A Review of the Antimicrobial Activity of Chitosan

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Abstract: Chitosan, a versatile hydrophilic polysaccharide derived from chitin, has a broad antimicrobial spectrum to which gram-negative, gram-positive bacteria and fungi are highly susceptible. In the current review, three possible and accepted antimicrobial mechanisms for chitosan are presented and briefly discussed. The activity dependence on polymeric molecular weight (MW) and degree of acetylation (DA) are described. The chitosan minimum inhibitory concentrations (MIC) are summarized according to recent data found in the literature. The potential to improve inhibitory growth of bacteria by using water soluble chitosan derivatives is also discussed. The data indicate that the effectiveness of chitosan varies and is dependent on species of target microorganisms.

Keywords: *Chitosan, polysaccharide, antimicrobial mechanisms.*

Introduction

Chitin is a polysaccharide of animal origin found abundantly in nature and characterized by a fibrous structure. It forms the basis of the main constituent of the outer skeleton of insects and crustaceans like shrimp, crabs and lobster[1]. The chemical structure of chitin is similar to cellulose, having one hydroxyl group on each monomer substituted with an acetylamine group (Figure 1). The extraction of chitin involves an acid removal of calcium carbonate (demineralization), generally by hot reaction with HCl, HNO_3 or HCl, etc., followed by a deproteinization (removal of proteins). This step usually performed by alkaline treatments (e.g. with NaOH)[1,2]. In its extracted crude form, chitin has a highly ordered crystalline structure, is translucent, resilient and quite tough. It has, however, poor solubility and low reactivity.

The chitin structure can be modified by removing the acetyl groups, which are bond to amine radicals in the C2 position on the glucan ring, by means of a chemical hydrolysis in concentrated alkaline solution at elevated temperature to produce a deacetylated form (Figure 1). When the fraction of acetylated amine groups is reduced to 40-35%, the resultant co-polymer, (1 → 4)-2-amine-2-deoxy-β-D-glucan and (1 → 4)-2-acetamide-2-deoxy-β-D-glucan, is then referred to as chitosan. Chitosan is primarily characterized by its molecular weight (MW) and the degree of acetylation (DA). Commercially chitosan is available with > 85% deacetylated units (DA < 15%), and molecular weights (MW) between 100 and 1000 kDa. There is no a specific standard to define MW, but it is accepted that Low MW < 50 kDa, Medium MW 50 – 150 kDa, and High MW > 150 kDa.

Chitosan is a weak base and is insoluble in water, but soluble in dilute aqueous acidic solutions below its pKa (~6.3), in which it can convert glucosamine units ($-NH_2$) into the soluble protonated form ($-NH^+_3$). The solubility of chitosan depends on its biological origin, molecular weight and degree of acetylation[3]. Since chitosan is soluble in diluted acid solutions, films can be readily prepared by casting or dipping, resulting in dense and porous structure[4,5].

Chitosan film is regarded as biofunctional material, well tolerated by living tissues, particularly applicable as edible coatings to prolong shelf-life and preserve quality of fresh foods[6]. In medical field, chitosan films have been tested as curative wound dressing and as scaffolds for tissue and bone engineering[7]. Additionally the reactive functional groups present in chitosan (amino group at the C2 position of each deacetylated unit and hydroxyl groups at the C6 and C3 positions) can be readily subjected to chemical derivatization allowing the manipulation of mechanical and solubility properties[8] enlarging its biocompatibility.

The Antimicrobial Models of Chitosan

Chitin and chitosan have been investigated as an antimicrobial material against a wide range of target organisms like algae, bacteria, yeasts and fungi in experiments involving *in vivo* and *in vitro* interactions with chitosan in different forms (solutions, films and composites). Early research describing the antimicrobial potential of chitin, chitosan, and their derivatives dated from the 1980-1990s[9-14]. Generally, in these studies the chitosan is considered to be a bacteriocidal (kills the live bacteria or some fraction therein) or bacteriostatic (hinders the growth of bacteria but does not imply whether or not bacteria are killed), often with no distinction between activities. Recent data in literature has the tendency to characterize chitosan as bacteriostatic rather than bactericidal[15], although the exact mechanism is not fully understood and several other factors may contribute to the antibacterial action[16].

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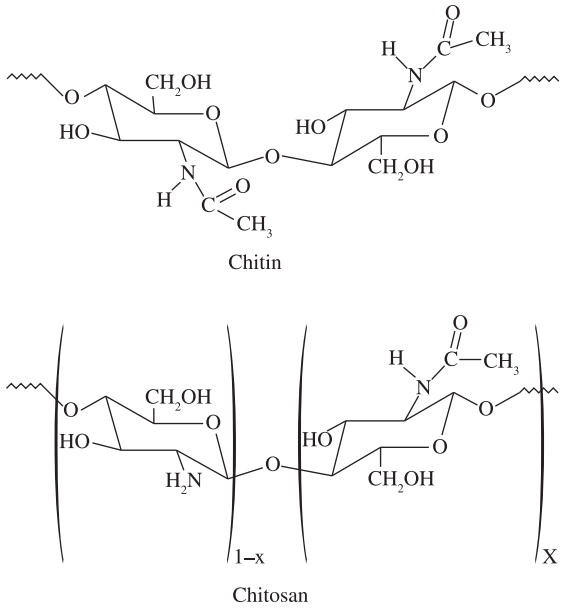

Figure 1. Schematic representations of the chemical structures of the chitin and chitosan.

Three models have been proposed, the most acceptable being the interaction between positively charged chitin/chitosan molecules and negatively charged microbial cell membranes. In this model the interaction is mediated by the electrostatic forces between the protonated NH^{+}_{3} groups and the negative residues[17], presumably by competing with Ca^{2+} for electronegative sites on the membrane surface[18].

This electrostatic interaction results in twofold interference: i) by promoting changes in the properties of membrane wall permeability, thus provoke internal osmotic imbalances and consequently inhibit the growth of microorganisms[10,12], and ii) by the hydrolysis of the peptidoglycans in the microorganism wall, leading to the leakage of intracellular electrolytes such as potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, nucleic acids, glucose, and lactate dehydrogenase)[9,11,13,19,20].

This model was investigated in a recent work by Raafat et al.[16], who observed under transmission electron microscope the ultrastructural changes of *S. simulans 22* cells upon exposure to positively charged chitosan. It was possible to observe and identify chitosan molecules attached on bacteria cell surfaces. In the interacting sites it was registered that the cell membrane became locally detached from the cell wall, giving rise to "vacuole-like" structures underneath the wall. The detachment generates ions and water efflux, provoking decreases on the internal bacteria pressure[16]. Visual confirmation of an effective membrane lysis been also reported on gram-negative and gram-positive bacteria[21-23].

Since such mechanism is based on electrostatic interaction, it suggests that the greater the number of cationized amines, the higher will be the antimicrobial activity[24,25]. This suggests that chitosan has higher activity than that found for chitin and this has been confirmed experimentally[17,24]. It is worth observing that the amount of polycationic chitosan available to bind to a charged bacterial surface is apparently reduced as the concentration of chitosan increases[15,26]. A possible explanation is that in the presence of a larger number of charged sites, the chains tend to form clusters by molecules aggregation while they are still in solution[4]. Observations have confirmed that at higher concentrations, the chitosan tends to form a coating over the bacteria, not necessary attached to the surface and independently of the bacteria type[13]. In such condition, adjustments on pH could be decisive for a good solubility and to keep the chains apart from each other.

Concerning the bacteria surface polarity, the outer membrane of gram-negative bacteria consists essentially of lipopolysaccharides containing phosphate and pyrophosphate groups which render to the surface a density of negative charges superior to that observed for gram-positive ones (membrane composed by peptidoglycan associated to polysaccharides and teichoic acids)[27]. This supports the evidence that the leakage of intracellular material observed by chitosan in gram-negative is superior to that reported in gram-positive bacteria[21-23].

The bacterial effectiveness on gram-positive or gram-negative bacteria is however, somewhat controversial. Some authors have stated that chitosan generally showed stronger effects for gram-positive bacteria (e.g. *Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, L. bulgaris, etc.*) than for gram-negative bacteria (e.g. *E. coli, Pseudomonas fluorescens, Salmonella typhymurium, Vibrio parahaemolyticus*, etc.)[28-31]. Conversely, it has been demonstrated that hydrophilicity in gram-negative bacteria is significantly higher than in gram-positive bacteria, making them most sensitive to chitosan[32]. These findings are confirmed by several *in vitro* experiments in which gram-negative bacteria appear to be very sensitive to chitosan, exhibiting increased morphological changes on treatment when compared to gram-positives[22,23,33-35]. The charge density on the cell surface is a determinant factor to establish the amount of adsorbed chitosan. More adsorbed chitosan would evidently result in greater changes in the structure and in the permeability of the cell membrane. This would suggest that the antibacterial mode of action is dependent upon the host microorganism[24].

Another proposed mechanism is the binding of chitosan with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms[10,13,36]. In this the chitosan molecules is assumed to be able to pass through the bacterial cell wall, composed of multilayers of cross-linked murein, and reach the plasma membrane. Observation by confocal laser scanning microscopy[7] confirmed the presence of chitosan oligomers (a chain with few number of monomer units) inside *E. coli* exposed to chitosan under different conditions. Raafat et al.[16] stated that in spite of been accepted as a possible mechanism, the probability of it occurring is

rater low. The prevailing contention is that chitosan acts essentially as an outer membrane disruptor rather than as a penetrating material[16,34].

The third mechanism is the chelation of metals, suppression of spore elements and binding to essential nutrients to microbial growth[37,38]. It is well known that chitosan has excellent metal-binding capacities where the amine groups in the chitosan molecules are responsible for the uptake of metal cations by chelation[23]. In general, such mechanism is more efficient at high pH in where positive ions are bounded to chitosan, since the amine groups are unprotonated and the electron pair on the amine nitrogen is available for donation to metal ions. A model proposed based on the system chitosan-Cu, relate the pH dependence on the proportion of available sites for interacting in polysaccharide backbone[39]. At pH < 6 the complexation involves only one NH_2 group and three hydroxyls or H_2O molecules, while at pH > 6.7 is likely to have two NH_2 involved in the complex formation. For higher pHs, i.e., 7-9, the deprotonation of hydroxyl groups are considered to occur and the predominant complexation is ruled by two $–NH_2$ and two hydroxyl groups dissociated. Similarly, in a recent model proposed by Wang et al.[40], the metal is arranged as an electron acceptor connected to one or more chitosan chains via $–NH_2$ and by forming bridges to hydroxyl groups, as illustrated in Figure 2.

It is unquestionable that chitosan molecules in bacteria surrounds might complex metals and blockage some essential nutrients to flow, contributing to cell death[1]. Nevertheless, this is, evidently, not a determinant antimicrobial action since the sites available for interaction are limited and the complexation reach saturation in function of metal concentration.

Influence of the Degree of Acetylation and Molecular Weight

Several studies have shown that the biological activity of chitosan depends significantly on its molecular weight (MW) and degree of acetylation (DA). Both parameters affect the antimicrobial activity of chitosan independently, though it has been suggested that the influence of the MW on the antimicrobial activity is greater then the influence of the DA[41].

To cite recent examples, studies carried out on *Bacillus cereus, E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella enterica, B. subtilis, Listeria monocytogenes* and *Klebsiella pneumoniae*[42-47], proved that for lower chitosan MW (LMW), greater is the observed effect on the reducing of microorganism growth and multiplication. The size and conformation appears to be fundamental to understand the effectiveness of LMW chitosan. The mobility, attraction and ionic interaction of small chains are easier than of big ones facilitating the adoption of an extended conformation and an effective binding to the membrane surface[1].

Similarly but in different intensity, chitosan antimicrobial effectiveness is improved as the degree of acetylation is lower[46,48]. Studies on chitin and chitosan with different DA were analyzed against fungi (*Aspergillus fumigatus, Aspergillus parasiticus, Fusarium oxysporum, Candida albicans*); Gram-positive *(Staphylococcus aureus, Staphylococcus saprophyticus, Bacillus cereus, Listeria monocytogenes) and* Gram-negative bacteria (*Escherichia coli, Samonella tiphymurium, Pseudomonas aeruginosa, Enterococcus faecailis, Aeromonas hydrophila, Shigella dysenteriae, Vibrio cholerae, Vibrio parahaemolyticus).* In all cases the antimicrobial activity also increased with decreasing DA[48-50].

As already mentioned, the DA is determinant in the solubility and charge development, where the $–NH_2$, –OH groups in the molecule of chitosan are considered as the

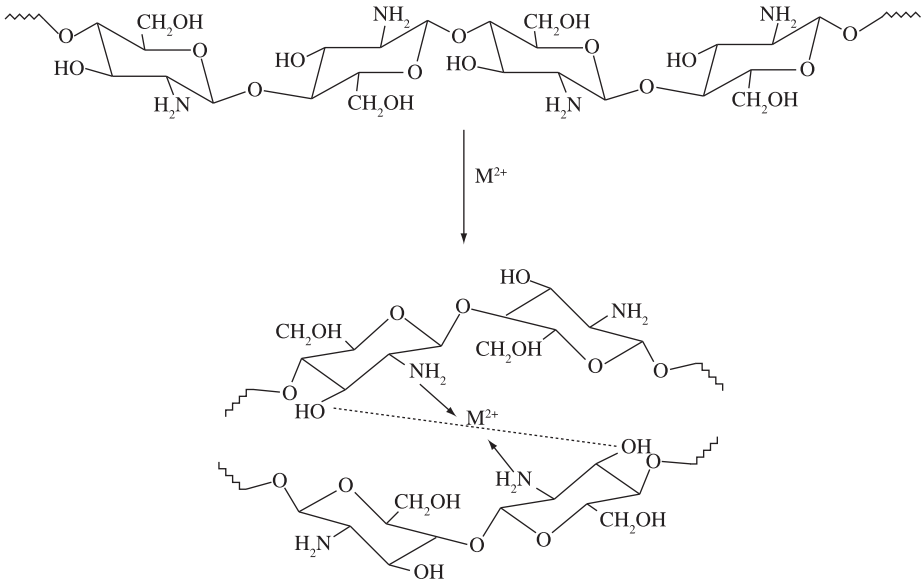

Figure 2. Metal-chitosan complexation model according to Wang et al.[40].

dominating reactive sites. Hence as the DA is reduced, higher will be the free amino groups present in chitosan and higher will be the antimicrobial effect[48].

Antifungal Activity

Similarly to bacteria, the chitosan activity against fungus is assumed to be fungistatic rather than fungicidal with a potential to communicate regulatory changes in both the host and fungus[16,51]. Generally chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation and radial growth[52,53]. Most of the studies have been done on yeasts and moulds associated with food and plant spoilage. For these, in the presence of chitosan, several biological processes are activated in plant tissue, where chitinases are induced with action on biotrophic and necrotrophic mycoparasites, entomopathogenic fungi and vesicular arbuscular mycorrhizal fungi[53].

The antifungic mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth, similarly to the effects observed in bacteria cells[52]. Microscopic observation reported that chitosan oligomers diffuse inside hyphae interfering on the enzymes activity responsible for the fungus growth[54]. The intensity of degradation action of chitosan on fungal cell walls is also dependant upon the concentration, DA and local pH[55]. Studies conducted in nutrient agar on cultures of *R. solani* and *S. rolfsii* reveled that the percentage of fungus germination decreased with increasing the chitosan concentration in the medium. Generally the primary observed influence is on the length of the lag phase. As the inhibition process takes place, the medium shifted toward alkalinity which reduces the effectiveness of the chitosan[55].

Inhibition rate in order of 80% against plant fungus such as *Phomopsis asparagi* and as high as 95% against *Fusarium oxysporum, Cucumernum owen, Rhizoctonia solani* and *Fusarium oxysporum* have been, however, known to occur with low chitosan concentration (20-150 $mg.L^{-1}$)[56].

Sensitivity of Microorganism Strains to Chitosan

Chitosan has several advantages over regular type of disinfectants owing to its broad spectrum of activity. Chitosan has been observed to act more quickly on fungi than on bacteria[57], and activity against typhoid organisms are comparable to the standard antibiotics used in clinical practice[26,33,57]. As discussed this antimicrobial activity has a strong dependence on MW and DA characteristics and also varied according microorganism strains.

There are many studies about the minimum inhibitory concentration (MIC) for chitin, chitosan, their derivatives or combination, with different results for different microorganism. MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. It is dependent of many factors and the non-standardized procedures make difficult to compare MIC results from author to author[58,59]. MIC however is useful as a practical indicator of a primary activity against a selected pathogenic microorganism. In Table 1 is a brief summarization

Table 1. Minimum inhibitory concentration (MIC) for chitosan against several microorganisms (concentration normalized to ppm).

Sensible organisms	MIC (ppm)	Reference
Gram negative		
Escherichia coli	20	[7]
	100	[50]
	468	[60]
	650	[49]
	1000	[31,61,62]
Xanthomonas campestris	500	[63]
Salmonella enterica	2000	[64]
	3000	[65]
Samonella tiphymurium	>1000	[31]
	1500	[50]
	2000	[61]
Pseudomonas aeruginosa	>200	[50]
	1700	[61]
Aeromonas hydrophila	1000	[50]
Shigella dysenteriae	>200	[50]
Vibrio cholerae	200	[50]
Vibrio parahaemolyticus	150	[50]
	1000	[31]
Pseudomonas fluorescens	250	[19]
	500	[7]
	~1000	[31]
Enterobacter aerogenes	250	[19]
Gram positive		
Bacillus cereus	<1000	[31]
	1000	[7,50]
Bacillus megaterium	800	[44]
Staphylococcus aureus	20	[7]
	100	[19]
	>800	[44]
	700	[61]
	>1250	[49]
Listeria monocytogenes	150	[50]
	250	[19]
	800	[31]
Candida lambica	250	[19]
Lactobacillus plantarum	<1000	[44]
	2000	[64]
Lactobacillus brevis	1000	[31]
Lactobacillus bulgaricus	>1000	[31]
Fungi		
Aspergillus fumigatus	>2000	[50]
Aspergillus parasiticus	>2000	[50]
Fusarium oxysporum	100	[7]
Botrytis cinerea	10	[7]
Byssochlamys spp.	1000-5000	[38]
Candida albicans	500	[50]
	600	[61]
	>1250	[49]
Drechstera sorokiana	10	[7]
Microsporum canis	1100	[61]
Trichophyton mentagrophytes	2200	[61]

of recent works showing some MIC values found for chitosan against several microorganisms.

Water Soluble Chitosan

Although chitin and chitosan have been confirmed as attractive biomacromolecules with relevant antimicrobial properties, applications are somewhat limited due to both being water-insoluble. Water soluble chitosan derivatives can be obtained by the introduction of permanent positive charges in the polymer chains, resulting in a cationic polyelectrolyte characteristic independently of the pH of the aqueous medium. This can be accomplished for instance by the quaternization of the nitrogen atoms of the amino groups. To attain this, an extensive methylation of chitosan is required that is carried out in suspension of dimethylsulfate, NaOH and NaCl resulting in N,N,N-trimethylchitosan (Figure 3)[66]. The synthesis of chitosan derivatives takes place by grafting methyl functionality onto chitosan amino groups at the C-2 position[67].

Studies with quaternary salts of chitosan reveled that the antimicrobial activity against bacteria is higher than that of chitosan[68]. Jia et al.[69], reported that the activity of N-propyl-N,N-dimethyl chitosan against *E. coli* is 20 times higher then that of chitosan, indicating that the derivatives with cationic charge exhibit particularly high activity. An important feature of the chitosan derivatives is the evidence that the alkyl moiety also plays an important role in the antimicrobial activity[69]. According to Xie et al.[70], at neutral pH, the degree of protonation of NH_2 is very low, so the repulsion of NH_3^+ is weak. Under such condition the intermolecular and intramolecular hydrogen bonds result in a formation of hydrophobic micro-area in the polymer chain rendering hydrophobic and hydrophilic parts, favoring the structural affinity between the bacteria cell wall and the derivative[26,70].

It would be expected that antimicrobial activity would increase as the content of the alkyl moiety increases, as confirmed by Rabea et al.[71], who found that the antibacterial activity had improved with an increasing on the chain length of the alkyl substituent. This better performance was attributed to the contribution of the hydrophobic portions of the derivatives.

Besides the quaternary salts of chitosan, other aqua-soluble derivatives such as hydroxypropyl and carboxymethyl chitosans exist. Hydroxypropyl chitosan derivatives with high degree of substitution (DS) are water insoluble, but after graftization with maleic acid they become soluble in neutral pH with antibacterial activity higher than that of the parent chitosan[70]. Studies with this kind of derivative are very important to help understand the mechanism of microbial-antimicrobial agent interaction. For example, it has been concluded that for neutral or alkaline media, the cationic nature of chitosan can no longer explain its antibacterial activity[70]. In this case, the strong coordination capability of NH_2 groups in chitosan chain might be one possible mechanism.

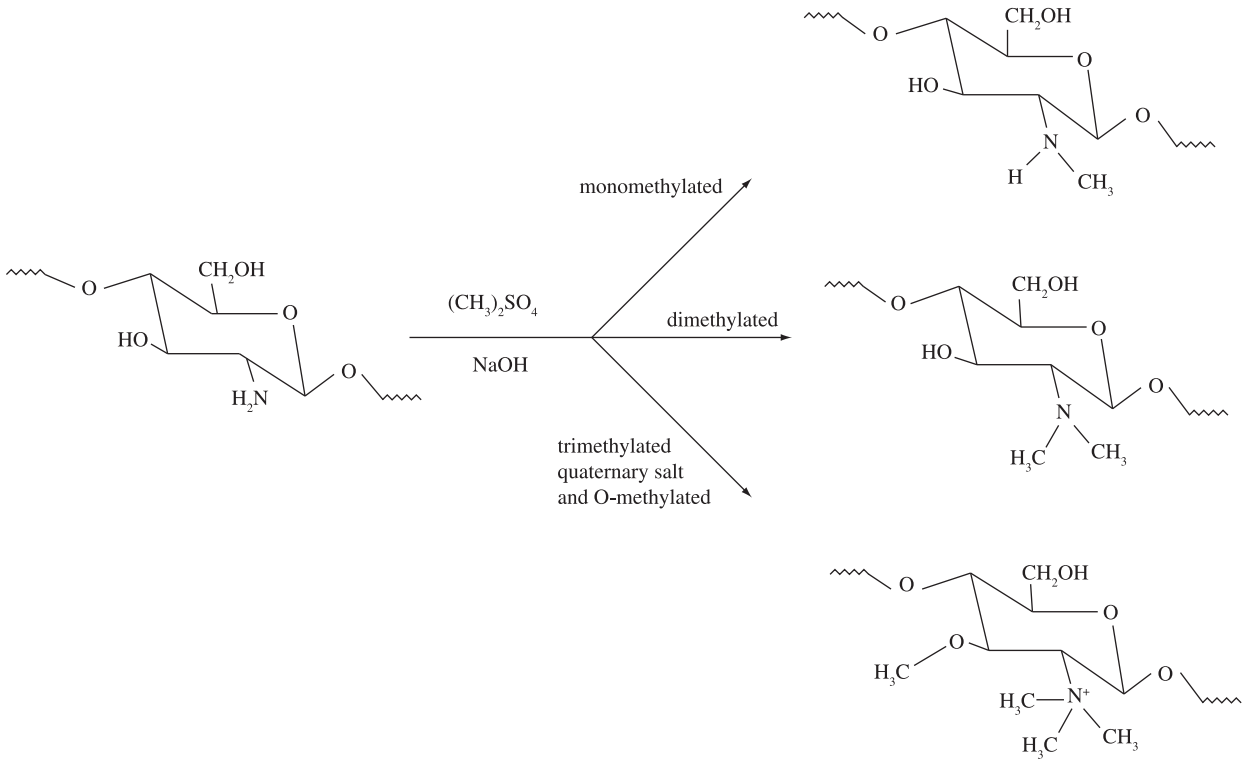

Figure 3. Schematic representation of the reaction leading to the quaternization of the amino groups of chitosan and resulting in N,N,N-trimethylchitosan[66].

The study with carboxymethyl chitosan realized by Sun et al.[72] is also very interesting since its derivative had both negative and positive substituint groups. They demonstrate that antimicrobial activities of carboxymethyl chitosan are affected either by the DS of quaternary group or by the MW, while no clear effect of the DS of carboxymethyl group was observed. A further and important conclusion was that when the derivative is complexed with calcium hydroxide as pulp-cap, it has better ability in inducing reparative dentine formation when compared to calcium hydroxide itself.

Conclusions

Chitosan is a versatile material with proved antimicrobial activity. Three antibacterial mechanisms have been proposed: i) the ionic surface interaction resulting in wall cell leakage; ii) the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms; and iii) the formation of an external barrier, chelating metals and provoking the suppression of essential nutrients to microbial growth. It is likely that all events occur simultaneously but at different intensities. The molecular weight (MW) and the degree of acetylation (DA) are also important factors in determining such activity. In general the lower the MW and the DA, the higher will be the effectiveness on reducing microorganism growth and multiplication. A study of previous work from the literature has not lead to any conclusive data as to whether the chitosan has higher activity on gram-positive or on gram-negative bacteria. On both species chitosan seems to act differently, though in both cases satisfactorily. Water soluble derivatives, which can be attained by chemical introduction of CH_3 in the main chain, enhancing the chitosan applicability in a large pH range and also improve the antimicrobial activity, opening up a broad range of possibilities.

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