Review

Biomedical Activity of Chitin/Chitosan Based Materials—Influence of Physicochemical Properties Apart from Molecular Weight and Degree of *N*-Acetylation

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Abstract: The physicochemical nature of chitin and chitosan, which influences the biomedical activity of these compounds, is strongly related to the source of chitin and the conditions of the chitin/chitosan production process. Apart from widely described key factors such as weight-averaged molecular weight (M_W) and degree of *N*-acetylation (DA), other physicochemical parameters like polydispersity (M_W/M_N), crystallinity or the pattern of acetylation (P_A) have to be taken into consideration. From the biological point of view, these parameters affect a very important factor—the solubility of chitin and chitosan in water and organic solvents. The physicochemical properties of chitosan solutions can be controlled by manipulating solution conditions (temperature, pH, ionic strength, concentration, solvent). The degree of substitution of the hydroxyl and the amino groups or the degree of quaternization of the amino groups also influence the mechanical and biological properties of chitosan samples. Finally, a considerable research effort has been directed towards developing safe and efficient chitin/chitosan-based products because many factors, like the size of nanoparticles, can determine the biomedical characteristics of medicinal products. The influence of these factors on the biomedical activity of chitin/chitosan-based products is presented in this report in more detail.

1. Introduction

Chitin and chitosan (Figure 1) are linear polysaccharides consisting of varying amounts of β-(1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose (GlcN) and 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) residues [1,2]. Chitin samples contain a high content of GlcNAc units, whereas chitosan mainly consists of GlcN units. Some authors believe that chitin is built up of at least 60% of GlcNAc residues [3], although a rigid nomenclature with respect to the degree of *N*-acetylation (defined as the average number of GlcNAc units per 100 monomers expressed as a percentage, DA) between chitin and chitosan has not been defined [4]. It should be mentioned that both compounds are insoluble in water (pH = 7) and common organic solvents, although they can be dissolved in specific solvents such as hexafluoro-2-propanol, *N,N*-dimethylacetamide or hexafluoroacetone [5-7].

Figure 1. Schematic representation of chitin and chitosan derivatives. Through functionalization of the chemical structure, the solubility of the almost insoluble biopolymer chitin **(a)** can be enhanced. Quaternized **(c)** and substituted **(d)** derivatives also show much better solubility under alkaline conditions than chitosan **(b)**.

Chitin is, next to cellulose, the second most abundant natural biopolymer. It is found predominantly in the shells of crustaceans such as crabs and shrimp, the cuticles of insects, and the cell walls of fungi [8]. Its biosynthesis by living organisms in the lower plant and animal kingdoms has been estimated at 10^{10}–10^{12} tons/year [9]. On the other hand, chitosan is produced only by some fungi *Mucoraceae* [10]. Commercial chitosan samples are typically prepared by the chemical de-*N*-acetylation of chitin under alkaline conditions [11-14]. Depending on the source of natural chitin [1,15,16] and the conditions of its production, chitosan preparations can differ in size (average molecular weight; M_W), DA and other physicochemical properties such as polydispersity (M_W/M_N), crystallinity or the pattern of acetylation (P_A) [16-20].

Chitin and chitosan possess very interesting biological properties (Figure 2); therefore, they have been used in many applications, mainly in the medical and pharmaceutical fields [4,17,19,21-30]. Not all biological activities have been observed with every chitin/chitosan preparation [31].
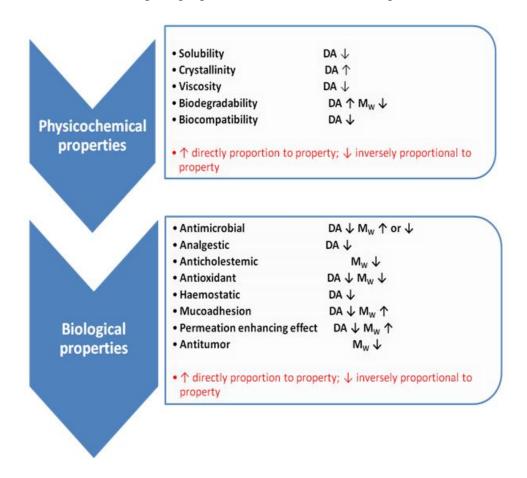
Figure 2. Biological properties of chitin and chitosan.

Biological properties of chitin and chitosan

- Non-toxity
- Biodegradality
- Biocompatibility
- Citocompatibility
- Antimicrobial activity
- Anticholesterolemic activity
- Antioxidant activity
- Anti-inflamatory action
- Analgestic action
- Haemostatic action
- Mucoadhesion
- Anginogenesis stimulation
- Macrophage activation
- Granulation and scar formation
- Adsorption enhancer

The biological properties of these compounds depend strongly on their solubility in water and other commonly used solvents. In its crystalline form, chitosan is normally insoluble in aqueous solutions above pH 7; however, in dilute acids, the protonated free amino groups facilitate the solubility of the molecule. The pKa of primary amino groups depends closely on DA, so the solubility of chitosan is also dependent on DA [6]. Being a highly insoluble and chemically rather unreactive material, chitin has a much lower applicability than chitosan. Recently, there has been growing interest in the chemical modification of chitosan and chitin (e.g., [32-35]) in order to improve their solubility and extend their applications (e.g., [7,19,36,37]).

Knowledge of the microstructure of chitosan samples is essential for understanding the structure-property-activity relationships of chitin and chitosan products [18,38-42]. To date, only the effect of weight-averaged molecular weight (M_W) and degree of *N*-acetylation (DA) on the physicochemical and biological properties of these compounds has been intensively investigated. The results are summarized in a number of papers, (e.g., [17,21,43-46]). Selected relationships between the structural parameters DA and M_W, and the physicochemical and biological properties are presented in Figure 3.

As already mentioned, the biological properties of chitin/chitosan samples can also be related to the polydispersity (M_W/M_N), crystallinity and distribution of GlcNAc and GlcN units along the polymeric chain described by the pattern of acetylation (P_A) [17,18]. When a chitosan solution is used in the investigations, factors such as concentration, the nature of salt counterion, pH, ionic strength, and the addition of non-aqueous solvent should be taken into account [47]. Apart from these factors, the use in medical chitin/chitosan-based products of finely divided powders, films, membranes, gels, coatings, suspensions and hydrogels (e.g., [30,37,48-50]) can influence their biomedical activity [6,51-54].

Figure 3. Influence of degree of *N*-acetylation (DA) and molecular weight (M$_W$) on the physicochemical and biological properties of chitin/chitosan samples.
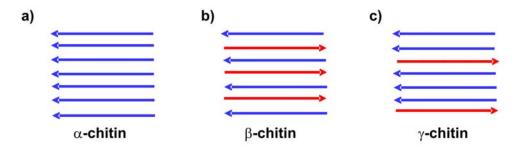

The aim of this review paper is to highlight the relationship between other key factors apart from the DA and M$_W$ properties of the chitin/chitosan-based materials and their biomedical activity. Special emphasis will be placed on the influence of such parameters as the source of chitin and chitosan, pH, ionic strength, concentration, degree of quaternization, degree of substitution, and the preparation techniques of chitin/chitosan-based products offered for biomedical and pharmaceutical applications.

2. Influence of the Sources of Chitin and Chitosan

Although chitin is biosynthesized by more than 10^6 species in three polymorphic configurations (α, β, and γ—see Figure 4), in laboratories or on an industrial scale it is usually isolated from the exoskeletons of crustaceans, particularly shrimps and crabs [16]. α-Chitin is obtained in this way [55]. β-Chitin can be extracted from squid pens, and γ-chitin from fungi and yeast [56]. α-Chitin is the most common form, whereas β-chitin is more reactive [57] and shows a higher affinity for solvents [58]. β-Chitin is easily converted to α-chitin by alkaline treatment followed by flushing in water [59].

Figure 4. Schematic representation of the three polymorphic forms of chitin **(a)** α-chitin. **(b)** β-chitin. **(c)** γ-chitin.

Youn *et al.* [60] demonstrated that selected physicochemical and functional properties of chitosans prepared from the shells of crabs harvested in three different years (2004, 2005, and 2007) differed according to the crab harvest and/or storage duration of shells. Chitosan prepared from the chitin collected in 2004 showed a higher degree of deacetylation, was less viscous, and less red in color than that prepared from the chitin harvested in 2005 and two years later. The shells of the crabs from 2004 and 2005 contained similar amounts of protein, chitosan and ash, whereas the sample from 2007 had higher protein and chitin but a lower ash content. Furthermore, the chitosan prepared from chitin isolated in 2004 exhibited a greater water-binding capacity, dye-binding capacity and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity than those from 2005 and 2007. This indicates that storage of crab shells for a certain period of time may be advantageous for chitosan production with improved functionality for particular uses.

It is well known that chitosan samples are usually prepared from α-chitin isolated from crab shells by partial or complete de-*N*-acetylation of the chitin in the solid (a heterogeneous process) [16,20]. Recently, more attention has been paid to the production of chitin and chitosan from fungal sources [61,62]. Wu *et al.* [61], determined the yield (alkali-insoluble material, crude chitin, and the glucosamine content in crude materials) and physicochemical properties (DA, crystallinity) of chitin and chitosan isolated from *Aspergillus niger* and *Mucor rouxii*; they examined the bioactivity of fungal chitin and chitosan against the food-borne pathogen *Salmonella* Typhimurium and the plant pathogens *Botrytis cinerea* and *Penicillium expansum*. The biological properties of these samples were compared with those obtained for commercial chitosan obtained from crustacean shells. The authors established that the mycelium of *M. rouxii* contained a higher level of glucosamine than *A. niger*, and that both chitin and chitosan were present in *M. rouxii* mycelia, but only chitin in *A. niger*. The degree of *N*-acetylation of chitin from *A. niger* was higher than that of chitin and chitosan isolated from *M. rouxii*. The crystallinity of fungal chitin and chitosan was less intense than in the corresponding materials from shrimp shells. Despite these physicochemical differences, the antimicrobial activity of the isolated chitin and chitosan against *Salmonella* Typhimurium and plant pathogens *B. cinerea* and *P. expansum* was similar and comparable with that obtained from commercially purified crustacean chitosan. Differences in the conditions of chitin de-*N*-acetylation (e.g., temperature, alkali concentration, ratio of alkali solutions to the shells) may exert a strong influence on chitosan preparations [20]. Kamil *et al.* [63] described the effect of chitosans prepared using different de-*N*-acetylation times (for 4, 10 and 20 h) on lipid autoxidation in a fish model system. These chitosan preparations were similar in their degree of *N*-acetylation, nitrogen content, ash, color,
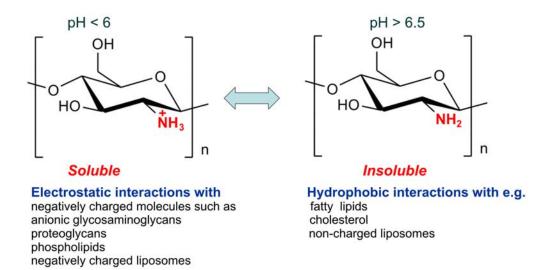
moisture, viscosity and molecular weight, but differed in their apparent viscosity. The viscosity was highest when de-*N*-acetylation was carried out for 4 h (360 centipoises units, 360 cP), less when this reaction lasted for over 10 h (57 cP) and least after 20 h (14 cP). The results showed that 14 cP chitosan was more effective than the higher viscosity chitosans in preventing lipid oxidation in the herring flesh model system. In other research [64], the effect of de-*N*-acetylation time on the preparation, properties and swelling behavior of chitosan films was tested. Five types of chitosan films were prepared in a single-step de-*N*-acetylation process by varying the alkaline treatment time from 2 to 10 h; the degree of *N*-acetylation, crystallinity index (CrI), swelling index, contact angle and the morphology using scanning electron microscopy were determined. DA turned out to be similar for all chitosans. The film prepared during 2 h de-*N*-acetylation has a lower CrI and maximum swelling index, whereas the film formed after 6 h deacetylation had the lowest DA with higher CrI and low contact angles, and was more suitable for biomedical applications.

Problems with the production of a large spectrum of chitin/chitosan structures with perfect control of the chemical architectures, molecular weights and molecular weight distributions were described by Domard [20]. In this interesting review the author summarized his team's almost 30-year long research into chitin and chitosan, e.g., the production of chitin, chitosan and series of co-polymers and co-oligomers and their characterizations. The control of the physicochemical characteristics of chitin and chitosan is important in all biomedical applications, e.g., in tissue engineering. The regulation of porosity and pore morphology of chitosan-based scaffolds is critical for controlling cellular colonization rates and organization within an engineered tissue [65]. In addition, the angiogenesis required for some scaffold application scenarios can be affected by scaffold porosity and pore morphology [66].

3. Influence of pH

The presence of amino groups means that the pH substantially alters the charged state and the properties of chitosan (Figure 5).

Figure 5. Schematic illustration of chitosan's versatility.

At pH > 6.5 chitosan solutions exhibit phase separation, while at pH < 6.5 chitosan is soluble, carrying a positive charge because of the presence of protonated amino groups [67]. At pH between 6.0 and 6.5 in solution, the free amino groups of chitosan molecules become less protonated and hydrophobicity along the chitosan chain increases. Therefore, chitosan self-aggregates could be formed in acetate buffer solutions by intra- and inter-molecular hydrophobic interactions. As a cationic polyelectrolyte, at low pH (less than about 6) chitosan can electrostatically interact with negatively charged molecules or polymers, e.g., anionic glycosaminoglycans, proteoglycans, and other negatively charged molecules. At higher pH (above about 6.5) chitosan's amino groups are deprotonated and hydrophobic interactions with several substrates (e.g., fatty acids and cholesterol) can appear (Figure 5) [21].

These pH-dependent properties of chitosan influence its biomedical activity and potential applications. One of them is the antimicrobial activity of chitosan [46]. The antibacterial mechanism of chitosan is generally considered to be due to its positively charged amino group at the C-2 position of the GlcN residue, which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms [68]. The presence of a large number of non-protonated amino groups as well as the poor solubility of chitosan at pH 7 [69-71] mean that chitosan's bactericidal activity is minimal. Helander *et al.* [72] reported that chitosan displayed antibacterial activity not only in an acid environment, although showed a stronger inhibitory effect at lower pHs with the inhibitory activity weakening with increasing pH. Kong *et al.* [73] and Yang *et al.* [74] observed that the antibacterial activity of the *N*-alkylated chitosan derivatives against *E. coli* increased as the pH rose from 5.0 reaching a maximum around pH 7.0–7.5. Also, the investigation of the antibacterial property of chitosan microspheres (CM) in a solid dispersing system showed that under neutral conditions, of the three tested CM samples with DA of 2.5, 16.5, 37.4% respectively, the highest inhibitory effect was observed for the CM sample with DA of 37.4% [73]. To date, there has been no positive information about the antimicrobial activity of chitosan under alkaline conditions.

The pH values of the medium also determines the stability of chitooligomers with the degree of polymerization (DP) < 20, which can be achieved by depolymerization of chitosan [75]. These compounds have various biological activities such as antitumor and immune enhancing effects [76], but can easily turn brown during shelf life, which influences their properties and limits their applications in many fields. It was established that the pH of chitooligomers for their optimal preservation should be below 4 or above 10, and in an oxygen-free environment. It was also reported that syneresis (the major properties of hydrogels, which allows a more stable state to be achieved than the initial state of chitin gels) depends on pH [77].

Chitosan micro/nanoparticles based on ionic interaction between drugs (insulin, diclofenac sodium and salicylic acid respectively) and chitosan were prepared and examined for their influence on pH [78]. Entrapment efficiency and the amount of drugs inside the particles were affected by the zeta potential and surface charge of the micro/nanoparticles produced, whereas immediate drug release was independent of the pH of the dissolution medium.

In another investigation, chitosan was blended with different amounts of polycaprolactone (PCL) and used to control the delivery of ofloxacin [79]. The swelling kinetics as well as the drug delivery systems using ofloxacin have also been studied at different pH (pH 3.4 and 7.4) and drug loading. The

percentage of swelling rises with pH increase from 3.4 to 7.4, and with increase in the percentage of drug loading.
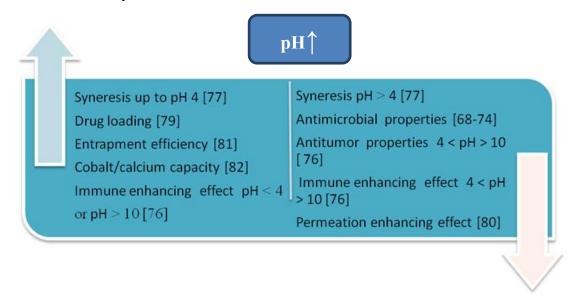
The influence of the pH of the release medium on the *in vitro* release of pDNA from chitosan microparticles was also investigated [80]. It was observed that the release of pDNA from chitosan microparticles in simulated gastric (pH 2.1), simulated intestinal (pH 6.47) and acidic PBS (pH 4.5) medium was pH-dependent: as the pH of the release medium increased, the release profile decreased. The differences in the release profiles were explained by the different solubilities of chitosan in the acidic and basic pH of the media.

In another investigation [81], the influence was studied of pH on the entrapment efficiency of lipase from *Candida rugosa* in photo-cross-linkable chitosan membranes modified by α-cyano-4-hydroxycinnamic acid. The efficiency of the immobilization was evaluated by examining the relative enzymatic activity of the free enzyme before and after the immobilization of lipase. The optimal pH for immobilized lipase was found to be 8.0, which was slightly higher than that of the free lipase (pH 7.5), and the immobilization resulted in enzyme stabilization over a broader pH range.

Bernkop-Schnürch and Krajicek [82] synthesized different chitosan-EDTA (EDTA, ethylenediaminetetraacetic acid) conjugates to evaluate their potential regarding mucoadhesion, viscosity and inhibitory effects on the proteolytic activity of enzymes. Although the complexing agent lost one of its carboxylic acid groups (formation of an amide bond between the amino groups of the polymer), the modification did not much affect its ability to form complexes with bivalent cations. The binding capacity of chitosan-EDTA conjugates was not only tested under intestinal pH-conditions but also at pH > 9.0. The results demonstrated that all chitosan-EDTA conjugates exhibited a greater capacity to bind zinc and cobalt than calcium. Whereas the capacity to bind calcium and cobalt could be enhanced in an alkaline medium, the pH had hardly any effect on the amount of zinc bound to polymers.

Schematic representation of influence of pH on the selected physicochemical and biological properties of chitin/chitosan samples is presented in Figure 6.
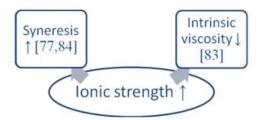
Figure 6. Influence of pH on the selected physicochemical and biological properties of chitin/chitosan samples.

4. Influence of Ionic Strength

Like pH, ionic strength plays an important role in the physicochemical properties of chitosan solutions and can strongly influence their biological behavior (Figure 7). Nevertheless, up to now the significance of this factor on the biomedical activities of chitin/chitosan-based products has been characterized very poorly. Rinaudo *et al.* [83] described the influence of ionic strength on chitosan chain expansion. The role of ionic strength on the electrostatic effects due to the existence of charged—NH_3^+ in acid conditions was tested by measurement of the intrinsic viscosity in solvents with different ionic concentrations.

Figure 7. Influence of ionic strength on the physicochemical properties of chitin/chitosan-based materials.

In another investigation [84], the effect of ionic strength on the stabilizing properties of chitosan in a model emulsion system containing whey protein isolate (WPI) as emulsifier and canola oil was studied. Syneresis was favored by the increasing ionic strength to 0.3 M.

Vachoud *et al.* [77] examined the role of the concentration of KCl initially present in the hydroalcoholic solution on gel formation, syneresis and the mechanical properties of the gel for two values of *R*—2.5 and 10—where *R* the molar ratio of Ac_2O-amine residues used for its preparation. It was established that the time necessary to achieve the gel point increased with KCl concentration independently of *R*. In the presence of salt, the rate of solvent exclusion during syneresis decreases when the concentration of salt increases for these two gels.
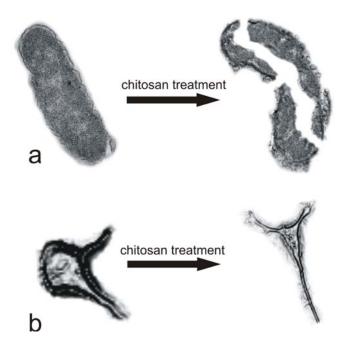
5. Influence of Concentration

The biomedical behavior of chitin/chitosan-based products also depends on the concentrations of these compounds.

The influence of concentration on the antimicrobial properties of these compounds is the most often described in literature (Figure 8). Ghaouth *et al.* [85] reported the effect of chitosan concentrations on the growth of *Alternaria alternate*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Rhizopus stolonifer*. Abou Sereih Neven *et al.* [86] described the influence of five different doses of chitosan on the linear growth (mm) of *Fusarium oxysporum* f. sp. *sesami* and *Trichoderma harzianum*. The relationship between the concentrations of 12 kinds of new hydroxylbenzenesulfonailide derivatives of chitosan sulphates and carboxymethyl chitosan and antimicrobial activity against *P. asparagi*, *A. solani*, *F. oxysporum f.* sp. *vasinfectum*, *C. gloeosporioides* (Penz.) *Saec* and *P. zingiber*

was presented by Zhong *et al.* [87]. The antibacterial activities of hydroxypropyl chitosan at different concentrations against S*taphylococcus aureus* and *Escherichia coli* have been explored by Xia *et al.* [88].

Apart from the influence of the chitosan concentration on its antimicrobial behavior, this parameter determines the properties of chitosan-based formulations for gene delivery systems [51,89,90]. It was established that the particle size of the chitosan/siRNA complex prepared by simple complexation increased when the concentration of chitosan was raised [51]. The comparative positive value of the surface charge (zeta potential) of chitosan-siRNA complexes also increased with the rising concentration of chitosan at a constant siRNA concentration. The transfection efficiency of the polyelectrolyte obtained depends on the mixing molar stoichiometry of DNA to chitosan (N/P ratio) [51,89,90]. Sajomsang *et al.* reported that the highest gene transfection efficiencies of all *N*-aryl chitosan derivative/DNA complexes were observed at N/P ratios 5 due to the smallest particle size (95–124 nm) [89].

Figure 8. Schematic representation of chitosan´s impact on the Gram-negative bacterium *E. coli* **(a)** and on brain cells **(b)**. Bacteria show lysis accompanied by death in the presence of chitosan, whereas brain cells show a change in their overall shape; their functionality and vitality are, however, not affected by the chitosan treatment.

Zhu *et al.* [91] investigated the aggregation behavior of *O*-carboxymethylchitosan in dilute solution to demonstrate its potential use in drug delivery systems. The critical aggregation concentration was established between 0.042 mg/mL and 0.050 mg/mL.

The influence of N/P ratio on the gene knockdown efficiency of chitosan 170 kDa (DA 16%) in H1299 human lung carcinoma cells was reported by Liu *et al.* [90]. They found that the level of EGFP knockdown increased at higher N:P ratios (50 and 150) in comparison to lower N:P ratios (2 and 10), and the nanoparticles formed at higher N:P (50 and 150) showed the greatest level (80%) of EGFP knockdown.

The hepatic cytotoxicity profile of chitosan nanoparticles in human liver progenitor cells with corresponding chitosan molecules as control was investigated by Loh *et al.* [92]. Both the chitosan molecules and nanoparticles induced CYP3A4 (the most abundant subfamilies of the cytochromes P450 in human liver (~40%) which metabolizes more than 50% of clinically used drugs)-mediated activity in the BHAL cells, but the chitosan molecules exhibited a concentration-independent effect, while the nanoparticles showed a concentration-dependent effect within the concentration range from 0.01 to 1%. The nanoparticles were less cytoadhesive than the chitosan molecules, although they were rapidly internalized by the BHAL cells. BHAL cells showed poor cell membrane integrity after exposure for 4 h to chitosan nanoparticles at concentrations ≥0.5% w/v, which correlated to a higher leakage of alanine transaminase from the cells into the extracellular space.
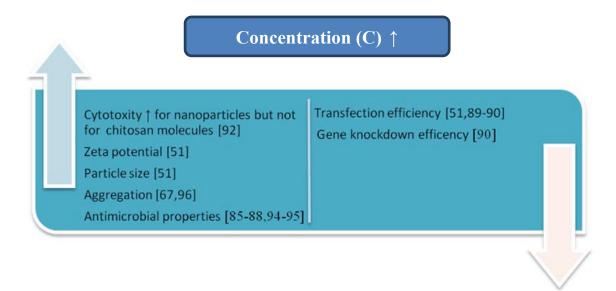
The different weight ratios of chitosan to chondroitin (CH/CS) influence the pore structures, mechanical properties and surface hydrophilicity of sulphate chondroitin sulphate-modified chitosan membranes [93]. Membranes with the ratio CH/CS 90/10 were chosen as the most promising biomaterials for tissue engineering applications. Cai *et al.* [94] produced composite nanofibrous membranes of chitosan (CS) and silk fibroin (SF) by electrospinning. The antibacterial activity against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) increased greatly with increasing proportions of chitosan.

Yang *et al.* [95] reported that the tensile strength of the composite cellulose membranes coated with different chitosan concentrations increased with increasing chitosan concentration.

The chitosan concentration should also be controlled when chitosan is used as a surfactant [67]. The concentration above which a surfactant starts to form micelles or aggregates is called the critical micelle concentration (CMC) or the critical aggregate concentration (CAC), and the determination of this value for chitin/chitosan-based products is very important [67,96]. The initial concentration of chitin also has an important effect on the syneresis of chitin gels [77].

Schematic representation of influence of concentration on the selected physicochemical and biological properties of chitin/chitosan samples is shown in Figure 9.

Figure 9. Influence of concentration on the selected physicochemical properties and biological activity of chitin/chitosan based materials.

6. Influence of the Degree of Quaternization

N,N,N-Trimethylammonium chitosan chloride (TMChC) was one of the quaternized chitosan derivatives firstly synthesized by Muzzarelli and Tanfani [97]. The influence of the degree of quaternization (DQ) on the mucoadhesive properties of TMChC was examined by Snyman, Hamman, and Kotze [98,99]. They demonstrated that the presence of quaternary ammonium groups decreased mucoadhesion and related this adverse effect to conformational changes in the TMChC. The opposite results were achieved by Sandri *et al.* (2005) [100]. Jintapattanakit *et al.* [101] established that TMChC with a relatively high degree of dimethylation (DD) exhibited higher mucoadhesion and cytotoxicity, and the mucoadhesive properties of these derivatives were influenced by the combination of molecular weight, the steric hindrance of the dimethyl groups on the polymer, and the positive charge density.

Sajomsang *et al.* [102] synthesized methylated *N*-aryl chitosan derivatives, methylated *N*-(4-*N,N*-dimethylaminocinnamyl) chitosan chloride (MDMCMCh) and methylated *N*-(4-pyridylmethyl) chitosan chloride (MPyMeCh) consisting of a variety of *N*-aryl substituents. The effects of the degree of quaternization (DQ) on mucoadhesion and cytotoxicity were investigated and compared to *N,N,N*-trimethylammonium chitosan chloride (TMChC). It was found that mucoadhesion and cytotoxicity increased when DQ was increased to 65%, but decreased with DQ > 65%. The influences of the quaternary and acyl groups on the *in vitro* anticoagulant activity of *N*-propanoyl-, *N*-hexanoyl- and *N,O*-quaternary substituted chitosan sulphate were also investigated [103]. It was established that the anticoagulant activity of the chitosan sulphate was enhanced by the *N*-acyl groups, whereas the quaternary groups decreased this activity. The influences of these groups may be attributed to the change in negative charge density and the arrangement of chitosan sulphate.
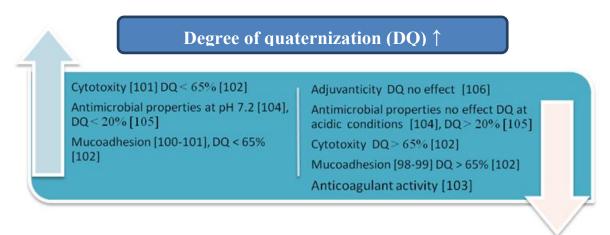
In another investigation [104] the antibacterial activity of a series of methylated chitosan and chitooligomer derivatives possessing various degrees of methylation against *Staphylococcus aureus* was examined. It was observed that *N*-quaternization was mainly responsible for the antibacterial effects at pH 7.2, whereas it did not contribute to the antibacterial activity under acidic conditions.

Sajomsang *et al.* [105] synthesized 17 derivatives of chitosan consisting of a variety of *N*-aryl substituents (ES parameter), and each of the derivatives was further quaternized using the quaternizing agent that reacted with either the primary amino or hydroxyl groups of the glucosamine residue of chitosan. All the quaternized derivatives of chitosan showed antibacterial activity, but derivatives with ES values higher than 20% exhibited low antibacterial activity. Probably a low quaternary ammonium moiety was obtained owing to the steric hindrance of the *N*-aryl substituents.

The influence of the DQ of *N,N,N*-trimethyl chitosan (TMC) on its adjuvanticity was also investigated [106]. The adjuvant properties of TMCs as an intranasal adjuvant are strongly decreased by the re-*N*-acetylation of TMC, whereas the DQ did not significantly affect this activity.

The most important results presented in this section are summarized in Figure 10.
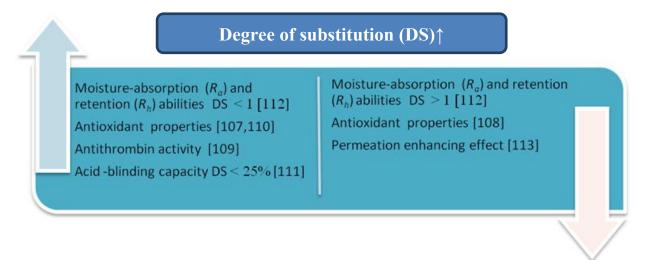
Figure 10. Influence of degree of quaternization on the selected physicochemical and biological properties of chitin/chitosan samples.

7. Influence of the Degree of Substitution

Chitin/chitosan contain three types of reactive functional groups: an amino/acetamido group at position C-2, and secondary and primary hydroxyl groups at positions C-3 and C-6 respectively. The degree of substitution of these groups may influence the biomedical properties of chitin/chitosan-based products (Figure 11).

Figure 11. Influence of degree of substitution on the physicochemical and biomedical properties of chitin/chitosan-based products.

The influence of DS on antioxidant activity of branched-chain chitosan derivatives (the Schiff base-type chitosan-fructose derivative and *N*-alkylated derivatives of chitosan) was investigated by Ying *et al.* [107]. The scavenging effect on DPPH increased with increasing DS, which was contrary to previously published data [108]. The scavenging effect of the *N*-alkylation chitosan derivative at concentrations lower than 0.5 mg/mL was negative, whereas for chitosan and Schiff base-type chitosan derivatives at any concentration was positive. Presumably, the increase in DS of the derivatives caused

the decrease in –NH$_2$ and the increase in –OH and –N=C (Schiff base-type chitosan derivatives) or –NH–C (*N*-alkylation of chitosan derivatives). This indicates that the –NH$_2$ and –C=N had a better antioxidant ability than –NH–C.

Drozd *et al.* [109] compared the antithrombin activity of sulphated chitosan (CP) with different degrees of polymerization and sulphation *in vivo* and *in vitro*. An average molecular weight of 20–123 kDa and sulphur contents of 8.8–16.9% were determined for these derivatives. The CPs with relatively low molecular weights (M$_W$ 61–82 kDa, degrees of polymerization 188–252) and the high sulphation patterns (sulphur contents 15.6–16.9%, sulphation degree 1.58–1.86) were characterized by a higher antithrombin activity (30–52 IU/mg). They recommend an *in vivo* system for evaluating the antithrombin activity of the CPs.

Xing *et al.* [110] described the effect of the preparation conditions of sulphated chitosans on their molecular weight and sulphur content. The sulphated chitosans they obtained contained 11.95–16.20% of sulphur, which corresponds to a degree of sulphation of 1.82–2.46 per glucosamine unit. The authors determined the antioxidant activity of high-molecular weight and high-sulphate-content chitosans (HCTS) and showed that HCTS could scavenge superoxide and hydroxyl radicals. Their IC$_{50}$ values were 0.012 and 3.269 mg/mL respectively.

Yoo *et al.* [111] selectively modified the C-6 primary alcohol on chitosan by sequential TEMPO (2,2,6,6-tetramethyl-1-piperidine oxoammonium ion)-mediated oxidation and monitored the changes in water solubility at different levels of oxidation (from 25 to 100%). Additionally, the biological functionality of 6-oxychitosan was examined by evaluating its bile acid-binding capacity. It was established that during the specific oxidation process, 25%-oxidized 6-oxychitosan had the highest solubility, and the solubility decreased substantially from 0.72 to 0.15% as the degree of oxidation increased from 25 to 100%. The binding capacity for 100%-oxidized chitosan samples was only 4.5% better than the control sample. It was observed that 25%-oxidized 6-oxychitosan displayed a much better binding capacity than any other sample groups.

The strongest cholic acid-retardation index (CRI, %) of highly soluble 25%-oxidized 6-oxychitosan was consistently observed until 24 h of dialysis, which means the CRI was closely related to the water solubility of 6-oxychitosan.

In another investigation, the relationships between the molecular structure and moisture-absorption and moisture-retention abilities of carboxymethyl chitosan with different degrees of *N*-acetylation (DA 5–72%) and carboxymethylation (DS 0.15–1.21) were studied by Chen *et al.* [112]. It was observed that the moisture-absorption (R_a) and -retention (R_h) abilities of carboxymethyl chitosans are closely related to the DA and DS values. Under conditions of high relative humidity, the maximum R_a and R_h were obtained at DA about 50%. When the DA value deviated from 50%, R_a and R_h decreased. Under dry conditions the R_h was the lowest when DA was 50%, and R_a and R_h increased with increasing DS value. Carboxymethyl chitosans with DS from 0.6 to 1.0 showed R_a and R_h values equal to or better than those of hyaluronic acid. A further increase in DS above 1.0 reduced the increasing tendency of R_a and R_h, and even some decreases in R_a and R_h were observed.

The effect of the *N*-betainate (DS 0.05–0.9) and *N*-piperazine derivatives (DS 0.15–0.9) of chitosan on the paracellular transport of mannitol and cell viability in the Caco-2 cell model were studied by Korjamo *et al.* [113]. The *N*-betainate derivative with the lowest degree of substitution (DS 0.05) was the most effective in the transport of mannitol at 1.0% (w/v) concentration. The effect was essentially

lost at DS > 0.15. The activity of *N*-piperazines depended both on the DS and the number of quaternary nitrogens. Derivatives with lower DS were generally more active than those with higher DS values, but the fastest mannitol transport was slower than that achieved with the best *N*-betainate derivative. In general, the relationship between the degree of substitution and activity suggests that an intact chitosan backbone is essential for the bioactivity of chitosan derivatives. Chitosan *N*-betainates should contain only the minimum number of substituents required to guarantee water solubility in order to reach the highest activity.

8. Influence of the Preparation Techniques of chitin/Chitosan-Based Products Offered for Biomedical and Pharmaceutical Applications

Chitin/chitosan-based products offered for biomedical and pharmaceutical applications are available in many different forms: nanoparticles, microspheres, hydrogels, films, fibers or tablets [30]. Considerable research efforts should be directed towards developing safe and efficient chitin/chitosan-based formulations. Agnihotri *et al.* [50] described the methods of preparing chitosan-based micro/nanoparticulate drug delivery systems. Chemically modified chitosan or its derivatives used in drug delivery systems are also reported. The choice of the method of preparation depends upon factors such as particle size requirement, the thermal and chemical stability of the active agent, reproducibility of the kinetic release profiles, stability of the final product and residual toxicity associated with the final product. All of these factors can influence the biomedical properties of chitin/chitosan-containing products (e.g., [114,115]).

For example, the drug release rates of theophylline, aspirin and griseofulvin from chitosan microspheres prepared by the emulsion cross-linking of a chitosan solution in paraffin oil as an external medium were influenced by cross-link density, particle size and initial drug loading [116].

Ko *et al.* [117] prepared chitosan microparticles with tripolyphosphate (TPP) by the ionic cross-linking method. Release behaviors of felodipine as a model drug were affected by various preparation processes. Chitosan microparticles prepared at a lower pH or a higher concentration of TPP solution resulted in slower felodipine release from microparticles. The release of this drug from TPP-chitosan microparticles decreased when the cross-linking time increased.

Katas and Oya Alpar [118] prepared chitosan nanoparticles using two methods of ionic cross-linking: simple complexation and ionic gelation using TPP. Both methods produced nanosized particles (<500 nm), depending on the type, molecular weight and concentration of chitosan. In the case of ionic gelation the TPP weight ratio and pH also affected the particle size. Chitosan-TPP nanoparticles with entrapped siRNA showed better properties as siRNA delivery vehicles than chitosan-siRNA complexes, possibly because of their high binding capacity and loading efficiency.

Yoskan *et al.* [114] described the preparation and evaluation of antimicrobial properties against silver nanoparticle-loaded chitosan-starch based films. The silver nanoparticles were prepared by γ-ray irradiation reduction of silver nitrate in a chitosan solution. They determined the minimum inhibitory concentration (MIC) of silver nanoparticles dispersed in chitosan solution. The films containing silver nanoparticles from 0.07 to 0.29% (w/w) exhibited antimicrobial activity against *Escherichia coli* and two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus cereus* (the MIC value for all test bacteria was 5.64 μg/mL). The author compared these results with other methods of silver nanoparticle

preparation. Silver nanoparticles prepared by the application of *Phytophthora infestans* showed lower MICs: 0.313 and 0.625 μg/mL against *E. coli* and *S. aureus* respectively [119], whereas the MICs of Ag nanoparticles synthesized by an electrochemical method were 5 and 2 μg/mL against *E. coli* and *S. aureus* respectively [120]. In contrast, nanoparticles prepared by a process involving photoassisted reduction showed higher MIC values: 6.25 and 12.5 μg/mL against *E. coli* and *S. aureus* respectively [121]. The authors suggested that the difference in MIC values could be related to the variation in nanoparticle size and shape. Silver/chitosan nanocomposites were characterized by Cao *et al.* [122] in terms of their particle sizes and morphology. The antibacterial activities of these nanocomposites against *S. aureus* and *E. coli* were investigated. When the concentration of the silver ions increased, the inhibition ability of the silver/chitosan nanocomposites against both *E. coli* and *S. aureus* decreased, and the MIC (defined as the lowest silver/chitosan nanocomposite concentration resulting in a lack of visible microorganism growth) of the composites increased. These authors thought that when the concentration of the silver ions increased, the size of the silver nanoparticles obtained would increase as well. The larger particles have a smaller surface area and a smaller infiltration rate, which could lead to an inferior antibacterial activity. The shapes of the silver nanoparticles ions also influence antibacterial activity. When the atomic density of the polyhedron surface was higher, the antibacterial effects were more obvious [123].

Foster and Butt [115] confirmed the statement that the morphology of the final biomaterial can strongly influence the antimicrobial activity of chitosan biomaterials. They showed that while native chitosan solutions demonstrated strong bactericidal activity against *S. aureus*, *S. epidermidis* and *E. coli* (the complete inhibition 98 ± 2%), this beneficial property was lost in thin films cast from the same solutions. Chitosan films (20 μm) showed no inhibitory effects against these bacteria. At pH 5, chitosan solutions exhibited higher bactericidal effects of this biopolymer than at pH 7.

In another study, Berger *et al.* [49] presented the physical properties of chitosan hydrogels formed by aggregation or complexation, which can be used in biomedical applications. It was noted that the preparation of grafted chitosan hydrogels was more complex and did not always improve biocompatibility compared to covalently cross-linked hydrogels. On the other hand, grafted chitosan hydrogels enhanced certain intrinsic properties of chitosan such as bacteriostasis tic and wound-healing.

The morphology and mechanical properties of chitosan fibers obtained by ageing gel-spinning were reported by Notin *et al.* [52]. They reported that ageing in the ambient atmosphere played an important role in the crystalline microstructure in relation to the kinetics of ammonium acetate hydrolysis, the formation of a weak fraction of the anhydrous allomorph of chitosan, and the increase in the crystallinity index, whereas Young's modulus (which quantifies the elasticity of the polymer) was higher and the tenacity slightly lowered. In addition, gel-jet-stretched or dry-jet-stretched fibers could be stored for at least 3 months in the ambient atmosphere without any significant degradation.

The antimicrobial activity of cotton fabrics treated with different cross-linking agents (butanetetracarboxylic acid (BTCA) and Arcofix NEC (low formaldehyde content)) and chitosan was tested by El-tahlawy *et al.* [124]. Both the type and concentration of the finishing agent in the presence of chitosan as well as the treatment conditions significantly affected the performance properties and antimicrobial activity of the treated cotton fabrics. The antimicrobial activity of cotton fabrics treated with BTCA was higher than that of the fabrics treated with Arcofix NEC. The maximum antimicrobial activity was obtained when the cotton fabrics were treated with 0.5–0.75% chitosan of molecular

weight 1.5–5 kDa, and cured at 160 °C for 2–3 min. Partial replacement of Arcofix NEC with BTCA enhanced the antimicrobial activity of the treated fabrics in comparison with that of Arcofix NEC alone.

González-Rodríguez *et al.* applied experimental statistics to study the formulation variables influencing the coating process of lidocaine hydrochloride (LID) liposomes by chitosan [125]. These variables were the concentration of the chitosan coating solution, the dripping rate of this solution on the liposome colloidal dispersion, the stirring rate, the time elapsing since the production of liposomes for the liposome coating and finally the amount of drug entrapped in the liposomes. The results indicated that the amount of LID was the predominant factor increasing the drug entrapment capacity (EE). The CE (%) response was affected mainly by the concentration of the chitosan solution and the stirring rate, although all the interactions between the main factors are statistically significant.

Germershaus *et al.* [126] studied the relationships between chitosan, trimethyl chitosan and polyethyleneglycol-graft-trimethyl chitosan/DNA complex structures, their physicochemical properties (hydrodynamic diameter, condensation efficiency and DNA release), the cytotoxicity of these polymers, and the uptake- and transfection efficiency of polyplexes *in vitro*. The formation of aggregates of about 1,000 nm and with reduced DNA condensation efficiency were observed for all chitosan polyplexes. These authors established that quaternization of chitosan strongly reduced the aggregation tendency and the pH dependence of DNA complexation. Apart from a reduction in cytotoxicity, PEGylation improved the colloidal stability of polyplexes and significantly increased cellular uptake compared to unmodified trimethyl chitosan.

Jayakumar *et al.* [127] described the preparation, properties and biomedical applications of chitin and chitosan-based nanofibers. The influence of many factors (e.g., the process production, the chitosan concentration, viscosity or PEO/chitosan ratio) on the physicochemical characteristics and biomedical properties of the nanofibers obtained was also presented.

9. Conclusions

Chitin and chitosan are natural aminopolysaccharides with unique structures, multidimensional properties and highly sophisticated functions that are widely used in biomedical applications. Their microstructure and biomedical activity are strongly dependent on the source of chitin and the conditions of chitosan preparation. Knowledge of the structural differences among chitin/chitosan products is very important in determining the properties of these biopolymers and is essential for the structure-activity-analysis of biological systems. For medical applications, various forms of chitosan-based products are available, like finely-divided powders, films, membranes, nanoparticles or hydrogels. The potential implementation of chitosan in medicine can only be explored if its utilized form is properly developed and prepared. It is very important for biotech companies, which have to control different parameters influencing the characteristics of chitin/chitosan materials offered for biomedical applications, and will help to accelerate their future applications. So far, only the influence of the most important factors, such as the weight-averaged molecular weight (M_W) and degree of *N*-acetylation (DA) on the biomedical activity of chitin/chitosan products, have been investigated in detail and summarized in some papers. On the basis of literature data it has been possible to present in this review important information about the influence of other parameters such as the source of chitin

and chitosan, pH, the ionic strength, the concentration, the degree of quaternization, the degree of substitution, and the condition of preparation of chitin/chitosan-based products on their biomedical activity. Influence of selected key factors mentioned in the text on the physicochemical and biological properties of chitin/chitosan samples is summarized in Table 1.

Table 1. Influence of selected key factors mentioned in the text on the physicochemical and biological properties of chitin/chitosan samples (DS—degree of substitution, DQ—degree of quaternization, IS—ionic strength, C—concentration, pH).

Physicochemical properties [References]	
Solubility	DQ ↑ [32-35]
Syneresis	pH < 4 ↑ pH > 4 ↓ [77]; IS ↑ [77,84]
Viscosity	DQ ↑ [98]
Cytotoxity	DQ ↑ [101,102]; C no effect for chitosan molecules [92]; ↑ for nanoparticles [92]
Moisture-absorption (R_a) and -retention (R_h) abilities	DS <1 ↑, DS >1 ↓ [112]
Zeta potential	C ↑ [51]
Intrinsic viscosity	IS ↓ [83]
Particle size	C ↑ [51]
Aggregation	C ↑ [67,96]
Drug loading	pH ↑ [79]
Entrapment efficiency	pH ↑ [81]
Biological properties	
Antimicrobial	DQ ↑ at pH 7.2, no effect at acidic conditions [104], DQ up 20% ↑ when ↓ [105]; C ↑ [85-88,94,95]; pH ↓ [68-74]
Antioxidant	DS ↑ [107,110], ↓ [108]
Mucoadhesian	DQ ↓ [98], ↑ [100,101], ↑ up to DQ 65% when ↓ [102]
Permeation enhancing effect	DS ↓ [113]; pH ↓ [80]
Antitumor	pH < 4 or pH > 10 ↑ [76]
Anticoagulant	DQ ↓ [103]
Adjuvanticity	DQ no effect [106]
Antithrombin	DS ↑ [109]
Acid-blinding capacity	DS ↑ up to 25% [111]
Immune enhancing effect	pH < 4 or pH > 10 ↑ [76]
Calcium/cobalt blinding capacity	pH ↑ [82]
Transfection efficiency	C↓ [51,89,90]
Gene knockdown efficiency	C ↓ [90]
↑ directly proportion to property; ↓ inversely proportional to property	

It should be also pointed that:

- when a chitosan solution is used in the investigations, factors such as concentration, pH, ionic strength, the nature of salt counterion, and the addition of non-aqueous solvent should be taken into account,

- chemical methods of modification can generate completely new chitin/chitosan-based biofunctional materials. For the obtained materials such parameters as the degree of quaternization and/or the degree of substitution should be determined and their influence on the investigated biological properties tested,

- the biological properties of chitin/chitosan materials used directly in biomedical applications (e.g., antimicrobial effects) could be different that observed for materials obtained after preparation of nanoparticles, microspheres, hydrogels, films, fibers or tablets,

- considerable research efforts should be directed towards developing safe and efficient chitin/chitosan-based formulations. As an example, such factors as particle size requirement, the thermal and chemical stability of the active agent, reproducibility of the kinetic release profiles, stability of the final product and residual toxicity associated with the final product depends upon the methods of preparing chitosan-based micro/nanoparticulate drug delivery systems,

- the influence of physicochemical properties apart from M_W and DA on chitosan-based formulation affecting the delivery of DNA and siRNA [51] and on antimicrobial properties of chitin/chitosan materials [68-74,85-88,94,95,104,105] is the best known. For other biological properties our knowledge is still not sufficient,

- chitin/chitosan properties are interrelated and many times they could influence the bioactivity in a conflicting manner, e.g., would healing properties and permeation enhancement properties are inevitably linked to other such as mucoadhesion and cytotoxicity. For these reasons, these types of investigations should be continued.

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