of chitosan is still ambiguous, although six main mechanisms, none of which are mutually exclusive, have been proposed, as follows: (1) interactions between the positively charged moieties on the chitosan molecules and those negatively charged ones on the microbial cell outer membranes leads to changes in the cell membrane structure and permeability inducing the leakage of proteinaceous and other intracellular constituents and so challenging the biochemical and physiological competency of the bacteria leading to loss of replicative ability and eventual death; (2) chitosan acts as a chelating agent that selectively binds trace metals and subsequently inhibits the production of toxins and microbial growth; (3) chitosan activates several defense processes in the host tissue, acts as a water binding agent, and inhibits various enzymes; (4) LMW chitosan penetrates the cytosol of the micro-organisms and, through the binding of chitosan with DNA, results in the interference with the synthesis of mRNA and proteins; (5) Chitosan on the surface of the cell can form an impermeable polymeric layer which alters the cell permeability and prevents nutrients from entering the cell; (6) finally, since chitosan can adsorb the electronegative substances in the cell and flocculate them, it

disturbs the physiological activities of the micro-organism leading to their death.

9. Concluding Remarks

The recourse to naturally occurring products with interesting antimicrobial and eliciting properties such as chitosan has been getting more attention in recent years. This product can be used in a number of ways to reduce plant disease levels and prevent the development and spread of pathogens, thus preserving crop yield and quality. The potent effect of chitosan on plant diseases control is from its antimicrobial properties and plant innate immunity elicited activity. The antimicrobial activity depend on several factors such as MW, DDA, solubility, positive charge density, chemical modification, pH, concentration, hydrophilic/hydrophobic characteristic, chelating capacity, and type of micro-organism. Chitosan has also become a postharvest promising treatment for fruits and vegetables due to its natural character, antimicrobial activity, and elicitation of defense responses. It possesses film-forming and barrier properties, thus making it a potential raw material for edible films or coatings and can be used to improve the storability of perishable foods. In spite of the chitosan advantages, the poor solubility, low-surface area, and porosity of chitosan are the major limiting factors in its utilization. Therefore, several research groups have started to modify a chitosan molecule to produce high-antimicrobial active derivatives.

Though there are many papers focused on chitosan and its derivatives in plant protection, there are still many problems that need to be studied. Examination of better ways to incorporate these products into Integrated Pest Management strategies remains to be pursued in many major crops especially against plant pathogenic bacteria and fungi. Interesting theoretical and applied findings were gathered in recent years, whereas more are needed to examine the mechanisms governing the mode of action of these compounds when applied at large scales. In the case of antimicrobial mode of action, future work should aim at clarifying the molecular details of the underlying mechanisms and their relevance to the antimicrobial activity of chitosan. Moreover, further investigations in this area, in particular with regard to microorganism resistance mechanisms against this compound, are warranted. In addition, participation and collaboration of research institutes, industry, and government regulatory agencies will be the key for the success of the antimicrobial mechanism.

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Research Article

Selective Modification of Chitosan to Enable the Formation of Chitosan-DNA Condensates by Electron Donator Stabilization

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Chitosan, a polyaminosaccharide, has been investigated for its use in the field of drug-delivery and biomaterial applications because of its natural biocompatibility and polycationic properties. Chemical modifications of chitosan have been attempted in an effort to increase the transfection efficiency with respect to gene delivery applications; however, it is unknown how these modifications affect the formation of the condensates. This study attempts to determine the effects of modification of the cationic center of chitosan on the ability to condense DNA. Specifically, electron-donating or -withdrawing groups were used as modifiers of the cationic charge on the chitosan backbone to stabilize the protonated form of chitosan, which is necessary to form condensates and increase the efficiency of the polymer to condense DNA by yielding condensates at a lower nitrogen to phosphorous (N : P) ratio. While an N : P ratio of 7 is needed to condense DNA with unmodified chitosan, phthalate-modified chitosan yielded condensates were obtained at an N : P ratio of 1.0.

1. Introduction

A natural polymer that has received increased research attention because of its inherent biocompatibility is chitin and its deacetylated derivative, chitosan [β (1–4)-2 amino-2-deoxy-D-glucose] [1, 2]. Depending on the degree of deacetylation, chitosan is a copolymer of N-acetyl-D-glucosamine and glucosamine units, with the glucosamine units predominating [3–5]. Due to its good biological activity, relative ease of solubility, biocompatibility, and biodegradability, chitosan and its derivatives have attracted attention as potential applicants in the field of biomedical polymers [3, 6, 7]. Additionally, the pH-dependent solubility of chitosan provides a convenient mechanism for processing under mild conditions and the high charge density of chitosan in solution allows for chitosan to form insoluble ionic complexes with a wide variety of water soluble polyanionic species, thus allowing for facile substitutions [8].

Chitosan and modified chitosans find a variety of applications in biomedical applications, including biomaterials for use in tissue engineering and drug delivery systems, and in bioseparation applications, including nano- and macro scale separations, and classical affinity- and nonaffinity-based chromatographic systems [2, 6, 7, 9–16]. Specifically in the area of targeted delivery, chitosan-based particles have been extensively investigated for the delivery of DNA macromolecules, proteins, peptides, and drugs [4, 17–19]. The abundance of literature to form chitosan particles with protein loadings is extensive and, generally speaking proteins are physically entrapped in chitosan chains to allow a latter release. However, in applications that seek to form chitosan-DNA nano- or microparticles with an intended application in gene therapy or gene transfection applications, there are three areas which must be optimized before it can be used effectively. First, effective loading of the DNA to the polymer must be achieved. Second, particles must be able to target specific cells within the body. Third, the particles must be able to effectively diffuse through or be actively transported across the cell membrane. As one might expect, all three of these areas are interrelated because of the polymer being used as the carrier agent is involved in all three steps. However, for research purposes, it is best to study each of these three areas

separately, to understand the principles behind each step so that the overall problem can be addressed. It is from this standpoint that this research project has been designed.

Chitosan-DNA polymer complexes are commonly attained via a condensation reaction, where the protonated amine (N) of chitosan forms an ionic attraction to the phosphate groups (P) found in the backbone of DNA; thus inducing a charge-induced titration leading to DNA condensation [19–23]. This charge-induced condensation is postulated to be influenced by the physical characteristics of the chitosan polymer as well as the experimental conditions. For example, the molecular weight of chitosan used was noted to have a minimal effect on the percentage of DNA bound by the polymer [24, 25]; whereas the degree of deacetylation (DA) of chitosan was shown to impact the N : P ratio required for the complete condensation of DNA [26, 27]. In this study, an N : P ratio of 9 was needed to yield DNA condensates when chitosan with a 62% DA was used, and N : P ratio of 3 was shown to yield DNA condensates with 90% DA chitosan. AFM analyses of polyplexes made from mixing plasmid DNA with chitosan were noted to yield a blend of toroids and rods, more toroids then rods were observed with higher DA chitosans and the amount of chitosan required to fully compact DNA into well-defined toroidal and rodlike structures was found to be strongly dependent on the chitosan molecular weight, and thus its total charge [22].

In this paper, we are interested in understanding and optimizing the structure-property correlations between chemical modification of the chitosan and the resultant biological activity, in charge titrating negatively charged biomolecules, for example, condensation of DNA. This study will attempt to determine the effects of modification of the cationic center of chitosan on the ability to condense DNA. The underlying hypothesis is that the use of electron donating or withdrawing groups as modifiers of the cationic charge on a polymer will lead to a more efficient condensation vehicle by stabilizing the ionization of the polymer. The study will look at two classes of modifications and its impact on DNA condensation, where the primary nitrogen on the chitosan backbone will be modified with groups that impart electron-donating or electron-withdrawing attributes. The overall strategy for the modification of the chitosan backbone is outlined in Figures 1 and 2. This paper will also seek to evaluate the effect of steric hindrance on the condensation of DNA.

2. Material and Methods

All chemicals were of analytical-grade or better. Chitosan with various degrees of deacetylation were purchased from Vanson Halosource (Remmond, WA, USA). In this study, chitosan with 81.7% of deacetylation (276 kDa, Lot # 01-CISQ-1702) was used in all experiments without further purification. All materials were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and were of the highest purity available unless otherwise noted. All methods described are the final optimized method for production.

3. Modifications of Chitosan

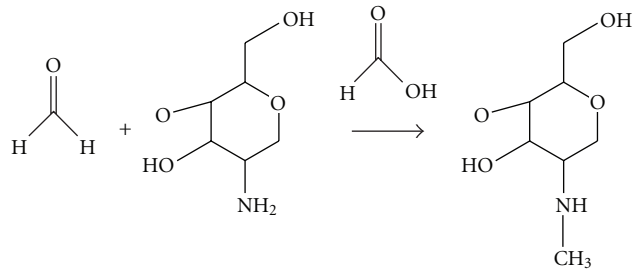
3.1. Methyl Addition to Chitosan. This procedure was adapted from the procedure detailed by Cignarella et al. [28] and is schematically represented in Figure 1, reaction 1. Briefly, 1.68 grams of 92.1% DA chitosan was dissolved into 75 mL of formic acid in a 3-neck 250 mL round bottom flask with a reflux attached. The solution was heated under an inert atmosphere to 110°C. 1.1 mL of 37% formaldehyde was added via syringe and the solution was stirred at temperature for 3 hours. The solution was allowed to equilibrate to room temperature and then evaporated under reduced pressure to remove the excess acid. The resulting solid was neutralized using 5 N sodium hydroxide. The solid precipitate was collected by centrifugation and washed 3 times with distilled water. The final solid was frozen at −80°C and lyophilized for at least 8 hours. The dry product was then analyzed by infrared spectroscopy.

3.2. Alcohol Addition to Chitosan. This procedure is based upon that described in Vogel's Textbook of Practical Organic Chemistry [29] and is schematically represented in Figure 1, reaction 2. Briefly, 1.68 grams of 92.1% DA chitosan was placed in a 3 neck round bottom flask with reflux attached and reduced to 0°C. 10 mL of 0.2 M hyperchloric acid (bleach, Clorox Company, Oakland, CA) was added with stirring. After 10 minutes, 4.5 mL tetrahydrofuran and 5.5 mL of a 1.8 M borane-tetrahydrofuran solution was added allowed to react for 1.5 hours while the mixture equilibrated to room temperature. 2 mL of 5 M sodium hydroxide was added to the reaction mixture and allowed to react for 5 minutes. A 20% theoretical mole excess of 20% hydrogen peroxide solution (Fluka, Buchs, SG, Sitzerland) was added very slowly to the reaction producing a very fast and violent reaction. The solid particles were collected by centrifugation and washed 3 times with distilled water. The final solid was frozen and lyophilized for at least 8 hours. The dry product was then analyzed by infrared spectroscopy.

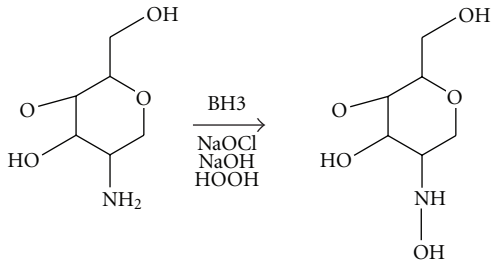
3.3. Chlorine Addition to Chitosan. This procedure is based upon that used by Favreau et al. [30] and is schematically represented in Figure 1, reaction 3. Briefly, 200 mL of 0.2 M hyperchloric acid (bleach) was cooled to 0°C. 3.4 grams of 92.1% DA chitosan was added and reacted with stirring for 3 hours. The mixture was separated by centrifugation at 4°C. The solid was collected and washed 3 times with chilled distilled water. The final solid was frozen and lyophilized for at least 8 hours. The dry product was then analyzed by infrared spectroscopy.

3.4. Cyclohexane Addition to Chitosan. This procedure is based upon that used by Kabalka and Wang [31] and is schematically represented in Figure 2, reaction 4. Briefly, 3.35 mL cyclohexene was added to 10 mL tetrahydrofuran with stirring and cooled to 0°C. 5.5 mL of hydroborane in THF was added at temperature and allowed to react under an inert atmosphere for 3.5 hours. In a separate flask, 1.68 g of 92.1% DA chitosan was cooled to 0°C. 10 mL of 2 M

Reaction 1: neutral group addition



Reaction 2: electron donating group addition



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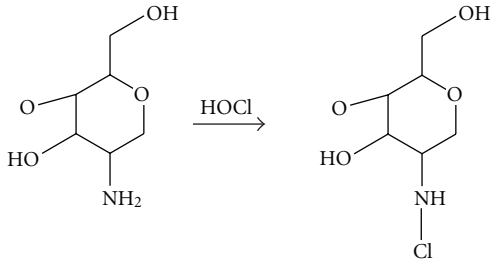
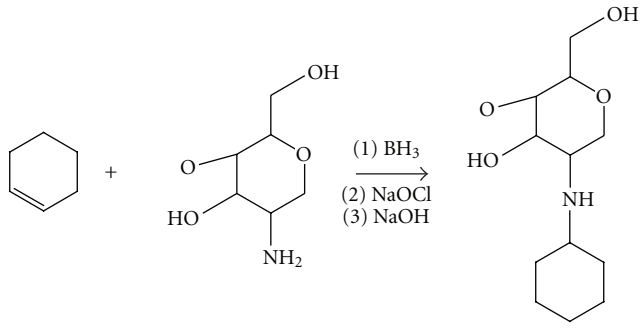
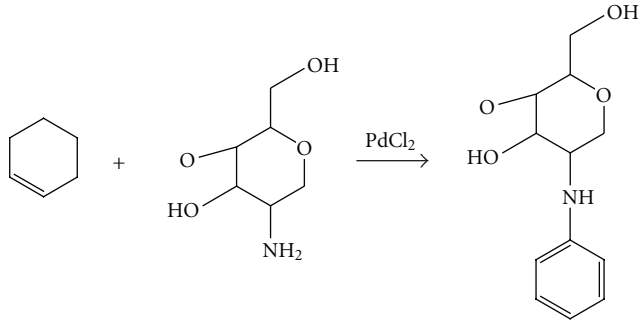


Figure 1: Proposed chemical modifications to chitosan. Reaction 1: methyl-group addition to chitosan, reaction 2: alcohol-group addition to chitosan, reaction 6: chlorine-group addition to chitosan.

Reaction 4: steric hindering, neutral group addition



Reaction 5: steric hindering, electron donating group addition



Reaction 6: steric hindering, electron withdrawing group addition

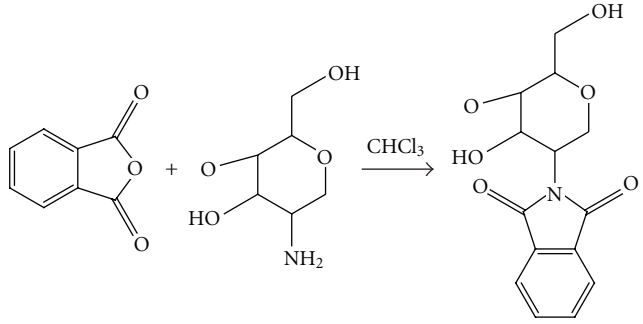


Figure 2: Proposed steric hindering chemical modifications to chitosan. Reaction 4: cyclohexane addition to chitosan, reaction 5: benzene addition to chitosan and reaction 6 phthalate group addition to chitosan.

hyperchloric acid was added dropwise with mixing. This reaction was allowed to proceed under inert atmosphere for 5 minutes. The complexed hydroborane was transferred via a double-sided needle to the flask containing the chlorinated chitosan. After transfer and initial mixing, the solution was allowed to warm to room temperature but maintained under an inert atmosphere and proceeded for 1 hour. After reaction the solution was decanted from the solid phase. The solid was transferred using a 2% solution of acetic acid and neutralized using 1 M sodium hydroxide. The solid was collected by centrifugation and washed 3 times with distilled water. The solid product was then frozen and lyophilized for at least 8 hours. The dry product was then analyzed by infrared spectroscopy.

3.5. Benzene Addition to Chitosan. This procedure is based upon that used by Ozaki and Akihiro [32] and is schematically represented in Figure 2, reaction 5. Briefly, 0.0183 mols of 92.1% DA chitosan was added to 0.055 mols cyclohexene and 0.56 mmols palladium (II) chloride. The mixture was heated to 60°C with reflux and reacted under an inert atmosphere with stirring. After 23 hours, the atmosphere was changed to a 5% mix of hydrogen/inert gas and reacted for three hours. The solid product was washed three times with distilled water and collected centrifugation. The solid product was frozen and lyophilized for at least 8 hours. The dry product was analyzed by infrared spectroscopy.

3.6. Phthalate Addition to Chitosan. This procedure is based upon that described in Vogel's Textbook of Practical Organic Chemistry [29] and is schematically represented in Figure 2, reaction 6. Briefly, 3 grams of 92.1% DA chitosan was added

to 150 mL of chloroform in a 3-neck 250 mL round bottom flask with a reflux attached. The mixture was heated to 70°C with stirring under an inert atmosphere. At temperature, 3 grams phthalic acid anhydride was added and reacted for 25 minutes. The mixture was allowed to cool to room temperature, and the solution evaporated under reduced pressure. The solid remaining was washed with a 2% acetic acid solution twice to remove unmodified chitosan. The residual solid was washed with 1 M sodium hydroxide to neutralize and collected by centrifugation. The solid was then washed three times with distilled water. The solid was frozen and lyophilized for at least 8 hours. The dry product was analyzed by infrared spectroscopy.

4. Analysis of Modification

4.1. Qualitative Analysis by Infrared Spectroscopy. Solid products were pressed into 2.5 cm diameter plates in a 1 : 10 ratio with analytical grade potassium bromide under 3 metric tons of pressure. The samples were analyzed by 100 scans from 600 to 3500 cm^{-1} with a 4 cm^{-1} resolution using either a Nicolete 20SXB FT-IR Spectrometer or a Nicolete 510P FT-IR Spectrophotometer.

4.2. Quantitative Analysis by Ninhydrin. This procedure is based upon that described by Curotto and Aros [33]. Solid samples and D-glucosamine were dissolved separately in 2% acetic acid at a concentration of 0.1 mg/mL. Different volumes of each sample (0.1–0.5 mL) were fractionated and to each 0.5 mL 4 M acetic acid/acetate buffer at pH 5.5 was added. The samples were brought to a 1 mL total volume using deionized water. 0.35 g of ninhydrin (Fluka) was dissolved in 100 mL of 200 proof ethanol. 2 mL of the ninhydrin solution was added to each 1 mL sample followed by heating at 90°C for 15 minutes. The samples absorbance was immediately taken following heating at a wavelength of 570 nm using a UV-Vis spectrophotometer. The absorbance for each sample was plotted as a function of concentration. The slope for each sample was calculated using a linear regression. The slope of each sample was then divided by the slope generated by D-glucosamine to give the concentration of free amine groups. The percent modification was determined by calculating the actual number of free amine groups of the sample and the total number of amine groups based upon the molecular weight of chitosan and the respective modification.

5. Production and Purification of DNA Plasmid

Plasmid DNA was supplied by the Biological Process and Development Facility at the University of Nebraska. The plasmid was transfected and grown in a *E. coli* culture for 2 days at 37°C. The cells were separated, lysed, and purified using a Qiagen Plasmid DNA Maxi Kit (Qiagen, Valencia, CA) preparation. The resultant DNA was quantitated by UV-Vis spectroscopy at 260 nm and by fluorescence using the fluorescent binding agent Pico Green (Molecular Probes, Inc., Eugene, OR).

6. Condensate Production and Characterization

6.1. Production of DNA-Polymer Condensates. The procedure was based upon that described by Leong et al. [34] and Mao et al. [17]. Approximately 1 gram of chitosan or modified chitosan products were dissolved in 10 mL of a 2% acetic acid solution. Any solid remainder was centrifuged out of the solution and dried under vacuum to determine the actual mass in solution. The solution was then diluted to 250 mL, and the total acetate concentration was adjusted to 25 mM using sodium acetate. These solutions were then mixed with 10 μg of either plasmid or Lambda DNA at a calculated Nitrogen : Phosphate ratio of 0.1, 1, 2, 5, or 7. The mixture was adjusted to 500 μL using 25 mM sodium acetate solution to maintain the same salt concentration for all experiments. The final mixtures were vortexed for 15 seconds and allowed to react for 1 hour at 4°C.

6.2. Quantitative Analysis of DNA Loading of the Condensates. DNA-Polymer condensates were prepared as described above. After production, the condensates were separated from noncomplexed DNA by centrifugation at 16 ×g for 1 hour. Supernatant solutions were collected and a portion reacted with Pico Green fluorescent dye. The DNA from each sample was standardized to a sample with 10 μg of DNA in 25 mM and a tube with 25 mM sodium acetate and no DNA. The loading was calculated from the mass balance.

6.3. Electrophoresis of DNA-Polymer Condensates. The DNA-polymer condensates were separated from plasmid DNA by a centrifugation of 6 ×g for 15 minutes. The supernatant was removed and discarded. The particles were resuspended in 250 μL of 25 mM sodium acetate solution. 10 μL of the resuspended particles were removed and added to 2 μL of a loading dye. The samples run for 2 hours in a 0.5% agarose gel which had been prepared with ethidium bromide. Agarose gels were developed under UV light.

6.4. Zeta Potential Analysis of Condensates. For this experiment, all stock solutions were filtered through a 0.22 μm pore size filter. DNA-polymer condensates were prepared as described above except that lambda DNA was used. The condensates were separated from uncondensed DNA by a centrifugation of 6 ×g for 15 minutes. The supernatant was removed and discarded. The particles were resuspended in 500 μL of 25 mM sodium acetate solution. The solution was diluted at a rate of 1 : 10 with deionized water. The samples were analyzed for zeta potential using a Zeta Plus zeta potential analyzer (Brookhaven Instruments Corp.).

7. Results

The number average molecular weight of the unmodified and modified chitosan samples used in this study was determined by gel permeation chromatography with internal standards ranging from 59 to 788 kDa. In certain cases, the molecular mass of the unmodified chitosan was also estimated using the experimentally determined intrinsic

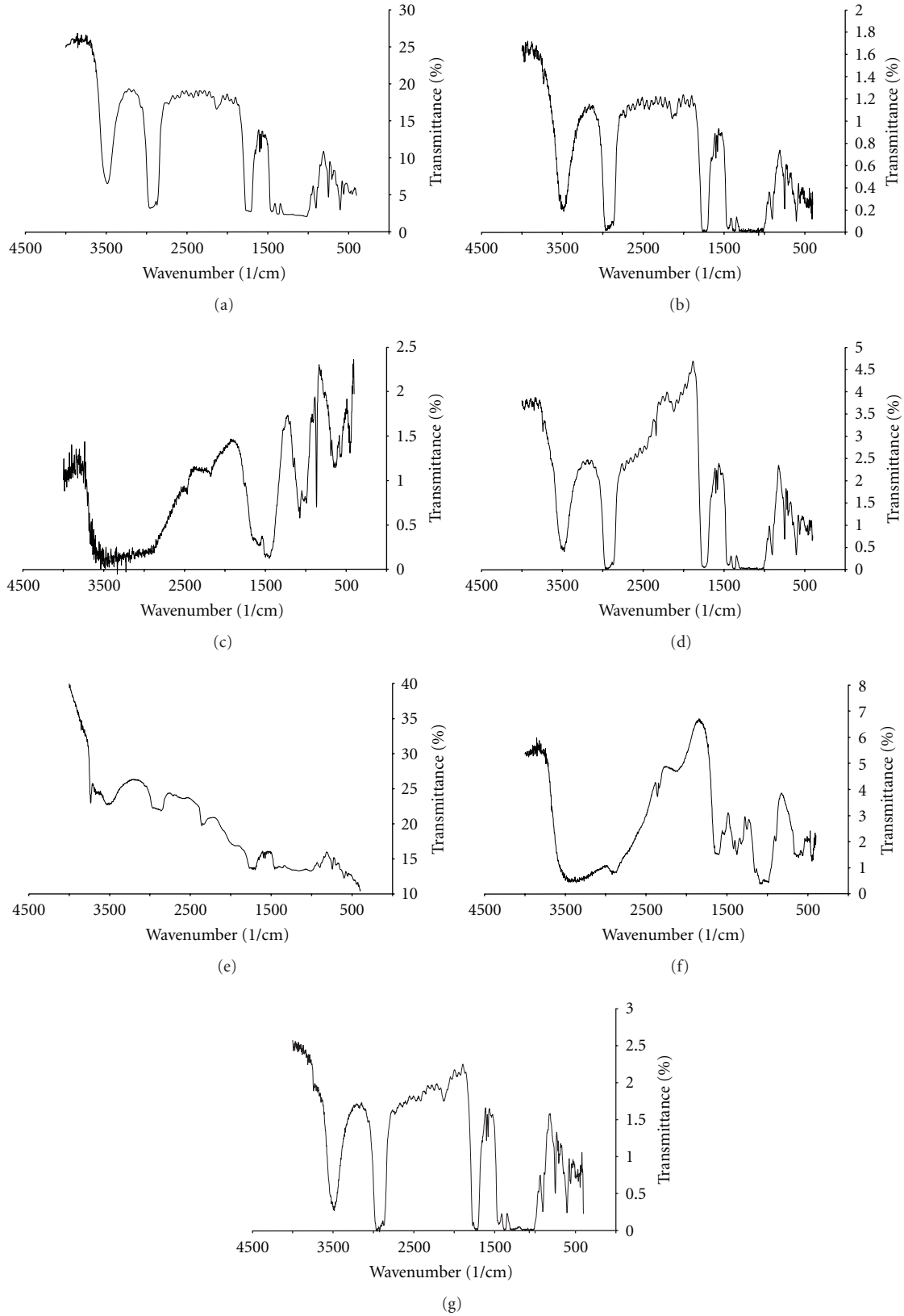


Figure 3: FTIR spectra (% transmittance) of unmodified and modified chitosan. (a) Unmodified chitosan, (b) methyl addition, (c) alcohol addition, (d) chlorine addition, (e) cyclohexane addition, (f) benzene addition, and (g) phthalate addition.

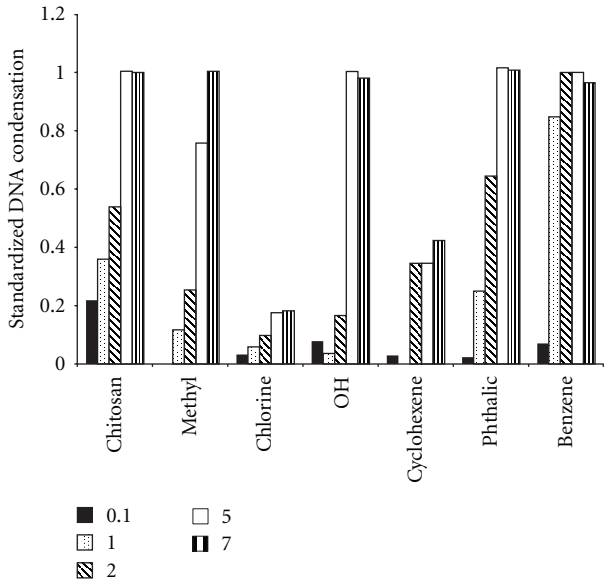


Figure 4: Plasmid DNA at a concentration of 10 μg/mL was vigorously mixed with an equal volume of modified chitosans in a pH 7.4 buffer to yield various stoichiometric charge ratio of modified chitosans to DNA. In the control, DNA was mixed with buffer, no chitosan present in the reaction system. Upon mixing, the reaction tubes were incubated at room temperature for 60 mins and centrifuged for 90 mins at 4°C. Addition chitosan-DNA complexes were separated by centrifugation. The supernatants were carefully pipetted off, and the pellet in each case was resuspended in intracellular saline. ABS 260 nm was measured for both the supernatants and pellets. Data was averaged over six independent experiments, with triplicates for each data point within each experiment. A standard error of less than 5% was obtained for data points reported.

viscosity, (η), using the Mark-Houwink-Kuhn-Sakurada equation (MHKS)[31]. The average molecular weight (MW) of chitosan was not found to change appreciably with the various chemical treatments employed in this study.

8. Analysis of Modifications to Chitosan

FTIR spectra of various modified chitosans prepared in this study are shown in Figure 3; transmittance spectrum is shown.

8.1. Pure Chitosan FTIR. First, an infrared spectrum of pure, underivatized chitosan was taken as a base measurement for comparison with modified chitosan products. The spectrum shows very strong peaks related to the alcohol groups which are centered at 3500 cm^{-1}, carbon–hydrogen bonds centered at 2750 cm^{-1}. A broad peak which stretches between 1000 and 1450 cm^{-1} is more than likely a combination of different bonds including amines, alcohols, and carbon-carbon bonding.

8.2. Methyl Addition to Chitosan FTIR. The spectrum of the methyl addition to chitosan reaction product contained many of the strong peaks observed in the spectrum of pure chitosan, also contained three new peaks centered at approximately 3970, 3740, and 405 cm^{-1}, with the peak at 3740 cm^{-1} being the most well defined. The peaks at 3970 and 3740 cm^{-1} are most likely related to the new amine bonds being formed. The peak at 405 cm^{-1} is difficult to assign due to position within the fingerprint region of an infrared spectrum.

8.3. Alcohol Addition to Chitosan FTIR. The spectrum the alcohol addition to chitosan reaction product contained a much different spectrum from that of the original chitosan. In this spectrum, the two large peaks at 3500 and 2750 cm^{-1} have been merged into a single broad peak. This characteristic is normally contributed to an increase in alcohol groups.

8.4. Chlorine Addition to Chitosan FTIR. The spectrum of the chlorine addition to chitosan reaction product contained two new peaks of interest in addition to the peaks from the original chitosan sample. New peaks were observed at 3740 and 715 cm^{-1}. These peaks were assigned as the secondary nitrogen group formed and an organic halogen, respectively.

8.5. Cyclohexane Addition to Chitosan. The sample of the cyclohexane addition to chitosan reaction product under standard conditions was too concentrated to take a proper spectrum, instead the mass percent of the sample had to be reduced by a factor of 5. A new peak was located at 3740 cm^{-1} which suggests a secondary nitrogen. Another new peak can be seen at 1490 cm^{-1}. This peak actually becomes merged into the large group of peaks between 1450 and 1000 cm^{-1} which can be seen in pure chitosan spectra. This new peak can be assigned to the large number of saturated aliphatic carbons found within the cyclohexane ring.

8.6. Benzene Addition to Chitosan. The spectrum of the benzene addition to chitosan reaction product did not include new peaks but instead showed a significant increase in the area between the peaks at 2750 and 3400 cm^{-1}. In addition, there is separation of the peaks that occurs between 1000 cm^{-1} and 1400 cm^{-1} with the greatest increase occurring at 1100 cm^{-1}. These increases occur in the areas at which a mono substituted benzene would occur.

8.7. Phthalate Addition to Chitosan. Two new peaks can be found in the spectra of the phthalate group addition to chitosan reaction product at 3745 and 1770 cm^{-1} as well as significant broadening of the peak centered at 3500 cm^{-1} and the large mass of peaks between 1310 and 1020 cm^{-1}. The new peak at 3745 cm^{-1} is due to the modification of the nitrogen group. The tertiary nitrogen is also the cause for the broadening of the mass of peaks between 1310 and 1020 cm^{-1}. The peak at 1770 as well as the broadening that is occurring at 3500 cm^{-1} is due to the ester groups which compose phthalate compounds.

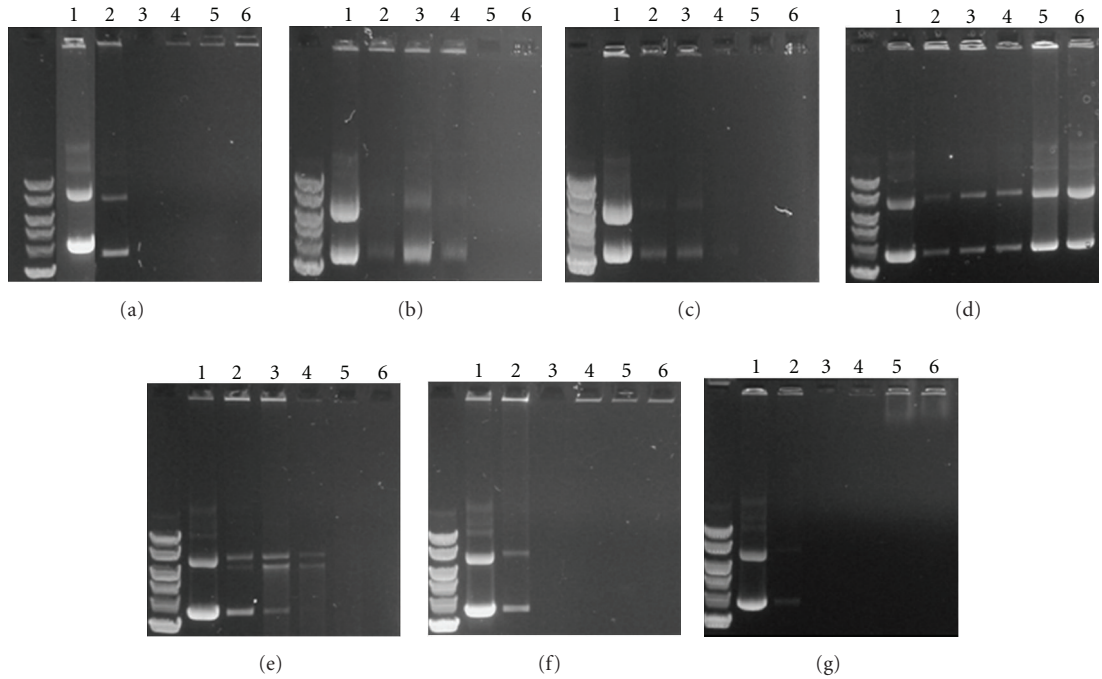


Figure 5: Mobility of DNA complexed in modified chitosan condensates. Frame (a) is pure chitosan, Frame (b) is methyl-modified chitosan, Frame (c) is alcohol-modified chitosan, Frame (d) is chlorine-modified chitosan, Frame (e) is cyclohexane-modified chitosan, Frame (f) is benzene-modified chitosan, Frame (g) is phthalate-modified chitosan. For all frames, Lane 1 is uncomplexed DNA; Lane 2 is a 0.1 N : P ratio. Lane 3, 1 N : P; Lane 4, 2 N : P; Lane 5, 5 N : P; Lane 6, 7 N : P.

9. Quantitative Analysis of Chitosan Modifications by Ninhydrin

Serial dilutions of each modified product and unmodified chitosan were prepared in acetic acid and reacted with ninhydrin. Upon heating, ninhydrin was expected to primarily react with the primary amine groups. Bonded ninhydrin produces a color change within the solution which can be measured by UV-Vis spectroscopy. The sample absorbance is plotted as a function of the concentration of modified chitosan in the solution. Using a linear regression tool, the percent modification can be calculated by the slope of the line for the sample divided by the slope of the line for the absorbance of a solution using D-glucoseamine, a sugar with the same structure as chitosan with only the primary amine present, Table 1. The substitution of chlorine gave the lowest conversion at 66%, while all other modifications proceeded at over 93% conversion.

10. Condensates Production and Characterization Condensation Experiments

Products from the chemical modifications and pure chitosan were mixed with plasmid DNA to condense condensates using nitrogen to phosphate ratios (N : P) of 0.1, 1, 2, 5, and 7. In each case 10 μg of DNA was used. The particles were separated by centrifugation and the supernatant solution was analyzed for DNA concentration using PicoGreen fluorescent dye. The quantity of DNA condensed was calculated by the mass balance and standardized to pure sodium acetate solution with no DNA and sodium acetate solution with 10 μg of DNA. Figure 4 shows the normalized amount of DNA condensed as a function of N/P ratio, for each of the reaction schemes studied.

Most of the substituted chitosans prepared in this study were able to yield near quantitative condensation efficiencies at a N/P ratio of 5 or greater. However, chlorine and cyclohexane substituted chitosan polymer was uneffective in inducing condensation at the N/P ratios studied. Benzene and phthalic acid substituted chitosans were able to yield a higher condensation efficiency and yield at lower N/P ratios studied.

10.1. Electrophoresis of Condensates. Electrophoresis was used to determine any change in the electrophoretic mobility of complexed DNA. Condensates were created at an N : P ratio of 0.1, 1, 2, 5, and 7 for pure chitosan and all chitosan modification products. The DNA used in these experiments had two fragments and can be seen as a doublet after electrophoresis. In many cases, bands can be seen in lanes with condensed DNA, these bands are most likely caused by partially condensed DNA or DNA which has been released postcondensation. A 0.5% agarose gel was used so that low mobility would not be an effect of high agarose content. Agarose gels, Figure 5, were developed by UV irradiation of an incorporated ethidium bromide stain. The concentration

Table 1: Slope and conversion based upon quantitative analysis by ninhydrin.

	Control	Chitosan modifications					
	D-Glucose amine	Methyl	Alcohol	Chlorine	Cyclo hexane	Benzene	Phthalate
Slope	1.53	0.00	0.03	0.53	0.04	0.11	−0.01
Conversion		1.00	0.98	0.66	0.98	0.93	1.01

of the DNA molecular weight standard was equal to that of the samples prior to condensation.

10.2. Zeta Potential of Condensates. The zeta potential analysis of condensed DNA was used to determine the degree to which the polymer charge neutralized the phosphate backbone DNA, and the values are listed in Table 2. The values are the means of between 3 and 5 samples dependant on the variability of the observed values.

Table 2: Mean zeta potential of condensed DNA particles formed using modified polymers and the standard deviation associated with the measurements.

	Zeta potential	
Condensing agent	Mean (mV)	Std Dev
Chitosan, pure	−0.64	1.0
Methyl addition	0.00	0.0
Alcohol addition	−2.59	2.29
Chlorine addition	−25.49	3.3
Cyclohexane addition	−3.20	4.6
Benzene addition	−5.21	3.0
Phthalic addition	−23.46	16.7

11. Discussion

Previous study has evaluated, in detail, the ability of various chitosans with varying degrees of acetylation (DA) and degree of polymerization (DP) to compact plasmid and linear DNA [22]. The amount of chitosan required to fully condense DNA was found to be dependent upon the chitosan molecular weight, hence the total charge at a given DA. Thus we have chosen to work with chitosan biopolymer with a MW of 217 kDa and a DA of 81.7%, to obtain effective condensation at medium to low concentrations of chitosan. The goal of this study was to evaluate the effects of electron donation and steric hindrance on the ability of chitosan to condense DNA into a chitosan_DNA complex. To evaluate these properties, different functional groups were chemical substituted onto the primary nitrogen of chitosan, as detailed in Figures 1 and 2. These functional groups were chosen based upon two factors. First, reactions would have to be in high yield. This aspect is of importance because most of the reactions occur on the interface of the solid phase and liquid phase, which in general decreases the degree of substitution. Second, the functional groups should be of similar size within their grouping, so that unbiased observations and conclusions could be made.

The experiments were conducted under the hypothesis that the addition of electron donator groups would help to stabilize the nitrogen in its protonated form ($R\text{-}NH_3^+$). It is hypothesized that the protonated form of chitosan is needed to create the ionic attraction that occurs between the nitrogen of chitosan and negatively charged phosphate groups of DNA which ultimately forms the nanoparticle. By stabilizing this protonated form, more nitrogen atoms should exist in the charged form, leading to a greater number of bonds or interactions with the phosphate backbone in the DNA molecule. The increase in ionic bonds should mean that DNA should be completely condensed at a lower nitrogen to phosphate ratio. Also the stabilization should show an increase in the effective zeta potential, since the zeta potential should also be related to the number of ionic bonds occurring between the polymer and DNA.

In contrast, chitosan modified with electron-withdrawing groups should show a lower bonding efficiency, if bonding can even occur. Chemically bonding electron-withdrawing groups to nitrogen is a common method in organic chemistry to protect or deactivate the reactivity of nitrogens within a compound. If the nitrogen groups of the polymer are fully protected, protonation and ultimately the ionic bonding to the phosphate groups of DNA are not likely to occur. Because of this decrease in intermolecular interactions, the zeta potential should be smaller or negative on any particles that do form.

Steric hindrance should also have an effect on the ability of the nitrogen from chitosan to bond to phosphate groups of DNA. By extending the distance between the two charged groups, this ionic bonding is weakened. The weakening of the bonding should decrease the efficiency of the condensation while increasing the zeta potential.

In the present study, the modifications to chitosan proceeded at a very high rate of substitution. The most misleading result from this section is the degree of substitution for the chlorine addition being lower than the two reactions using boronation in which the chlorine modification is a step. It is important to realize that because of the nature of chlorine as a leaving group and especially given the decay that can be physically seen over time that the results are reasonable for reactions that would be occurring simultaneously. The degree of substitution for the chlorine addition might also be influenced by the temperature that the ninhydrin reaction occurs at, which could also increase the degradation rate, releasing chlorine and producing error in the reading.

12. Particle Condensation and Characterization

It is important to note that the production of condensates was not optimized in this study. All other factors, such as temperature, salt concentration, pH, and mixing times were kept constant between experiments. The pH was specifically maintained near neutral so as to not artificially increase the charge density of the polymers.

All modified polymers were able to precipitate DNA; however, the efficiency was dependant on the modification. Efficiency for the purpose of this topic will be defined as the percent of DNA precipitated by a polymer at a certain nitrogen to phosphate (N : P) ratio. Previously reported work has noted the transfection efficiency to be optimal when condensates were prepared at an N : P ratio of 5.6 : 1 [35]. More important than the actual number is the proposed theory that by needing a greater number of polymer subunits, more nonspecific binding occurs. Only two modified polymers were able to precipitate DNA at the same or greater efficiency as bare chitosan, the addition of benzene and phthalate. Benzene-modified chitosan was able to precipitate nearly 85% of the DNA present at an N : P ratio of 1. At that same ratio, bare chitosan could only precipitate 36% of present DNA. For all N : P ratios greater than 1, benzene-modified chitosan was able to precipitate all DNA present. Chitosan was able to fully precipitate present DNA at N : P ratios of 5 and 7. Only phthalate and alcohol-modified chitosan could match this efficiency with that of the alcohol modified chitosan dropping significantly at lower ratios.

In contrast, very slight modifications such as the methyl addition could have a large effect in the precipitation efficiency. Neutral charge group modification showed a drop in efficiency with methyl-modified chitosan only able to fully precipitate present DNA at an N : P ratio of 7. Cyclohexane-modified chitosan at an N : P ratio of 7 could only precipitate 42% of present DNA. The least efficient modification for precipitation was the chlorine addition to chitosan. Chlorine-modified chitosan could only precipitate 18% of present DNA at an N : P ratio of 7, less than the 21% of precipitated DNA for bare chitosan at an N : P ratio of 0.1.

The data from the precipitations correlated well with the gels from the mobility studies. As described in the methods section, the DNA was precipitated at different N : P ratios and separated by centrifugation from uncondensed DNA. The condensates that were formed were resuspended into solution and separated by electrophoresis. While DNA bands were seen in condensates created from the modified polymers and bare chitosan at an N : P ratio of 0.1; bands from other ratios were only seen in polymers which were less efficient. For example, only a very faint band was seen in the gel for condensates created using phthalate-modified chitosan and benzene-modified chitosan, where as bands were seen in all lanes of chlorine-modified chitosan and cyclohexane-modified chitosan. The most interesting result from this study may be in the alcohol-modified chitosan gel. In this experiment, bands can be seen in the lanes corresponding 0.1, 1 and slightly in the lane corresponding to 2 N : P ratios; however, no bands can be seen in the higher ratios. This phenomena can be used as a secondary method of determining efficiency as it is most likely caused by weakly condensed DNA.

Another interesting result is that the polymer which hypothetically should have been the least efficient at precipitating DNA, phthalate addition to chitosan, was actually just as efficient at an N : P ratios of 5 and 7 or more so at a ratio of 2 than bare chitosan. This effect could be caused by the oxygen groups on the phthalate group forming hydrogen bonds to the DNA as opposed to the ionic-type bonding normally occurring with polycationic polymers. This theory is supported by data reported elsewhere [36] which showed a thermodynamic link between hydrogen bonds forming the condensates.

Finally, the sizes of the chitosan-DNA complexes were analyzed by Dynamic Light Scattering (DLS) using DP801 instrument by Protein solutions Inc. using a calibration with latex standards (spheres) of 21 to 300 nm in diameter. Our results with light scattering indicate the size of DNA condensates to be about 140–160 nm. These condensates appear to stable as judged by the size from DLS (DLS readings were taken over a period of 3-4 days).

13. Zeta Potential

The zeta potential was strongly influenced by the type of modified polymer used for precipitation. Chitosans substituted with an electron-withdrawing group produced particles with strongly negative charges. This data would seem to influence the idea that the precipitation is based upon the hydrogen bonding as opposed to ionic interaction which would produce particles which are charge neutralized. All other modified polymers and bare chitosan produced particles with the zeta potential near zero. This lack of influence on the zeta potential could be a likely result of experimental procedure in which the samples are prepared in a neutral solution. The overall use of negatively charged condensates is still debated, many researchers believe that to be able to transfect through a cell membrane the zeta potential must be positive and transfection efficiency is increased as the magnitude of the potential increases [20] while other studies have shown successful transport of negatively charged particles [37, 38]. These results should instead be interpreted as the need for hydrogen bonding as a secondary source of binding for efficient polymer carriers.

14. Conclusions

Polymer design and modification for the production of condensates is a field which will continue to be strong during the development of gene therapy. With in this field, chitosan has been shown to be useful because of biological and chemical properties. The study has also shown that the efficiency of chitosan to create condensates can be increased through targeted chemical modifications via nucleophilic reactions. The increase in efficiency caused by the modification has been shown to be related to two different factors, electron donation and hydrogen bonding. These factors were shown to also affect the zeta potential. In contrast, this study also

showed that neutral modifications produce condensates at a significantly lower efficiency. Finally the study showed that steric hindrance has very little effect on the formation and particle size of condensates. Work in progress also includes characterizing the shape and dimensions of the structures formed.

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Review Article

Chitosan-Based Macromolecular Biomaterials for the Regeneration of Chondroskeletal and Nerve Tissue

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The use of materials, containing the biocompatible and bioresorbable biopolymer poly(1 → 4)-2-amino-2-deoxy-β-D-glucan, containing some N-acetyl-glucosamine units (chitosan, CHI) and/or its derivatives, to fabricate devices for the regeneration of bone, cartilage and nerve tissue, was reviewed. The CHI-containing devices, to be used for bone and cartilage regeneration and healing, were tested mainly for *in vitro* cell adhesion and proliferation and for insertion into animals; only the use of CHI in dental surgery has reached the clinical application. Regarding the nerve tissue, only a surgical repair of a 35 mm-long nerve defect in the median nerve of the right arm at elbow level with an artificial nerve graft, comprising an outer microporous conduit of CHI and internal oriented filaments of poly(glycolic acid), was reported. As a consequence, although many positive results have been obtained, much work must still be made, especially for the passage from the experimentation of the CHI-based devices, *in vitro* and in animals, to their clinical application.

1. Introduction

Chitosan (CHI) is a poly(1 → 4)-2-amino-2-deoxy-β-D-glucan, containing some N-acetyl-glucosamine units (Figure 1), obtained by partial deacetylation of chitin, the main component of the exoskeleton of crustaceans, and it is generally considered as biocompatible and biodegradable [1, 2]; chitin and CHI are the most abundant polysaccharides among those containing amino sugars [3]. CHI was used some years ago, by the authors' group, as a template for the polymerization of acrylic acid and sodium 4-sty-renesulfonate [4]; the polyelectrolyte complex obtained with the first monomer showed a good cytocompatibility, while that with the second one seemed to influence negatively the cell proliferation [5]. Very many studies have been done on CHI and its derivatives as materials for the fabrication of scaffolds, used for tissue engineering and regeneration. The early studies about the possibility of using CHI and its derivatives in the food and biomedical sciences sand industries regarded mainly the immobilization of enzymes on the polysaccharide [6, 7]. The results obtained in this field up to 1980 were the argument of a review by A. Muzzarelli [3]. Another interesting argument of these early studies regarded the chelating ability of CHI towards metal cations [8–10], which was found later to facilitate the tissue mineralization in tooth implantation [11]; furthermore, it was proposed more recently that the CHI-metal interaction modes might be involved in the controlled bioactivity of CHI [12]. In 2005, R. A. A. Muzzarelli and C. Muzzarelli thoroughly reviewed the researches made on CHI and its derivatives as biomaterials [13]. In 2008, Korean biotechnologists reviewed the use of CHI and CHI derivatives for tissue engineering of various organs, between which there were bone, cartilage, and nerves [14]. This paper will summarize mainly the current body of growing literature, where a use of CHI for cartilage, bone, and nerve tissue

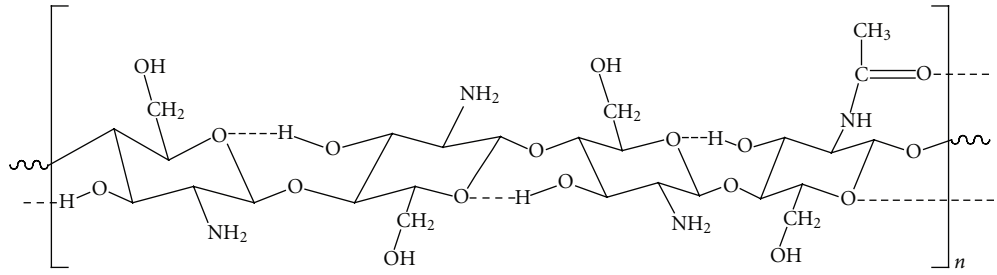

Figure 1: Structure of a chitosan with about 25% of acetylated repeating units.

regeneration was reported, because of their importance in the mobility and sensitivity of human body. The use of CHI, a completely bioresorbable material [1, 2], permits solving the main problems arising in the orthopaedic and neurological surgery: first, the substitution of damaged cartilage and bone with permanent prostheses of foreign biomaterials could not assure the same tribological and mechanical properties as the natural bone and cartilage, whose complete regeneration is preferable, when possible; second, the unique function of the nerve tissue can be fully restored only by regenerating it.

2. Characterization of CHI-Based Biomaterials

Regarding the analytical studies on CHI and its derivatives, some of them were carried out in view of a use of the polysaccharide in the biomedical field. Japanese researchers prepared and characterized polyion complexes formed by the reaction between CHI and the anionic polyelectrolyte gellan [15]; the complexes were structured in fibres and capsules, which were said to be able, when filled with either drugs or growth factors, to release them into the injured part of the body, during the biodegradation of the component biopolymers [16]. However, in a following study of the same authors, the biodegradation of the polyion complex fibres was found to occur via soil filamentous fungi, so that they were proposed as environmentally biodegradable materials [17]. This fact throws many doubts on the possible use of these fibres as bioresorbable materials within the body. The biochemistry, the histology, and the clinical uses of chitins and chitosans for the treatment of leg ulcers, the use of skin substitutes, and the regeneration of nerve, meniscus, and bone tissues were thoroughly reviewed by A. Muzzarelli et al. in 1999 [18]. Regarding the nerve regeneration, they cited an early paper of Zielinski and Aebischer, who found that the fibroblast cells R208N.8 released nerve growth factor, when sequestered in 60 : 40 acrylonitrile-vinyl chloride copolymer containing precipitated CHI as an internal matrix [19]. Regarding the meniscus, Muzzarelli et al. found that CHI stimulated its repair by providing the necessary tissue elements and humoral factors. Regarding the bone tissue, they reviewed all the work then made, both *in vitro* and *in vivo*. A short review by Babensee et al., regarding the growth factor delivery also from CHI-albumin microspheres, for musculoskeletal, neural, and hepatic tissue engineering, appeared in the following year. However, CHI-albumin microspheres were used only for the growth of hepatocytes [20]; then, they were not useful for cartilage, bone, and nerve regeneration.

CHI was grafted onto silk fibroin by means of mushroom tyrosinase through the reaction of the amino groups of CHI with the tyrosyl residues of the protein, oxidized enzymatically to *o*-quinone groups; the resulting copolymer was analyzed, with the aim of enhancing the biocompatibility of silk-based biomedical devices in the hosting biological environment [21]. However, products of the same reaction, carried out under heterogeneous conditions, seemed to be, for their authors, more interesting as structural polymers than as biomedical ones [22]. The interactions with bovine serum albumin of two CHI macromolecules, having acetylation degrees of 1% and 12%, were measured by means of the turbidity change with varying pH, in 0.1 M NaCl solution. The results were presented as a model for enzyme immobilization on CHI, but no test with enzymes was reported [23]. Biodegradable blend membranes composed of CHI, and either poly(D,L-lactide) or poly(L-lactide) was prepared using a solution-casting and solvent-extracting processing technique. The membranes were examined by means of SEM, FTIR, TGA, DSC, DMA, and X-ray diffraction to test the miscibility of the polymers, which depended strongly on the polymer concentration and on the composition ratio of the mixed solvents, as well as on the drying technique. In the blends prepared under optimized processing conditions, FTIR showed hydrogen bond interactions between the polymers, which also caused a lowering of their crystallinity, detected by X-ray, thermal and dynamical testing, so indicating a good miscibility [24]. Fluorescein was attached, via its epoxy derivative, to water-soluble CHI, and the temperature/pH-sensitive qualities of fluorescence were investigated; the results obtained indicated that this modified CHI could be able to provide a convenient way to prepare low-cost and multifunctional macromolecular biomaterial for determining pH and temperature changes in biological systems simultaneously [25]. Bioartificial biodegradable materials were prepared, by the authors' group, by mixing CHI and poly(vinyl alcohol); then they were manufactured as films and finally cross-linked with pentane-2,5-dial (glutaraldehyde), both in the absence and in the presence of the edible plasticizer sorbitol, with the aim of using them as biomaterials and, in particular, as localized drug carriers. The materials were characterized by means of FTIR, DSC, TGA, X-ray diffraction, SEM, and tensile test

[26]. The blends showed a good biodegradability [27] and, as toughened by dehydrothermal treatment, no cytotoxicity toward murine fibroblasts [28]; their ability for drug release and permeation, tested for ascorbic acid, paclitaxel, D(+)glucose, vitamin B12, and bovine serum albumin, was found to depend on the chemical structures and properties of the tested molecules [27, 28]. The results obtained indicate that these blends could be useful to make both scaffolds for tissue engineering and devices for drug delivery. Canadian researchers fractionated and characterized CHI by size-exclusion chromatography and ^{1}H NMR, to produce homogeneous monodisperse chitosans in the molecular weight range of 5–100 kDa, which were said to be particularly useful in biomedical applications such as gene delivery; however, no results in the biomedical field were reported [29]. Japanese researchers modified an AFM probe by depositing on its tip, through a micropipette controlled by micromanipulator, first an acetone solution of poly(lactic-*co*-glycolic acid) and then a CHI solution in ethanol. The modified probe was used to study the interaction between the polymers and a mucin film, to understand the mucoadhesive mechanisms of CHI, when used for oral mucosal drug delivery. It was revealed that when a poly (lactic-*co*-glycolic acid) probe is retracting from the mucin film, a repulsive force appeared; however, after the probe was further overcoated with CHI, the force became attractive if the amount of CHI was enough, such as at CHI concentrations of 0.2% w/v [30]. Chinese researchers prepared a stable and translucent novel composite film consisting of CHI and cortical cells, extracted from waste wool fibres and characterized it by SEM, FTIR, DSC, X-ray diffraction, and tensile test. This film has a potential utilization in many fields, such as food packaging, wound dressing, and also tissue engineering [31]. Canadian researchers used AFM and AFM-based force spectroscopy, with CHI-modified tips, to investigate desorption of individual CHI polymer chains from substrates with varying chemical composition. They concluded that the experimental results reported in their paper might be used as a basis to investigate the interaction of CHI with surfaces, which may later be used as coatings in biomaterial applications, although no application in this field has been reported at the present time [32]. A new label-free amperometric immunosensor was developed for detection of human chorionic gonadotrophin, based on a multiwall carbon nanotubes-CHI complex film, electrodeposited on a glassy carbon electrode, and a three-dimensional Au nanoparticles-TiO_2 hybrid. The ease of the nonmanual technique and the promising features of this composite were presented by these researchers as a possible versatile platform for constructing other biosensors [33]. Direct formation of porous CHI structures, to be used as scaffolds for cell culture, by supercritical carbon dioxide method was presented to an international conference in India in 2009 [34]. In the same year, a review appeared, regarding chitins and chitosans for the repair of wounded skin, nerve, cartilage, and bone [35]. Generally, these analytical studies pointed out the suitability of CHI and of its derivatives to be used in the biomedical field, mainly for tissue engineering and regeneration, as well as for drug and gene delivery.

3. Bone and Cartilage Regeneration

An early work in the field of the chondroskeletal tissue engineering regarded the use of a CHI ascorbate gel for the treatment of periodontitis, according to current dental surgery; in ten patients, two months after the treatment, CHI was completely reabsorbed and the periodontium well regenerated [36]. The positive results of this pioneering clinical application encouraged the researches on the use of CHI containing biomaterials for bone and cartilage regeneration.

3.1. Bone. Methyl pyrrolidinone CHI was used to favour the formation of new bone tissue within the large alveolar cavity, remaining after the avulsion of a wisdom tooth [37]. The use of imidazolyl and 2-methyl-imidazolyl CHI for bone lesion healing was tested on sheep femoral condyles [38]. A particularly interesting feature was the use of CHI and its derivatives for the treatment of osteoporosis, a possible physicochemical component which was recently studied by the authors' group [39]. The release, as a consequence of CHI biodegradation, of the bone morphogenetic protein BMP-7 (OP-1), linked to an N,N-dicarboxymethyl CHI matrix in the form of a polyelectrolyte complex, was tested on the femoral condyles of four female osteoporotic rats. After 30 days, a complete new bone formation was observed in the surgical bone defects [40]. In another research [41], the same osteoporotic rats were treated with only hydroxyapatite and two biological glasses, with rather scarce results, whilst a CHI-hydroxyapatite composite was successfully inserted in femoral condyles of healthy New Zealand rabbits. N,N-dicarboxymethyl CHI was found to chelate calcium and phosphate ions, forming a gel, which favoured osteogenesis while promoting bone mineralization. Bone regeneration was observed in bone defects surgically made in sheep femoral condyle and trochanter [41, 42], as well as in human mandible after tooth avulsion and cyst removal [42]. Surgical lesions in rat condylus were treated with N,N-dicarboxymethyl CHI and the sodium salt of 6-oxychitin. Morphological data indicate that 6-oxychitin promoted the best osteoarchitectural reconstruction, even though healing was slower compared to that with N,N-dicarboxymethyl CHI. Complete healing was obtained with N,N-dicarboxymethyl CHI within three weeks [43]. A blend of CHI gel, ionically cross-linked with ascorbic acid, ZnO, CaO, crystalline carbonate-hydroxyapatite, and NaF, was prepared and tested by chemical, physical, and crystallographic measurements, with the aim of obtaining an efficient dressing for use during regeneration of the periodontal barrier [44]. A review focused on the manufacture of CHI-inorganic composites, based on calcium carbonate, calcium phosphate, and silica, pointed out their importance in the field of blood compatible materials, bone substitutes, and cements for bone repair and regeneration [45]. Biodegradable polylactide/CHI blends were used to fabricate scaffolds with well-distributed and interconnected porous structures. The porosity and the pore

dimension were monitored to obtain scaffolds suitable for applications in cartilage or bone tissue engineering [24, 46]. A more recent work regarded a good influence of the CHI component on the interactions between those blends and rat osteoblasts [47]; however, at the present time no clinical application of them has been reported. The bioactivity of a novel composite of carbonate-containing low-crystallinity nanoparticle hydroxyapatite and a CHI–phosphorylated CHI polyelectrolyte complex was evaluated *in vitro* and *in vivo.* The material was cocultured with rat osteoblasts *in vitro* and implanted into rabbit femur marrow cavities. The results indicated that the composite promoted osteoblast adhesion, morphology, proliferation, and differentiation *in vitro;* the bone tissue response *in vivo* to the material showed that the composite provided a suitable environment for active bone formation, with marrow cell infiltration and new bone deposition around the powder; then, it was bioactive as well as biodegradable [48]. Chitin and CHI were used to fill the defects in fractured segments of radius and ulna of dogs after stabilizing with dynamic compression plates. The study revealed that the fracture healing was better in CHI group of dogs [49]. Taiwan researchers produced a CHI surface-bonded recombinant human bone morphogenetic protein 2 via amide bond formation between the components. The surface-bonded protein did not denature but expressed sustained biological activity, such as osteoblast cell adhesion, proliferation, and differentiation, so making the material useful for bone tissue regeneration [50]. Korean researchers prepared porous, biodegradable and biocompatible scaffolds, using CHI, CHI with natural hydroxyapatite derived from *Thunnus Obesus* bone, and CHI grafted with functionalized multiwalled carbon nanotube, via freeze-drying method, and characterized them physiochemically as bone graft substitutes. Cell proliferation in composite scaffolds was twice than in pure CHI when checked *in vitro* using a human osteosarcoma cell line [51]. The preparation and characterization of CHI-blended polyamide-6 nanofibres by a new single solvent system via electrospinning process for human osteoblastic cell culture applications were carried out. The *in vitro* cytotoxicity evaluation of these nanofibres indicated that this scaffold material was nontoxic for the osteoblast cell culture [52]. A Brazilian research group synthesised a porous chitosan-gelatin scaffold cross-linked by glutaraldehyde and characterized it by both physicochemical and morphological tests, as well as investigating its effects on growth and osteogenic differentiation of rat bone marrow mesenchymal stem cells. Free-cell scaffolds were implanted into tooth sockets of Lewis rats after upper first molars extraction; on the 21st day, alveolar bone and epithelial healing were completely established [53]. Recently, Muzzarelli reviewed the use, for bone regeneration, of CHI composites with inorganic materials, morphogenetic proteins, and stem cells [54]. As a whole, despite the numerous positive results obtained from the tests made both *in vitro* and in animals, at the present time CHI-containing biomaterials have not yet reached the clinical application for human bone tissue regeneration, with the only exception of dental surgery. Also a very recent Russian patent, regarding a porous sponge of chitosan-gelatin composite with octacalcium phosphate, suitable for filling of bone defects, does not contain, in its abstract, any mention of having obtained the government approval for its use in clinical orthopaedic practice [55].

3.2. Cartilage. N-Carboxybutyl CHI was used to promote the tissue repair of the meniscus in rabbits [56]. Recombinant bone morphogenetic protein BMP-7 (OP-1), linked to a N,N-dicarboxymethyl CHI matrix in the form of a polyelectrolyte complex, was used to induce or facilitate the repair of articular cartilage lesions, produced in 21 adult male New Zealand white rabbits [57]. *In situ* gelling CHI-disodium β-glycerol phosphate-glucosamine solution, containing chondrocytes, was injected into cartilage defects in rabbits. The results showed that the delivered cells could gradually produce a viable and mechanically stable repair tissue at the defect site [58]. Another group inserted a hydrogel of CHI-hyaluronan polyelectrolyte complex into the patella articular cartilage of rabbits, obtaining some promising results. The implants were capable of developing hyaline-appearing tissue, maintained at 24 weeks postoperatively; the presence of chondrocytes was also observed [59]. CHI-glycerol phosphate/blood implants applied in conjunction with drilling, compared to drilling alone, elicited a more hyaline and integrated repair tissue associated with a porous subchondral bone repleted with blood vessels, in New Zealand white rabbits. Concomitant regeneration of a vascularized bone plate during cartilage repair could provide progenitors, anabolic factors, and nutrients that aid in the formation of hyaline cartilage [60]. An analogous treatment, made on identical rabbits subjected to bilateral arthrotomies, with each trochlea receiving a cartilage defect bearing four microdrill holes into the subchondral bone, favored intramembranous bone formation in the microdrill holes and resulted in a cartilage repair strategy that modulates acute and intermediate events in the subchondral bone in order to improve final cartilage repair outcome [61]. The proliferation *in vitro* of New Zealand white rabbit chondrocytes on porous poly(DL-lactide)/chitosan scaffolds was studied using scanning electron microscopy, histological observations, and proteoglycan measurements; the results indicated that the resulting scaffolds exhibited increasing ability to promote the attachment and proliferation of chondrocytes and also helped the seeded chondrocytes to spread through the scaffolds and distribute homogeneously inside them [62]. Korean researchers obtained thermosensitive gels grafting N-isopropylacrylamide onto CHI and coupling CHI with Pluronic, a commercial poly(ethylene oxide-*b*-propylene oxide) triblock copolymer [63]. The first copolymer induced the chondrogenic differentiation *in vitro* of human mesenchymal stem cells [63]; the second one favoured the proliferation *in vitro* of bovine chondrocytes [64]. Temperature-responsive CHI hydrogels were prepared by combining CHI, β-sodium glycerophosphate, and hydroxyethyl cellulose. Tissue-engineered cartilage-regenerating scaffolds were made *in vitro* by mixing sheep chondrocytes with a CHI hydrogel. To collect data for *in vivo* repair, scaffolds cultured for one day were transplanted to the freshly prepared defects

of the articular cartilage of sheep. The results showed that the chondrocytes in the regenerated cartilage survived and retained their ability to secrete matrix when cultured *in vitro*. The scaffolds induced complete cartilage defects repair within 24 weeks after being transplanted *in vivo* [65]. Canadian researchers successfully investigated temporal and spatial modulation of chondrogenic foci in subchondral microdrill holes, made in 32 New Zealand white rabbits, by CHI-glycerol phosphate/blood implants. The chondrogenic foci bore some similarities to growth cartilage and could give rise to a repair tissue having similar zonal stratification as articular cartilage [66]. In the field of cartilage regeneration, the research on CHI is less advanced than in that of bone, considering the absence of clinical applications in humans.

4. Nerve Tissue Regeneration

Chinese researchers studied the ability of materials made with CHI and CHI derivatives to facilitate the *in vitro* growth of nerve cells for nerve tissue regeneration. Their results suggested that, after being precoated with laminin and fibronectin solution or serum, all materials have better nerve cell affinity [67]. More recently, the same group found that films of carboxymethyl chitosan, cross-linked with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride, enhanced the spread of Neuro-2a cells and provided a good proliferation substratum for Neuro-2a cells, as compared to chitosan films [68]. In a much later research, that group investigated the application of the CHI/glycerol-β-phosphate disodium salt hydrogel in peripheral nerve regeneration, in 24 female adult Sprague Dawley rats. Contrary to former *in vitro* reports, they found that the implanted hydrogel actually impeded nerve regeneration; then, many further studies are necessary in this matter [69]. Japanese researchers coated apatite CHI tubes prepared from crab tendons and then used them as nerve-regenerating guides for the sciatic nerves of male Sprague Dawley rats, with successful results [70]. Researcher of the University of Texas reviewed the strategies for repair and regeneration of damaged nerves, among which there was the use of nerve guides made by bioresorbable materials, including CHI [71]. Chinese researchers developed a dual-component artificial nerve graft comprising an outer microporous conduit of CHI, made from crab tendons, and internal oriented filaments of poly(glycolic acid). The novel graft was used for bridging sciatic nerve across a defect of 3 cm length in six Beagle dogs; they were compared with other six dogs subjected to autograft, as a positive control, and with five not grafted dogs, as a negative control. At six months postoperatively, the dogs grafted with the artificial nerve showed motion ability comparable to that of the positive control ones, unlike the not grafted ones [72]. Italian researchers made *in vitro* neuroblast adhesion test on films of CHI-gelatin blends and on nerve guides of CHI-poly(ε-caprolactone) blends; the cells adhered better to the first materials than to the second ones [73]. A US research team prepared nerve guides with a blend of CHI and type I collagen from rat-tail tendon, as well as guides of pure CHI. Each guide group was used to bridge a 10 mm sciatic nerve gap of 24 female Lewis rats; equal numbers of rats were subjected to autograft and left unrepaired, respectively, as positive and negative controls. Both guides gave quite good results, although less than autograft; the researchers concluded that further investigation was necessary [74]. A Chinese group fabricated nerve conduits of a CHI-poly(lactic acid) blend, using a mold casting/infrared dehydration technique. The conduits were inserted in 10 mm gaps of the sciatic nerves of ten 4-month-old Sprague Dawley rats; equal numbers of rats were treated with autograft and silicone conduits, respectively. The nerve regeneration with the blend was not different from that with the autograft, and much better than with the silicone [75]. A group of the Purdue University, USA, performed *ex vivo* and *in vivo* experiments on spinal cord injuries made in guinea pigs. Their results demonstrated that the application of CHI was able to immediately restore compromised membrane integrity, CHI was a potent neuroprotective agent, even though it did not show any ability to scavenge either reactive oxygen species or acrolein, and that CHI clearly targeted the area of tissue damage, where uninjured spinal cord exhibited a very weak affinity for CHI. Then, the CHI approach for damaged membranes provides novel therapeutic potential through site-specific delivery following traumatic spinal cord and head injury [76]. Canadian researchers found that CHI could be promising in transplantation strategies of neural stem and progenitor cells, to treat an injury to the central nervous system, such as a spinal cord injury. Four amine-functionalized hydrogels, comprised CHI, were screened *in vitro* for the viability, the migration, and the differentiation of adult murine neural stem and progenitor cells. Only CHI supported survival of multipotent stem cells and the differentiation of the progenitor cells into neurons, astrocytes, and oligodendrocytes. Then, CHI appeared as a promising material for the therapies involving adult neural stem and progenitor cells [77]. Conductive polycaprolactone/CHI/polypyrrole composites were prepared and characterized in view of their possible use for peripheral nerve repair [78]; conductive conduits made with polypyrrole particles dispersed in a CHI-*g*-polycaprolactone matrix were prepared and characterized by the same group, then implanted into rabbits for various periods, to test the variations of their properties during bioresorption *in vivo* [79]; however, no data about a test of them as nerve repair conduits have been reported. To design a novel kind of scaffolds for blood vessel and nerve repairs, random and aligned nanofibrous scaffolds based on collagen-CHI-thermoplastic polyurethane blends were electrospun to mimic the componential and structural aspects of the native extracellular matrix. Vascular grafts and nerve conduits were electrospun or sutured based on the nanofibrous scaffolds; the results indicated that nanofibrous scaffolds, made blending collagen, CHI, and thermoplastic polyurethane might be a potential candidate for vascular repair and nerve regeneration [80]. Another group developed an aligned CHI-polycaprolactone fibrous scaffold and investigated how the fibre alignment influenced nerve cell organization and function in comparison with randomly oriented fibrous scaffolds and cast films of the same material. Schwann cells grown on the aligned CHI-polycaprolactone fibres exhibited a

Table 1: CHI-containing devices used for bone and nerve tissue regeneration in humans.

Device	Tissue	Time	Reference
CHI ascorbate gel	periodontium	2 months	[35]
Me-pyrrolidinone-CHI gel	tooth alveolar bone	6 months	[36]
DCMC-CaP[a] gel or solution	mandible bone	15 days	[41]
CHI conduits with PGA[b] filaments	right arm median nerve	36 months	[86]

[a] *N,N*-dicarboxymethyl CHI with calcium phosphate;
[b] poly(glycolic acid).

bipolar morphology that oriented along the fibre alignment direction, while those on the films and randomly oriented fibres had a multipolar morphology. Similarly, the CHI-polycaprolactone material supported neuron-like PC-12 cell adhesion, and the aligned fibres regulated the growth of PC-12 cells along the fibre orientation. Additionally, PC-12 cells cultured on the aligned fibres exhibited enhanced unidirectional neurite extension along fibre orientation and significantly higher β-tubulin gene expression than those grown on CHI-polycaprolactone films and randomly oriented fibres. The results reported suggest that the aligned CHI-polycaprolactone fibres can serve as a suitable scaffold for improved nerve tissue reconstruction [81]. The differentiation of bone marrow stromal cells in three-dimensional scaffolds consisting of collagen, poly(lactide-*co*-glycolide), and CHI was also investigated. The induction with neuron growth factor inhibited osteogenesis and guided the differentiation of bone marrow stromal cells towards neurons in the constructs. Therefore, the combination of collagen-functionalized poly(lactide-*co*-glycolide)/CHI scaffolds, neuron growth factor, and bone marrow stromal cells can be promising in neural tissue engineering [82]. Indeed, bone marrow stromal cells are generally known to be useful for bone tissue regeneration [83]. To have found a possible use of them also for nerve tissue regeneration opens new ways to neural surgery.

Regarding the aim of using bioresorbable macromolecular materials to make nerve regeneration conduits to be employed in the clinical practice of human neural surgery, it has been reached since quite several years, both with only synthetic bioresorbable polyesters [84, 85] and with blends of them with CHI [86]. In the latter case, a 37-year-old Chinese man, having a 35 mm-long nerve defect in the median nerve of the right arm at elbow level, underwent a surgical repair with an artificial nerve graft, comprising an outer microporous conduit of CHI and internal oriented filaments of poly(glycolic acid). Suitable functional recovery of the hand ability proceeded slowly, but regularly, with time, together with nerve regeneration, and was near complete 36 months after the implantation [86]. The same Chinese surgeon group reviewed the construction of tissue-engineered nerve grafts and their application in peripheral nerve regeneration very recently [87]. As regarding the use of CHI-containing grafts in clinical neural surgery, only their preceding intervention was reported, in comparison with many equally successful ones with other biomaterials. This fact might be a sign that CHI-containing nerve grafts still need much theoretical and laboratory study before becoming of common practice in surgery.

5. Conclusion

The importance of the biomaterials containing either CHI or CHI derivatives for the regeneration of damaged bone, cartilage, and nerve tissue appears evident from the studies carried out near towards the entire world. However, as it can be seen from the data summarized in Table 1, only few applications to the human health in this field have been done at the present time. Then, much work must still be made, especially for the passage from the experimentation of the CHI-based devices *in vitro* and in animals, in which many successful results were obtained, to their general application in clinical practice.

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Research Article

Consequences of Neutralization on the Proliferation and Cytoskeletal Organization of Chondrocytes on Chitosan-Based Matrices

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In tissue engineering strategies that seek to repair or regenerate native tissues, adhesion of cells to scaffolds or matrices is essential and has the potential to influence subsequent cellular events. Our focus in this paper is to better understand the impact of cellular seeding and adhesion in the context of cartilage tissue engineering. When scaffolds or surfaces are constructed from chitosan, the scaffolds must be first neutralized with sodium hydroxide and then washed copiously to render the surface, cell compatible. We seek to better understand the effect of surface pretreatment regimen on the cellular response to chitosan-based surfaces. In the present paper, sodium hydroxide concentration was varied between 0.1 M and 0.5 M and two different contacting times were studied: 10 minutes and 30 minutes. The different pretreatment conditions were noted to affect cell proliferation, morphology, and cytoskeletal distribution. An optimal set of experimental parameters were noted for improving cell growth on scaffolds.

1. Introduction

The successful cellular colonization of scaffolds for use in tissue engineering applications often relies on an important first step: cell seeding, where cells in suspension adhere to scaffolds or matrices [1]. Poor or inadequate cell adhesion often yields low starting cell densities that can result in lower cellular yields upon completion of the *in vitro* cell culture step. While in general, cellular adhesion is a critical step in most tissue engineering strategies that seek to repair or regenerate the native tissue, our focus in this study is to better understand the impact of cellular seeding and adhesion in the context of cartilage tissue engineering. Scaffold parameters that impact tissue engineering strategies include chemical [2–5] and mechanical properties [6], geometry (2D [7] versus 3D [7, 8]; micro [9–11] versus nano [12]), environment, and morphology (pore size and pore shape) [6, 13].

Most materials that are used in the preparation of scaffolds for use in tissue engineering applications are either derived from natural origin (collagen, gelatin, chitosan, and agarose) or prepared from synthetic polymers (poly(lactic acid) (PLA), poly(L-lactic acid)-polyglycolic acid (PLLA-PGA), poly(ε-caprolactone) (PCL), etc.) [14–16]. Cellular response to biomaterial interfaces is often directed by surface characteristics. For example, cell adhesion has been shown to be influenced by substrate chemistry, which partly modulates the pattern, conformation, and extent of protein adsorption on biomaterial surfaces [2, 3, 17, 18]. For example, $-CH_3$, polyethylene glycol (PEG) and OH-terminated surfaces bind low levels of fibronectin (FN), which correlates with low cell-surface interactions and were noted to fail to organize the extracellular fibronectin matrix [1].

Another important parameter impacting cellular adhesion during cell seeding is the scaffold conditioning treatment. Briefly, most scaffolds currently used in tissue engineering applications are made from polymeric solutions or suspensions using very well-established manufacturing methods, such as freeze drying lyophilization (FL) [19], solvent casting (SC) and particulate leaching [20], gas foaming [21], rapid-prototyping [22], and electrospinning (ES) [23].

Scaffold conditioning is needed to create a cell-friendly environment with pH ranges close to neutral or adjust mechanical properties of the material to favor cell viability. The effect of scaffold pretreatments on cell attachment and proliferation has been reported for several materials [24–32]. For example, 3D poly(L-lactide-co-glycolide) scaffolds treated with 1 M sodium hydroxide have been shown to enhance osteoblast adhesion and differentiation but had impeded proliferation [25]. Such cellular changes are mainly attributed to a nanostructural alteration of the scaffold surface as a result of the NaOH treatment. Scaffolds prepared from varying ratios of alginate/hydroxyapatite (HAP) via the phase separation technique required pretreatment with a crosslinking agent to slow scaffold dissolution rates [31]. Also, when woven poly(ethylene terephthalate) fabrics were coated with PLGA and an artificial glycopolymer poly [N-p-vinylbenzyl-D-lactamide] (PVLA) [30], hepatocyte cells were noted to be spread and in closer contact when compared to uncoated scaffolds, where hepatocytes were rounded, with no noticeable spreading or aggregation. Electrospun PGA fibers, when subjected to acid hydrolysis, have shown an improvement in cell proliferation and in vivo compatibility and in contrast, untreated scaffolds demonstrate low cell proliferation and pronounced inflammation and tissue ingrowth [24]. It is postulated that the surface hydrolysis of ester bonds, as a result of the acid pretreatment step, exposes the carboxylic acid and alcohol groups, thus improving vitronectin binding, hereby increasing cell adhesion.

The general emphasis of our research is to evaluate chitosan scaffolds as substrates for growth and proliferation of chondrocytes. Chitosan (poly 1,4 D-glucosamine), a biocompatible polymer, is a partially deacetylated derivative of chitin and is structurally similar to glycosaminoglycan (GAG). Its structure resemblance to the glycosaminoglycan structure makes it a very suitable candidate for scaffold material [33], as it has been shown to support the expression of ECM proteins [34–38]. However, chitosan requires dissolution in inorganic or organic acids due to its limited solubility in aqueous solutions. Thus postprocessing steps often require the removal of acid remnants by neutralization with sodium hydroxide (NaOH) [36, 39–41], sodium bicarbonate ($NaHCO_3$) [42], or NaOH-sodium salts mixtures [43, 44]. The underlying hypothesis of this study is that the conditions of neutralization impacts cellular fate; thus in this study chitosan scaffolds that were prepared by freeze drying and lyophilization were subjected to the following pretreatments: sodium hydroxide concentration was varied between 0.1 M and 0.5 M and two different contacting times were studied: 10 minutes and 30 minutes. Subsequently, the attachment of chondrocytes to these scaffolds was analyzed.

2. Materials and Methods

2.1. Isolation of Chondrocytes. Intact cartilaginous tissues were obtained from the hip-joint of young calves provided by a local abattoir and were isolated using procedures detailed elsewhere [45, 46]. Briefly, cartilage slices were incubated with pronase for 1 h at 37°C, washed, and incubated in collagenase for 12 h at 37°C. Chondrocyte suspension was filtered, spun down and cell pellet was washed and resuspended in DMEM/F12 with 10% FBS; 100 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.29 mg/mL of L-glutamine and counted in a hemacytometer. Chondrocytes were plated in a 75 cm^2 T-flask at a cell density of 10^4 cells/cm^2 in RPMI medium (RPMI 1640 containing 10% FBS, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL of amphotericin B, and 0.29 mg/mL of L-glutamine). Bovine chondrocytes from passage 2 were used in all experiments. Cell culture media and other reagents were obtained from Invitrogen (CA).

2.2. Preparation of Films and Scaffolds

2.2.1. Films. A 2% w/v solution of chitosan (degree of deacetylation: 81.7%, molecular weight = 276 kDa, Vanson HaloSource, WA) in 1% acetic acid was freshly prepared. Glass slides (25 × 75 mm) or rounded cover slips (12 mm diameter) were coated (one side) with a 1 mL and 0.5 mL of chitosan solution, respectively, allowed to dry at room temperature overnight, with subsequent heating at 80°C for 24 h to prevent the detachment of the chitosan films. Dried films were then neutralized with NaOH solutions, copiously rinsed with deionized (DI) water and used immediately.

2.2.2. Scaffolds. Scaffolds were prepared via freezing and lyophilization (FL) using procedures detailed elsewhere [47]. Briefly, a 2 mL of freshly prepared 2% w/v chitosan solution in 1% acetic acid was pipetted into each well of a 24-well tissue culture polystyrene plate, frozen at −20°C for 24 h and then lyophilized at $P < 0.1$ torr for 24 h in a Thermo-Savant Lyophilizer (model no. RVT 4104). Scalpel and biopsy punches were used to cut out scaffolds with the following dimensions: 5 mm × 5 mm. The scaffolds were then neutralized with indicated NaOH solutions, copiously rinsed with deionized (DI) water, and used immediately.

2.2.3. Neutralization Conditions for Films and Scaffolds. Chitosan films and scaffolds were subjected to sodium hydroxide (NaOH) neutralization. Three different concentrations of NaOH solutions (0.1 M, 0.25 M and 0.5 M) and two different contacting times (10 min and 30 min) were used to neutralize the samples.

2.3. Surface Characterization

2.3.1. Contact Angle Measurement. Advancing contact angle measurement was performed following sessile drop method with OCA 15, SCA 20, Data Physics Instrument GmbH (Filderstadt, Germany). Water contact angles (θ) were measured (1 μL with flow rate of 0.1 μL/s), and to avoid spreading of the drops and droplet shape variation, contact angle values were recorded within 15–20 seconds after placing the drop. The average contact angle values were collected from at least ten drops on different areas of a test surface and three test surfaces were used for each condition. All measurements

were conducted in air and at a temperature of 23°C. In separate experiments, contact angles were measured for chitosan films that were exposed (16 h) and were not exposed to RPMI medium.

2.3.2. Scanning Electron Microscopy. Morphology of the cells on either coverslips or scaffolds was visualized by SEM. Briefly, samples were rinsed with PBS and cross-linked with 2.5% glutaldehyde/PBS solution for 30 min. The glutaraldehyde was subsequently rinsed off with PBS and samples were sequentially dehydrated in ethanol solutions (from 20 to 100%), where samples were incubated for 15 min in each ethanol concentration. The 100% ethanol was removed with hexamethyl disilazane (HMDS, Sigma) and finally samples were air-dried. Cast films and scaffolds prepared by FL were coated with gold-palladium (Au-Pd) and viewed under a scanning electron microscope (SEM, s-3000N, Hitachi, Tokyo, Japan).

2.3.3. Fourier Transform Infrared Spectroscopy (FTIR). FTIR spectra were recorded in the transmittance mode using a Nicolet 20XB spectrometer, in the range of 4000–400 cm^{-1}, with a spectral resolution of 2 cm^{-1}. Chitosan films were removed from the glass slide and subjected to indicated treatments with NaOH, and dried overnight at 40°C.

2.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Chitosan scaffolds ($n = 8$) that were neutralized with indicated concentrations of NaOH were copiously rinsed with DI water followed by three 10-minute washes with PBS. Scaffolds were then incubated in RPMI media for 24 h at room temperature. After that, they were copiously rinsed with multiple washes of PBS and surface adsorbed proteins were then eluted with 200 μL of 2% SDS (Invitrogen) for 4 h. The protein concentration in the eluate was measured by the bicinchoninic acid (BCA) method and a volume equivalent to a total protein (8 μg/well) was subjected to SDS-PAGE analysis on a 10% NuPAGE gel under denaturing conditions.

2.5. Cell Seeding

2.5.1. On Films. Chitosan-coated coverslips were placed in 24 well TCP plates and were first disinfected with 70% ethanol for one hour, rinsed with sterile DI water and PBS, and incubated with RPMI-1640 medium (Gibco, NY) that was supplemented with 10% FBS (Gibco, NY) and containing 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL of amphotericin (Gibco, NY) for 24 hours. Bovine chondrocytes were seeded onto the Chitosan-coated coverslips at seeding density of 1×10^4 cells/cm^2 and maintained for 3 h in CO_2 incubator at 37°C, 5% CO_2, 95% humidity followed by addition of RPMI medium. Medium was changed every other day.

2.5.2. On Scaffolds. Cells were seeded on chitosan scaffolds at a cell seeding density of 3.3×10^4 cells/scaffold and maintained in an incubator for 3 h in CO_2 incubator at 37°C, 5% CO_2, 95% humidity, and, subsequently, 8 mL of medium was added to each well. Typically, 6 to 8 scaffolds were placed per well in a 6-well TCP plate and will be indicated as a test plate. Cells were cultured in RPMI medium. Test plates were placed in a cell culture incubator at 37°C, 95% humidity and 5% CO_2, and the medium was changed every two days.

2.5.3. Cell Number Estimation. At the end of the culture period, the scaffolds were removed and placed in 1.5 mL centrifuge tubes (3 scaffolds/tube), and 800 μL of a 0.5% trypsin (Gibco, NY) was added. Tubes were then incubated for 15 minutes in the CO_2 incubator at 37°C with intermittent manual inversion of the tubes. At the completion of the trypsin step, a 0.4 mL of RPMI medium was added to each tube and the suspension was centrifuged ($100 \times$ g) to obtain a pellet, which was resuspended with RPMI medium and cells were counted with a hemacytometer.

2.6. Cytoskeletal Organization by Immunochemistry. Cells grown on coverslips or chitosan films were fixed in 4% paraformaldehyde (PFA) in PBS (solution freshly made) for 2 h at room temperature, blocked and permeated with block/perm solution (BPS: 0.25% Triton X-100, 10 mg/mL of bovine serum albumin, 50 μL/mL of normal goat serum in PBS) and incubated with 1 : 200th dilution of mouse monoclonal antivinculin antibody (Sigma-V9131) in BPS for 12 h, rinsed with PBS supplemented with 0.1% BSA (rinsing buffer-RB) and incubated with 1 : 200th dilution of Alexa-488 (Invitrogen A11001) labeled antirabbit polyclonal antibodies for 12 h at room temperature and copiously rinsed with RB. Subsequently, specimens were labeled with 1 : 100th dilution of Alexa 633 phalloidin (Invitrogen A22284) and 1 : 100th dilution of Anti-Vimentin-cy3 (Sigma C9080) in BPS for 12 hours at room temperature and finally rinsed extensively with RB. Counterstaining with 0.05 mg/mL of DAPI (Inivtrogen-D1306) was performed for 3 minutes prior to mounting with aqueous mounting medium on coverslips. A confocal laser scanning microscope (Olympus FV500 Inverted_Olympus IX 81) was used to obtain the images.

2.7. Statistical Analysis. Student's t-test was used for statistical analysis, and statistical differences were declared as $P < 0.05$.

3. Results and Discussion

In order to make chitosan-based surfaces or scaffolds, it is often necessary to first dissolve chitosan in an acidic solution, and remove the solvent after the scaffold or film preparation step, leaving behind a chitosan acetate material. Thus neutralization steps are undertaken to remove acid remnants, regenerate the NH_2 groups on the chitosan, and render the surface hydrophilic and cell compatible. However, the conditions of this neutralization step have not been completely evaluated, leading to variability in the extent of cellular adhesion obtained during the cell-seeding step. Typically, *in-vitro* tissue engineering methods rely on good initial cell

adhesion to yield optimal starting cell densities. Thus our objective was to find optimal regimens of scaffold pretreatment for chitosan-based scaffolds such that reliable starting cell densities, the maximum cell attachment without losing the chondrocyte phenotype can be obtained. Hence, in this paper, chitosan scaffolds or films that were prepared from 2% (w/v) solution of chitosan in acetic acid were neutralized with varying concentrations of sodium hydroxide for different incubation times. As 3D scaffolds present technical difficulties (i.e., lack of a flat surface) in surface characterization measurements, chitosan films were used when estimating surface properties.

Figure 1 shows the FTIR spectra of chitosan films that were neutralized with varying concentrations of NaOH, and FTIR spectra of nonneutralized films were included as controls. In the FTIR spectra for nonneutralized films, peaks at 1560 cm^{-1} and 1400 cm^{-1} correspond to the asymmetric and symmetric stretching of the carboxylate ion [48], characteristic of the presence of acetate ion in the chitosan acetate salt. These two peaks are replaced by the amide I (C=O stretching) and amide II (N–H bending) peaks at 1656 cm^{-1} and 1546 cm^{-1}, respectively, in the FTIR spectra of neutralized films, denoting the appearance of the amide group characteristic of chitosans not completely deacetylated. The most important feature of the FTIR spectra of neutralized films is the presence of a peak at 1591 cm^{-1} which corresponds to the N–H stretching of the NH_2 (amine) group. The removal of the acetate counterion is further confirmed by the appearance of peaks at 1419 cm^{-1}, 1380 cm^{-1}, and 1320 cm^{-1} corresponding to the CH_2 bending, CH_3 deformation; symmetric CH_3 deformation and CH bending and CH_2 wagging, respectively [49]. From these spectra we were able to conclude that under any of the tested neutralizations conditions, the films were completely neutralized based on the confirmation of the removal of the acetate counterion.

Contact angle measurement was used to assess the polarity of the surfaces after the indicated NaOH treatments. Measurements were also taken after the surfaces were exposed to medium containing 10% FBS. Figure 2(a) shows the contact angle of chitosan surfaces that were neutralized with varying concentrations of NaOH for either 10 or 30 minutes, and subsequently rinsed with DI water and sterile PBS. Contact angle values in the range of 43° to 65° were obtained and these values were noted to be lower to that reported elsewhere [41]. For a given treatment time, contact angle values were observed to decrease with an increase in the concentration of NaOH, indicating an increase in the hydrophilic nature of the surface. It was not possible to measure the contact angle of nonneutralized films, as the films resolubilized in aqueous solutions. Figure 2(b) shows the contact angles of chitosan films that were first neutralized with varying concentrations of NaOH, rinsed, and subsequently exposed to FBS supplemented RPMI medium. Contact angle values in the range of 75° to 95° were obtained. Films that were neutralized for 30 minutes had lower contact angles when compared to films that were neutralized for 10 minutes implying that longer contacting times between the chitosan acetate surface and sodium hydroxide solution renders more hydrophilic

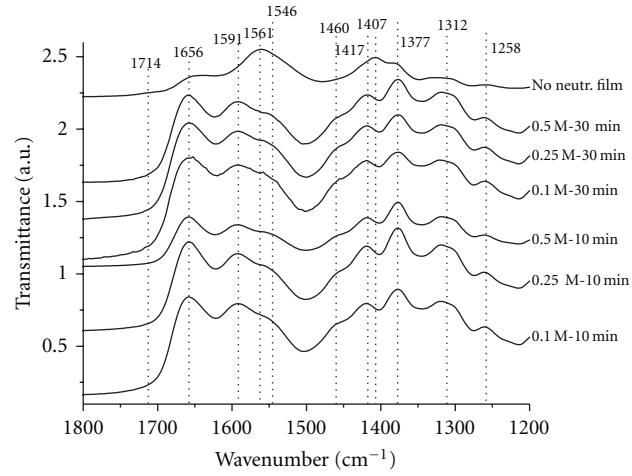

Figure 1: FTIR spectra for nonneutralized films showing the peaks at 1560 cm^{-1} and 1400 cm^{-1} corresponding to the asymmetric and symmetric carboxylate ion stretching [48] characteristic of the presence of acetate ion in the chitosan acetate salt. FTIR spectra of chitosan films that were neutralized with varying concentrations of NaOH show the amide I (C=O stretching) and amide II (N–H bending) peaks at 1656 cm^{-1} and 1546 cm^{-1}, respectively, characteristic of chitosans not completely deacetylated. Also they show the presence of a peak at 1591 cm^{-1} which corresponds to the N–H stretching of the NH_2 (amine) group.

surfaces, comparatively Figure 3(a) shows the SDS-PAGE gel electrophoresis of eluates obtained from chitosan scaffolds that were first neutralized with varying concentration of NaOH, and exposed to media supplemented with FBS. Figure 3(b) shows the semiquantitative evaluation of the bands noted in Figure 3(a) by using ImageJ software. Intensities of the protein bands detected at 55 kDa, 65 KDa, and 75 kDa were further evaluated and compared between different treatment conditions. The observed protein bands at 66-67 kDa, and 55 kDa can be assigned to albumin and the heavy chain of IgG (samples were run under reducing conditions) whereas 75 kDa band is more likely to be vitronectin [1]. The total amount of protein adsorbed on scaffolds was observed to decrease with increasing sodium hydroxide concentration.

Surface pretreatments can alter the surface properties and render them hydrophilic or hydrophobic and may also impact the pattern of protein adsorption in an independent manner. Cell and protein adhesion to surfaces has been shown to be modulated by surface chemistry [2, 3, 17] and additionally, adsorbed proteins may act as intermediates in cell attachment. In this study, treatments with increasing concentration of NaOH and higher incubation times were noted to increase the hydrophilicity of the surfaces. Interestingly, surfaces preexposed to 10% FBS in RPMI medium exhibited higher contact angles or increased hydrophobicity when compared to chitosan surfaces that were not exposed to FBS containing medium, possibly due to the nature of the protein adsorbed and also to the conformation status of the proteins on the surface.

An increase in contact angle suggests an increase in the hydrophobic nature of the protein adsorbed surface.

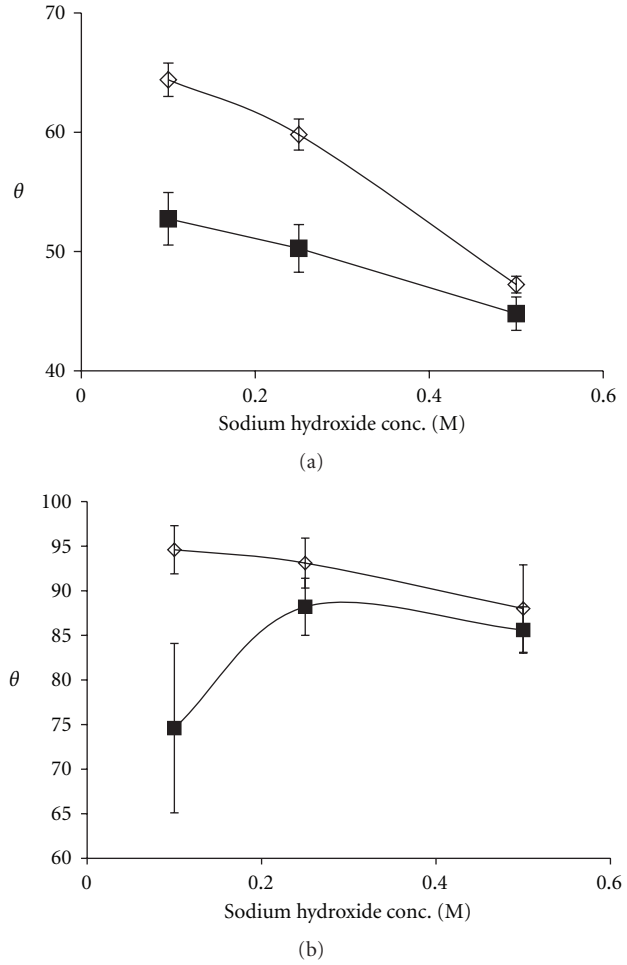

Figure 2: Contact angle of chitosan surfaces. Water contact angles of the various surfaces prepared were measured. (a) surfaces that were neutralized with varying concentrations of NaOH for either 10 (◊) or 30 (■) minutes and rinsed with sterile PBS, (b) contact angle of chitosan films neutralized and exposed to RPMI medium supplemented with 10% FBS for 16 hours.

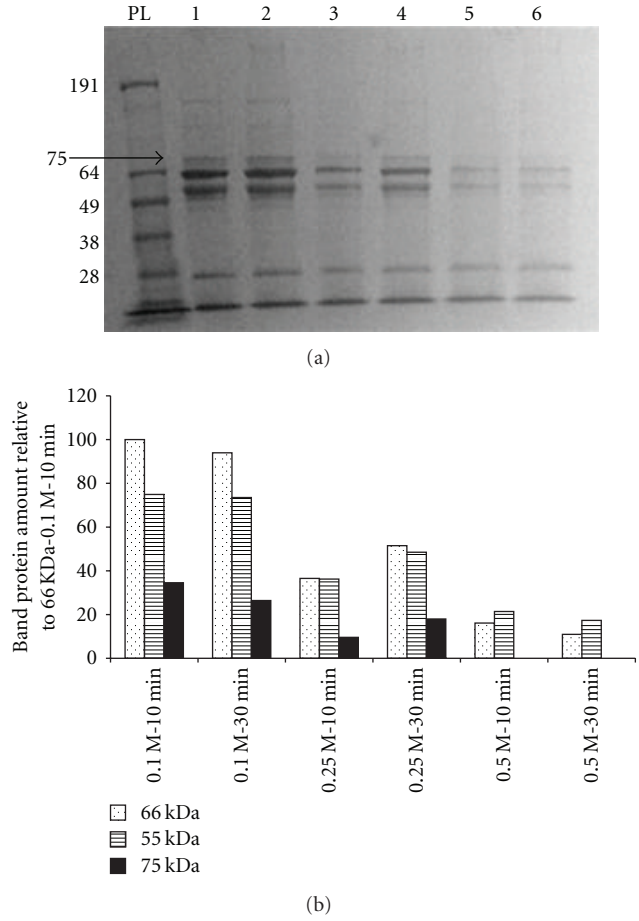

Figure 3: SDS-PAGE analysis of surface adsorbed proteins. Scaffolds that were subjected to the indicated pretreatments were exposed to serum and the surface adsorbed proteins were eluted and analyzed by gel electrophoresis. Typically, 3 to 4 scaffolds were treated as independent experiments. (a) SDS-PAGE of proteins eluates. PL: protein ladder. Lane 1: 0.1 M NaOH concentration for 10 min., Lane 2: 0.1 M NaOH concentration for 30 min, Lane 3: 0.25 M NaOH concentration for 10 min., Lane 4: 0.25 M NaOH concentration for 30 min., Lane 5: 0.5 M NaOH concentration for 10 min., and Lane 6: 0.5 M NaOH concentration for 30 min. (b) Semiquantitative evaluation of the gel image in (a) with ImageJ software.

The concentration of NaOH used, and the duration of the neutralization step was observed to influence the total amount of protein adsorbed. Previous investigations have shown that the attachment and spreading of cells are enhanced on moderately wettable surfaces while hydrophobic or nonionic hydrophilic surfaces inhibit interactions with cells. In this work we have used SDS-PAGE electrophoresis to visualize the proteins in the extracts obtained from scaffolds that were preexposed to serum-rich medium. It has been reported elsewhere [50] that serum albumin and immunoglobulins preferentially adsorb onto hydrophobic surfaces while adhesive proteins like FN and VN had higher absorbed amounts on hydrophilic surfaces. The adsorption of fibronectin on tissue culture polystyrene (TCPS) was inhibited by other serum proteins [51, 52] while vitronectin was noted to competitively adsorb to the surface in the presence of other plasma proteins. These findings suggested that the attachment of vitronectin to surfaces was more important than fibronectin for the initial attachment of endothelial cells and fibroblasts [51]. It has also been reported that vitronectin was the main adhesive protein for chondrocyte attachment to TCP and the PEMA/THFMA system in a complete medium [53]. The differential adsorption of vitronectin is also observed, based on the SDS-PAGE gel results shown in Figures 3(a) and 3(b). Figure 3(a) lanes 5 and 6 show the presence of weak 66–55 kDa bands, corresponding to the films neutralized with 0.5 M NaOH (10 and 30 min). The corresponding contact angles for these films before contacting with medium were 47° and 45°, respectively. Contact angles after contacting with medium were 88°–86°, respectively and similarly to Faucheux et al. [1] no vitronectin band (75 kDa) was detected under these

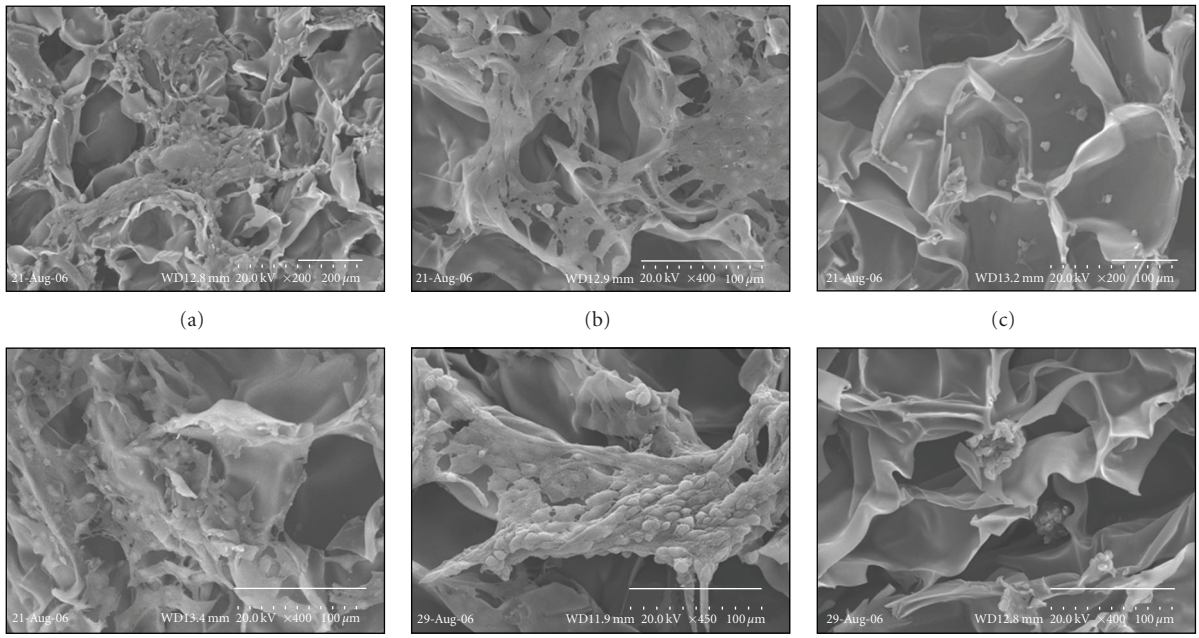

Figure 4: SEM pictures of neutralized 3D scaffolds. (a) Scaffolds neutralized with 0.1 M NaOH for 10 minutes. (b) Scaffolds neutralized with 0.25 M NaOH for 10 minutes. (c) Scaffolds neutralized with 0.5 M NaOH for 10 minutes. (d) Scaffolds neutralized with 0.1 M NaOH for 30 minutes. (e) Scaffolds neutralized with 0.25 M NaOH for 30 minutes, and (f) Scaffolds neutralized with 0.5 M NaOH for 30 minutes. Scale bar: 100 μm.

conditions. Surface wettability has shown to effect protein adsorption, fibronectin was shown to adsorb better on hydrophilic surfaces while albumin was shown to adsorb better on hydrophobic surfaces [54] when exposed to human serum, and similar trends were noted in this study; higher amounts of albumin adsorbed on hydrophobic surfaces compared to more hydrophilic surfaces were noted. It is possible that a balance between surface adsorbed proteins is required for improving cell attachment and proliferation.

Cellular morphology, attachment and proliferation of cells on chitosan surfaces prepared in this study under varying neutralization regimens were evaluated by scanning electron microscopy [55]. Figure 4 shows the representative SEM pictures of adherent cells on neutralized 3D scaffolds. Panel (a) shows cells on scaffolds neutralized with 0.1 M NaOH for 10 minutes where an elongated cell shape (spindle shape) was observed and a high rate of proliferation was noted. Panel (b) shows cells on scaffolds neutralized with 0.25 M NaOH for 10 minutes cells also look elongated, proliferation also was high but cell attachment to the scaffold walls was fair, the cells formed a layer and stayed detached from the scaffold. Panel (c) shows cells on scaffolds neutralized with 0.5 M NaOH for 10 minutes cells look more rounded, proliferation was very low; attachment was poor with few cellular processes, less than 3 extending to the scaffold surface. Panel (d) shows cells on scaffolds neutralized with 0.1 M NaOH for 30 minutes presented elongated cell shape (spindle shape), proliferation was high, cell attachment was good. Panel (e) shows cells on scaffolds neutralized with 0.25 M NaOH for 30 minutes, cells look rounded, high proliferation, and good cell attachment to the scaffold. Panel (f) shows cells on scaffolds neutralized with 0.5 M NaOH for 30 minutes, cells look more rounded and they formed clumps, proliferation was very low. Elongated cells are characteristic of fibroblastic shape or in this case dedifferentiated chondrocytic shape, while rounded shape is characteristic of differentiated chondrocytes. Neutralization with 0.25 M NaOH for 30 min., 0.5 M for 10 and 30 min rendered rounded cell shape consistent with differentiated chondrocytes, although cells on scaffolds neutralized with 0.5 M NaOH did not show high proliferations.

Cell density was calculated from 6 to 8 images with the cell counter plug-in available in ImageJ software, where the cell nuclei were counted and their number was divided by the calculated area of the image. Cell proliferation observed in these images is consistent with cell counting in Figure 5. Figure 5 shows the cell counts obtained from scaffolds with indicated pretreatments. The highest cell counts were obtained for scaffolds neutralized with 0.25 M NaOH for 30 min, followed by scaffolds neutralized with 0.1 M NaOH for 10 min. Number of cells in scaffolds treated with 0.25 M NaOH for 30 min were significantly ($^{*}P < 0.005$) higher than number of cells on scaffolds treated with 0.5 M sodium hydroxide for 30 mins. Number of cells in scaffolds treated with 0.1 M sodium hydroxide for 10 minutes is significantly ($^{**}P < 0.01$) higher than number of cells on scaffolds treated with 0.5 M sodium hydroxide, 10 min. In separate experiments, scaffold morphology (in the absence of cells) was determined before and after neutralization and no visible change in scaffold morphology was noted (data not included).

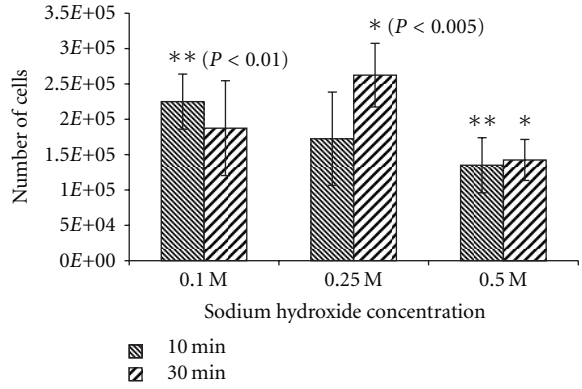

Figure 5: Cell counts obtained from scaffolds after 7 days in culture. Cells were released from scaffolds by trypsinization. Significance was considered to be $P < 0.05$.

To render the surface cell compatible, acid solubilized chitosan surfaces have been subjected to surface treatments with nitrogen or argon plasma treatment at 20 W for 10–40 min. [41]. These modifications were noted to produce an increase in surface roughness as a consequence of the etching process and resulted in improved cell adhesion and proliferation. In this study, the neutralization protocol employed was not observed to impact the gross scaffold morphology; however, we will in a future study characterize the surfaces by atomic force microscopy (AFM).

We have also evaluated the effect of scaffold pretreatments on the cellular morphology and the formation of focal adhesions. The organization of cytoskeletal filamentous actin was examined along with vinculin distribution, and localization of the two. The presence of vinculin is indicative of formation of focal adhesions [56]. Focal adhesions are large protein complexes that act as connectors between the cell and the extracellular matrix. Focal adhesions are important also as signal transduction sites by binding integrins and cytoplasmic proteins as vinculin paxillin, tallin and α-actinin. It has been reported that IL-1 can physically associate to focal adhesions and affect calcium signaling in chondrocytes [57] and can respond to surface roughness [58]. Figure 6 shows the superimposed confocal images for actin-vinculin in cells that were grown on chitosan films and glass slides, respectively. Again, chitosan films were used instead of scaffolds, as they present better surfaces for microscopy. Representative pictures that were obtained upon neutralization for 10-minutes are shown. Panels (a), (b), and (c) show the distribution of actin-vinculin distribution in cells cultured on glass slides. Focal adhesions were apparent by the presence of vinculin, green spots on the cell ends, marked by yellow arrows, on glass slides regardless of NaOH concentration used and time of incubation. Similar images were obtained for other neutralization times (data not included). Some colocalization of vinculin and actin was evident from the yellow ends in the cells marked as white arrows in the figures. Panel (d) shows the confocal images of cells grown on chitosan films that were neutralized with 0.1 M NaOH for 10 minutes. Cells were noted to present a well-spread appearance, and the presence of focal adhesions was also evident by the presence of vinculin. Panel (e) shows the confocal images of cells grown on chitosan films that were neutralized with 0.25 M NaOH for 10 minutes, where the formation of focal adhesions and the presence of cytoskeletal filamentous actin were not noted. Panel (f) shows the confocal images of cells grown on chitosan films that were neutralized with 0.5 M NaOH for 10 minutes, where the cells look also spread with no detection of filamentous actin or focal adhesion. Panel (g) shows the confocal image of cells grown on chitosan films that were neutralized with 0.1 M NaOH for 30 minutes cells show spread morphology, no stress actin fibers or focal adhesions; panel (h) shows the confocal image of cells grown on chitosan films that were neutralized with 0.25 M NaOH for 30 minutes, cells look rounded, high proliferation and good cell attachment to the scaffold, the formation of focal adhesions and the presence of cytoskeletal filamentous actin were not noted; panel (i) shows the confocal image of cells grown on chitosan films that were neutralized with 0.5 M NaOH for 30 minutes, cells look more rounded, smaller in size and they formed clumps, proliferation was very low. Actin was just detected on the cell membrane and no focal adhesions were detected. The presence of focal adhesions is directly or indirectly related to nature and amount of surface adsorbed and it is postulated that integrin-mediated attachment of cells to surfaces is protein dependent. In our study, only those surface that presented higher amounts of vitronectin (0.1 M NaOH, 10 min) developed focal adhesions.

The effect of scaffold pretreatment on the distribution of intermediate filaments (IFs) and cell morphology was also evaluated. IFs also form a mesh that spans from the cell nucleus to the cell membrane but its function is more related to mechanical signal transduction via integrins and also by directly transducing cell deformation to nuclear deformation [59]. Chondrocytes contain vimentin as IF, its assembly is regulated by phosphorylation and it is also in a dynamical equilibrium between polymerized and unpolymerized forms [60], the rounded shape of differentiated chondrocytes is preserved by vimentin [61].

Cytoskeletal intermediate filament vimentin was examined along with actin distribution. Figure 7 shows the superimposed confocal images for actin-vimentin. Representative pictures that were obtained upon neutralization for 10 minutes are shown. Figures 7(a), 7(b), and 7(c) shows actin-vimentin distribution of cells grown on glass slides. Vimentin was noted to be present as a very well-spread network of filaments surrounding the nucleus, and expanding throughout the cytoplasm. The distribution of vimentin was not impacted by the concentration of NaOH used or the time of the neutralization step. Panel (d) shows the confocal image of cells grown on chitosan films that were neutralized with 0.1 M NaOH for 10 minutes, where cells were noted to be well-spread and the vimentin network was noted to resemble the distribution seen on glass slides; panel (e) shows the confocal image of cells grown on chitosan films that were neutralized with 0.25 M NaOH for 10 minutes, vimentin filaments were noted to surround the nucleus in a rounded shape but filament distribution around the nucleus

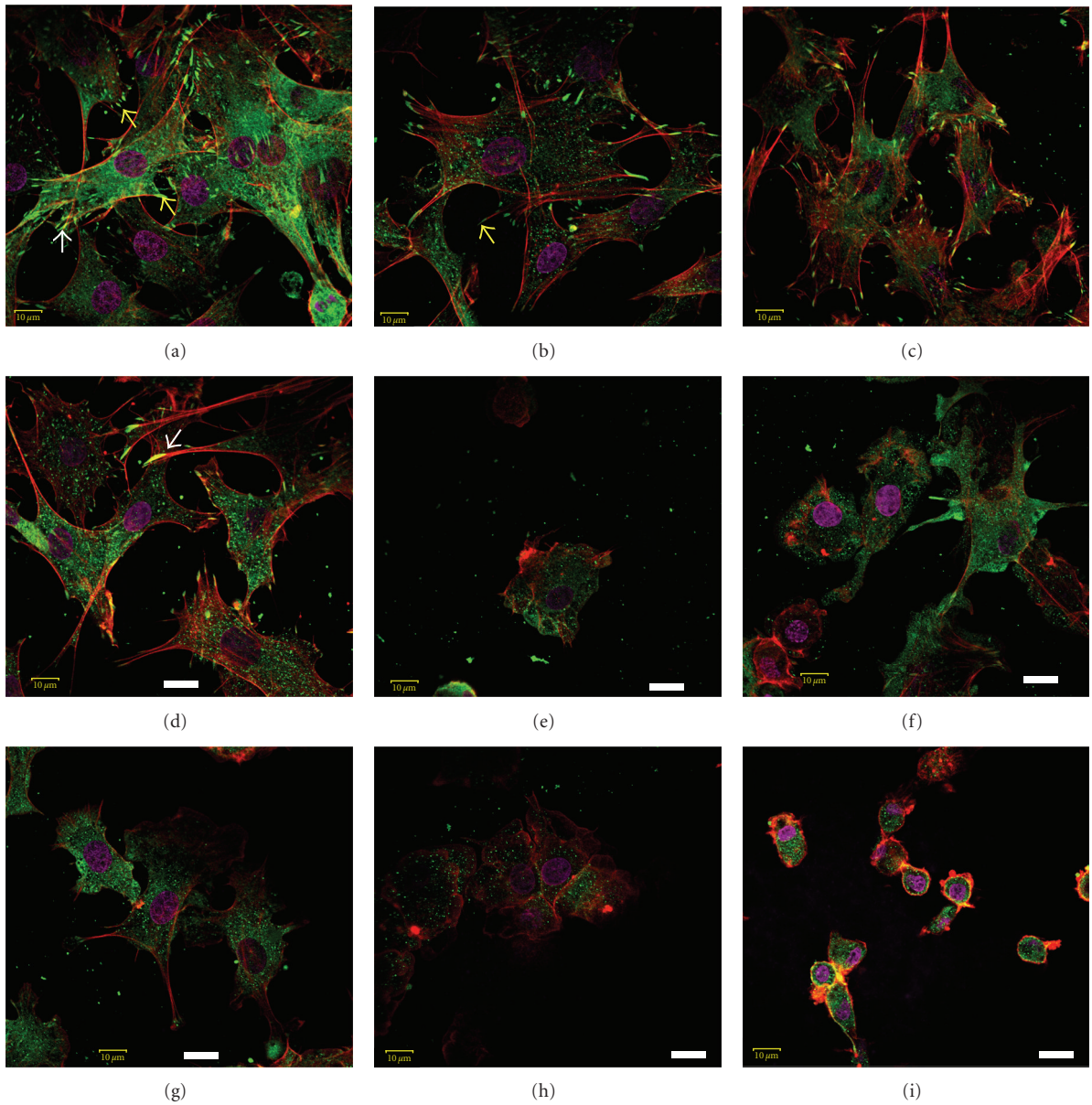

Figure 6: Visualization of actin and vinculin distribution by confocal microscopy. Cells on either glass slides or chitosan films were stained for actin or vinculin as detailed in the methods section. Panels (a), (b), and (c) shows the distribution of actin/vinculin on glass slides. Panels (d), (e), (f), (g), (h), and (i) show the distribution of actin/vinculin on chitosan films. Panel (d) shows chitosan films neutralized with 0.1 M NaOH for 10 minutes, panel (e) shows chitosan films neutralized with 0.25 M NaOH for 10 minutes, and panel (f) shows chitosan scaffolds neutralized with 0.5 M NaOH for 10 minutes, panel (g) shows chitosan films neutralized with 0.1 M NaOH for 30 minutes, panel (h) shows chitosan films neutralized with 0.25 M NaOH for 30 minutes, and panel (i) shows chitosan films neutralized with 0.5 M NaOH for 30 minutes. Similar distributions were noted at different incubation times evaluated. Scale bar: 10 μm.

was asymmetrical, the cytoskeleton does not posseses actin filaments; panel (f) shows the confocal image of cells grown on chitosan films that were neutralized with 0.5 M NaOH for 10 minutes where the cell population is a mixture between well-spread cells and rounded shape, vimentin distribution consistent with shape; panel (g) shows the confocal image of cells grown on chitosan films that were neutralized with 0.1 M NaOH for 30 minutes, where the cells were well-spread and vimentin cytoskeleton was also well-spread; panel (h) shows the confocal image of cells grown on chitosan films that were neutralized with 0.25 M NaOH for 30 minutes, cells look rounded, high proliferation and good cell attachment to the scaffold vimentin network surrounds the nucleus in a symmetric fashion; panel (i) shows the confocal image of cells grown on chitosan films that were neutralized with 0.5 M NaOH for 30 minutes, cells look more rounded and they formed clumps, proliferation was very low. Vimentin network encloses the nucleus but the network looks more

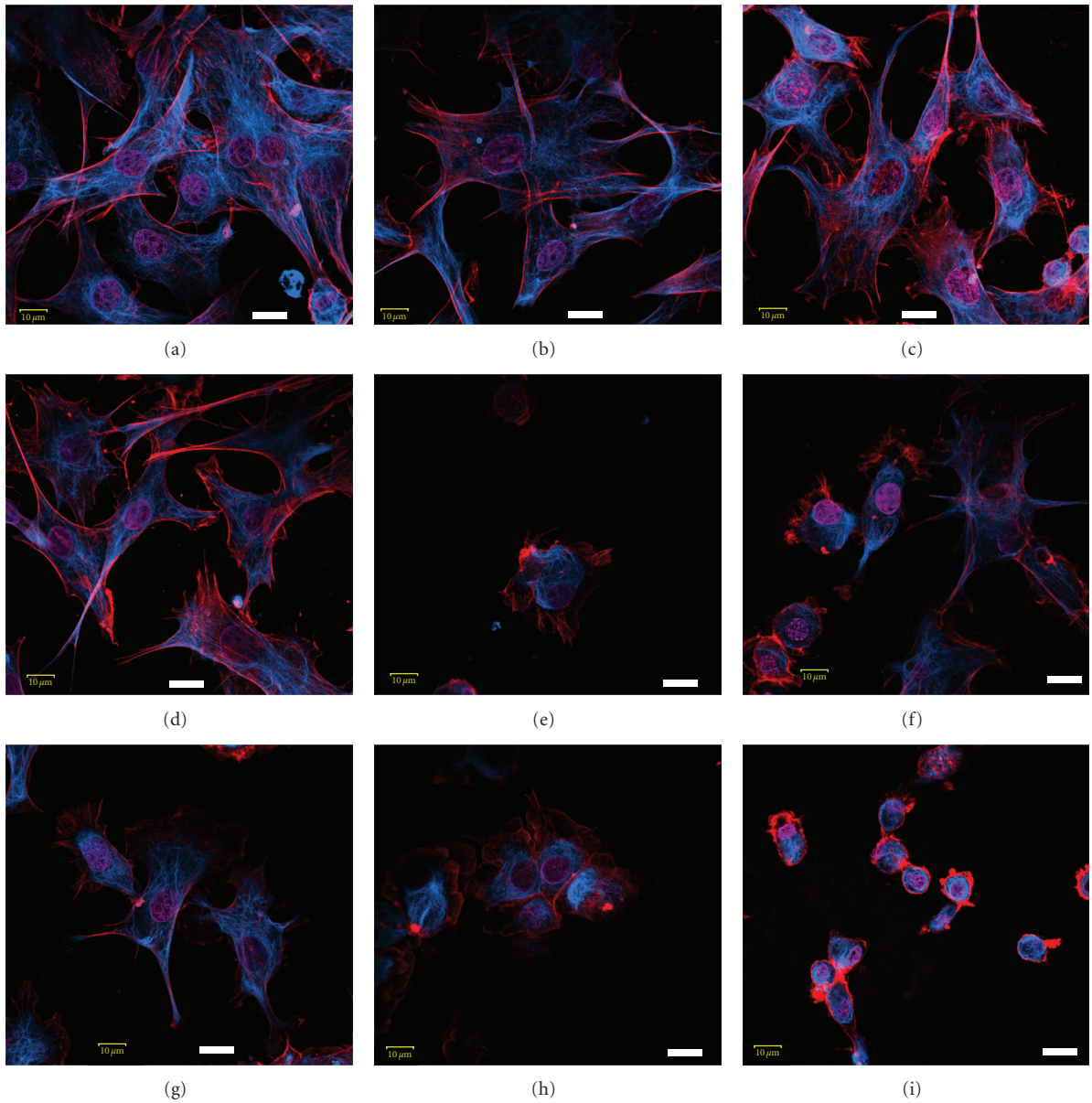

Figure 7: Visualization of the distribution of actin and vimentin by confocal microscopy. Cells on either glass slides or chitosan films were stained for actin or vimentin as detailed in the methods section. Panels (a), (b), and (c) show the distribution of actin/vimentin on glass slides. Panels (d), (e), (f), (g), (h), and (i) show the distribution of actin/vimentin on chitosan films. Panel (d) shows chitosan films neutralized with 0.1 M NaOH for 10 minutes, panel-E shows chitosan films neutralized with 0.25 M NaOH for 10 minutes, and panel (f) shows chitosan scaffolds neutralized with 0.5 M NaOH for 10 minutes, panel (g) shows chitosan films neutralized with 0.1 M NaOH for 30 minutes, panel (h) shows chitosan films neutralized with 0.25 M NaOH for 30 minutes, and panel (i) shows chitosan films neutralized with 0.5 M NaOH for 30 minutes. Similar distributions were noted at different incubation times evaluated. Scale bar: 10 μm.

compact due to cell size reduction. In the present study, the distribution of vimentin intermediate filaments was noted to be significantly impacted by the different treatments.

To assess the impact of surface pretreatment employed on cellular morphology, Figures 6 and 7 were quantitatively analyzed by using ImageJ software. The cell shape was estimated by the circularity of the cell, defining circularity as 4∗pi∗Cell Area (A)/Cell perimeter (P). The shape and size of the nuclei was also determined in a similar manner. The longest distance between two points in a cell, which give us an idea of the longest dimension in a cell, is often measured by the Feret's diameter [62]. A rounded cellular morphology has been reported to be characteristic of a chondrocytic phenotype whereas elongated cellular shapes are reported to more closely resemble a fibroblastic phenotype. Therefore we assign a circularity value of one or close to one to cells typical of chondrocytic phenotype. Figure 8 shows cell circularity, nuclei circularity, cell spread area/nuclei projected area, cell

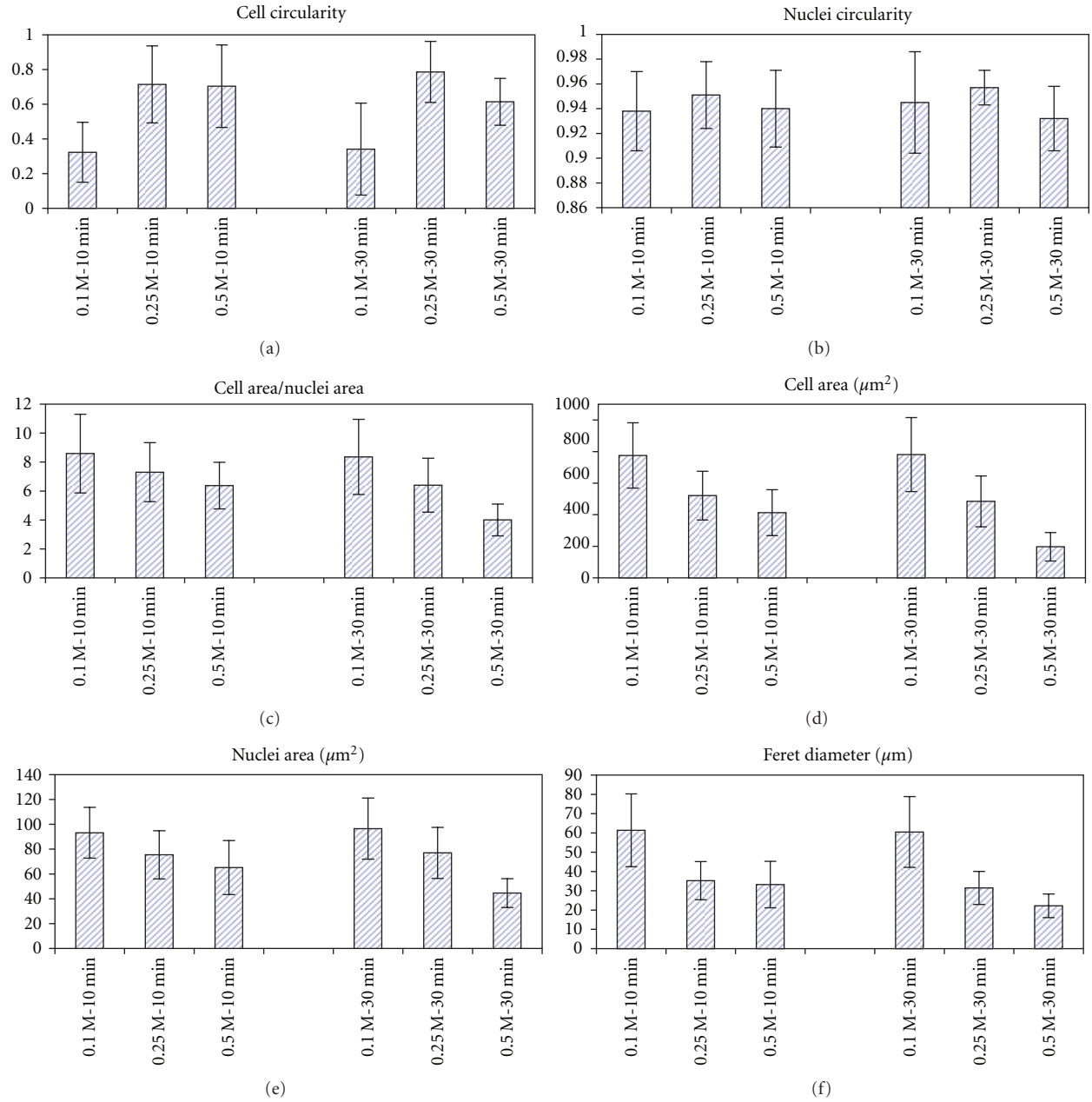

Figure 8: Quantitative analyses of Figure 6. Cell circularity (a), nuclei circularity (b), cell spread area/nuclei projected area (c), cell spread area (d), nuclei projected area (e), and feret diameter (f) for all conditions, the data were extracted from confocal images from 2 different experiments, five sections from each experiment were analyzed. Reported values are mean values ± S.D.

spread area, nuclei projected area and Feret's diameter for all pretreatment conditions evaluated. The data were extracted from confocal images from 2 different experiments; five sections from each experiment were analyzed. Figure 8(a) shows that cell shape is close to circular shape in samples treated with 0.25 M and 0.5 M sodium hydroxide for 10 min, also for 0.25 M sodium hydroxide for 30 min. with values of 0.8 ± SD, while in cells growing on chitosan surfaces treated with 0.1 M NaOH for 10 minutes or 30 minutes, cell circularity decrease to 0.3 ± SD indicating a loss of rounded shape and therefore of chondrocytic morphology. Cell projected area is also reported in Figure 8(d), and a correlation in the reduction of the projected area or cell size with increasing NaOH concentrations can be noted. Nuclei circularity was higher than 0.9 in all cases as shown in Figure 8(b). Interestingly, the size of the nuclei was noted to change, as determined from the changes in the projected area of the nuclei shown in Figure 8(e), and this trend was noted to mimic or be in agreement with the observed changes in the cell projected area.

In monolayer culture, chondrocytes dedifferentiate into fibroblastic morphology, losing their rounded shape and acquiring a flat and spread shape [63]. Actin in freshly isolated chondrocytes has been found to be focally organized at

the cell surface resembling a cortical structure. Vimentin in contrast forms a dense network of filament beneath the cell surface and surrounds the nucleus [61, 64]. Differences in cell spreading were confirmed by the analysis of confocal images. Focal adhesion formation (yellow arrows in panels 6(a) and 6(b)) was evident in cells cultured on glass slides. Vinculin and filamentous actin was observed to colocalize as evidenced by the yellow color formed from the presence of actin (red label) and vinculin (green label) in the same spot at the focal contacts in well-spread cells (see Figure 6, panel (d), white arrow), for cells seeded and cultured on chitosan surfaces that were pretreated with 0.1 M NaOH for 10 minutes. The vimentin network was widespread and seems to be localized following the actin cytoskeleton shape under these conditions in accordance with observations detailed elsewhere [65], suggesting a direct interaction between the tail domain of vimentin and F-actin. This interaction it was proved to enhance the mechanical properties of mixed actin/vimentin filament networks compared with F-actin or vimentin only networks which would be the cell response to the attachment of the cell through focal contact to the surface, the cell is trying to match stiffness of the surface or balance the forces applied by these focal contact to the cytoskeleton in accordance with Ingber's tensegrity model [66–68]. Blain et al. [64] have reported a distinct shrinkage of chondrocytes associated with vimentin cytoskeleton disruption by acrylamide treatment. A shrinkage of approximately 50% was seen across their experiments. In our case a decrease of approximately 30% and 50% was detected for cells seeded over films pretreated with different sodium hydroxide treatment and 10 minutes of exposure as shown in Figure 8(d). A decrease of approximately 38% and 75% was seen over surfaces exposed to sodium hydroxide for 30 min, see Figure 8(d).

Polyelectrolyte behavior of chitosan in acidic solutions is governed by the chelating capacity of amino groups. In acidic solution amino groups are protonated to NH^{3+} with a pKa value of 6.1 to 6.4. Viscosity in the absence of NaCl increases with protonation of amine group, in contrast when NaCl is present viscosity is nearly independent of pH [69]. This polyelectrolyte behavior can be the answer to the differences found in our study. When a chitosan acetate surface is contacted with sodium hydroxide solution, acetate ions tend to associate to its counterion (Na^+) and at the same time NH^{3+} looses a proton to form a molecule of water, forming a microenvironment on the film surface that assumes the characteristics of a polyelectrolye. The layer in contact with the film present higher viscosities at lower pH (0.5 M NaOH concentration) and lower viscosities at higher pH (0.1 M) rendering a surface of different characteristics for each value of sodium hydroxide concentration used. With concentrations higher than 0.5 M the effect of high viscosity is more pronounced and can only be alleviated by addition of some sodium salt, a practice that has been applied by some group with success [43, 44]. In conclusion, pretreatment of chitosan surfaces was noted to impact cell proliferation and differentiation and our studies have helped identify a set of scaffold pretreatment conditions which favor the chondrocytic shape.

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Review Article

Chitosan-Grafted Copolymers and Chitosan-Ligand Conjugates as Matrices for Pulmonary Drug Delivery

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Recently, much attention has been given to pulmonary drug delivery by means of nanosized systems to treat both local and systemic diseases. Among the different materials used for the production of nanocarriers, chitosan enjoys high popularity due to its inherent characteristics such as biocompatibility, biodegradability, and mucoadhesion, among others. Through the modification of chitosan chemical structure, either by the addition of new chemical groups or by the functionalization with ligands, it is possible to obtain derivatives with advantageous and specific characteristics for pulmonary administration. In this paper, we discuss the advantages of using chitosan for nanotechnology-based pulmonary delivery of drugs and summarize the most recent and promising modifications performed to the chitosan molecule in order to improve its characteristics.

1. Introduction

Alongside the successful market launch of different products over the last decades, a continuous effort to formulate delivery systems for the pulmonary administration of a wide variety of drugs has been extensively described in the literature [1, 2]. The particular anatomical, physiological, and pathophysiological features of the respiratory tract pose enormous challenges that need to be overcome in order to obtain effective lung deposition, uniform distribution, *in loco* retention (main challenge is to circumvent mucocilliary clearance), and stability (particularly to enzymatic degradation) of therapeutic agents [1, 3].

Nevertheless, particular attention has been dedicated not only to drugs themselves but also to excipients required to improve the bioavailability of drugs administered pulmonarily. In this context, excipients that could transiently enhance the absorption of drugs are on the spot. Chitosan, a polysaccharide with structural characteristics similar to glucosamines and obtained by the alkaline deacetylation of chitin, derived from the exoskeleton of crustaceans, is one of such appealing excipients.

The safety and tolerability of chitosan are synergistic characteristics towards its application in drug delivery by different administration routes. Despite the natural properties, some drawbacks are associated with the poor solubility at physiologic pH and the passive targeting effect. Thus, chemical modifications of chitosan by conjugating various functional groups allow the control of the hydrophilicity and the solubility at neutral and basic pH and open new opportunities to expand the application of this biopolymer.

In this paper, we revise some of the most recent and promising modifications performed to chitosan with special focus on its employment in the pulmonary delivery of drugs.

2. Advantages of Chitosan for Pulmonary Delivery of Drugs

Chitosan possesses different beneficial properties that make it an attractive option for designing adequate dosage forms and advanced drug delivery systems to be administered to or through the lung. General advantages include the well-established biocompatibility and biodegradability of chitosan [4]. Moreover, antimicrobial [5] and antioxidant [6, 7] activities have been reported for different types of chitosan and derivatives, which can also be regarded as potentially useful for the development of pulmonary drug delivery systems. The processability of chitosan and several derivatives allow obtaining different types of systems (powders or well-structured micro- and nanocarriers) that can be optimized in order to present optimal aerodynamic particle diameters for lung deposition and retention [8–11]. Also, the presence of reactive amine groups grants chitosan the chemical versatility for modification and functionalization (Figure 1) [12].

Chitosan is a cationic mucoadhesive polymer. The ability to establish ionic, hydrogen, and hydrophobic bonding with negatively charged chains of mucin [13], the structural component of mucus fluids, evidences its potential for increasing lung retention of drug carriers comprising chitosan. The mucoadhesive properties of the native polymer can be further increased by chemical modification. In particular, thiolation (i.e., attachment of side chains containing thiol groups) has been proved an interesting strategy for this last purpose [14]. In one recent study, Makhlof et al. [15] reported on the enhanced mucoadhesion of nanoparticles composed of thioglycolic acid-glycol chitosan, as compared to nanoparticles based on the nonthiolated polymer (i.e., glycol chitosan), after intratracheal administration to rats. More importantly, the increment in pulmonary mucoadhesion observed by these researchers was correlated with the greater bioavailability of calcitonin when this peptide was associated with nanoparticles. However, mucoadhesive properties of chitosan and derivatives may also be detrimental, since adhesion of delivery systems at the upper respiratory tract and airways can also occur, thus limiting the amount of carrier that effectively reaches the deep lung. Formulating scientists should keep this phenomenon in mind when developing chitosan-based systems.

Chitosan is able to enhance absorption of drugs by the paracellular route, in particular macromolecules, due to the transiently disruption of tight junctions (Figure 2) [16].

In order to confirm this effect in pulmonary drug delivery, Yamamoto et al. [17] performed *in vivo* experiments in guinea pigs by comparing the pulmonary absorption of different model drugs in solution (carboxyfluorescein and fluorescein isothiocyanate dextran with molecular weight (MW) varying from 4 to 70 kDa), either in the presence or absence of chitosan. Results showed significantly higher permeation for all the investigated model drugs in the presence

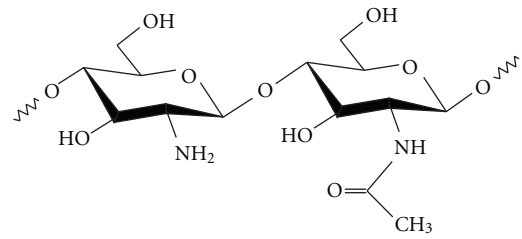


Figure 1: Chemical structure of chitosan, comprising *N*-acetyl-D-glucosamine (right) and D-glucosamine (left) units.

of chitosan, as assessed by blood drug levels. Absorption enhancement was higher for higher MW isothiocyanate dextran, which is mainly absorbed by the paracellular route, providing evidence for the mechanism of intercellular tight junction disruption.

Toxicological data on chitosan has been extensively reviewed and is regarded to be favorable when considering its use as a pharmaceutical excipient [4]. In the particular case of pulmonary drug delivery, *in vitro* cytotoxicity experiments were conducted for chitosan presenting different degrees of deacetylation and MW using human embryonic lung cells (L132 cells) [18]. Results revealed that chitosan presented 50% inhibitory concentration (IC_{50}) values higher than 1 mg/mL. Studies have also been conducted for derivatives of chitosan or chitosan-based formulations intended for pulmonary administration. For instance, Grenha et al. [19] showed that chitosan nanoparticles obtained by ionotropic gelation with tripolyphosphate and entrapped in mannitol microparticles presented reduced toxicity (concentrations of up to 1.3 mg/mL of nanoparticles were tested) to adenocarcinoma epithelial lung cell lines (Calu-3 and A549). In another study conducted in A549 cells, stearic acid-*g*-chitosan oligosaccharide micelles presented an IC_{50} value of approximately 369 μg/mL [20]. However, chitosan and derivatives may also induce immune responses by lung cells/tissue. In an *in vitro* study, Calu-3 cells exposed to chitosan microparticles were able to elicit the release of inflammatory cytokines (IL-2 and IL-8) [21]. Florea et al. [22] performed *in vivo* studies in rats by administering intratracheally chitosan and two brands of *N*-trimethylated chitosan (TMC), presenting different degrees of substitution (20% and 60%). Histopathological analysis of lung tissue showed that chitosan elicited neutrophil infiltration and structural damage in the lung parenchyma; however, this effect may be attributable not to the inherent toxicity of chitosan but most probably to the physical obstruction of the bronchioles due to higher viscosity of the chitosan formulation, thus causing local asphyxiation. In the case of TMC, these effects were milder, which could be associated with lower viscosity when compared to chitosan. Comparable inflammatory effects were also observed in rats after the intratracheal administration of chitosan microparticles [23]. Moreover, the production of several proinflammatory cytokines has been observed for hydrophobically modified glycol-chitosan nanoparticles *in vivo* after intratracheal instillation in mice [24]. Even if generally regarded as detrimental, immune stimulation may

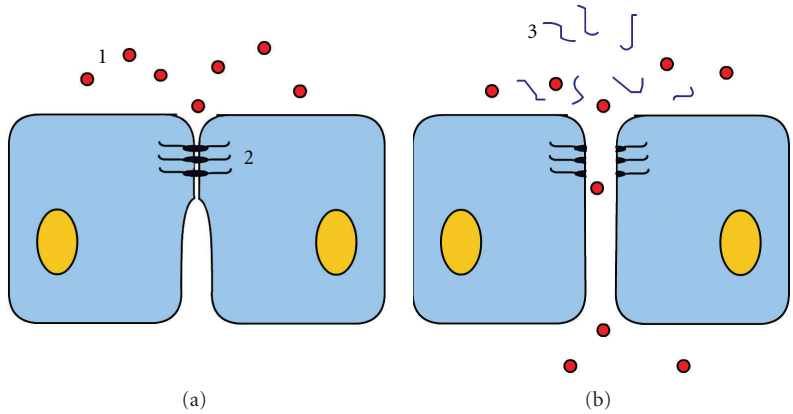


Figure 2: Effect of chitosan on the absorption of drugs by the paracellular route. (a) Normal epithelium. (b) Transient disruption of tight junctions by chitosan with enhancement of drug absorption. 1: represents the drug, 2: represents the tight junction, and 3: represents chitosan.

be a particularly interesting feature for vaccine development [25].

3. Chitosan and Chitosan-Grafting Copolymers for Nanoparticle-Based Pulmonary Drug Delivery Systems

The first nanoparticles with drug delivery purposes began in the late 1960s [26]. Nanoparticles are solid colloidal particles made of macromolecular materials ranging in size from 1 to 1000 nm, although sometimes the term identifies particles in the 1 to 200 nm range, depending on the application field [27]. For pharmaceutical and medical applications, nanoparticles can be used therapeutically as carriers, either by dissolving, entrapping, or encapsulating the active substance (drug or biologically active material) or by adsorbing or attaching the active substance on the surface.

Different nanoparticles have been developed for pulmonary administration of various drugs to treat diseases such as tuberculosis (TB) [28–30] and other pulmonary infections [31] and diseases [31, 32]. Since this natural polymer offers remarkable advantages over other natural and synthetic polymeric carriers, in this section, we will focus on chitosan-grafting-based polymeric nanoparticles as drug carriers.

3.1. Trimethyl Chitosan (TMC). Trimethylated chitosan (TMC) is a partially quaternized chitosan derivative that is freely soluble in aqueous solutions over a wide range of pH as compared to other chitosan salt derivatives (Figure 3). TMC is obtained by reductive methylation of chitosan using methyl iodide in the presence of a strong base (e.g., NaOH) at 60°C [33, 34]. This soluble chitosan derivative has mucoadhesive properties and displays excellent absorption-enhancing properties, even at neutral pH [35, 36]. This capability as enhancer is due to opening the tight junctions between adjacent epithelial cells through interactions between the protonated (positively charged) amino groups on the C-2 position and the negatively charged sites on the cell membrane and/or in the tight junctions [37]; TMC has

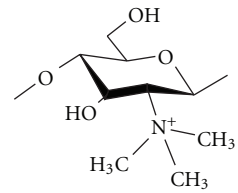


Figure 3: Chemical structure of trimethyl chitosan (showing the modification at the D-glucosamine unit).

positive charges, independently of the pH, at all degrees of quaternization [38]. An increase in the degree of quaternization leads to increase of the permeation-enhancing effect of TMC [34].

TMC-based nanoparticles have consistently shown their feasibility for mucosal immunization. Studies have addressed oral vaccination against *Helicobacter pylori* using urease [39]. Intranasal administration of the influenza antigen elicited local and systemic immune responses in mice [40]. A similar approach was assessed with the tetanus toxoid by nasal instillation in mice [41]. TMC nanoparticles were loaded with FTIC ovalbumin, and their transport across the nasal epithelium of rat was studied [42]. A recent study has also shown that the delivery of the model antigen ovalbumin (OVA) to the cervical lymph nodes in a nanoconjugated form with TMC (~30 nm diameter) was twice more effective than the nasal administration of ovalbumin-containing TMC nanoparticles with size of one order of magnitude greater (~300 nm diameter) [43]. In turn, synergistic effects of the TLR9 ligand CpG in TMC particles have been reported after nasal vaccination [44].

Florea et al. observed that 20% and 60% N-TMC (TMC20 and TMC60) enhanced the permeation of octreotide *in vitro* by 21-, 16-, and 30-fold. Also, the bioavailability was enhanced by 2.4-, 2.5-, and 3.9-fold, respectively. Cell viability and histology studies showed that the TMCs are safer than chitosan and that Calu-3 cell monolayers are a valuable model for predicting the paracellular transport kinetics in

the airway epithelia. Taking into account these results, they sustain that cationic polysaccharides are promising enhancers for peptide drug absorption with prospect for sustained-release formulations [22]. Slutter et al. reported on the conjugation of an antigen, OVA, to TMC and the preparation of nanoparticles for subunit vaccination. The uptake of TMC-OVA conjugates by dendritic cells was similar to the uptake of TMC/OVA nanoparticles and over 5-fold greater when compared to a solution of OVA and TMC. Conjugation of the antigen to TMC and TMC/OVA is therefore a viable strategy to increase the immunogenicity of subunit vaccines [45].

3.2. Carboxymethyl Chitosan (CMC). CMC is prepared by adding a carboxymethyl group in the structure of chitosan. This modification increases its solubility in neutral and basic solutions without affecting other important characteristics [46]. CMC is prepared by carboxymethylation of the hydroxyl and amine chitosan groups [47]. Different substitutions patterns can be obtained according to the reaction temperature used (Figure 4). At room temperature the O-substitution is favored, while at higher temperature the N-substitution is the preferred pathway. Taking into account reaction conditions and reagents, different derivatives can be produced, that is, N-, O-, N,O-, or N,N-dicarboxymethyl chitosan [48].

CMC nanoparticles were prepared as carriers for some anticancer drugs. For example, Shi et al. used different kinds of CMC with various molecular weights and degrees of substitution to prepare nanoparticles through ionotropic gelification with calcium ions. These results showed the feasibility of CMC nanoparticles to entrap doxorubicin and the potential to deliver it following a controlled profile [49]. Anhitha et al. prepared curcumin-loaded O-CMC nanoparticles (curcumin-O-CMC Nps) as a novel carrier in cancer drug delivery applications. In L929, MCF-7, and PC-3 cell lines, the O-CMC NPs without drug showed no cytotoxicity, whereas curcumin and curcumin-O-CMC NPs resulted in considerable cell death. Cellular uptake was analyzed by fluorescence microscopy. Control cells without any exposure to NPs and cells incubated with O-CMC NPs showed no fluorescence. Conversely, cells incubated with curcumin-O-CMC NPs displayed green fluorescence, confirming the internalization of the particles [46]. In another work, tea polyphenols (TPs) were loaded into carboxymethyl chitosan and chitosan hydrochloride [50]. *In vitro* studies showed that TPs were controlled released from nanoparticles in PBS at pH 7.4. In addition, the cell apoptosis rate was increased from 30% after 24 h to 62% at the end of 72 h, induced by loaded nanoparticles.

CMC-based nanoparticles of varying average size (40–400 nm diameter) were developed for intranasal immunization [41]. An interesting finding of this study was that TMC-MCC composite nanoparticles obtained by electrostatic complexation of the two polymers with a positive surface charge exhibited higher immune responses when compared to chitosan, TMC, and MCC nanoparticles.

Li et al. prepared nanoparticles with oleoyl-carboxymethyl chitosan encapsulating rifampicin as drug delivery systems [51]. These nanoparticles were not tested for any specific route of administration. But their pulmonary administration could be a possibility to treat tuberculosis. Therefore, it is necessary to carry out studies to test the feasibility of these NPs as inhaled drug delivery systems.

3.3. N-Succinyl-Chitosan (NSC). NSC is a chitosan derivative obtained by the incorporation of succinyl moieties into the N-terminal group of the glucosamine units (Figure 5) [52]. Like other derivatives, NSC displays good water solubility in a broad pH range, and it is considered biocompatible both *in vitro* and *in vivo*. NSC was initially developed as a wound dressing material combined with collagen. It is also recognized as an excellent cosmetic ingredient (Moistfine liquid, INCI name Chitosan Succinamide).

Some authors have used this chitosan derivative to prepare anticancer-drug-loaded NPs. For example, Hou et al. synthesized a new NSC derivative by means of microwave irradiation. They entrapped successfully hydroxycamptothecin (HCPT) into the NSC nanoparticles and observed tumor targeting and significant suppression of tumor growth after s.c. injection (close to the tumor) in mice bearing S180 sarcoma tumor [53]. Yan et al. prepared similar nanoparticles loaded with 5-fluorouracil (5-FU) [54]. They evaluated biodistribution and tumor targeting after i.v. administration in Sarcoma 180-bearing mice. The 5-FU-loaded NPs were biodistributed mainly in the tumor and liver, being found small quantities in kidney and spleen [54]. Luo et al. evaluated antitumor effects of NSC nanoparticles (NSCNPs) without drugs in K562 cells [55]. The results revealed that NSCNP could inhibit the proliferation of K562 with an IC_{50} of 37.78, 14.26, 10.93, and 9.78 υg/mL at 12, 24, 36, and 48 h, respectively. According to a cytomorphology study and the analysis of DNA fragments, the antitumor effect of NSCNP is achieved by necrosis and apoptosis induction in K562 cells.

3.4. PEGylated Chitosan. Grafting of hydrophilic polymers such as PEG onto chitosan is a well-known strategy to improve the solubility and biocompatibility of chitosan as well as to achieve lower recognition by the host immune system and increased blood circulation time (Figure 6) [56]. These PEG chains create a barrier layer to prevent the adhesion of opsonins present in the blood, so that the particles can be "invisible" to phagocytic cells.

Due to the advantages that PEG confers to chitosan, chitosan-g-PEG copolymer has been prepared and utilized to develop various types of nanocarriers for transmucosal drug delivery. Chitosan-g-PEG nanoparticles have been prepared by ionotropic gelation with TPP [57]. This system displayed a high association efficiency (>78.6%) leading to insulin loading values as high as 38.6%. Results of *in vivo* studies after intranasal administration to healthy rabbits showed that the plasma glucose levels fell sharply and remained at a low concentration for, at most, 2-3 h and returned to baseline after 5 h. Other studies have shown that, apart from the reduction of the cytotoxicity, PEGylation of TMC led to improved colloidal stability of polyplexes and significantly increased

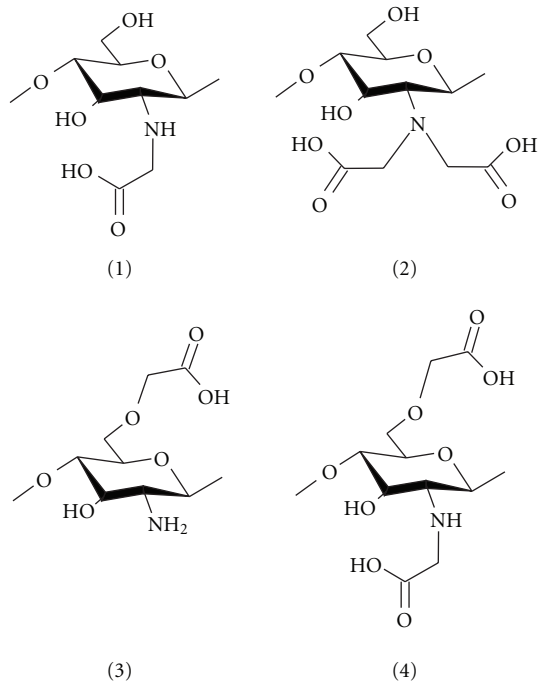


Figure 4: Chemical structure of different types of carboxymethyl chitosan (CMC): (1) *N*-CMC, (2) *N,N*-CMC, (3) *O*-CMC, and (3) *N,O*-CMC (showing the modification at the D-glucosamine unit).

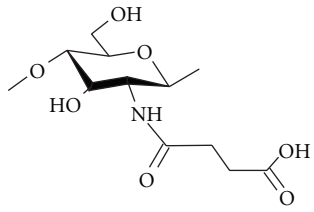


Figure 5: Chemical structure of *N*-succinyl-chitosan (showing the modification at the D-glucosamine unit).

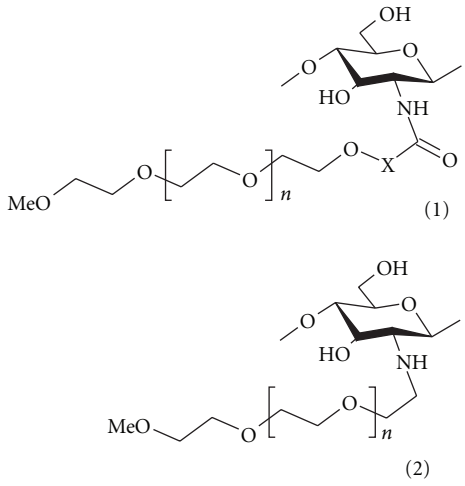
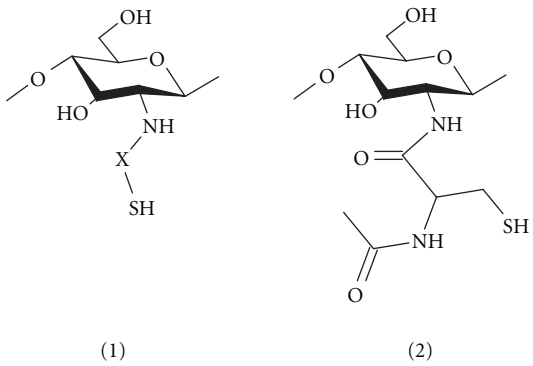


Figure 6: PEG-*g*-chitosan as obtained by the two most commonly used synthetic routes: (1) reaction with an active ester derivative and (2) reductive amination. X: linker.

cellular uptake compared to unmodified TMC [58]. These improvements resulted in a significant, up to 10-fold, increase of transfection efficiency in NIH/3T3, L929, and Me-Wo cells.

Mao et al. prepared chitosan-DNA nanoparticles using a complex coacervation process [59]. They conjugated PEG on the nanoparticles and observed that the clearance of the PEGylated nanoparticles in male AKR/J mice (6–8 weeks) following intravenous administration was slower than that of unmodified nanoparticles at 15 min, with higher depositions in kidney and liver [59]. Other authors developed a functional nanoparticulate carrier for DNA transfection in asialoglycoprotein receptor overexpressed in HepG2 cells. For this, they grafted a methoxy PEG (MPEG) and a receptor ligand, lactobionic acid (LA) in chitosan [60]. They observed that the system with ligand chitosan-(O-MPEG)-(N-LA) showed better transfection efficiency (45.3%) than ligand-free chitosan-(O-MPEG) (19.8%).

3.5. Thiolated Chitosan. Thiol modifications to chitosan and nanoparticles derived from it have been aimed to localize a drug delivery system at a given target site (Figure 7). Recently, it was documented that thiolated chitosan has strong mucoadhesive properties ascribed to the formation of disulfide bonds with cysteine-rich domains of mucus glycoproteins, leading to an improvement in mucoadhesion of up to 140-fold when compared to unmodified chitosan [61]. Insulin-loaded nanoparticles prepared with chitosan-N-acetyl-L-cysteine were found to improve the systemic absorption of insulin after nasal administration [62]. Although the exact mechanism for the enhanced effectiveness of this



(1) (2)

Figure 7: Thiolated chitosan: (1) general structure of thiolated chitosan as modified by an –SH group and (2) chitosan-*N*-acetylcysteine (showing the modification at the D-glucosamine unit). X: linker.

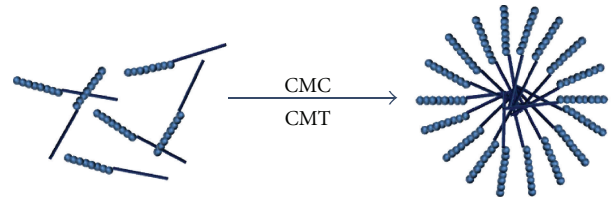


Figure 8: Formation of micelles by self-assembly of polymer monomers above critical micelle concentration (CMC) and critical micellization temperature (CMT).

system remains unclear, it was speculated that the mucoadhesive and permeation-enhancing properties based on disulfide bond "anchorage" increase the accessibility of insulin molecules to the epithelial membrane and, hence, facilitates the absorption of the protein across the nasal epithelium.

4. Chitosan-Based Polymeric Micelles for Pulmonary Drug Delivery

Micelles are spherical nanosized colloidal dispersions with a hydrophobic core and hydrophilic shell, composed by am-phiphilic molecules which self-assembly under certain concentration and temperature conditions (Figure 8). The concentration and the temperature above which the monomers turn in micelles are called critical micelle concentration (CMC) and critical micellization temperature (CMT), respectively [63]. The self-assembly of amphiphilic molecules in water is driven by a gain in entropy of the solvent molecules and a decrease of free energy in the system as the hydrophobic components withdraw from the aqueous media [64]. Two forces are involved in the micelle formation, an attractive force that leads to the association of molecules and a repulsive force that prevents unlimited growth of the micelles [65]. Micelles can be used as drug delivery systems, especially for poorly water-soluble drugs that are incorporated into the micelle core. Like liposomes, polymeric micelles allow the encapsulation of drugs with different polarities. Water-soluble drugs are adsorbed on the micelle surface, and drugs with intermediate polarity are distributed along the amphiphilic molecules [66]. However, they are more stable than liposomes. Furthermore, due to its general small size, micelles have the ability to penetrate tissues, showing also a high encapsulation efficiency and possibility of sterilization by filtration [63, 66, 67].

Recently, much attention has been given to polymeric micelles formed by lipid polymer or block copolymers, generally di- and triblock copolymers, as drug delivery systems. Compared to micelles prepared from conventional detergents, polymeric micelles present a lower CMC value, making them more physically stable, both *in vitro* and *in vivo*, even under dilutions to final concentration below the CMC [68]. Many studies have been published in the last years regarding the use of polymeric micelles as solubilization agents and bioavailability enhancers of different drugs, with emphasis on poorly soluble anticancer drugs [69–73]. They have also been studied as carriers of genetic material [74] and diagnostic agents [75]. Due to its size, generally lower than 200 nm, and hydrophilic surface, micelles are poorly recognized by the reticuloendothelial system (RES) and present long circulation times in bloodstream with enhanced permeability and retention effects (EPR effect) at solid tumor sites or another areas with leaky vasculature such infarcts, infections, and inflammations, for passive targeting [76, 77]. It is also possible to promote the targeting of drugs to specific organs and tissues using stimuli-responsive micelles [77–79] or modulating the micellar surface with active-targeting ligands [77, 80]. By sharing some structural and functional features with natural transport systems, for example, virus and lipoproteins, polymeric micelles can be a useful strategy to solve the problem of drug resistance [81].

Different polymeric micelles have been developed, in the last years, for pulmonary administration of various drugs to treat different diseases such as cancer [82], inflammation conditions [83], and pulmonary infections [84–86]. They have been also studied as carriers of genetic material to treat cystic fibrosis [87]. In this section, we will focus on chitosan-based polymeric micelles as drug carriers.

4.1. Chitosan-Based Micelles. Due to its cationic nature, chitosan can be complexed with negatively charged DNA and be used as nonviral vector for gene therapy. Hu and coworkers synthesized stearic acid- (SA-) grafted chitosan oligosaccharide (CSO) in order to produce polymeric micelles to delivery pDNA (pEGFP-C1) [88]. The SA-CSO/DNA micelles efficiently protected the condensed DNA from enzymatic degradation by DNAse I and presented lower cytotoxicity and comparable transfection efficiency in A549 cells compared to Lipofectamine 2000 [88], making these micelles a promising gene delivery system in the treatment of pulmonary diseases. The same research group developed SA-CSO micelles for the delivery of drugs like paclitaxel [89], doxorubicin [90, 91], and proteins [92]. The encapsulation of doxorubicin in SA-CSO micelles resulted in higher uptake and accumulation by A549 cells and a decrease in the IC_{50} [20]. Recently, SA-CSO micelles were developed by another

research group, for the pulmonary administration of amphotericin B (AmB) [93]. The local administration of AmB to treat invasive pulmonary fungal infections, present in some patients receiving immune suppressive treatments, avoids the systemic side effects and improves the bioavailability of the drug [94]. After encapsulation into micelles, AmB presents the same antifungal activity of Fungizone but lower toxicity [93], a phenomenon intimately related to its aggregation state [95]. The micelles possessed positive charges with mean diameters between 100 and 250 nm and were efficiently nebulized using an Air-jet nebulizer presenting up to 52% of fine particle fraction, making them a suitable choice for pulmonary delivery of AmB [93].

In the field of block copolymers, Liu and coworkers developed an triblock copolymer consisting of poly(ε-caprolactone)-*b*-chitooligosaccharide-*b*-poly(ethylene glycol) (PCL-b-COS-b-PEG) for delivery of drugs, using doxorubicin as model drug [96]. The obtained polymer presents the capacity to form micelles with encapsulation efficiency of doxorubicin close to 50%. Genipin after crosslinking did not affect the macroscopic characteristics of the micelles but delayed the *in vitro* release of doxorubicin from the micellar reservoir [96]. Similar results were obtained by Chen et al. using chitosan-poly(ε-caprolactone)-poly(ethylene glycol) to encapsulate paclitaxel and rutin with glutaraldehyde after crosslinking [97]. Wu and co-workers also synthesized chitosan-based copolymers for drug delivery [98]. Polylactide- (PLA-) chitosan copolymers with different molar ratios were developed and characterized. Rifampicin was used as lipophilic drug model to be encapsulated. As PLA molar ratio increased, the micelle size and drug-loading content increased, and the rifampicin release rate decreased [98]. The present systems were not tested for any specific route of administration.

Besides the creation of lipid-chitosan and polymer-chitosan conjugates, it is also possible to produce chitosan derivatives with amphiphilic characteristics that may self-assemble in aqueous environment and form polymeric micelles [99–101]. Zhang and co-workers synthesized different chitosan derivatives composed by long-chain alkyl groups as hydrophobic moieties and sulfated groups as hydrophilic moieties to delivery of paclitaxel [102]. Between the different derivatives, N-octyl-O-sulfate chitosan presented the best results in terms of solubilization of paclitaxel [102]. Paclitaxel-loaded micelles prepared with N-octyl-O-sulfate chitosan had high drug-loading capacity and encapsulation efficiency, were shown to be safe for intravenous injection, and presented similar antitumor efficacy as Taxol, but significantly reduced toxicity and improved bioavailability [103]. They also synthesized N-alkyl-N-trimethyl chitosan derivatives to deliver 10-hydroxycamptothecin. The best results in terms of encapsulation efficiency, stability, release behavior, and pharmacokinetic properties were obtained with N-octyl-N-trimethyl chitosan (degree of octyl and trimethyl substitution is 8% and 54%, resp.) [104]. Another research group studied the feasibility of N-succinyl-N-octyl chitosan as delivery system of doxorubicin [105]. Although these systems have been tested for intravenous administration, they present antitumor efficacy against lung cancer cell lines such as Lewis lung cancer cells [103] and A549 cells [103, 105]. Their pulmonary administration could be a possibility to treat locally lung cancer; however, studies are required to access its feasibility as inhaled drug delivery systems.

Chitosan-based polymeric micelles have also been developed as drug delivery systems for other routes of administration than pulmonary, with emphasis on intravenous administration of paclitaxel. Examples are the N-mPEG-N-octyl-O-sulfate chitosan micelles produced by Qu and co-workers [106], the N-lauryl-carboxymethyl-chitosan micelles developed by Miwa et al. [107], and the N-octyl-N-(2-carboxyl-cyclohexamethenyl) chitosan micelles produced by Liu and co-workers [108]. Other chitosan-based micelles have been developed such as the doxorubicin-loaded linoleic acid-grafted chitosan oligosaccharide micelles produced by Du et al. for intercellular antitumor drug delivery to drug resistance tumor cells [109, 110] or the methoxy poly(ethylene glycol)-grafted chitosan micelles for delivery of methotrexate to treat colon carcinoma [111, 112].

5. Chitosan-Ligand Conjugates for Nanoparticle-Based Active Target Drug Delivery Systems

As discussed in previous sections, chitosan is an ideal natural polymer for the design and development of drug delivery systems structured at micro- and nanoscopic scales. Particularly, its mucoadhesiveness [113, 114], biocompatibility [115, 116], and capacity to promote the absorption of poorly absorbable macromolecules across epithelial barriers by transient widening of cell tight junctions thus modifying the parallel transport [117–120] have been exploited in the development of nanocarrier systems for transmucosal delivery. A recognized feature of chitosan-based nanostructured systems is their capacity to protect sensitive therapeutic macromolecules against degradation and their ability to overcome mucosal barriers. As a consequence, their application has been centered particularly in noninvasive routes of administration including transmucosal administration of proteins [121–125] and genetic material [126–130].

The capacity of chitosan to undergo multiple chemical modifications has been exploited to increase the active targeting of chitosan-based nanocarriers particularly for protein and genetic material delivery towards specific cells [126, 131]. The modifications have comprised from derivatization with small functional groups or substructures (e.g., thiolated derivatives, grafted PEG) to conjugation with biologically active ligands such as carbohydrates (e.g., galactose, mannose) or specific ligands such as folate, transferrin, or KNOB viral protein. Nanocarrier systems of this kind with improved biological and biopharmaceutical performance have been the subject of active research in the past decade or so, as discussed, with reference to specific systems, in the following sections.

5.1. Conjugation of Sugar Ligands. Strategies to improve the targeting potential of chitosan have addressed its functionalization with sugar moieties, mostly with D-galactose and D-mannose. Galactose groups were chemically bound to

chitosan aiming to achieve liver target delivery, while dextran was grafted to enhance the stability of the complex in aqueous media. The system was found efficient to transfect liver cells expressing asialoglycoprotein receptor (ASGRr), which specifically recognizes the galactose ligands on modified chitosan [132]. Galactosylated chitosan-graft-PEG (GCP) was developed for the same purpose. GCP-DNA complexes were found to be stable due to hydrophobic shielding by PEG and increased the stability against DNAse degradation. This system was found to enhance the transfection of HepG2 cells having ASGRr, thus indicating that galactosylated chitosan is an effective hepatocyte-targeted gene carrier [132]. This same system was tested *in vivo* by a different group. Glycoconjugated chitosan was designed for ASGRr-directed delivery to liver parenchymal cells. It was found that this system has the potential to be a vector for targeting to Kupffer cells *in vivo*. Other chitosan glycoconjugates have incorporated lactose to develop gene nanocarriers. HeLa cells were effectively transfected by this nanocarrier system, but neither HepG2 nor BNL CL2 cells. TEM evidence was consistent with the proposal that the nanocomplexes were internalized by HeLa cells and located inside endocytotic vesicles and endosome-like compartments [133]. In turn, efforts have been made to target mannose receptors on dendritic cells residing in tumors; hence, chitosan has been functionalized with mannose. Using these nanoparticles in the delivery of a plasmid encoding IL-12 resulted in enhanced IL-12 gene transfer efficiency, suppressed tumour growth and angiogenesis in the carcinoma BALB/c mouse model [134]. A trisaccharide branch was attached onto chitosan chain in order to target lectins on the cell surface in lung tissues [135]. This modification increased the carrier uptake and transfection efficiencies in various *in vitro* assays as well as in mouse lung tissue as recently reviewed elsewhere [136].

5.2. Conjugation of Folate. Folic acid (FA) is appealing as a ligand for targeting cell membrane and allowing nanoparticle endocytosis via the folate receptor (FR) for higher transfection yields. Importantly, the high affinity of folate to bind its receptor (1 nM) [137] and folate small size allows its use for specific cell targeting. Moreover, the ability of FA to bind its receptor to allow endocytosis is not altered by covalent conjugation of small molecules [138]. Folate receptor (FR) is over-expressed on many human cancer cell surfaces, and the nonepithelial isoform of FR (FRb) is expressed on activated synovial macrophages present in large numbers in arthritic joints [139]. Hence, folate conjugation to the surface of chitosan and chitosan-derivatives-based nanoparticles has been one of the actively studied strategies to vectorize drugs over the past few years [140–149]. These systems have been developed with a view to achieve targeting effect in the delivery of cytostatic drugs to tumor cells, genetic material, or antiarthritis therapies and also for diagnostic and imaging purposes. To this end, the majority of *in vitro* studies have been conducted in various types of cell lines well known to overexpress the human folate receptors (FRa and FLRb), such as HeLa [145], HT29 [143], Caco-2 [143], B16F1 [150], KB [151, 152], HepG-2 [153], and SKOV3 [141, 154] cells.

The evidence from most of these studies is consistent to indicate that the folic acid modification promotes the uptake of nanoparticles by FR-positive tumor cell lines most likely via receptor-mediated endocytosis but has little impact on other cells without FR [155]. Results of transfection studies showed that folate-chitosan-based nanoparticles enhanced the reporter gene expression against a cell line overexpressing FR (SKOV3 cells) compared to an FR-deficient cell line (A549 cells) and did not induce obvious cytotoxicity against HEK 293 cells [154]. In turn, NPs made out of folate-grafted chitosan were produced to transfect interleukin-1 receptor antagonist (IL-1Ra) in synovial mononuclear cells and CD14+ cells via the targeting of the folate receptor-b [156]. Compared to unmodified chitosan or naked DNA, this system allowed for an increase in IL-1Ra expression combined with a diminution of cytotoxicity *in vitro* and reinforced protection against inflammation and abnormal bone metabolism *in vivo*.

5.3. Conjugation of Protein Ligands. Proteins, such as transferrin and viral KNOB, have been conjugated at the surface of chitosan-based nanoparticles intended for DNA delivery, as a strategy to achieve active targeting and thus high transfection efficiency [59, 157]. The transferrin receptor (TfR) is found in many mammalian cells, responsible for iron import to cells, and it is known to enhance the transcytosis of viral vector [158, 159]. Transferrin (Tf) or antibodies against TfR were conjugated to oligonucleotides or polycations, which then complexed with pcDNA. Two different synthetic approaches were tested to couple Tf to the surface of chitosan nanoparticles, achieving a conjugation in both cases about 33–43% (transferrin to chitosan moL%) [59]. In this study HEK293 cells were transfected using luciferase reporter gene. Tf-conjugated nanoparticles invariably showed threefold greater transfection efficiency than unmodified nanoparticles. However, the Tf-decorated nanoparticles did not show significant enhancement of the transfection of HeLa cells [160].

Tf decoration of chitosan-based DNA-loaded nanoparticles has been found to enhance the transport across polarized monolayer Caco-2 cells known to have abundant Tf receptors on their surface [159] and extensively used as a model of normal intestinal epithelium transport [161]. It was demonstrated that Tf conjugation could enhance the transport of nanoparticles through Caco-2 alone and Caco-2-PPL cocultures by 3- to 5-fold. One drawback of these systems is that they only proved to be ineffective in the presence of added serum medium. An interesting contribution of this study was to address the behavior of a coculture of polarized Caco-2 cells infiltrated with lymphocytes that induces the differentiation of M-type phenotype, a model much closer to the real intestinal epithelium. The results led to suggest that uptake in the lymphoid follicles of the duodenum could play a more significant role compared to Peyer's patches [157].

A second related strategy has been to decorate the surface of DNA-loaded chitosan-based nanoparticles with KNOB protein so as to enhance the uptake and overcome one of the major rate-limiting steps for transfection mediated by

chitosan nanoparticles via a specific receptor-mediated endocytosis mechanism [59]. KNOB conjugation to the nanoparticles significantly improved the transfection efficiency (increased 130 times) when transfecting HeLa cells but still not to a level that could rival Lipofectamine transfection.

5.4. Hybrid Nanoparticles Comprising Other Polysaccharides. Yet another approach to enhance the targeting capacity of chitosan-based nanocapsules has been to develop hybrid cogelled systems including other polysaccharides with known affinity toward specific receptors. Among these, nanoparticles comprising hyaluronan, alginate, and glucomannan have deserved attention, particularly for the delivery of insulin, vaccines, and DNA via transmucosa. The reader is referred to recent comprehensive reviews on the subject [125, 127, 162].

6. Conclusion

Pondering advantages and drawbacks, chitosan-based materials represent a very versatile and appealing technological platform to address the design of drug delivery systems that combine unique features such as mucoadhesiveness and enhanced drug absorption for the localized and systemic delivery of drugs through the respiratory system. Furthermore, in a more global perspective that values the potential of the bench-to-bedside translation, relatively low price, commercial availability in a broad spectrum of molecular weights and degrees of deacetylation and chemical versatility are outstanding properties that make of chitosan an excellent candidate in an industrial setting. On the other hand, the lack of reproducibility between batches usually displayed by natural polymers might lead to a less robust production process and remains a challenge to be addressed and overcome. Finally, to date chitosan is approved only as food supplement classified as a "generally recognized as safe" (GRAS) material [4]. In the context of pharmaceutical and biomedical products, a diversity of systems (e.g., micro- and nanoparticles, films, scaffolds, etc.) is under investigation. Remarkably, none of them has been approved by regulatory agencies yet. The diversity of molecular aspects that might affect the biodegradability, biodistribution, and biocompatibility needs to be carefully investigated as straight extrapolations among derivatives seem very complex, if not impossible. Hence, the regulatory phase will also need to be comprehensively addressed if an effective technology transfer and novel endeavors want to be ensured for chitosan and its derivatives.

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