Minireview

Chitosan and its antimicrobial potential – a critical literature survey

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Summary

Chitosan, an aminopolysaccharide biopolymer, has a unique chemical structure as a linear polycation with a high charge density, reactive hydroxyl and amino groups as well as extensive hydrogen bonding. It displays excellent biocompatibility, physical stability and processability. The term 'chitosan' describes a heterogenous group of polymers combining a group of physicochemical and biological characteristics, which allow for a wide scope of applications that are both fascinating and as yet uncharted. The increased awareness of the potentials and industrial value of this biopolymer lead to its utilization in many applications of technical interest, and increasingly in the biomedical arena. Although not primarily used as an antimicrobial agent, its utility as an ingredient in both food and pharmaceutical formulations lately gained more interest, when a scientific understanding of at least some of the pharmacological activities of this versatile carbohydrate began to evolve. However, understanding the various factors that affect its antimicrobial activity has become a key issue for a better usage and a more efficient optimization of chitosan formulations. Moreover, the use of chitosan in antimicrobial systems should be based on sufficient knowledge of the complex mechanisms of its antimicrobial mode of action, which in turn would help to arrive at an appreciation of its entire antimicrobial potential.
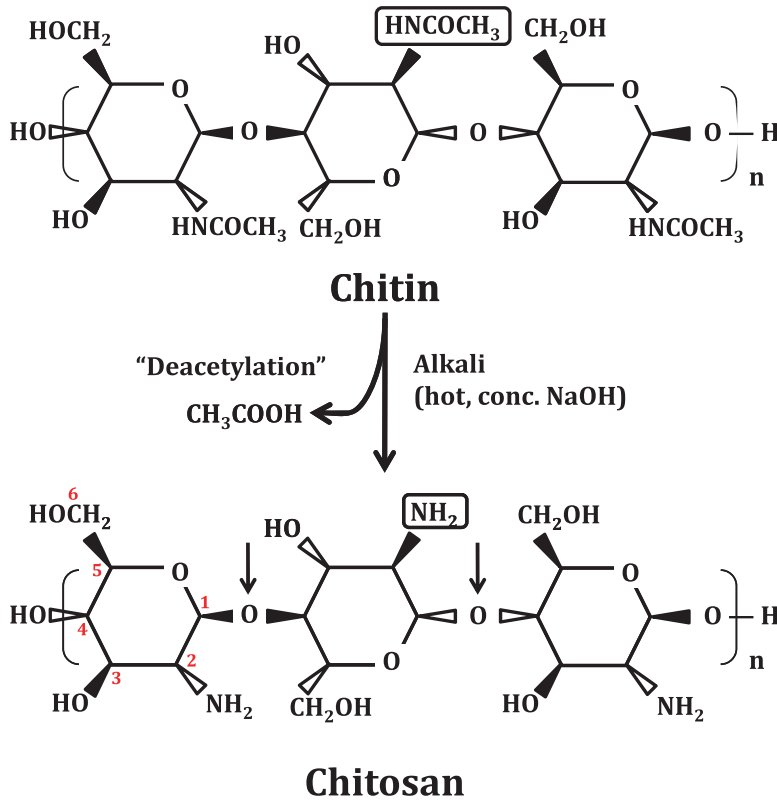
Introduction

Chitosan is primarily produced from chitin, which is widely distributed in nature, mainly as the structural component of the exoskeletons of arthropods (including crustaceans and insects), in marine diatoms and algae, as well as in some fungal cell walls. Structurally, chitin is an insoluble linear mucopolysaccharide (Fig. 1) consisting of *N*-acetyl-D-glucosamine (Glc*N*Ac) repeat units, linked by β-(1→4) glycosidic bonds (Tharanathan and Kittur, 2003). Technically, the structure of chitin is highly related to that of cellulose and may be regarded as cellulose where the hydroxyl [—OH] at the C-2 position is replaced by an acetamido [—NHCOCH₃] group (Suzuki, 2000).

Resources of chitin for industrial processing are crustacean shells and fungal mycelia (Shigemasa and Minami, 1995). However, its commercial production is usually associated with seafood industries, such as shrimp canning, where the processing of crustacean shells mainly involves the removal of proteins (deproteinization in a hot basic solution, usually sodium or potassium hydroxide) and calcium carbonate (demineralization with diluted acid), both present in crustacean shells in high concentrations, encasing the chitin microfibrils (Kumar, 2000). Chitin has aroused great interest not only as an underutilized resource, but also as a new functional material of high potential in various fields (Kumar, 2000). Several chitin derivatives have been prepared, but none was as commonly studied, on both the academic and industrial level, as chitosan.

One area of intense research activity has been the use of natural compounds, including chitosan, for preservation purposes; it has evolved from a need for safer and economically priced control of microbial stability of food and pharmaceutical systems, which is fast becoming a new research frontier (Roller, 2003). The present review will therefore report on some of the major aspects of chitosan as a promising and versatile biopolymer, including its physicochemical and biological properties, recent developments related to its applications, as well as economic aspects of its industrial utilization. Since a thorough investigation of the antimicrobial potential of chitosan is of significant importance in the design of antimicrobial

systems of industrial value, particular attention will be made to its *in vitro* antimicrobial activity and to its mode of action. Where possible or feasible, we will concentrate on aspects that could gain practical importance while developing antimicrobial systems that can be implemented into industrial applications.

Nature and sources

Chitosan, discovered by Rouget (1859), is a technologically important polysaccharide biopolymer. Chemically, it is a high-molecular-weight linear polycationic heteropolysaccharide consisting of two monosaccharides, Glc*N*Ac and D-glucosamine (GlcN), linked together by β-(1→4) glycosidic bonds (Fig. 1). The relative amount of the two monosaccharides in chitosan may vary, giving samples of different degrees of deacetylation (75–95%), molecular weights (MWs) (50–2000 kDa), viscosities, pK$_a$ values, etc. (Singla and Chawla, 2001; Tharanathan and Kittur, 2003).

The production of chitosan from chitin primarily takes place through exhaustive alkaline deacetylation (Fig. 1): this involves boiling chitin in concentrated alkali for several hours (40–45% sodium hydroxide, 120°C, 1–3 h). Since this *N*-deacetylation is almost never complete, chitosan is considered as a partially *N*-deacetylated derivative of chitin. Consequently, a sharp distinction between

chitin and chitosan on the basis of the degree of *N*-deacetylation cannot be drawn (Kumar, 2000; Rabea *et al.*, 2003).

Chitosan is also found in nature, such as in cell walls of fungi of the class *Zygomycetes*, in the green algae *Chlorella* sp., yeast and protozoa as well as in insect cuticles (Singla and Chawla, 2001; Pochanavanich and Suntornsuk, 2002). Advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan (Rabea *et al.*, 2003). However, chitosan from both sources differs slightly: whereas the acetyl groups in chitosan produced from crustacean chitin are uniformly distributed along the polymer chain, a chitosan of similar degree of deacetylation (DD) isolated from fungal cell walls would possess acetyl residues that are grouped into clusters. In contrast to most of the naturally occurring polysaccharides, e.g. cellulose, dextran, pectin, alginic acid, agar, agarose and carragenans, which are neutral or acidic in nature, chitosan is an example of a highly basic polysaccharide, with a nitrogen content varying between 5% and 8%, depending on the extent of deacetylation (Kumar, 2000).

Physicochemical aspects

Chitosan is commercially available from a number of suppliers in various grades of purity, MWs and MW distribu-

tions, chain lengths, degrees of deacetylation, charge densities and charge distributions, salt-forms, viscosities and water retention values. These properties greatly affect its physicochemical characteristics, which in turn govern almost all of its applications. Therefore, the choice of the most suitable grade for use is related to the application intended.

Molecular weight

Although the chemical and physical processes underlying some of the applications of chitosan and its derivatives are still not known in detail, considerable evidence has been gathered indicating that most of their physiological activities and functional properties depend on their MW (Rabea *et al.*, 2003). The MW distribution of a raw chitosan preparation is influenced by variable conditions employed in the deacetylation process; weight-average MWs (M_w) of several hundreds to over one million Dalton are common, with a mean molecular mass of up to 1 MDa, corresponding to a chain length of approximately 5000 U (Rhoades and Roller, 2000). Because of the influence of polymer composition and MW range on the various physicochemical properties of chitosan, it is very important to adequately characterize each batch of polymer produced. The MW of chitosan can be determined by several methods, such as light scattering spectrophotometry, gel permeation chromatography and viscometry (Kumar, 2000). Unlike monodisperse substances, no exact MW is indicated, but rather a number of different means are defined, to describe the sample statistically.

Degree of deacetylation

An important parameter to examine closely is the DD of chitosan, i.e. the ratio of Glc*N*Ac to GlcN structural units. The DD of chitosan is influenced by the preparation procedure; for example, increasing proportionally with increasing treatment time. It has an impact on the extent of moisture absorption, charge distribution, intrinsic viscosity and chitosan solubility in aqueous solutions (Singla and Chawla, 2001). A number of analytical tools have been used to define the DD, such as FTIR spectroscopy, UV spectrophotometry, ^{1}H-NMR and ^{13}C solid-state NMR spectroscopy, various titration methods, equilibrium dye adsorption, elemental analysis, acid degradation followed by HPLC, as well as thermal analysis (Kumar, 2000).

Solubility and solution properties

The main difference between chitin and chitosan lies in their solubility; chitosan is therefore said to be chitin that has been *N*-deacetylated to such an extent that it becomes soluble in dilute aqueous acids. Pure, native

chitosan ($pK_a \sim 6.3$) is insoluble in water, in alkaline medium and even in organic solvents. However, water-soluble salts of chitosan may be formed by neutralization with organic acids (e.g. 1–10% aqueous acetic, formic, succinic, lactic, glutamic and malic acids) or inorganic acids such as hydrochloric acid (Henriksen *et al.*, 1996; Singla and Chawla, 2001). The pH-dependent solubility of chitosan is attributed to its amino groups (—NH_2), which become protonated upon dissolution at pH 6 or below to form cationic amine groups (—NH_3^+), increasing intermolecular electric repulsion and resulting in a polycationic soluble polysaccharide, with a large number of charged groups on a weight basis. Upon dissolution, chitosan forms viscous solutions, which could function as thickeners, stabilizers or suspending agents. In addition, chitosan solutions show pseudoplastic and viscoelastic properties; their viscosity is affected by chitosan's DD, MW and concentration, concentration and types of solvents, the prevailing solution pH and ionic strength, as well as temperature (Chen and Tsaih, 1998; Singla and Chawla, 2001). The viscosity range of commercial chitosans [1% (w/v) in 1% acetic acid at 25°C] is from 10 to 1000 mPa·s (Kumar, 2000).

Chemical reactivity and derivatization

Chitosan possesses three types of reactive functional groups: an amino group at the C-2 position of each deacetylated unit, as well as primary and secondary hydroxyl groups at the C-6 and C-3 positions, respectively, of each repeat unit (Fig. 1). These reactive groups are readily subjected to chemical derivatization under mild conditions, to allow for conjugation with some drugs, as well as the manipulation of mechanical and physicochemical properties, for example, improving chitosan's solubility at neutral pH range (Singla and Chawla, 2001).

Miscellaneous properties

Chitosan is a promising cationic mucoadhesive polysaccharide at pH < 6.5. Several factors affect the mucoadhesive properties of chitosan, including its concentration, MW, DD and cross-linking, in addition to contact time, environmental pH and ionic strength (Henriksen *et al.*, 1996). Moreover, the superior solubility makes chitosan more easily manageable than chitin. It could be simply processed into a variety of useful forms such as gels, membranes, sponges, films, fibres and beads, by controlling factors such as acid solvent, DD and MW, to address a variety of applications. Chitosan-based films and gels also display good oxygen/moisture transmission coefficients and substantivity (Hirano *et al.*, 1991). In addition, chitosan and its derivatives are endowed with permeation- and absorption-enhancing effects, are able

Table 1. Examples of identified chitosanases.

Sources	Chitosanase family	References
Gram-positive microorganisms		
Amycolatopsis spp.	46	Okajima *et al.* (1994)
Bacillus spp.	46	Saito *et al.* (1999); Rivas *et al.* (2000)
	8	Izume *et al.* (1992); Mitsutomi *et al.* (1998)
	N/A	Fukamizo *et al.* (1994); Omumasaba *et al.* (2000)
Nocardia spp.	N/A	Sakai *et al.* (1991)
Nocardioides spp.	46	Masson *et al.* (1995)
Paenibacillus spp.	8	Kimoto *et al.* (2002)
Streptomyces spp.	46	Fukamizo *et al.* (1995)
	5	Tanabe *et al.* (2003)
	N/A	Ohtakara (1988)
Gram-negative microorganisms		
Acinetobacter spp.	N/A	Shimosaka *et al.* (1995)
Burkholderia spp.	46	Shimosaka *et al.* (2000)
Enterobacter spp.	N/A	Yamasaki *et al.* (1993)
Matsuebacter spp.	80	Park *et al.* (1999)
Myxobacter spp.	N/A	Pedraza-Reyes and Gutierrez-Corona (1997)
Pseudomonas spp.	N/A	Yoshihara *et al.* (1992)
Sphingobacterium spp.	80	Matsuda *et al.* (2001)
Fungi		
Aspergillus spp.	75	Cheng and Li (2000)
Fusarium spp.	75	Shimosaka *et al.* (1996)
Penicillium spp.	N/A	Fenton and Eveleigh (1981)
Viruses		
Chlorella virus	46	Sun *et al.* (1999)

N/A, not available.

to enhance the dissolution and bioavailability of poorly absorbable drugs, and are capable of strongly binding transition metals *in vitro* through a chelation process, thus lending themselves to a variety of applications (Singla and Chawla, 2001; Smith *et al.*, 2004).

Biological properties

Much of the recent commercial interest in chitosan and its derivatives arises from the fact that they combine several favourable biological characteristics, including biodegradability, biocompatibility and non-toxicity, making them valuable materials for pharmaceutical, biomedical as well as industrial applications.

Biodegradation

Whereas chitosan solutions are highly stable over a long period (Cuero, 1999), there is sometimes a need for degrading chitosan to a level suitable for a particular application, or as a way to confer solubility to chitosan at neutral pH. Several methods for producing chitosan oligomers ('chitosanolysis') have been described in literature, including radiation (ionizing radiation or ultrasound) (Ulanski *et al.*, 2000), chemical (acid hydrolysis or oxidative-reductive degradation) (Kendra and Hadwiger, 1984) and enzymatic methods, of which the latter are preferred, since reaction and thus product formation could be controlled by means of pH, temperature and reaction

time (Rhoades and Roller, 2000). Chitosan is susceptible to enzymatic degradation by non-specific enzymes from a variety of sources, such as lysozymes, chitinases, cellulases or hemicellulases, proteases, lipases and β-1,3-1,4-glucanases (Vårum *et al.*, 1997; Rhoades and Roller, 2000; Kumar *et al.*, 2005). In addition, it is hydrolysed by chitosanases (chitosan *N*-acetyl-glucosaminohydrolases, EC 3.2.1.132), enzymes that attack chitosan but not chitin, catalyzing the endohydrolysis of β-(1→4)-glycosidic linkages between GlcN residues in partly acetylated chitosan (Rivas *et al.*, 2000; Kimoto *et al.*, 2002) (Fig. 1).

Chitosanase activities with different substrate specificities have been reported in a variety of microorganisms, including bacteria (an estimated 1–7% of heterotrophic soil bacteria) and fungi as well as plants; genes encoding chitosanases have also been identified in some viruses. They have been found to belong to five glycoside hydrolase families: 5, 8, 46, 75 and 80 (Table 1). Interestingly, the majority of the sequenced chitosanases are produced by Gram-positive microorganisms; the crystal structures of *Streptomyces* sp. N174 (Marcotte *et al.*, 1996) and *Bacillus circulans* MH-K1 (Saito *et al.*, 1999) chitosanases are available.

Fukamizo and colleagues (1994) proposed the classification of chitosanases into three distinct classes according to their substrate specificities: (i) class I chitosanases split the Glc*N*Ac–GlcN linkage in chitosan, e.g. *Bacillus pumilus* BN262 (Fukamizo *et al.*, 1994), *Penicillium*

islandicum (Fenton and Eveleigh, 1981) and *Streptomyces* sp. strain N174 (Fukamizo *et al.*, 1995), (ii) class II chitosanases, where cleavage specificity is exclusively restricted to the GlcN–GlcN linkage, e.g. *Bacillus* sp. No.7M (Izume *et al.*, 1992), and (iii) class III chitosanases, which can split both GlcN–GlcN and GlcN–Glc*N*Ac linkages, such as *Streptomyces griseus* HUT 6037 (Tanabe *et al.*, 2003), *B. circulans* MH-K1 (Saito *et al.*, 1999), *Nocardia orientalis* (Sakai *et al.*, 1991) and *B. circulans* WL-12 (Mitsutomi *et al.*, 1998).

Biocompatibility

One of the most important biological properties of any implantable biomaterial is its biocompatibility; i.e. it should not be affected by the host and at the same time should not elicit any undesirable local or systemic effects. Chitosan is well tolerated by living tissues, including the skin, ocular membranes, as well as the nasal epithelium, and has thus been proven valuable for a wide range of biomedical applications (Shigemasa and Minami, 1995; Felt *et al.*, 1999).

Safety

The low toxicity profile of chitosan compared with other natural polysaccharides is another of its many attractive features. It has been reported that the purity of chitosan influences its toxicological profile, yet its safety in terms of inertness and low or no toxicity has been demonstrated by *in vivo* toxicity studies. Its oral LD_{50} (median lethal dose) in mice was found to be in excess of 16 g day^{-1} kg^{-1} body weight, which is higher than that of sucrose. Nonetheless, it is contraindicated for people with shellfish allergy (Singla and Chawla, 2001).

In their review article, Ylitalo and colleagues (2002) reported the absence of significant side-effects following chitosan ingestion in human studies (for up to 12 weeks). However, Tanaka and colleagues (1997) cautioned that special care should be taken in the clinical use of chitosan over a long period of time, due to possible disturbances in intestinal microbial flora. Concerns have also been raised that chitosan could cause the loss of fat-soluble vitamins, decrease mineral absorption and bone mineral content and block absorption of certain medicines (Deuchi *et al.*, 1995). We were unable to identify any epidemiological studies or case reports investigating the association of chitosan exposure and cancer risk in humans, any carcinogenicity studies on chitosan in animals and any *in vitro* or *in vivo* studies evaluating chitosan for mutagenic effects in the available literature.

Applications

Although extensive resources were involved in both research and development of processes and applications for chitosan, only the last two decades have witnessed serious developments in a variety of technologies aiming for the commercial utilization of chitosan and its derivatives. Chitosan, its oligomers and a number of its derivatives emerged as new biomaterials for a number of applications ranging from pharmaceutical, cosmetic, medical, food and textile to agricultural applications, representative examples of which are summarized in Table 2. Due to the wide scope of applications, only a number of them will be further discussed in this section.

Introduced to the market in the 1990s, chitosan has been the subject of much research regarding its potential as a useful and promising pharmaceutical excipient in various pharmaceutical formulations. Next to the more traditional formulations, chitosan has found use in novel applications such as vaccine delivery, peptide and gene delivery, in addition to its use in tissue engineering (Singla and Chawla, 2001). One of the most recent applications of chitosan involves targeted delivery for biological applications, where it is used in the preparation of magnetic nanofactories, which allow the local synthesis and delivery of active cargo at a target site, thus minimizing non-specific effects (Fernandes and Bentley, 2009).

In spite of the promising use of chitosan in the pharmaceutical industry, most of the chitosan researches are directed towards medical applications. For example, some studies showed that chitosan, whether it is used as an immune adjuvant, drug delivery agent or dietary fibre, could effectively promote local immune response and enhance antigen presentation (Porporatto *et al.*, 2005; Xie *et al.*, 2007).

Probably one of the most prominent commercial applications of chitosan is its use as a hemostatic. Several chitosan-based wound dressings are available on the market for clinical use, including HemCon® Bandage and ChitoFlex wound dressings (HemCon Medical Technologies, UK), as well as CELOX™ (Medtrade Products, England); both of which stated to be FDA approved (www.hemcon.com and www.celoxmedical.com, respectively).

Moreover, chitosan is implicated as a component of host–fungal interactions; it acts as a potent elicitor of plant defence responses, activating the expression of plant defensive genes and inducing the production of pathogen-related proteins, such as chitinases and other hydrolytic enzymes. These enzymes can hydrolyze chitin and chitosan in fungal cell walls, consequently leading to growth inhibition and/or death (Doares *et al.*, 1995; Mason and Davis, 1997). The induction of chitosanases and chitinases through genetic engineering has also been proposed (Cuero, 1999). Moreover, Beauséjour and colleagues (2003) suggested the use of chitosan together with a biocontrol strain exhibiting chitosanolytic activity as a promising biocontrol tool against plant pathogens, claiming that the presence of a chitosan-hydrolyzing

Table 2. Applications of chitosan.

Applications	Benefits/advantages	References
Pharmaceuticals and cosmetics		
Conventional formulations		
Tablet manufacture	Binder; disintegrant; coating; lubricant; diluent	Nunthanid *et al.* (2004)
Gels	Sustained drug release; enhanced absorption	Kofuji *et al.* (2004)
Films and membranes	Controlled drug release	Yan *et al.* (2001)
Emulsions	Stabilizer	Hino *et al.* (2000)
Microspheres, microcapsules	Mucoadhesive; increased bioavailability; sustained drug delivery; penetration enhancement	Dias *et al.* (2008)
Ophthalmic formulations	Ocular tolerance; mucoadhesive; wetting and penetration-enhancing properties; antibacterial; prolonged precorneal drug residence	Felt *et al.* (2000)
Transdermal delivery systems	Enhancement of penetration across epithelia; controlled drug release	He *et al.* (2008)
Colon-specific drug delivery	Biodegradable by colonic bacteria	Yamamoto *et al.* (2000)
Targeted cancer therapy	Antitumour; long systemic retention and tumour accumulation, due to enhanced permeability and retention (EPR) effect	Dass and Choong (2008)
Vaccine delivery		
Mucosal vaccination	Induction of mucosal and systemic immune responses; penetration into intestinal and respiratory mucosae	Illum *et al.* (2001)
Oral vaccination	Protection of antigens from gastric juice, bile acids and salts and from proteolytic enzymes of the gastrointestinal tract	van der Lubben *et al.* (2001)
Peptide drug delivery	Improving oral bioavailability of peptides and proteins	Bernkop-Schnürch (2000)
Gene/nucleic acid delivery	Safe, non-viral system	Fernandes *et al.* (2006)
Deodorant formulations	Dermatological compatibility; non-irritating; enhancing fragrance adhesion; deodorizing	Hohle and Griesbach (1999)
Hair and skin care products	Preservative; emulgator; thickener; moisturizer; soothing effect on skin	Pittermann *et al.* (1997)
Medical and biomedical		
Antacid and anti-ulcerogenic	Demulcent and protective effect on stomach mucosa	Anandan *et al.* (2004)
Hypoglycaemic, antihypertensive	Lowering of blood glucose level; increasing glucose tolerance and insulin secretion	Lee *et al.* (2003)
Antioxidant	Scavenging of radicals and chelation of divalent metals	Xie *et al.* (2001)
Antitumour	Induction of apoptosis in tumour cells	Hasegawa *et al.* (2001)
Anticoagulant	–	Park *et al.* (2004b)
Haemostatic	Biological adhesive for soft tissues	Malette *et al.* (1983)
Spermicidal	Strong binding to mammalian cells	Shigemasa and Minami (1995)
Hypocholesterolaemic; nutritional aid for weight loss	Prevention of fat absorption; reduction of blood lipid levels	Hossain *et al.* (2007)
Wound dressings; products for wound treatment	Inhibition of fibroplasia; promotion of tissue regeneration and wound-healing with minimal scar formation	Mi *et al.* (2002)
Contact and bandage lenses	Optical clarity; wound-healing; antimicrobial; mechanical stability; immunological compatibility; optical correction; gas permeability; wettability	Kumar (2000)
Dentistry and oral medicine	Bioadhesive; viscosity-enhancer; prolonged drug release in buccal cavity; permeabilizer; antimicrobial; anti-adhesive; anti-dental caries; treatment of periodontal diseases/oral candidiasis/tooth mobility; reduction of plaque formation	Decker *et al.* (2005)
Anti-inflammatory	–	Shigemasa and Minami (1995)
Immunopotentiator	Augmenting immunogenicity of co-administered antigens; stimulation of immune system	Okawa *et al.* (2003)
Surgical sutures and implants	Biodegradable	Suzuki (2000)
Haemodialysis membranes; coating for biomedical devices	Thromboresistance; compatibility with blood; anti-biofilm properties	Shigemasa and Minami (1995); Carlson *et al.* (2008)
Tissue engineering		
Scaffold for tissue engineering applications	Promoting tissue growth and differentiation	Kawase *et al.* (1997)
Artificial skin grafts	Non-antigenic; biodegradable template for synthesis of neodermal tissue	Kumar (2000)
Agriculture		
Soil and plant revitalizer	Preventing microbial infection; promoting growth of plants	Mulawarman *et al.* (2001)
Preservative coating and biofungicide	Sprayed on seeds to extend their storage life	Hirano (1996)
Food industries		
Food processing	Enhances safety, quality and shelf life of food; clarification of liquids; preservative; thickener	Rhoades and Roller (2000); Roller (2003)

Table 2. *cont.*

Applications	Benefits/advantages	References
Coatings for vegetables, fruits and fish	Improving shelf life; preventing moisture loss; delaying fungal growth	Cagri *et al.* (2004)
Edible antimicrobial films for food packaging	–	Durango *et al.* (2006)
Textile industries		
Finishing of textiles	Antimicrobial; moisture control and dye absorptive properties	El Tahlawy *et al.* (2005)
Novel fibres for textiles	–	Kumar (2000)
Wastewater treatment		
Food and beverage processing plants	Coagulation and flocculating agent; resulting dried sludge used in animal feeds	Chi and Cheng (2006)
Industrial waste	Removal of heavy metal ions through adsorption and chelation	Babel and Kurniawan (2003)
Textile effluents	Sorption of dyes (high affinity to many classes of dyes)	Crini and Badot (2008)
Miscellaneous		
Photographic paper	Resistance to abrasion; favourable optical characteristics; film-forming ability	Kumar (2000)
Paper finishing	Imparts wet strength to paper	Kumar (2000)

activity is virtually synonymous with resistance towards chitosan. However, we found little evidence to support this assumption, since we were unable to detect a direct correlation between the chitosanolytic ability of 28 test strains (*Bacillus* and related strains) and their *in vitro* susceptibility to the antimicrobial activity of chitosan (our unpublished data). Nonetheless, chitosan-based plant growth stimulators found their way into the market (e.g. ChitoPlant® and SilioPlant®; ChiPro GmbH, Germany). They presumably stimulate the plant immune response against pathogens and have a growth-promoting activity.

Unfortunately, a survey of the available literature revealed that there are only relatively few specific and objective research studies to support claims ascribing a range of rather impressive pharmacological properties to chitosan; most of these studies are indeed very difficult to verify. For example, chitosan is often being heralded, and sold, as a 'revolutionary' weight loss supplement, a 'fat magnet', although this presumptive property is often discredited in recent studies (Mhurchu *et al.*, 2004; Gades and Stern, 2005). Given the large number of proclaimed medicinal benefits of chitosan, it comes as no surprise that the literature is filled with conflicting reports.

Economic aspects and regulatory status

Since a large amount of the crustacean exoskeleton is readily available as a by-product of the seafood processing industry, the raw material for chitosan production is relatively inexpensive, and thereby the production of chitosan on a large scale from this renewable bio-resource is economically feasible (Rabea *et al.*, 2003). Chitosan is commercially produced in different parts of the world (North America, Poland, Italy, Russia, Norway, Japan and India) on a large scale (with an estimated 10^9–10^{10} tons annually produced in nature); it is also widely used in foods in Italy, Finland, Korea and Japan (Peter, 1997; Singla and Chawla, 2001). Another important aspect to be considered is that utilizing the shellfish waste for chitin production provides a solution for the waste disposal problem, and provides an alternative for the use of this oceanic resource.

'Generally Recognized As Safe (GRAS)' is a designation used by the FDA to indicate that a chemical or substance added to foods and beverages is considered safe. Chitosan has not been officially proclaimed GRAS by the FDA, although it has approved chitosan for medical uses such as bandages and drug encapsulation. However, one Norwegian company (Primex Ingredients ASA), which manufactures shrimp-derived chitosan, has announced in 2001 that its purified chitosan product (ChitoClear®) has achieved a GRAS self-affirmed status in the US market.

Chitosan's antimicrobial activity

The modern era of chitosan research was heralded by publications in the 1990s describing the antimicrobial potentials of chitosan and its derivatives, which exhibit a wide range of activities towards human pathogens as well as food-borne organisms (Muzzarelli *et al.*, 1990; No *et al.*, 2002; Savard *et al.*, 2002; Rabea *et al.*, 2003). In fact, a number of commercial applications of chitosan benefit from its antimicrobial activity, including its use in food preservation, in dentistry and ophthalmology, as well as in the manufacture of wound-dressings and antimicrobial-finished textiles (Table 2).

In recent years, there was a growing demand for a more rational use of chemicals in food preparations, thus shifting the attention to natural substances that might function as preservatives. In that respect, much attention has focused on the safety and efficacy of chitosan as a natural preservative to be included in pharmaceutical as well as

food preparations (Rhoades and Roller, 2000; Helander *et al.*, 2001; No *et al.*, 2002). For instance, Jumaa and colleagues (2002) suggested chitosan's use as an antimicrobial preservative in emulsion formulations for mucosal as well as for parenteral applications. Similarly, Sagoo and colleagues (2002) proposed its use as an adjunct in the potentiation of the biocidal efficacy of antimicrobial compounds such as benzoates. Moreover, we have previously demonstrated using a challenge test that chitosan displayed adequate preservative efficacy, for up to 28 days, with respect to potential bacterial contaminants, which might allow its use as an antimicrobial preservative in pharmaceutical preparations. In addition, it was capable of potentiating the antimicrobial activity of a number of preservatives, including phenethyl alcohol, benzoic acid and phenylmercuric acetate against a number of test strains (Raafat, 2004). Therefore, investigations of the antimicrobial potential of chitosan and its derivatives have recently gained momentum.

Antimicrobial spectrum

The spectrum of antimicrobial activity of chitosan and its derivatives extends to include filamentous fungi, yeasts and bacteria, being more active against Gram-positive than Gram-negative bacteria (Muzzarelli *et al.*, 1990; Rhoades and Roller, 2000; Jeon *et al.*, 2001; No *et al.*, 2002). More interestingly, chitosan seems to hold some promise in dentistry, since it was shown to exhibit a potent plaque-reducing action as well as *in vitro* antibacterial activity against several oral pathogens implicated in plaque formation and periodontitis, including *Actinobacillus actinomycetemcomitans*, *Streptococcus mutans* and *Porphyromonas gingivalis* (Choi *et al.*, 2001; İkinci *et al.*, 2002). However, chitosan's activity is mostly growth-inhibitory, where resistant subpopulations might emerge, as a result of physiological adaptation of the cells to chitosan stress (Raafat *et al.*, 2008). When Jarry and colleagues (2001) tested the antimicrobial activity of chitosan against several microorganisms, they found that the bacteria could rapidly grow after separation from the chitosan solution by membrane filtration. Therefore, this bacteriostatic activity, with regrowth of bacterial cultures treated with chitosan, might be explained by the irreversible binding of chitosan to microbial cells or medium particles, which renders it inactive against the remaining unbound microorganisms (Rhoades and Roller, 2000).

Factors affecting the activity

There are numerous reports on the antimicrobial potency of different chitosans and chitosan derivatives, from various sources and tested under diverse conditions. In many instances discrepancies in the results obtained were observed, which were not surprising, since chitosan's *in vitro* antimicrobial activity is influenced by various intrinsic and extrinsic factors, related to both chitosan itself (type, MW, DD, viscosity, solvent and concentration) and the environmental conditions (test strain, its physiological state and the bacterial culture medium, pH, temperature, ionic strength, metal ions, EDTA, organic matter) respectively. Underestimation of any of these factors would inevitably lead to false conclusions regarding the potency of the chitosan preparation under test. In the following part we will present a general account of the main criteria that should be closely observed while developing antimicrobial systems to be implemented into industrial applications.

It is presently impossible to pinpoint the influence of MW or DD on the antimicrobial activity of chitosan; reliable methods must therefore first solve the task of accurately determining these values. For instance, the antimicrobial activity of chitosan was found to be greatly influenced by its MW; oligosaccharides and D-glucosamine possessed weak or no antibacterial activity (Rhoades and Roller, 2000; No *et al.*, 2002). Jeon and colleagues (2001) even went as far as to suggest that a MW of more than 10 kDa is required for proper inhibition of microorganisms. Interestingly, we have found in a previous study after testing a large number of chitosan preparations that they did not differ appreciably in their antimicrobial activity at a molecular size above 10 kDa (Raafat *et al.*, 2008). Therefore, there seems to be a minimum degree of polymerization required for antimicrobial activity. Moreover, chitosans with a high DD were more effective than those with a low degree in inhibiting bacterial growth, probably due to the higher percentage of protonated amine groups (Shigemasa and Minami, 1995; Liu *et al.*, 2001).

The antimicrobial activity of chitosan is inversely affected by pH, with higher activity observed at lower pH value; on the other hand, it increases with increasing temperature, and in presence of EDTA (Tsai and Su, 1999; Jumaa *et al.*, 2002; No *et al.*, 2002; Raafat, 2004). Results regarding the effect of ionic strength on chitosan's activity are still contradictory. While Chung and colleagues (2003) propose that higher ionic strength might enhance the solubility of chitosan and thus increase its antibacterial activity, regardless of the test strain, Tsai and Su (1999) suggest that the presence of sodium ions (100 mM) reduces chitosan's activity against *Escherichia coli*. We, however, observed no detectable effect of NaCl (10 or 25 mM) on the antimicrobial activity of chitosan against several indicator strains (our unpublished data). More importantly, the addition of metal ions results in a dramatic reduction in chitosan's antibacterial activity, probably due to complex formation between chitosan and these metal ions (Tsai and Su, 1999; Bhatia and Ravi, 2003). Analogous findings have been made for plant cells (*Glycine max*), where chitosan-induced permeability changes were

strongly inhibited by divalent cations (Young *et al.*, 1982). The lone pair electrons present on the amino nitrogen can establish coordinate covalent bonds with transition metal ions. In addition, deprotonated hydroxyl groups can participate in coordination with metal ions, thus functioning as second donors (Micera *et al.*, 1985; Udaybhaskar *et al.*, 1990). On the other hand, the choice of chitosan's solvent, whether inorganic (HCl) or organic acids (HAc), had no significant effect on the antimicrobial activity of chitosan (our unpublished data).

It is still unclear at which physiological state bacterial cells are most susceptible to the antimicrobial activity of chitosan. Whereas we found that chitosan-treated *Staphylococcus simulans* 22 cells exhibited lower viable counts at the stationary phase (our unpublished data), Tsai and Su (1999) observed that *E. coli* cells were most susceptible in the late exponential phase.

In an *in vitro* setting, the potency of an antimicrobial agent may be assessed through an estimation of the MIC (minimum inhibitory concentration). In preliminary studies we found evidence that the experimental setting used for susceptibility assessment could account for variations in the reported MIC values of chitosan in the available literature (our unpublished data). We reached the conclusion that the broth microdilution technique would be the most suitable for such a purpose, as opposed to agar-based methods (Hirano and Nagao, 1989; No *et al.*, 2002), and that the bacterial growth medium should be well chosen, in order to prevent unfavourable interaction with chitosan.

These examples serve to illustrate the reasons for the vast variability in reported data, and underline the need for vigorous criteria to be applied in any study that is aimed at elucidating the efficacy of chitosan as an antimicrobial agent in any given system, such as food or pharmaceutical formulations. Therefore, conclusions relying solely on *in vitro* investigations, which are dependent on a number of variables in the experimental methodology, should always be treated with caution. Investigations should rather take into consideration the specific environmental context in which this biopolymer is to be used, by paying attention to changes in chitosan sensitivity that might accompany the respective methodological setting.

Antimicrobial mode of action

The overall mechanism(s) of action of an antimicrobial may be defined according to the target component of the bacterial cell against which it has its main activity. Thus, three levels of interaction can be described: (i) interaction with outer cellular components, (ii) interaction with the cytoplasmic membrane and (iii) interaction with cytoplasmic constituents. The mechanisms underlying the antimicrobial activity of chitosan have only been studied comparatively recently and the amount of information available is limited, although increasing. Several studies purport to have identified such mechanisms; but only few were supported by experimental evidence. In this section we will highlight the main mode of action mechanisms suggested, and discuss some of them in more detail. We would like to reiterate that these mechanisms of action are not mutually exclusive, since microbial inhibition by chitosan is thought to be a result of a sequence of molecular processes, resulting in random multiple detrimental events that ultimately lead to cell inhibition/killing.

The properties and structure of the bacterial cell envelope play an important role in chitosan's antimicrobial activity. As stated above, Gram-positive bacteria are markedly more sensitive to the antimicrobial activity of chitosan, compared with Gram-negative ones (MIC $\geq$ 1000 μg ml^{-1}, our unpublished data). This difference in sensitivity is largely ascribed to the different architectures of their cell envelopes – an issue that will be considered below, on the level of the mode of action of chitosan. Therefore, we will discuss in this section chitosan's mode of action, with reference mainly to staphylococci, occasionally pointing out fundamental differences to Gram-negative bacteria.

Structure of the staphylococcal cell envelope

The term cell envelope comprises both the cell wall and the cytoplasmic membrane of a bacterial cell; it also includes the semi-permeable lipid bilayer (outer membrane) of Gram-negative bacteria, which acts as an additional diffusion barrier. The staphylococcal cell wall is composed of multilayers of murein, where glycan strands of alternating β-1→4-linked Glc*N*Ac–Mur*N*Ac disaccharides are cross-linked by short peptides. Extending to the surface of the peptidoglycan layer are teichoic acids, which are essential polyanionic polymers found only in the cell wall of Gram-positive bacteria that contribute to the negative charge of the cell wall (Fig. 2); similar polymeric structures, referred to as lipopolysaccharides (LPS), are found in the outer membrane of Gram-negative bacteria. The *Staphylococcus aureus* cell membrane consists of three major phospholipid (PL) species: negatively charged phosphatidylglycerol and cardiolipin, and positively charged lysyl-phosphatidylglycerol (LPG), the latter accounting for 14–38% of the total PL content of the *S. aureus* cytoplasmic membrane (Peschel *et al.*, 2001).

Interaction of chitosan with the bacterial cell surface

The mode of action of cationic antibacterial agents is widely believed to be the interaction with and disruption of the cell envelope. Electron microscopical examinations of various chitosan-treated microorganisms suggest that its site of action is indeed at the microbial cell surface

Extracellular space
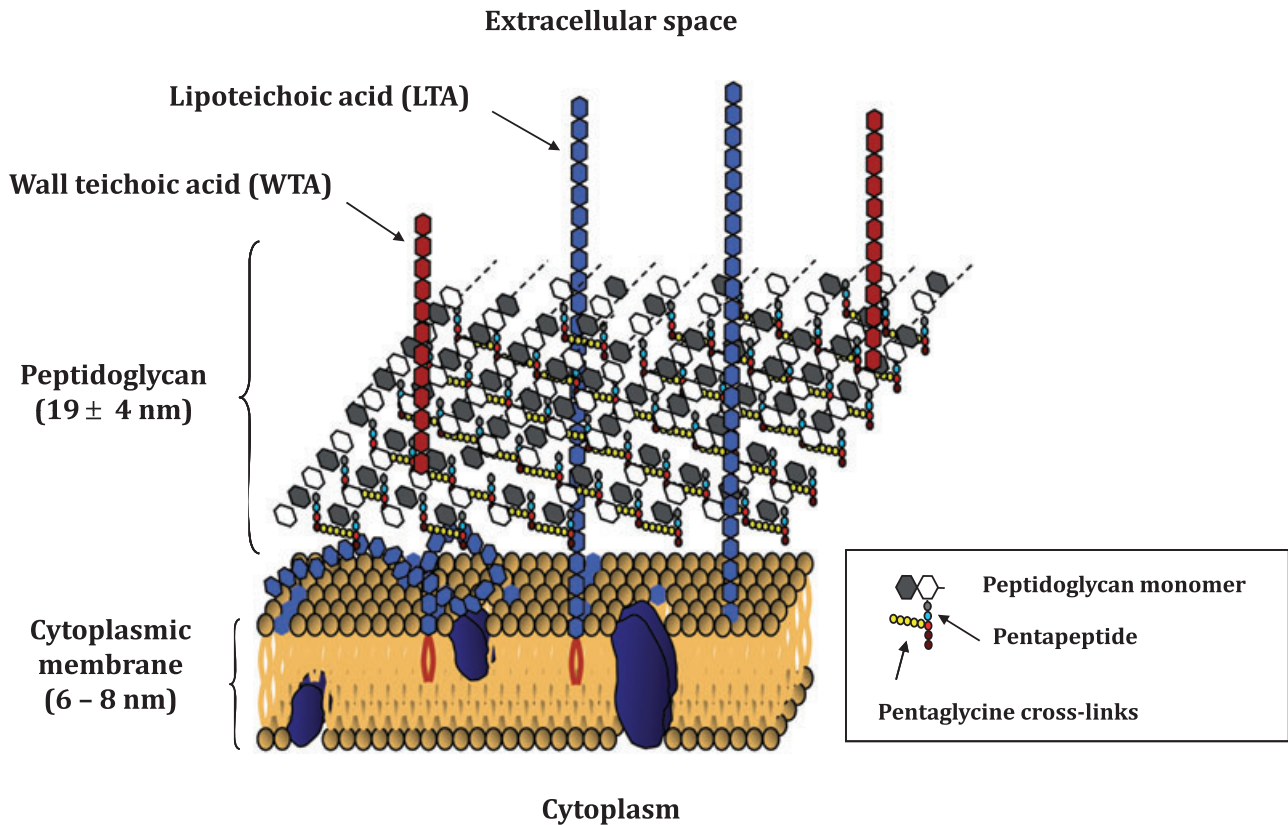
Fig. 2. The staphylococcal cell envelope. The staphylococcal cell wall is composed of multilayers of glycan strands of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramyl-pentapeptide (Mur*N*Ac-PP), cross-linked by pentaglycine side-chains. For clarity, only one layer of peptidoglycan is depicted here. Teichoic acids, polyanionic surface polymers extending through the peptidoglycan layer, contribute to the negative charge of the cell wall. The cytoplasmic membrane is a selectively permeable membrane lying internal to the cell wall.

(Muzzarelli *et al.*, 1990; Cuero, 1999; Savard *et al.*, 2002; Raafat *et al.*, 2008). It is generally assumed that the polycationic nature of chitosan, conveyed by the positively charged —NH₃⁺ groups of glucosamine, might be a fundamental factor contributing to its interaction with negatively charged surface components of many fungi and bacteria, causing extensive cell surface alterations, leakage of intracellular substances, and ultimately resulting in impairment of vital bacterial activities (Helander *et al.*, 2001; Zakrzewska *et al.*, 2005; Je and Kim, 2006). This hypothesis is backed by several lines of evidence, including:

(i) Chitosan loses its antimicrobial activity at pH 7.0, assumably due to the deprotonation of amine groups, as well as poor solubility in water at this pH (Sudarshan *et al.*, 1992; Liu *et al.*, 2001).

(ii) The antimicrobial activity of chitosan was found to be directly proportional to its DD, which in turn is related to the number of its protonated amine groups (Liu *et al.*, 2001; Park *et al.*, 2004a).

(iii) The *N*-acetylation of chitosan oligomers effectively destroyed their fungistatic activity, since the 2-amino

groups could no longer become protonated (Torr *et al.*, 2005).

(iv) Mutants of *Salmonella typhimurium* with strongly reduced negative cell surface charge were found to be more resistant to chitosan than the parent strains (Helander *et al.*, 2001). This mirrors data generated from our laboratory using various staphylococcal mutants displaying different overall cell surface charges, where we could establish that a highly anionic bacterial surface greatly enhances the antimicrobial activity of chitosan against *S. aureus* (Raafat *et al.*, 2008).

(v) The fact that a stable chitosan-resistant *S. aureus* variant, with more than 50× reduced susceptibility compared with the wild-type, displayed an increased content of positively charged membrane PLs (our unpublished data) led us to hypothesize that the overall surface charge of a microorganism plays an important role in chitosan's activity.

In sum, all these findings are consistent with the above-mentioned hypothesis; they also add weight to the

conclusion that the cell envelope charge does contribute to chitosan's antimicrobial activity.

However, the nature of the surface components involved in this interaction with chitosan was rarely accurately defined. While working with plants, Young and colleagues (1982) suggested that chitosan might bind to polygalacturonate, a component of plant cell walls, thereby increasing the membrane permeability of plant cells. Regarding bacteria, on the other hand, several research groups later hypothesized that an electrostatic interaction takes place between chitosan and either (i) negatively charged cell membrane components (i.e. phospholipids or proteins) (Liu *et al.*, 2004), (ii) amino acids in the Gram-positive bacterial cell wall (Kumar *et al.*, 2005), or (iii) various lipopolysaccharides in the outer membrane of Gram-negative bacteria (Davydova *et al.*, 2000; Helander *et al.*, 2001), thereby affecting membrane integrity and permeability. Morimoto and colleagues (2001) reported the specific binding of a chitosan derivative to a receptor on the cell surface of *Pseudomonas aeruginosa*. In a previous investigation, we showed that the initial contact between the polycationic chitosan macromolecule and the bacterial cell is most probably mediated through electrostatic interaction with the negatively charged teichoic acids of Gram-positive bacteria (Raafat *et al.*, 2008). This finding is consistent with the much lower activity of chitosan against Gram-negative bacteria, since a possible binding of chitosan to LPS would not significantly influence the dynamics of the cell envelope, since these molecules are confined to the outer membrane.

Chitosan is considered by several researchers to be a membrane-perturbing compound (Helander *et al.*, 2001; Zakrzewska *et al.*, 2005; Je and Kim, 2006), although we do not consider membrane destabilization to result from a direct interaction of chitosan with the membrane itself, since chitosan was unable to induce the efflux of small marker molecules from liposomes; membrane effects rather constitute a secondary event triggered by interference with cell wall dynamics via immobilization of anionic cell wall polymers (Raafat *et al.*, 2008). The interaction of chitosan with cell surface polymers, e.g. membrane-anchored lipoteichoic acids, may interfere with dynamic processes within the cytoplasmic membrane and hence alter its optimal functioning, causing a partial dissipation of the membrane diffusion potential and a generalized disruption of membrane-associated functions, finally resulting in leakage of small cellular components from staphylococci, without pore formation (Raafat *et al.*, 2008). The transition from sublethal injury, caused by disruption of the cell permeability barrier and leakage, to cell death might be mediated by metabolic imbalance and impaired ionic homeostasis following chitosan challenge. While some researchers observed a frayed cell wall (Muzzarelli *et al.*, 1990), and even the appearance of protoplasts (Didenko *et al.*, 2005) upon chitosan treatment, we observed no effect on cell wall integrity (Raafat *et al.*, 2008).

As opposed to our own data, Kumar and colleagues (2005) claimed that the binding of chitosan to cell surface structures of *Bacillus cereus* resulted in pore formation. An analogous hypothesis was put forward by Young and colleagues (1982), who suggested that quite large 'pores' can be induced in the plant membrane by chitosan, through displacing cations (such as Ca^{2+}) from complexes that stabilize the cell membrane of *G. max* cells, resulting in destabilization of the membrane and leakage of low- and high-MW proteins, arguing that large polycations such as DEAE-dextran do penetrate the cell wall to interact with the plasma membrane. They further hypothesize that polyanions (polygalacturonate) on plant cell walls help protect the plasma membrane, the actual target of chitosan action, by binding to the polycationic chitosan, thus preventing the contact with the cell membrane.

Intracellular chitosan targets?

It was suggested that the positively charged chitosan (or its oligosaccharides) might be taken up by cells, where they interact with cellular DNA of fungi and bacteria, consequently inhibiting DNA transcription, as well as RNA and protein synthesis (Tarsi *et al.*, 1997; Liu *et al.*, 2001; Rabea *et al.*, 2003). It has also been previously reported that chitosan can penetrate plant cells, being detected 15 min after its application to the surface of the plant tissue within the plant cytoplasm and conspicuously detectable within the plant nucleus (Hadwiger *et al.*, 1981). If this same logic were to be applied to the situation with bacteria, for instance, staphylococci, this contention would be provocative, because it would imply that chitosan is able to circumvent two barriers, namely the multilayered staphylococcal cell wall and the plasma membrane (Fig. 2), to afford access to the intracellular space. However, a consideration of the molecular size of a native chitosan molecule (up to 1000 kDa; Fig. 3) would render such a notion rather unlikely. Moreover, we have found no evidence that chitosan is broken down by staphylococci into smaller fragments, which might pass the cell wall (Raafat *et al.*, 2008).

Other mechanisms of action

The chelating activity of chitosan has also been implicated in its antimicrobial mode of action; for instance, it might selectively bind essential trace metals and thereby inhibit the production of toxins and microbial growth (Chung *et al.*, 2003). Moreover, growth inhibition through blockage of nutrient flow has been suggested by several researchers (Sudarshan *et al.*, 1992; Tokura *et al.*, 1997;
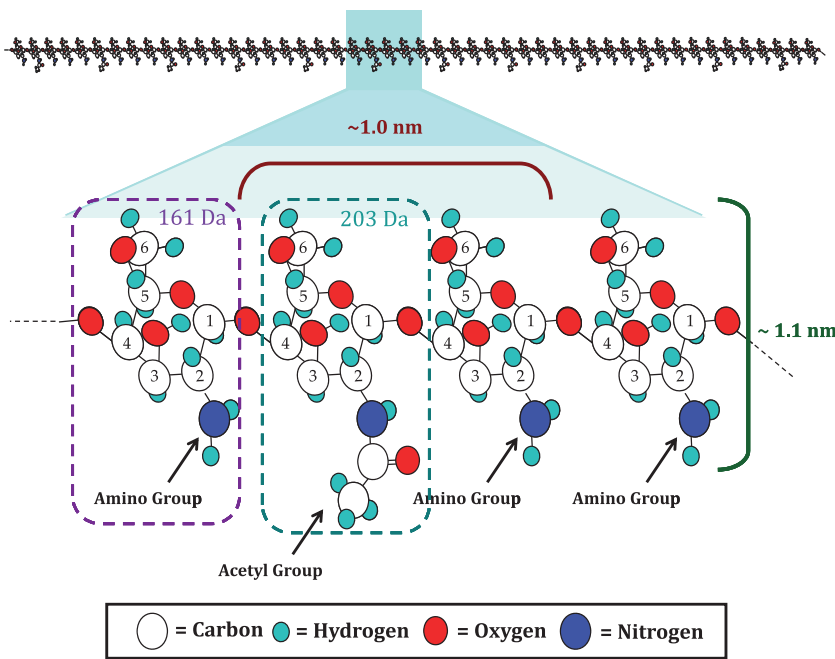
Fig. 3. Detailed structure of a chitosan molecule. Shown is an enlarged portion of a chitosan molecule, with relevant dimensions. For a chitosan molecule of a MW of 240 kDa and a DD of 87%, an average chain would consist of around 1400 units, with an average length of about 700 nm.

Kumar *et al.*, 2005), who attribute the antibacterial activity of chitosan to its deposition (stacking) onto the surface of bacteria, thereby impeding mass transfer and suppressing the metabolic activity of bacteria.

Future prospects

Food and pharmaceutical industries are increasingly confronted with fundamental challenges regarding the appropriate choice of preservative systems. The discovery of new preservatives, and the refinement of traditional ones, is creating unprecedented excitement in the field of applied sciences.

Nowadays, one might attest that a gap has been bridged between basic and applied research in the field of natural polymers. There is now a growing body of work demonstrating the utility of chitosan in antimicrobial systems, aimed for industrial use. For instance, it could be used in conjunction with classical preservatives to provide microbial stability to food and pharmaceutical systems.

This review might therefore help to clarify what may have been considered disparate conclusions about the antimicrobial activity of chitosan in the literature, and might thereby extend our understanding of this industrially important natural polymer. A noteworthy fact is that a major source of these dissimilarities is the lack of standardized microbiological procedures when dealing with this natural biopolymer. The potential value of chitosan as a preservative for pharmaceutical and food preparations would open a new avenue for the use of this natural product. However, the detailed application setting has to

be well defined, in order to avoid unfavourable interactions, or loss of its activity.

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