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Review

Chitin/chitosan: modifications and their unlimited application potential—an overview

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Use of natural biopolymers for diversified applications in life sciences has several advantages, such as availability from replenishable agricultural or marine food resources, biocompatibility, biodegradability, therefore leading to ecological safety and the possibility of preparing a variety of chemically or enzymatically modified derivatives for specific end uses. Polysaccharides, as a class of natural macromolecules, have the tendency to be extremely bioactive, and are generally derived from agricultural feedstock or crustacean shell wastes. Cellulose, starch, pectin, etc. are the biopolymers derived from the former while chitin and chitosan are obtained from the latter. In terms of availability, chitin is next to cellulose, available to the extent of over 10 gigatons annually. The application potential of chitosan, a deacetylated derivative of chitin, is multidimensional, such as in food and nutrition, biotechnology, material science, drugs and pharmaceuticals, agriculture and environmental protection, and recently in gene therapy too. The net cationicity as well as the presence of multiple reactive functional groups in the molecule make chitosan a sought-after biomolecule. The latter offers scope for manipulation for preparing a broad spectrum of derivatives for specific end use applications in diversified areas. The biomedical and therapeutic significance of chitin/chitosan derivatives is a subject of significant concern to many all over the world. An attempt is made in this overview to consolidate some of the recent findings on the biorelated application potential of chitosan and its derivatives.

Introduction

Of late, a tremendous awareness of the suitability of using natural biopolymers for diversified applications in life science is increasing (Tharanathan, 2003). The present day folks, being better educated and well informed, prefer to go for organic/natural products, be it a food or a drug, and are prepared to pay a premium price for anything natural and safe. In this regard natural biopolymers have several advantages, such as availability from replenishable agricultural or marine food resources, biocompatibility, biodegradability, therefore leading to ecological safety and the possibility of preparing a variety of chemically or enzymatically modified derivatives for specific end uses. Polysaccharides, as a class of natural macromolecules, have the tendency to be extremely bioactive and are generally derived from agricultural feedstock or crustacean shell wastes (Ramesh & Tharanathan, 2003). Cellulose, starch, pectin, etc. are the biopolymers derived from the former, while chitin and chitosan are derived from the latter. In terms of availability, chitin is next to cellulose, available to the extent of over 10 gigatons (1×10^{13} kg). To a considerable extent chitin is also obtained from fungi and bacteria (see Fig. 1; Tharanathan & Kittur, 2003).

It is believed that both chitin and chitosan are linear copolymers of D-GlcN and D-GlcNAc residues distributed randomly and not blocked together, the residues are linked entirely in the β-1,4-configuration (Fig. 2); the various depolymerization products and structural and spectroscopic studies provide ample proof for this. The β-1,4-configuration results in a rigid and unbranched structure. The abundance of hydroxyl groups (1 primary hydroxyl at C-6 and 1 secondary hydroxyl at C-3) and highly reactive amino group (at C-2) or its *N*-acetyl counterpart (wholly in chitin) with concomitant tendency for intra- and intermolecular hydrogen bonds results in the formation of linear aggregates with extensive crystallinity. The latter contributes to the strength shown by chitinous structures, and also to the virtual

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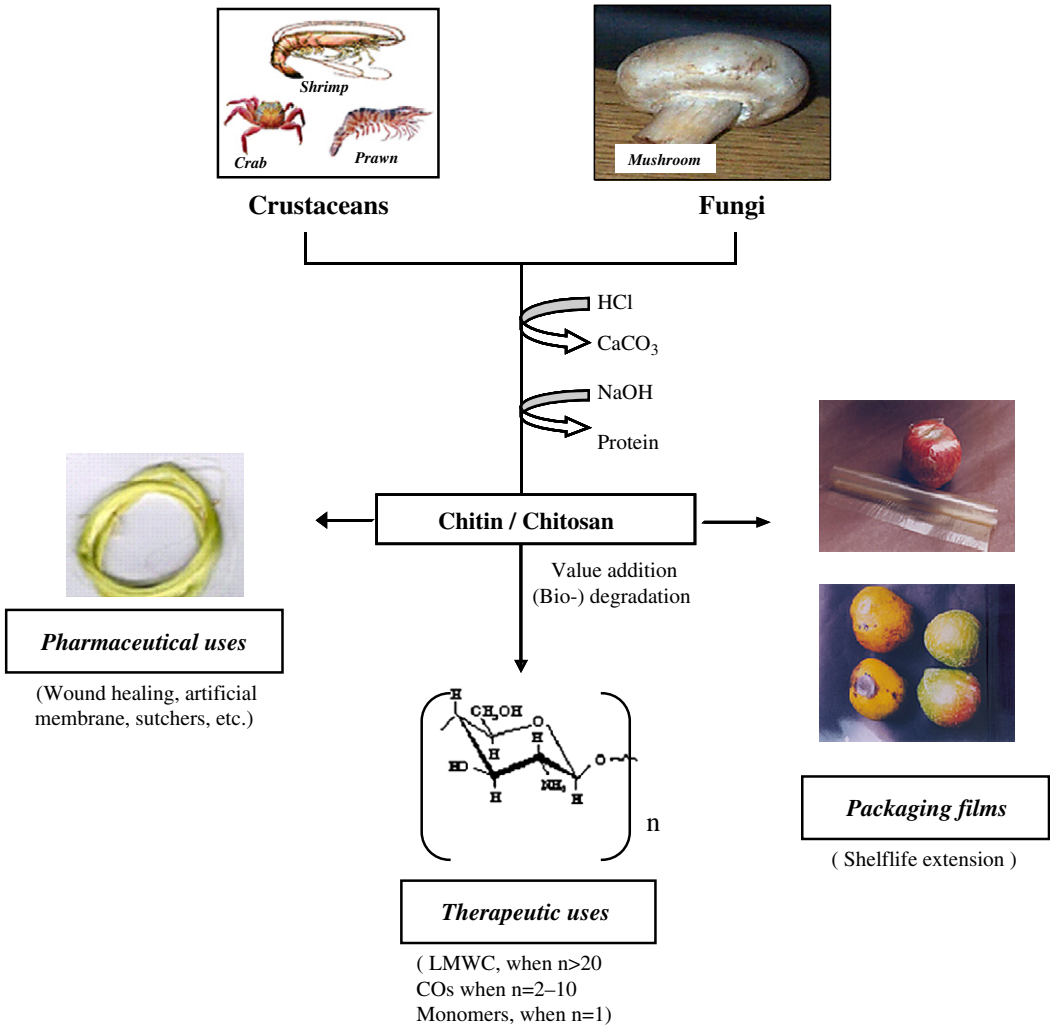

Fig. 1. Production of and value addition to chitin/chitosan.

insolubility of chitin in common solvents, particularly water at neutral pH. Their molecular weight (Mw) can be as high as 10^6 Da.

Chitosan, a de-*N*-acetylated analog of chitin, is a heteropolysaccharide consisting of linear β-1,4-linked GlcN and GlcNAc units. Both the content and sequence of these units will determine the physico-chemical and the biological properties of the polymer. It is known that heterogeneous conditions during deacetylation provide a block-wise distribution whereas under homogeneous conditions a random distribution of acetyl groups appear in chitosan. Due to its rigid and specific crystalline structures, possible through intra- and intermolecular hydrogen bonding, chitosan has the ability to exist in nature in different polymorphic forms, whose properties vary considerably.

In native as well as modified forms both chitin and chitosan are used in a wide range of applications, such as in food, biotechnology, material science, drugs and pharmaceuticals, and recently in gene therapy too. The net cationicity as well as the presence of reactive functional groups (1 amino and 2 hydroxyl groups per GlcN residue) in the molecule make chitosan a sought-after biomolecule. The free amino group present in each monomeric unit affords ammonium group, due to protonation, in aqueous acidic media. The latter offers scope for manipulation for preparing a broad spectrum of derivatives (Fig. 3) for specific end use applications in diversified areas (Fig. 4). In this review, an attempt is made to consolidate some of the recent findings on the biorelated (biotechnological and biomedical) application potentials of chitosan and its derivatives.

Acylation

A variety of acylation reactions are possible with both chitin and chitosan. Acylation with long chain aliphatic carboxylic acid chlorides such as hexanoyl, dodecanoyl, and tetradecanoyl chlorides give derivatives with a degree of acylation of 3. Such a thoroughly acylated product is soluble in chloroform (Fujii, Kumagai, & Noda, 1980). IR and thermal analyses data have revealed that chitosan with a higher degree of deacetylation was more susceptible for acylation owing to a decrease in hydrogen bonding.

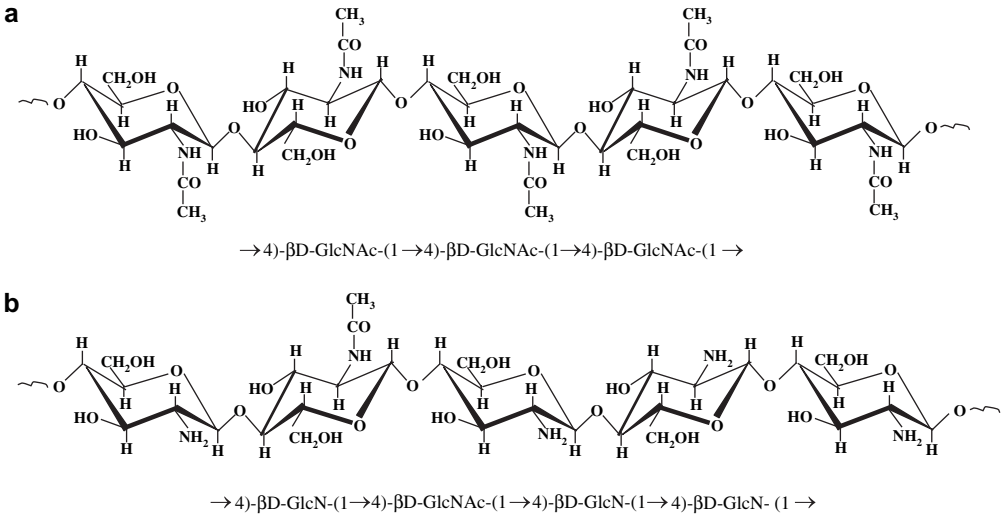

Fig. 2. Primary structure of (a) chitin and (b) chitosan.

Acylation of chitosan with a cyclic ester (lactone) such as β-propiolactone or γ-butyrolactone in an appropriate solvent gives derivatives having *N*-hydroxyalkanoyl groups (Loubaki, Sicsic, & Le Goffic, 1989).

Graft copolymerization

The possibility of grafting synthetic polymers to chitin and chitosan has attracted worldwide attention as a new and exciting way to modify and extend their use against

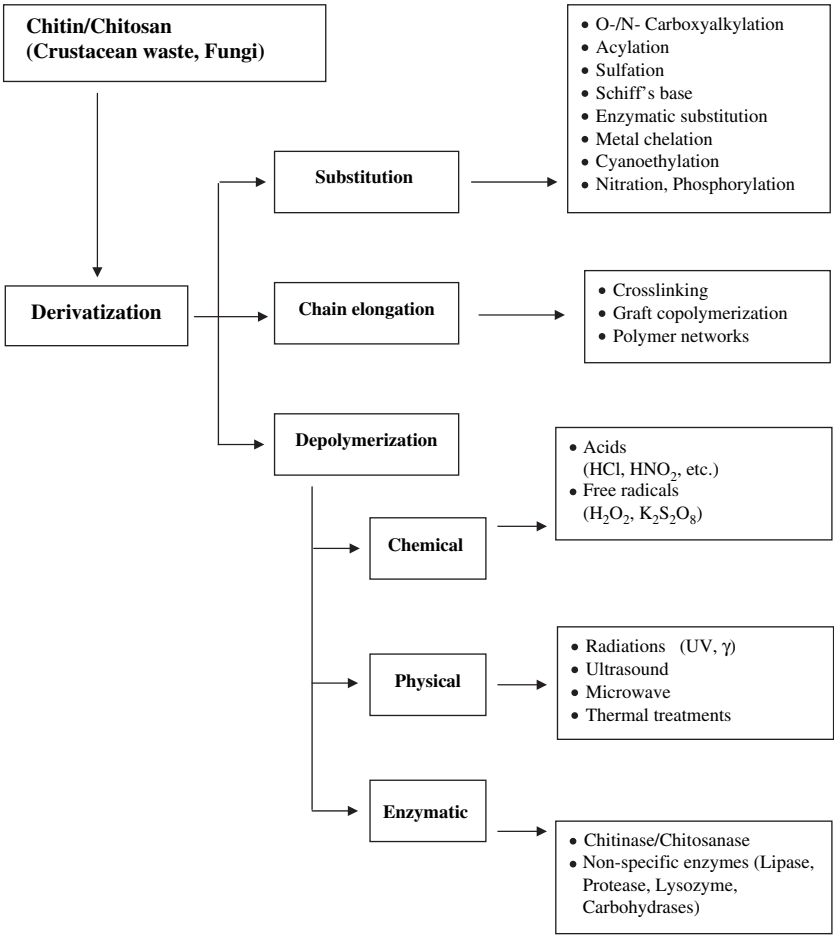

Fig. 3. Multifaceted derivatization potential of chitin/chitosan.

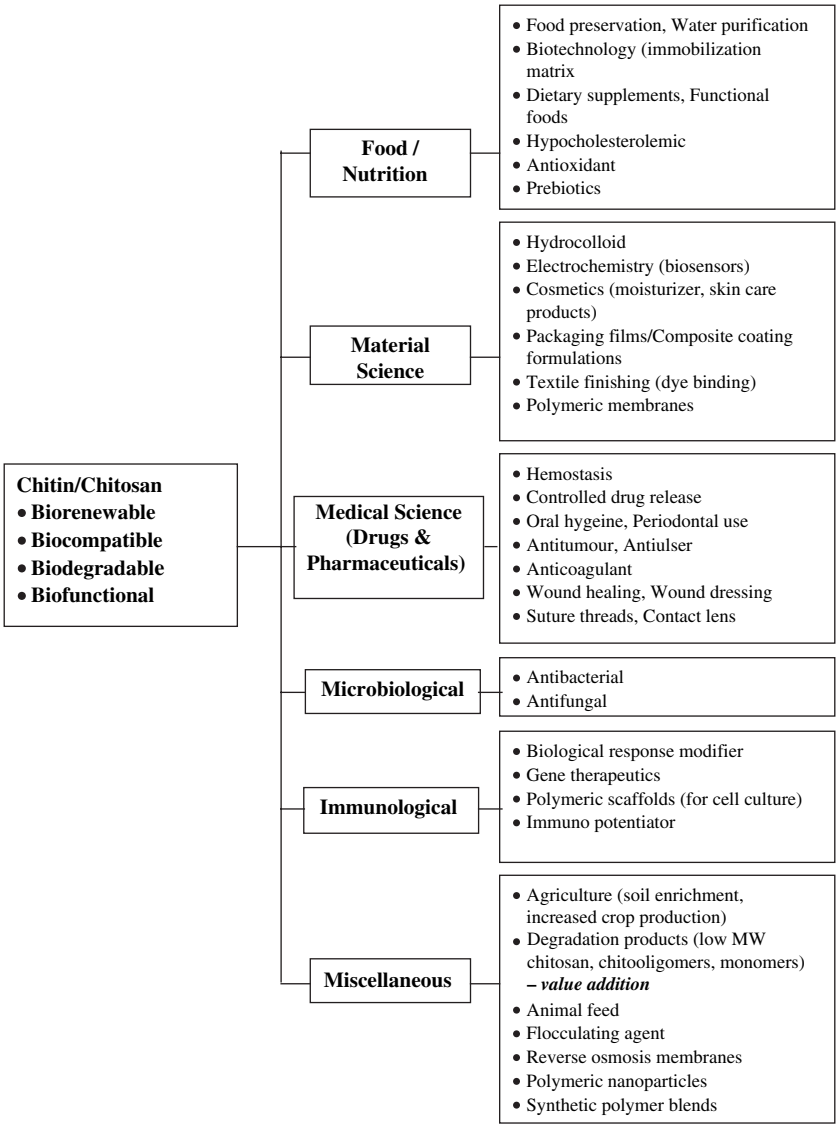

Fig. 4. Application potential of chitin/chitosan.

the rapidly growing competition from synthetic polymers themselves. Research in this field has blossomed quickly and is still an extremely active subject of study. In spite of enormous efforts, there is still no large-scale commercial application of chitin/chitosan graft copolymers. Graft copolymerization onto chitin and chitosan has not yet been explored extensively; it is a rapidly advancing field in polymer modification (Table 1; Kurita, 1997). Graft copolymerization reaction introduces side chains and makes various molecular designs possible, thus affording novel types of tailored hybrid materials composed of natural polysaccharides and synthetic polymers. The properties of the graft copolymers may be controlled by molecular structure, length and number of side chains attached. It is thus one of the most attractive approaches toward constructing versatile molecular environments. Grafting behavior is generally discussed in terms of grafting percentage, which is a ratio of the

Table 1. Types of chitosan-graft-copolymers

Type	Initiator	Monomers grafted
Radical-induced	Ce^{4+}, $K_2S_2O_8$, Fenton's reagent ($Fe^{2+} + H_2O_2$), tributyl borane	Acrylonitrile, *N*-isopropyl acrylamide, methyl methacrylate, vinyl monomers
Radiation-induced	γ-rays, ^{60}Co	Styrene, 2-hydroxyethyl methacrylate
Microwave irradiation		Polyacrylamide
Grafting onto method	Various catalysts are used [4,4′-Azobis (4-cyanovaleric acid)]	Telechelic polymers, polyethylene glycol, poly-dimethyl siloxane
Dendronization	(Reductive *N*-alkylation)	Polyamidoamine, styrene

weight of introduced side chains to the weight of the main chain. Several types of chitosan-graft-copolymers with acrylic, vinyl, nonvinyl, etc. have been prepared for use as flocculants, paper binder-strengthener, slow-release drug carrier, etc. Conventionally, such graft copolymerizations are being carried out using a variety of redox systems (see Table 1), although simultaneous homopolymerization leading to low grafting yield is the major constraint.

Grafting onto chitin

Cerium(IV), sometimes used for grafting onto cellulose, is also effective for grafting onto chitin. In its tetravalent state, Ce(IV) is an excellent oxidizing agent; it forms a redox pair with the NH_2 group of chitosan to give macroradicals. Typical vinyl monomers such as acrylamide and acrylic acid were graft-copolymerized onto powdery chitin in aqueous suspensions. The amount of Ce(IV) affected the polymerization markedly, and the grafting percentages reached 240% and 200% with acrylamide and acrylic acid, respectively. The resulting graft copolymers showed improved solubilities and swelling behaviors. Those having poly(sodium acrylate) side chains were soluble in dichloroacetic acid, whereas those having polyacrylamide chains swelled highly. Both the copolymers were much more hygroscopic compared to chitin (Kurita, Kawata, Koyama, & Nishimura, 1991).

Methylacrylate and methylmethacrylate (Ren, Miura, Nishi, & Tokura, 1993) were similarly graft-copolymerized with chitin using Ce(IV). The extent of swelling of chitin-graft-poly(methylmethacrylate) was dependent on the grafting percentage. At a grafting percentage of above 400%, the swelling became pronounced to give transparent gels from which films could be cast. The glass transition temperature of the copolymer was 130 °C as determined by differential scanning calorimetry (Ren *et al*., 1993). Grafting of methylmethacrylate onto chitin was also possible with tributylborane in water, although the grafting efficiency was not that high. Redox initiators could also be used for grafting (Yazdani-Pedram & Retuert, 1992).

γ-ray irradiation on powdery chitin initiates the polymerization of styrene as in the case of cellulose but the grafting percentage is low. Water is essential for initiation (Kurita & Inoue, 1989). On addition of a Lewis acid such as $SnCl_4$ or $TiCl_4$ to iodo-chitin in nitrobenzene, reactive carbenium species were formed, with which styrene was efficiently graft-copolymerized by a cationic mechanism in the swollen state (Kurita, 2001). The grafting percentage was up to 800%. The grafted side chains could be isolated from the copolymers by hydrolytic cleavage of the chitin main chain with hydrochloric acid to elucidate the grafting behavior. The polystyrene liberated from the graft copolymer with a grafting percentage of 650% was characterized for M_n, Mw and Mw/M_n values of 58,000 Da, 87,000 Da and 1.50, respectively. The values of grafting percentage and M_n indicated that one polystyrene chain was attached to one out of every 44 GlcNAc units on an average. The resulting chitin-graft-polystyrene was nearly soluble in aprotic polar solvents such as DMSO and DMAc containing 5% LiCl when the grafting percentage was above 100%. They showed considerable swelling even in common low boiling organic solvents.

Another candidate for the polymeric radical initiator to prepare well-defined graft copolymers is 6-mercapto-chitin (Kurita, Yoshino, Nishimura, & Ishii, 1993). Although mercapto-chitin is not soluble, it is expected to efficiently initiate graft copolymerization owing to the presence of readily dissociating mercapto groups and its swelling in organic solvents. Styrene was copolymerized onto mercapto-chitin efficiently in DMSO at 80 °C, and the grafting percentage was almost 100%. Similarly, methylmethacrylate was graft-copolymerized onto mercapto-chitin efficiently in DMSO at 80 °C to give chitin-graft-poly(methylmethacrylate). The grafting percentage increased with the amount of monomer and was above 120% under appropriate conditions. The resulting graft-copolymers exhibited remarkable affinity for various common organic solvents (Kurita, 2001).

Grafting onto chitosan

Graft copolymerization onto chitosan has also been attempted by various methods (Jalal Zohuriaan-Mehr, 2005; see Table 1), but it is performed typically with 2,2′-azo bisisobutyronitrile, Ce(IV), or a redox system. Vinyl monomers such as acrylonitrile, methylmethacrylate, methylacrylate and vinylacetate were graft copolymerized onto chitosan with 2,2′-azobisisobutyronitrile in aqueous acetic acid solution or in aqueous suspension. The grafting percentages were generally low. The chitosan-graft-poly (vinylacetate) was converted into chitosan-graft-poly(vinylalcohol) by hydrolysis (Blair, Guthrie, Law, & Turkington, 1987). Ce(IV) is also a suitable initiator, for graft copolymerization of polyacrylamide, poly(acrylic acid), and poly(4-vinylpyridine) with chitosan (Yilmaz, Hasopoglu, Caner, & Yilmaz, 1998). Fenton's reagent (Fe^{2+}/H_2O_2), as a redox initiator, graft-copolymerized methylmethacrylate with chitosan. Here, the reaction between the Fe^{2+} and H_2O_2 leads to free $OH^{\bullet}$ radicals, which in turn creates macroradicals on the polymer backbone by means of hydrogen abstraction. This facilitates grafting. Use of Fe^{2+} is advantageous, as it favours binding to chitosan, which in turn facilitates better yield of radicals, and minimizes any homopolymerization. In addition to higher yield of grafting, the reaction takes place at much lower temperatures.

Potassium persulphate (KPS) is another potential free radical initiator, wherein the grafting reaction takes place by two step single electron transfer mechanism followed by propagation. At 60 °C, KPS actually undergoes thermal degradation generating anionic persulphate free radicals, which then attack the cationic amino groups (NH_3^+) at C-2 of the GlcN moiety, ultimately resulting in glycosidic bond cleavage, leading to the formation (Harish Prashanth & Tharanathan, 2004; Hsu, Don, & Chiu, 2002)

of water-soluble low molecular weight chitosan (LMWC) and chitooligosaccharides (COs). The molecular weight of LMWC was found to be ~37 kDa and HPLC analysis of the chitooligomeric fraction showed pentamer, hexamer and higher oligomers. The effect of these degradation products on the growth of Ehrlich ascites tumor cells and tumor-induced neovascularization revealed COs (50 μg) to be more effective compared to LMWC (100 μg) and proved them to be potent angioinhibitory and antitumor compounds as shown by inhibition of angiogenesis and inducing apoptosis as a function of DNA fragmentation (Harish Prashanth & Tharanathan, 2004). These degradation products also showed scavenging of $OH^{\bullet}$ and $O_2^{\bullet}$ radicals and offered protection against calf thymus DNA damage (Harish Prashanth *et al*., unpublished data). Fluorescence study showed binding of LMWC in the minor groove, forming H-bonds to the backbone phosphates without distorting the DNA double helix structure. It was also observed that the fragmented chitosan chains upon cooling at 4 °C overnight led to extensive crosslinking (radical-induced self association), which was reported for the first time, and which involved several fragmented chains of depolymerized chitosan (Harish Prashanth & Tharanathan, 2006). In fact, secondary interactions such as hydrogen bridges and hydrophobic interactions between acetylated and non-acetylated units of chitosan fragments lead to a higher degree of crystallinity and as a result induce conformational changes in the molecule. Scanning electron micrograph of crosslinked chitosan revealed modification of fibrous native chitosan to crystalline granule bundles. The latter was highly refractory for swelling and dissolution. The possibility of exhibiting specific biofunctionalites by such crosslinked chitosan needs further investigations.

Recently, KPS-initiated graft-copolymerization of acrylonitrile and methylmethacrylate (MMA) onto chitosan was reported (Harish Prashanth & Tharanathan, 2003). Maximum graft yield of C-g-PAN (249%) was obtained with 0.12 M of acrylonitrile and 0.074 mM of KPS at 65 °C for 2 h. C-g-PMMA, 0.14 M of MMA at 65 °C gave maximum grafting (276%). No residual monomers were found by HPLC (Saroja, Gowda, & Tharanathan, 2000). The graft-copolymers could be thermopressed to thin membranous films, which were very fragile and brittle. *In vitro* biodegradability tests on the graft-copolymer revealed preferential (bio-)degradation of the chitosan moiety, leaving the synthetic polymeric chains, undegraded (Harish Prashanth, Lakshman, Shamala, & Tharanathan, 2005).

Microwave irradiation, is yet another efficient source of thermal energy that is used in a variety of modern synthetic reactions. Advantages of using microwave irradiation are that the reaction could be achieved in a very short duration of time, the workup procedures are simple and the product yields are fairly good. Its utility in the context of *green chemistry* has been widely accepted and appreciated for improved selectivity and eco-friendly reaction conditions. The enhancement of reaction rate under microwave irradiation is explained on the basis of dielectric heating of the molecule, which involves rapid energy transfer from the functional groups (-OH and $-NH_2$ groups) to reacting molecules. Possibly, such a high energy store may be responsible for the breakage of covalent and glycosidic linkages between the GlcN residues. Microwave irradiation is also known to lower Gibbs energy of activation of the reaction, thus ultimately effecting a free radical-type reaction mechanism. The grafted chitosan derivative was shown to be an efficient adsorbent for Ca^{2+} and Zn^{2+} ions from effluent water.

Grafting polyethylene glycol (PEG) onto chitosan is a convenient approach to prepare water-soluble chitosan derivatives, to be used as a carrier of anticancer drugs. In spite of their easy solubility, the PEG-g-chitosans, obtained by the use of carbodiimide, were found to aggregate in aqueous medium, due to hydrogen bonding. Whereas when such derivatives were prepared via reductive alkylation by PEG monoaldehydes, followed by further crosslinking, their water solubility was found restricted, and they gradually reaggregated, finally becoming insoluble (Dal Pozzo *et al*., 2000). Such a property is highly useful in wound dressings to prevent tissue adhesion in internal surgery. Ideally, such materials are insoluble when positioned in the surgical wound, but undergo progressive bioerosion leading to complete resorption when they are no longer needed. Based on choice of PEG substitution and crosslinking, derivatives having high swelling capacity with enhanced hydrophilicity can be obtained.

Aldehydes are generally used for protecting (via Schiff's base formation) the amino groups of chitosan and to allow hydroxyl groups to be modified. Glutaraldehyde, a dialdehyde, is frequently used for the crosslinking of chitosan (Fig. 4). Crosslinking affects the permeability characteristics. Crosslinking through covalent or ionic interactions results in the formation of chitosan hydrogels, which are useful as drug delivery systems allowing the release of bioactive materials by diffusion, and also as permanent networks, for example, as polymeric scaffolds in cell culture.

Depolymerization of chitin and chitosan

The very high molecular weight and therefore a very high viscosity of chitosan precluded its use in several biological applications. For some specific applications, more than the chitosan, its degradation products, viz. LMWC, COs and monomers, were found to be much more useful. A variety of degradation methods, viz. chemical, physical and enzymatic, are being worked out to generate these degradation products (Fig. 5). Both chitin and chitosan oligomers possess additional functional properties such as antitumor activity (Suzuki, 1996; Suzuki *et al*., 1986; Tsukada *et al*., 1990), immuno-enhancing effects, and enhancing protective effects against infection with some pathogens in mice (Tokora *et al*., 1989), antifungal (Hirano & Nagao, 1989; Kendra, Christian, & Hadwiger, 1989) and antimicrobial (Uchida, Izume, & Ohtakara, 1989) activities. Additionally, they have lower viscosity, low molecular

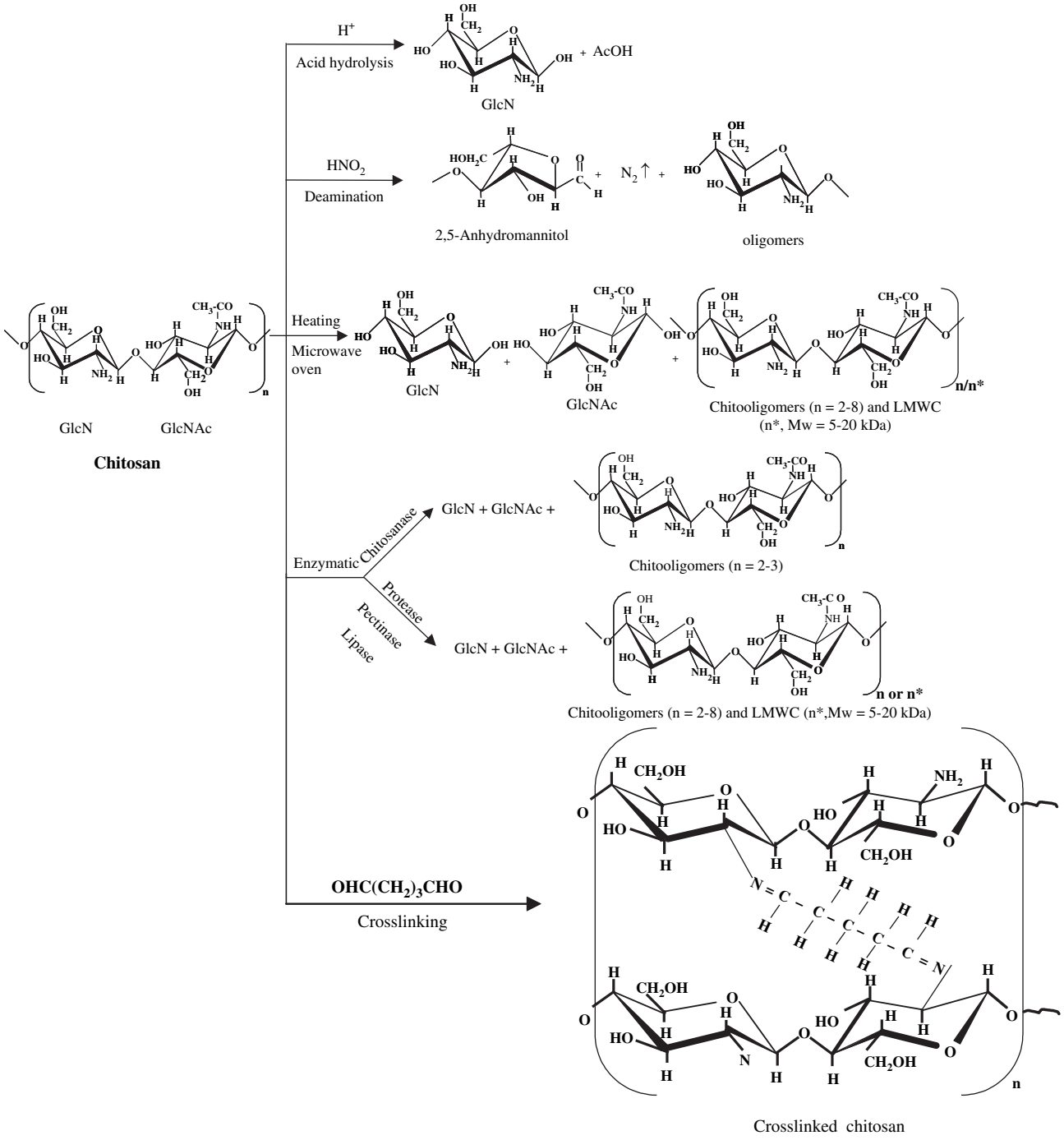

Fig. 5. Possible depolymerization products and crosslinking of chitosan.

weights and short chain lengths and are soluble in neutral aqueous medium. Subsequently, they seem to be readily absorbed *in vivo*.

Depolymerization process

Chemical methods

Acid degradative methods are not specific, the hydrolysis goes randomly generating a large amount of monomers, and later the removal of acid poses problems and also it is not economical. Chemical treatment using strong acids (viz. nitrous acid and HCl) is a very common and fast method to produce a series of chitooligomers, but the method has some disadvantages such as high cost, low yield and residual acidity. Recently, LMWC have been prepared by salt-assisted acid hydrolysis under microwave irradiation (Xing *et al*., 2005). The mechanism is explained as due to direct absorption of thermal energy by salt molecules, which causes localized superheating of solution.

Increase in the conductivity of the solution as well as dielectric loss and microwave coupling of the solvents have a dramatic influence on the rate of heating. The Mw of chitosan changed drastically in the presence of salt (from 10×10^4 to $\sim 3 \times 10^4$ Da), and the composition of the electrolyte followed the order $K^+ > Ca^{2+} > Na^+$, which was related to the ionic radius of the metals.

Depolymerization of chitosan by the use of nitrous acid (HNO_2) is a homogeneous reaction where the number of glycosidic bonds broken is roughly stoichiometric to the amount of nitrous acid used (Allan & Peyron, 1995). The mechanism involves deamination of deacetylated glucosamine residues (D-units) forming 2,5-anhydro-D-mannose (M-units) at the new reducing end. Since the latter is unstable, the standard procedure has been to reduce to 2,5-anhydro-D-mannitol by the use of $NaBH_4$. Nitrous acid induced depolymerization has been used previously to study the distribution of *N*-acetylated units in partially *N*-acetylated chitosan. It has been found to be specific in the sense that HNO_2 attacks the amino group of D-units, with subsequent cleavage of the adjacent glycosidic linkage.

There have been very few reports on the degradation of chitin or chitosan by free radicals. Nordtveit, Varum, and Smidsrod (1994) demonstrated that the viscosity of chitosan solution decreased rapidly in the presence of hydrogen peroxide (H_2O_2) and $FeCl_3$, probably due to random depolymerization of chitosan. Tanioka *et al.* (1996) showed that Cu(II), ascorbate, and UV$-H_2O_2$ systems also gradually reduced the molecular weight of chitosan. They postulated that the hydroxyl radicals generated in the experimental system caused polymer degradation and that this phenomenon may help to explain the disappearance of chitosan *in vivo* during biomedical applications. There are several other methods to degrade chitosan molecules including thermal degradation and ultrasonic treatment (Allan & Peyron, 1995; Chen, Chang, & Shyru, 1997).

Physical methods

Radiation can provide a useful tool for degradation of biological polymers and it is often viewed as being the last process after packaging to control pathogenic and spoilage organisms. Recently, radiation effect on carbohydrates such as chitosan, sodium alginate, carrageenan, cellulose, and pectin has been shown to enhance the use for recycling these bioresources and reducing the environmental pollution (Hien *et al.*, 2000; Nagasawa, Mitomo, Yoshii, & Kume, 2000). The irradiation effect on chitosan in acetic acid solution with various dose rates and the yield of chitosan oligomers have been investigated (Choi, Ahn, Lee, Byun, & Park, 2002). In another method using 85% phosphoric acid, low molecular weight chitosans were prepared by irradiation at different reaction temperatures and reaction time intervals, wherein the viscosity average molecular weights of chitosan decreased to 7.1×10^4 from 21.4×10^4 after 35 days of treatment (Jia & Shen, 2002).

Enzymatic methods

Although various degradation products of chitosan could be produced by a variety of methods, enzymatic methods are gaining importance because they allow regioselective depolymerization under mild conditions. Nevertheless, the undesirable level of pyrogenicity caused by the presence of protein admixtures in such preparations cannot be discounted. In the case of enzymatic degradation of chitosan, LMWC with high water solubility were produced by chitinase, chitosanase, glucanase, lipase and some proteases (Pantaleone, Yalpani, & Scollar, 1992; Vishu Kumar, Gowda, & Tharanathan, 2004; Vishu Kumar, Varadaraj, Lalitha, & Tharanathan, 2004). Non-specific enzymes (Muzzarelli, 1997), including lysozyme, cellulase, lipase, amylase, papain and pectinase (Grigolon, Azevedo, Santos, & Franco, 2001; Nordtveit, Varum, & Smidsrod, 1996) that are capable of depolymerizing chitosan are known, and among these papain, a cysteine protease, is particularly attractive because of its plant origin, wide industrial use in meat tenderization, use in medication for wound debridement, and its inhibition by human salivary cystatin.

Biological activity of LMWC and COs

Partially depolymerized chitosans, with an average molecular weight of 10 kDa, seem to have enhanced biochemical significance compared to native chitosan. Their superior antibacterial activity has been explained in terms of inhibition of the transcription from DNA (Liu, Guan, Yang, Li, & De Yao, 2001). LMWC modulated plant resistance to disease (Vasyukova *et al.*, 2001), stimulated murine peritoneal macrophages, showed antitumor activity (Seo *et al.*, 2000) and were useful in functional food formulations (Jeon, Shahidi, & Kim, 2000). LMWC of 20 kDa prevents progression of diabetes mellitus and exhibits higher affinity for lipopolysaccharide than chitosan of Mw 140 kDa (Hadwiger, Chiang, Victory, & Horovitz, 1989; Kondo, Nakatani, Hayashi, & Ito, 2000).

D-Glucosamine oligosaccharides have long attracted much attention, as they have physiological functions in a great variety of living organisms, including induction of phytoalexins (Hadwiger *et al.*, 1989), hemostatic effects (Malette & Quigley, 1984) and antitumor activities (Toda, Shimoji, & Sasaki, 1987). It is thought that the greatest physiological activities are shown by oligosaccharides with a chain length greater than the pentasaccharide. Hexa-*N*-acetylchitohexaose [$(GlcNAc)_6$] has immunopotentiating and antitumor functions (Suzuki *et al.*, 1986). They inhibit the growth of fungi and phytopathogens (Kendra & Hadwiger, 1984) and elicit defense mechanisms in plants (Roby, Gadelle, & Toppan, 1987). They also affect the mitogenic response and chemotactic activities of animal cells (Usami, Minami, Okamoto, Matsubashi, & Shigemasa, 1997). Their lipid binding (Ikeda *et al.*, 1993) properties make them useful ingredients for dietary and food preservation applications. LMWC was also shown to reduce blood glucose and serum triglyceride levels in obese

diabetic KK-Ay mice (Hayashi & Ito, 2002). It was reported that oligochitosans (Mw 1–3 and 3–5 kDa) prevent oxidative stress in mice (Shon, Park, Moon, Chang, & Nam, 2002).

Hypocholesterolemic activity

Due to its beneficial plasma cholesterol level lowering effect, which plays an important role in the alleviation and treatment of cardiovascular diseases, chitosan has become a useful dietary ingredient. The hypocholesterolemic action of chitosan has been explained to be due to decreased cholesterol absorption and interference with bile acid absorption, a mechanism similar to those of dietary fiber constituents. The cholesterol-lowering action of oral chitosan has been reported by many (Sugano, Watanabe, Kishi, Izumi, & Ohtakara, 1988), whereas chitin, although exhibiting higher excretion of triglycerides in feces, does not display cholesterol-lowering action. Information regarding digestion and absorption of chitin and chitosan in the GI tract is limited. In an *in vivo* study in canine GI tract, itwas shown that chitin did not under go any changes in weight and shape, whereas chitosan showed ~10% decrease in weight and formed a film.

Enzyme immobilization

The fact that an enzyme can coexist in various oligomeric forms is of major importance for its catalytic expression. Enzyme immobilization is a technique of significant practical utility, especially to enhance the catalytic potential, resistance to pH and temperature, and continued reusability. It is known that chitosan is an excellent base material for immobilization of several carbohydrate degrading enzymes, as it exhibits increased thermostability compared to the free enzyme. Urease has been immobilized covalently onto glutaraldehyde crosslinked chitosan membrane, especially to provide resistance to the influence of inhibitors, such as boric acid, thioglycolic acid, sodium fluoride and acetohydroxamic acid (Zaborska, 1995). Similarly, resistance to mechanical stirring of D-amino acid oxidase (a flavoprotein using FAD as cofactor) has been provided by enzyme immobilization on crosslinked chitosan matrix (Lemainque, Braun, & LeGoffie, 1988).

Antioxidant property

The trend to go for potent, naturally derived antioxidant molecules over those of synthetic origin is ever increasing. To this class belong chitosan and several of its derivatives, which being safe and non-toxic offer protection from free radicals, thus retarding the progress of numerous chronic diseases (Tiwari, 2004). It is known that the antioxidant effect of chitosan varies with its molecular weight and viscosity, as shown in cooked comminuted fish flesh model systems (Kamil, Jeon, & Shahidi, 2002; Xie, Xu, & Liu, 2001). It is attributed to differences in the availability of net cationic amino groups in the molecule, which impart intermolecular electrostatic repulsive forces leading to increase in the hydrodynamic volume of the extended chain conformation. The highly unsaturated fatty acids commonly found in seafood are particularly sensitive to oxidative change during storage. Treatment of herring fish samples with chitosan, however, showed lower peroxide values and total volatile aldehydes than the untreated samples. The low viscosity chitosan showed the strongest antioxidative effect (Lin & Chou, 2004).

Wound healing property

Wound healing is a process for promoting rapid dermal regeneration and accelerated wound healing. A novel asymmetric chitosan membrane consisting of skin surface on top layer supported by a macroporous sponge-like sublayer has been designed. The chitosan membrane showed controlled evaporative water loss, excellent oxygen permeability and promoted fluid drainage ability, at the same time effectively inhibiting invasion of exogenous microorganisms (Mi *et al.*, 2001). Wound covered with such membrane was hemostatic and healed quickly. Histopathological examination confirmed increased epithelialization rate as well as well organized deposition of collagen in the dermis. Chitosan-based wound dressing reduced scar tissue (fibroplasias) by inhibiting the formation of fibrin in wounds and it was hemostatic and formed a protective film coating (Lloyd, Kennedy, Methacanon, Paterson, & Knill, 1998). Being a substrate for lysozyme, chitosan degradation products were internally absorbed, which in turn affected macrophage activity.

Chitosan-alginate polyelectrolyte complexes, prepared *in situ* in beads and microspheres, caste as films showed good wound dressing capability (Yan, Khor, & Lim, 2000). Two chitosan films, viz. Chit-LA and Chit-AA, treated for wound healing efficiency, showed complete wound closure, good epithelialization and no scar formation (Khan & Peh, 2003). As an effective wound dressing agent with antibacterial properties, chitosan-cellulose blend membranes have been prepared. They may protect wounds from excessive dehydration and infection (Wu *et al.*, 2004).

Membranes

Chitosan and several of its innumerable derivatives have the ability to form thin membranous films of use in packaging (Kittur, Kumar, & Tharanathan, 1998; Srinivasa, Baskaran, Ramesh, Harish Prashanth, & Tharanathan, 2002; Srinivasa, Ramesh, Kumar, & Tharanathan, 2004), encapsulation and drug delivery systems. Due to drug–polymer interactions, high viscosity chitosan films showed better sustainable release; and the mechanism of release followed Fiekian diffusion control with subsequent zero order release (Puttipipatkhachorn, Nunthanid, Yamamoto, & Peck, 2001). A novel organic (chitosan) and inorganic (tetraethyl orthosilicate) composite membrane has been prepared, which is pH sensitive and drug permeable (Park, You, Park, Haam, & Kim, 2001). The latter possibly involved ionic interactions. By plasma source ion implantation

technique, the adhesion between linear low density polyethylene and chitosan could be improved (Shin *et al*., 2002). Such bilayer films showed 10 times lower oxygen permeability, a property of use in food packaging applications. These multilayer films were easily recyclable.

Periodontal use

In periodontal and implant surgery, bone regenerative procedures employing bone graft materials have become essential. For tissue engineered regeneration of bone, a variety of polymeric scaffolds such as, collagen type-1 matrix, porous poly-(lactide/gylcolide)/hydroxyapatite 3-D polymer matrix, chitosan have been attempted. The results have shown that artificial bone formation using tissue-engineering technology is possible and may be a promising strategy to regenerate bone tissue. The results of a preliminary *in vitro* experiment suggest that chitosan potentiates the differentiation of osteoprogenitor cells and may facilitate the formation of bone (Lee *et al*., 2000a). Growth factors, especially platelet T-derived growth factor are known to enhance periodontal regeneration, when administered using a biodegradable carrier that provides a sustainable release of therapeutic concentration over a period of time (Lee *et al*., 2000b). Chitosan-tricalcium phosphate sponges have shown promise as very good scaffold material for transplantation into a site for bone regeneration *in vivo*.

Due to disadvantages of the systemic administration, of late considerable attention is given to local delivery systems of antimicrobial agents for treatment of periodontal diseases. An ideal formulation should exhibit ease of delivery, a good retention at the application site and preferably a controlled release of the drug. Bioadhesive chitosan gels fulfill all these properties; it stays long in the oral cavity, has adequate drug penetration, shows excellent antimicrobial activity, and also shows high efficiency and acceptability. Studies, using chitosan formulations either in gel or film form, were conducted against a periodontal pathogen, *Porphyromonas gingivalis* (Ikinci *et al*., 2002). The viscosity, bioadhesive properties and antimicrobial activity of chitosans of different molecular weights and degree of deacetylation were evaluated in the presence or absence of chlorohexidine gluconate, incorporated into formulations at 0.1% and 0.2% levels. The flow property of the gels was found to be excellent and local therapy at the site of infection provided complete protection.

Controlled drug-release

Chitosan is a versatile carrier for biologically active species such as drugs due to the presence of free amino groups as well as its low toxicity. In view of the development of potent anticancer and antimetastasis agents, the pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR) is rated high as an active peptide, since it is a partial sequence of laminin that is known to be involved in metastasis of tumor cells. Though the pentapeptide itself inhibits experimental metastasis formation, it is digested with proteases rapidly *in vivo*, suggesting that its conjugation with appropriate polymeric material would be promising. Acyl-Tyr-Ile-Gly-Ser-Arg-β-Ala-OH, where β-Ala stands for β-alanine as a spacer arm could be conjugated with chitosan. An attempt to couple directly with chitosan was unsuccessful, but the introduction of the spacer arm enabled coupling with the peptide in the presence of a water-soluble carbodiimide (Hojo *et al*., 2000). The resulting conjugate, chitosan-(Gly)-β-Ala-Arg-Ser-Gly-Ile-Tyr-Ac, exhibited significant inhibitory activities toward experimental lung metastasis with B16-BL6 melanoma cells in mice. This was higher than that of the parent pentapeptide YIGSR. The enhanced activity may be partly attributable to a protective effect against enzymatic digestion *in vivo* as a result of its conjugation with chitosan.

With reference to pharmaceutical excipient for directly compressed tablets, chitosan-alginate combination showed an extended drug release property. Dry coated tablets having a long induction period in drug release have been prepared by an ion-complex of alginate-chitosan (Takeuchi, Yasuji, Yamamoto, & Kawashima, 2000). The drug release profiles showed a long induction period followed by a rapid drug release phase in the artificial intestinal fluid. With an increase in the degree of deacetylation and in the amount of chitosan in the formulation the induction period could be prolonged.

Sustained intestinal delivery of drugs such as 5-fluorouracil (choice drug for colon carcinomas) and insulin (for diabetes mellitus) seems to be a feasible alternative to injection therapy. For the latter, the drug should be delivered at proper sites (intestine) for long duration for producing maximum pharmacological activity. The property of bioadhesiveness through encapsulation of alginate with polyacrylic acid and chitosan has been made use of, which using bromothymol blue as the model drug, released the drug for an 8-day period *in vitro* (Ramdas, Dileep, Anitha, Paul, & Sharma, 1999). Sustained release of oxytetracycline, an antibiotic agent, from chitosan microspheres (5–30 μ) for both oral administration and injection has been reported (Mi, Wong, & Shyu, 1997). The latter was prepared by spray hardening and interfacial acylation methods.

Antimicrobial property

The antimicrobial property of chitosan and its derivatives, with conflicting results, has received considerable attention in recent years due to imminent problems associated with synthetic chemical agents. Such an application stems from the cationic charge of chitosan molecule to give rise to aggressive binding onto the microbial cell surface, leading to gradual shrinkage of cell membrane and finally death of the cell. Several possible explanations have been proposed for antimicrobial activity, viz. polycationic chitosan molecule interacting with the predominantly anionic cell wall components (lipopolysaccharides and proteins) of the microorganism, which results in the leakage of intracellular components due to changes in permeability barrier;

preventing nutrients from entering the cell; upon entry into the cell (especially LMWC), binding to DNA, and thus inhibiting RNA and protein synthesis; binding through hydrophobic interactions, etc. Chitosan shows a broad-spectrum antimicrobial activity against both gram-positive and gram-negative bacteria and fungi (Vishu Kumar, Varadaraj, Gowda, & Tharanathan, 2005).

In a study on the mode of antimicrobial action of chitosan (250 ppm at pH 5.3) by monitoring the uptake of the hydrophobic probe 1-*N*-phenylnaphthylamine, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* showed significant uptake which was reduced (in *coli, Salmonellae*) or abolished (*aeruginosa*) by $MgCl_2$. Chitosan also sensitized *P. aeruginosa* and *Salmonellae* to the lytic effect of sodium dodecyl sulfate. Electrophoretic and chemical analyses of the cell-free supernatants revealed no release of LPS or other membrane lipids. Electron microscopic observations showed that chitosan caused extensive cell surface alterations and covered the outer membrane with vesicular structures, resulting in the loss of the barrier functions (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001). This property of chitosan is useful in food preservation and food protection. To enhance the antibacterial potency of chitosan, thiourea chitosan was prepared by reacting chitosan with ammonium thiocyanate followed by its complexing with Ag+ (Chen, Wu, & Zeng, 2005).

It has been reported that quaternary ammonium salt of chitosan exhibits good antibacterial activities, for example, diethylmethylchitosan chloride showed higher antibacterial activity than chitosan. Novel *N*,*O*-acyl chitosan derivatives were more active against the gray mould fungus *Botrytis cinerea* and the rice leaf blast fungus *Pyricularia oryzae*; hydroxypropyl chitosan grafted with maleic acid sodium killed over 99% of *Staphylococcus aureus* and *E. coli* within 30 min of contact at a concentration of 100 ng/ml; hydroxypropyl chitosan was a potent inhibitor of *Azatobacter mali*, *Clostridium diplodiella*, *Fusarium oxysporum* and *Pyricularia piricola*. The degree of substitution of hydroxypropyl group also influenced their antifungal activity. With regard to their antifungal mechanisms, it was reported that these chitosan derivatives directly interfered with fungal growth and activated several defense processes, such as accumulation of chitinases, synthesis of proteinase inhibitors and induction of callous synthesis. It was also noted that the antibacterial activity of chitosan derivatives increased with increasing chain length of the alkyl substituent, and this was attributed to the increased hydrophobicity.

Anticoagulant activity of sulfated chitosan derivatives

The phenomenon of blood coagulation involves the sequential activation of a series of serine proteinases, which culminates in the generation of thrombin and subsequent thrombin-catalysed conversion of fibrinogen into insoluble fibrin. The ability of certain sulfated glycosaminoglycans to interfere with blood coagulation process is a subject of extensive clinical trials and practical utility (Vongchan, Sajomsang, Subyen, & Kongtawelert, 2002). Heparin, as an efficient antithrombotic agent has a long standing clinical usage (Bourn & Lindhal, 1993). Compared to heparin, the sulfated chitosan has been shown to possess high anticoagulant potency. Sulfation was carried out using chlorosulfonic acid in *N*,*N*-dimethylformamide at room temperature to avoid degradation of chitosan. Unlike heparin, sulfated chitosan does not show anti-platelet activity, which causes excessive bleeding in patients. A higher degree of sulfation was shown to be beneficial for the anticoagulant activity, with respect to thrombin time. The arrangement of sulfate groups was found to have tremendous influence on the anticoagulation process, for example, C-6 sulfate group was a key requirement, as its desulfation led to loss of activity (Nishimara *et al*., 1998).

Chemical modification of sulfated chitosan is also of interest because it enhances the structural similarity of sulfated chitosan to that of heparin (Huang, Du, Yang, & Fan, 2003). The anticoagulant activity of sulfated polysaccharides generally results from the interaction between the negatively charged sulfated groups and positively charged peptide sequences. *N*-acetyl groups are also shown to improve the anticoagulant activity.

Miscellaneous applications

Chitosan is a natural cationic polymer that has recently emerged as an alternative nonviral gene delivery system. Conceptually, gene therapy involves the introduction of an extraneous gene into a cell with the aim of tackling genetic disease, slowing down the progression of tumors and fighting viral infections. Out of the various nonviral vectors tried, such as liposomes, cationic polyelectrolytes, chitosan excels in its biocompatibility and nontoxicity even at escalating doses with no side effects. Due to superior mucoadhesive properties chitosan facilitates the transport of various drugs across cellular membranes. Chitosans in which over 2 out of 3 monomer units carried a primary amino group formed stable colloidal polyplexes with pDNA. Only the protonated form of soluble chitosan having an uncoiled configuration can trigger the opening of the tight junction zones of DNA and thereby facilitate the paracellular transport of hydrophilic compounds. Chitosan behaved more like a Gaussian coil instead of the worm-like chain model found in other polyelectrolytes. Also the molecular size of chitosan and pH of the medium are two other important parameters that dictate its permeabilizing and perturbing effects on the cell membrane. A combined electrostatic-hydrophobic driving force from chitosan might induce the destabilization of cell membranes. In addition to ionic interactions, non-ionic interactions between the carbohydrate backbone of chitosan and cell surface proteins might exert an important role in the chitosan-mediated transfection of cells (Koping-Hoggard *et al*., 2001).

DNA condensation provides a promising means whereby DNA containing genes of therapeutic interest can be prepared for transfer from solution to target cells for gene

therapy applications. In addition to synthetic condensation agents (PEG), multivalent cations (chitosan) may also facilitate binding. Such chitosan-based transfection systems are advantageous because of non-immunogenicity, lack of biohazards and the possibility of introducing larger DNA fragments into targets over viral vectors in gene therapy. However, the high molecular weight of chitosan precluded its usage because of toxicity problems. Nevertheless, the LMWCs are neither toxic nor haemolytic and they are shown to form complexes with DNA and protect against nuclease degradation, thereby validating LMWC as components of a synthetic gene delivery system (Tiwari, 2004).

Chitosan and its amino acid derivatives (poly D, L-lactic acid) have been explored as an extracellular matrix-like surface to promote cell adhesion and growth (Zhu *et al*., 2002). Four kinds of chitosan-amino acid derivatives were prepared to minimize the carbohydrate moieties of cell matrix glycoproteins. From detailed cell cultural studies these chitosan derivatives were shown to promote chondrogenesis.

Chitosan nanoparticles are shown to enhance oral bioavailability and intestinal absorption of peptide and protein formulations. By ionotropic gelation of chitosan with tripolyphophate anions insulin-loaded nanoparticles have been prepared (Pan *et al*., 2002). Enhanced intestinal absorption as well as relative increase in the pharmacological bioavailability of insulin was investigated by monitoring the plasma glucose level of alloxan-induced diabetic rats. The nanoparticle, having a size in the range of 250–400 nm and polydispersity index <0.1, positively charged and remaining stable, showed insulin association of over 80% and its *in vitro* release showed a great initial burst with a pH-sensitivity. It showed an increased absorption of insulin *in vivo* ($\sim$15%), and the hyperglycemia was prolonged for over 15 h.

Chitosan-poly(acrylic) acid polyionic complexes have been prepared for prolonged gastric antibiotic delivery (Torrado, Prada, de la Torre, & Torrado, 2004). Different polyionic complexes of amoxicillin, chitosan and polyacrylic acid were prepared and employing a non-invasive method the gastric residence time of the formulations was evaluated, by means of ^{13}C-octanoic acid breath test. All the complexes showed extensive swelling, and diffusion of the antibiotic was controlled by the degree of polymer-drug interaction.

In the construction of heart valve substitutes, bovine pericardium fixed in buffered glutaraldehyde is presently being used. Calcification limits the durability of such heart valve substitutes. As an alternative, crosslinking of biomacromolecules with glutaraldehyde was tried, which creates void spaces in the fiber matrix leading to exposure of potential binding sites for calcification. By hydrogen peroxide degradation LMWC (2000 Da) was prepared for coupling onto polymer grafted glutaraldehyde crosslinked pericardial tissue to prevent calcification in rat subcutaneous model (Shanthi & Panduranga Rao, 2001).

The capacity to preconcentrate anions has allowed use of chitosan and its derivatives in modified electrodes, for application in sensor and biosensor electrochemistry (Rodrigues, Laranjeira, Stadler, & Drago, 2000).

To facilitate improving water solubility of biologically useful chitosan derivatives, *N*-methylene phosphonic chitosan has been prepared using a one step reaction that allowed homogeneous modifications (Heras, Rodrigues, Ramos, & Agullo, 2001). The resulting NH_2-CH_2-PO_3^{2-} combines its strong donor effect with a monodentate ligand as PO_3^{2-}, thus increasing its metal-binding properties, especially for calcium. The derivative also shows good filmogenic nature.

Phosphated chitin (P-chitin) has been used as an anti-inflammatory agent in a mice model of chitosan-induced acute respiratory distress syndrome (Khanal *et al*., 2001). The interstitial pneumonia was thus successfully blocked by a simultaneous intravenous injection of P-chitin. Intravenous infusion of some P-chitin formulations dramatically reduced lung injury and diminished the accumulation of neutrophils in the interstitial and alveolar spaces of the lungs. P-chitin with a Mw of 24000, DS of 58% and DA of 4% was found to be most effective in the prevention of pneumonia.

Attempts have been made to develop new types of anti HIV-1 reagents having different inhibitory mechanisms from those of nucleoside analogs, which are toxic, show side effects and are not effective against drug-resistant strains (Nishimara *et al*., 1998). A regioselective sulfation of C-2 and/or C-3 groups of chitin has been developed employing 6-0-trityl chitosan, as a protected intermediate. Sulfation at both 2 and 3 positions showed the highest inhibitory effect, whereas sulfation at C-6 seems to decrease the anti HIV-1 activity, but nevertheless the latter showed high anticoagulant activity. These new inhibitors of retrovirus infection showed both low cytotoxicity and low anticoagulant activity.

Chitooligosaccharides stimulate purportedly beneficial gut species (*Bifidobacterium* and *Lactobacillus* sp.) opening up the possibility of them acting as prebiotics. Despite this property, in a mixed culture system no increase in the bifidus counts was observed (Vernazza, Gibson, & Rastall, 2005). Nevertheless, through pure culture studies chitooligosaccharides were shown to be stimulatory to *Bifidobacterium bifidum* and *Lactobacillus* sp., in low concentrations they led to increased cell numbers and showed prebiotic effects (Lee, Park, Jung, & Shin, 2002). *In vitro* studies have shown that chitooligosaccharides can bind 4 to 5 times its weight of micellar lipids, leading to claims on some brands of slimming pills for blocking fat absorption, and therefore of use in obesity control (Nauss, Thompson, & Nagyvary, 1983).

Final remarks

In brief, it is very much evident that chitin/chitosan and their modified derivatives exhibit an unlimited application potential for use in a wide range of faculties. The recent paradigm shift from synthetic packaging materials to biodegradable packaging films made out of biobased polymers opens up a lot of opportunities for chitosan-derived

packaging films. Such films are advantageous because of their user-friendly and eco-friendly characteristics, and also the raw materials are generally derived from replenishable natural resources. A continuous but sustainable use of such hydrocolloid-based biodegradable packaging films augurs minimizing plastic wastes, whose volume (over 30% of total wastes generated) and disposal are causing new challenges and serious threats for the generations to come. The annual per capita utilization of plastics in India is ~2 kg/person/year compared to 60 kg/person/year in developed countries. Although plastic packaging films cost less (~$2 per kg), chitosan as a raw material is priced at ~$15 per kg, whereas other biopolymers (e.g., corn starch and cellulose at ~$ 0.8–1.8 per kg) are much cheaper. Nevertheless, for some specialized applications (pharmaceutical and therapeutic) such premium prices for biodegradable, safe packaging are well tolerated. In the area of nanoscience, the use of chitosan and its derivative is immense. The biopolymer chitosan is especially very useful as it can be made available in a variety of morphologies including fibers, films, hydrogels, membranes, nanoparticles and microspheres. Although chitosan has received the GRAS (generally recognized as safe) status by the Food and Drug Administration, USA, its full-fledged usage in food formulations (functional foods) needs official clearance. For the latter, additional studies to address regulatory issues of concern need to be done to substantiate unequivocally the various health-related claims put forth already. Further basic and application oriented studies are expected to fully utilize the potential of chitin/chitosan for still additional uses and for the benefit of the society.

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