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Trimethyl chitosan and its applications in drug delivery

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Abstract Chitosan, a polymer obtained by deacetylation of chitin is widely studied for its pharmaceutical and nonpharmaceutical applications. Recommendations about uses of this polymer although could not be always realized due to limited solubility. Chitosan, for example, has been extensively evaluated for its mucoadhesive and absorption enhancement properties. The positive charge on the chitosan molecule gained by acidic environment in which it is soluble seems to be important for absorption enhancement. However chitosan is not soluble in medium except below pH 5.6. This limits its use as permeation enhancer in body compartments where pH is high. In this regard there is a need for chitosan derivatives with increased solubility, especially at neutral and basic pH values. Trimethylation of chitosan is an effort in this direction. Despite the abundance of the research related to trimethyl chitosan (TMC), the overview of the topic is not available. Hence an attempt is made in this review to cover the recent findings pertaining to synthesis, characterization and applications of TMC especially in pharmaceutical field. TMC has been synthesized by different ways and characterized by FTIR, NMR, DSC etc. This quaternized derivative of chitosan possesses a positive charge and is soluble over a wide range of pH. TMC, being a derivative of cationic polymer enriched with positive charge shows better mucoadhesive, permeation enhancement, drug delivery and DNA delivery properties. TMC can be further derivitized or grafted for modulating properties as solubility, cytotoxicity or cell recognition ability. Apart from these applications, TMC itself and its derivatives exhibit antimicrobial properties also. Quaternization of chitosan not only with methyl group but higher group as ethyl or along with spacer or quaternization of modified chitosan can be of interest too.

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1 Introduction

Chitin and chitosan are aminoglucopyrans composed of *N*-acetylglucosamine and glucosamine residues. These polysaccharides are renewable resources which are currently being explored intensively for their applications in pharmaceutical, cosmetics, biomedical, biotechnological, agricultural, food, and non-food industries as well (water treatment, paper, and textile) [1]. These are recommended as suitable resource materials because these biorenewable polymers have excellent properties, such as biocompatibility, biodegradability, and nontoxicity [2].

Chitin is a linear cationic heteropolymer of randomly distributed 2-*N*-acetyl-2-deoxy-glucose (*N*-acetylglucosamine) and 2-amino-2-deoxy-glucose (glucosamine) residues with β-1,4-linkage which is one of the most abundant polysaccharides in nature and is mostly derived from the exoskeleton of crustaceans [3] (Fig. 1). Depending on the source and preparation procedure, the number of glucosamine residues denoted as the degree of deacetylation in isolated chitin ranges from 5 to 10% and the molecular weight of this linear polysaccharide can be as high as $1–2 \times 10^6$ Da corresponding to a degree of polymerization of ca. 5,000–10,000. Chitosan is a product derived from alkaline N-deacetylation of chitin. In chitosan, degree of deacetylation is above 60% and the molecular weight ranges from 2,000 Da (oligomers) to $10^4 - 2 \times 10^6$ Da [4, 5]. The recommendations about uses of these polymers however

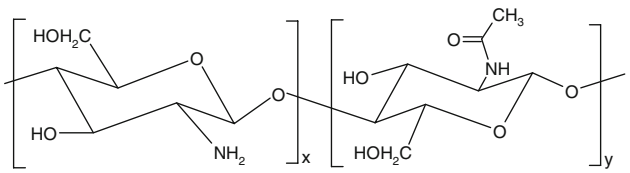

Fig. 1 Repeat residues for chitin and chitosan. Chitin is composed predominantly of (y) units and chitosan is composed predominantly of (x) units distributed in random fashion

could not be always realized due to their limited solubility. Chitin is insoluble in water and almost all commonly used organic solvents. Chitosan, in its crystalline form, is normally insoluble in aqueous solutions above pH 7; however, in dilute acids (pH < 6.0), the protonated free amino groups on glucosamine facilitate solubility of the molecule. In aqueous acidic solution chitosan swells and in the swollen state demonstrates mucoadhesive properties [6]. This has further drawn into its use as absorption enhancer. In recent years, chitosan has been extensively studied as a potential absorption enhancer.

2 Chitosan as permeation/absorption enhancer

Illum et al. were the first to report that chitosan at 0.5% (w/v) concentration was able to promote the absorption of small polar molecules as well as peptide and protein as insulin across nasal mucosa in rats and sheep [7]. Immediately afterwards Artursson et al. reported that chitosan can increase the paracellular permeability of [^{14}C]-mannitol (a marker for paracellular routes) across Caco-2 intestinal epithelia [8]. Aspden et al. confirmed the absorption enhancement in vivo by nasal route [9]. Lueßen et al. evaluated the potential of chitosan glutamate to improve the intestinal transport of 9-desglycinamide, 8-L-arginine vasopressin (DGAVP) in vitro by using Caco-2 cell monolayers as well as a vertically perfused rat intestinal loop model [10]. The team also evaluated in vivo absorption enhancing effects of chitosan hydrochloride (1.5% w/v) in a gel formulation of the peptide drug buserelin on intraduodenal administration [11]. It resulted in the highest absolute bioavailability for intraduodenally administered buserelin in rats (5.1%) in comparison to no polymer or Carbopol 934P containing formulations. Chitosan glutamate and chitosan hydrochloride, at a pH of 6.2, leads to a pronounced reduction in transepithelial electrical resistance (TEER) across Caco-2 cell monolayers [12]. In agreement with the reduction in TEER with 1.5% (w/v) of the chitosan salts, the increase in transport of the peptide drug buserelin and insulin was seen.

In all the studies that have been mentioned, absorption enhancement was found only in acidic environments in which the pH was less or of the order of the pKa value of chitosan (5.5–6.5). However this was not the case in pH above this value. Borchard et al. investigated chitosan glutamate solutions at pH 7.4 for their effect in increasing the paracellular permeability of [^{14}C]-mannitol and fluorescently labeled dextran (MW 4,400 Da) in vitro in Caco-2 cells. No effect on the permeability of the monolayer could be observed, indicating that at neutral pH value chitosan is not effective as permeation enhancer [13]. The pH dependency of chitosan's effect on epithelial permeability was further studied by Kotzé et al. [14]. Two chitosan salts (hydrochloride and glutamate) were evaluated for their ability to enhance the transport of [^{14}C] across Caco-2 cell monolayers at two pH values 6.2 and 7.4. At low pH both chitosans showed a pronounced effect on the permeability of the marker, leading to 25-(glutamate salt) and 36-fold (hydrochloride salt) enhancement. However, at pH 7.4 both chitosans failed to increase the permeability, due to solubility problems. (It was shown that the effect of chitosan on the transport of the marker molecule, [^{14}C]-mannitol, is optimal when the pH is well below the pKa of 6.5). This is due to nature of chitosan, a weak base, which requires a certain amount of acid to transform the glucosamine units into the positively charged water-soluble form. Due to their charge loss in neutral and basic environments, chitosan precipitates from solution rendering it unsuitable as an absorption enhancer. Chitosan is practically insoluble at higher pH values. At these higher pH values, the chitosan molecules exist in a more coiled configuration but as the pH decreases and the molecule becomes more ionized; the molecule uncoils and assumes a more elongated shape. Hence, at lower pH values the chitosan has a higher charge density and will have a better solubility and a better possibility for intimate contact with the epithelial membrane. This suggests that charge density might be of importance for enhancement of mucosal drug absorption [8]. The suggestion has been confirmed since addition of the highly anionic heparin in the test solutions inhibited the permeation enhancing effects [15].

Besides pH, varying the degree of acetylation also controls the charge density of chitosan, since only the GlcN-units are positively charged. Schipper et al. used chitosan with a degree of acetylation between 1 and 49%, each prepared in a low and high molecular weight form to reveal that the structural properties of chitosan, i.e. molecular weight and degree of acetylation, dictated absorption enhancing properties [16]. Chitosans with a low molecular weight (22 kDa) and a high degree of acetylation ($\geq$35%) lacked absorption enhancement activity whereas chitosans with a low degree of acetylation and/or high molecular weight increased intestinal epithelial permeability. From the results of the study, it is clear that

chitosans with a low degree of acetylation (1 and 15%) are active as absorption enhancers at low and high molecular weights. However, these chitosans displayed a clear dose-dependent toxicity, which seemed to be influenced more by the degree of acetylation than by the molecular weight of the chitosans. The chitosans with degrees of acetylation of 35 and 49% enhanced the transport of [^{14}C]-mannitol at high molecular weights only, with low toxicity. One chitosan, with a degree of acetylation equal to 35% and a molecular weight of 170 kDa, was found to have especially advantageous properties such as an early onset of action. This study also attempted to correlate structural features of chitosan with toxicity and concluded that the structural features of chitosans determining absorption enhancement are not correlated with those determining toxicity, which makes it possible to select chitosans with maximal effect on absorption and minimal toxicity.

The absorption enhancing effects of chitosan are dependent on the concentration of the chitosan administered. However, an unlimited increase in concentration did not lead to an unlimited increase in absorption enhancement. This saturable effect suggests a mechanism of absorption enhancement, namely paracellular absorption enhancement that depends on the opening of tight junctions. The increase in the transport of large hydrophilic compounds thus probably occurs by paracellular absorption enhancement attributed to an interaction of a positively charged amino group on the C-2 position of chitosan with negatively charged sites on the cell membranes and tight junctions of the mucosal epithelial cells to allow opening of the tight junctions [8]. Confocal laser scanning microscopy has confirmed that chitosan is able to open the tight junctions to allow the paracellular transport of large hydrophilic compounds. It has also been reported that pharmacological agents, which interact with cytoskeletal F-actin simultaneously, increase the paracellular permeability. The redistribution of F-actin after the administration of chitosan was visualized by staining the F-actin with the fluorescent probe, rhodamine phalloidin [15]. The positive charge on the chitosan molecule seems to be important again for absorption enhancement. The bio/mucoadhesive property of chitosan also contributes to the permeation enhancement effect by increasing the residence time at the biological layer.

3 Need of derivatives of chitosan

Chitosan in its protonated form facilitates the paracellular transport of hydrophilic drugs by combination of bioadhesion and a transient widening of the tight junctions in the membrane. Although it is incapable of enhancing absorption in more basic environment of small intestine as jejunum, ileum—the main absorption area in the gastrointestinal tract as well as of large intestine and colon. Kotzé et al. put forward the hypothesis that polymers such as unmodified chitosan with a primary amino group may not be the optimal ones in opening tight junctions, but the polymers or derivatives with different substituents, different basicities, or different charged densities will have the same or even increased efficacy in this respect [17]. Kotzé et al. further stated in this regard that there was a need for chitosan derivatives with increased solubility, especially at neutral and basic pH values, for use as absorption enhancers aimed at the delivery of therapeutic compounds in the more basic environments [18, 19].

Attempts to boost up the positive charge on the polymer chain appear to be consistent with this rationale. The quaternization of amino group with simplest alkyl group i.e. methyl is the unambiguous step towards comprehending the hypothesis.

4 Synthesis of trimethyl chitosan

Quaternization (methylation) of amino groups in chitosan can be achieved with methyl iodide at elevated temperature in strong alkaline environment to bind the acid being generated during the reaction taking place and to avoid protonation of the unreacted primary amino groups (Fig. 2). The degree of quaternization (DQ) can be altered by increasing the number of reaction steps or by increasing the reaction time or by controlling the reaction steps or by using different deacetylation grades of chitosan. At higher degrees of quaternization however evidence of O-methylation on the 3 and 6 hydroxyl groups of chitosan is found. In general, O-methylation led to less soluble products. It is desirable hence to prepare trimethyl chitosan (TMC) polymers with a high DQ but with a low degree of O-methylation.

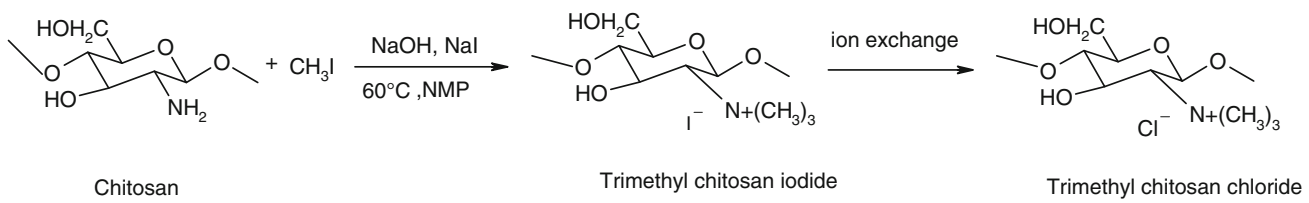

Fig. 2 Synthesis of *N,N,N*-trimethyl chitosan

The synthesis of *N*,*N*,*N*-trimethyl chitosan was reported by Domard et al. based on the dispersion of 5 g chitosan in 250 ml *N*-methyl-2 pyrrolidinone reacting with CH_3I and NaOH (chitosan:CH_3I:NaOH in molar ratio 1:15:2) for 3 h at 36°C [20]. This method however caused extensive depolymerization of chitosan. The process was modified by Le Dung with respect to the ratio of reactants (chitosan:CH_3I:NaOH in molar ratio 1:15:3.45) to reduce polymer degradation and control the different parameters affecting quaternization. The ^{1}H-NMR examination however suggests that such procedure would mainly result in dimethylated polymer with only 10–15% of quaternization. Sieval et al. modified the process with respect to the solvent/reagent addition sequence and reported one step and two step syntheses. In one step synthesis, chitosan was dispersed in NMP with CH_3I, and NaI and then the mixture was made alkaline by adding aqueous NaOH solution [21]. In two step synthesis, chitosan was dispersed in aqueous NaOH with NaI and then CH_3I mixed with NMP was added. The resultant product was washed with ethanol and ether and subjected to methylation again but with less quantity of CH_3I this time. Dimethylation is significantly decreased by repeating the basic reaction. To get the higher degree of substitution Snyman et al. carried out the reaction in repetition of one, two, three or four times with same or different quantity of CH_3I: the starting polymer for each subsequent step was the product of previous reaction step washed with ethanol [22]. The modifications were done by Hamman and Kotzé [23] and employed by Polnok et al. [24] for varying the number of reaction steps and the type of base. The bases used were NaOH and dimethylaminopyridine along with NMP. The degrees of quaternization of TMC polymers obtained from the processes using dimethylaminopyridine as the base were lower than those using sodium hydroxide. The polymer degradation also was lower. This may be explained by the weaker alkaline properties of dimethylaminopyridine compared to sodium hydroxide. A combination of the two bases did not reduce polymer degradation, while the DQ was limited to relatively low values (12.5–34.4%). The attempts to increase the DQ by increasing the number of steps are accompanied by O-methylation. Moreover an increased number of reaction steps decreased dimethylation also. However an extended duration of reaction increased both DQ and dimethylation [25].

Runarsson et al. changed the solvent system to DMF/H_2O mixture (50:50) and performed the reaction without the aid of a catalyst-sodium iodide [26]. This significantly reduced O-methylation since DMF/water seems to lowers the reactivity of the hydroxyl group enough to keep the O-methylation down. The DQ, however, was always low in the materials obtained. The DQ varied from 0 to 74% depending on the reaction conditions accompanied by monomethylation, dimethylation and O-methylation (chitosan:CH_3I:NaOH in molar ratio 1: 6 or 12:1.5 to 9, time 0.5 to 48 h, temperature 21, 50, 75 C). Based on this solvent system recently they also claimed to get high degree of substitution 81 to 88% by 'one pot' synthesis procedure (chitosan:CH_3I:NaOH in molar ratio 1:6:3, time 48 h, room temperature) [27]. They suggested protection group strategy for more selective N-quaternization (sequence of N-phtahloylation, O-tritylation, N-deprotection, N-methylation and O-deprotection).

The exchange of counter ion iodide with chloride was done finally by dissolving the quaternized polymer in a small quantity of water followed by the addition of HCl in methanol or by dissolving in NaCl solution. The exchange can be achieved by dialysis too against NaCl solution and water.

All these methods of methylation make use of methyl iodide which despite being efficient is a highly volatile, carcinogenic and expensive reagent. In addition it offers limited control over a perilously chemical reaction. In an attempt to overcome these disadvantages, an alternative sequence for the synthesis of chitosan quaternized derivatives is proposed by Britto et al. using dimethylsulfate as the reactive agent wherein the polymer in solution of NaOH and NaCl is mixed and refluxed with methylating agent at RT or at 70°C [28]. Here also the quaternization intensity was time and temperature dependent. The undesirable O-methylation and polymeric degradation were observed to take place for the reaction also.

Other synthetic strategies have been reported to produce TMC derivatives but are not as widely used as the Domard reaction. One such method utilizes sequence of formation of Schiff's base and reduction reported by Muzzarelli and Tanfani (Fig. 3) [29]. The trimethylation up to 60% of the amine groups could be accomplished by Schiff's base formation with formaldehyde, followed by reduction with sodium borohydride and quaternization in alkaline condition with methyl iodide. This two-step method likely prevents chain scission and deacetylation of remaining N-acetyl groups, and might result in TMC without O-methylation. With this, quaternization with different alkyl groups is also possible as in synthesis of *N*-diethylmethylchitosan [30] *N*-*N*-propyl-*N*,*N* dimethyl chitosan and *N*-furfuryl-*N*,*N*-dimethyl chitosan [31], *N*-butyl *N*,*N* dimethyl chitosan [32] and *N*-phenyl or *N*-(substituted phenyl) *N*,*N*-dimethyl chitosan [33]. In an attempt to synthesize O-methyl free TMC, Verheul et al. synthesized dimethylated chitosan first and quaternized it [34]. The procedure was based on the method of Muzzarelli and Tanfani, with modifications in solvent and reducing agent system as use of a formic acid-formaldehyde methylation (Eschweiler–Clarke) and quaternization by CH_3I in NMP without assistance of catalyst.

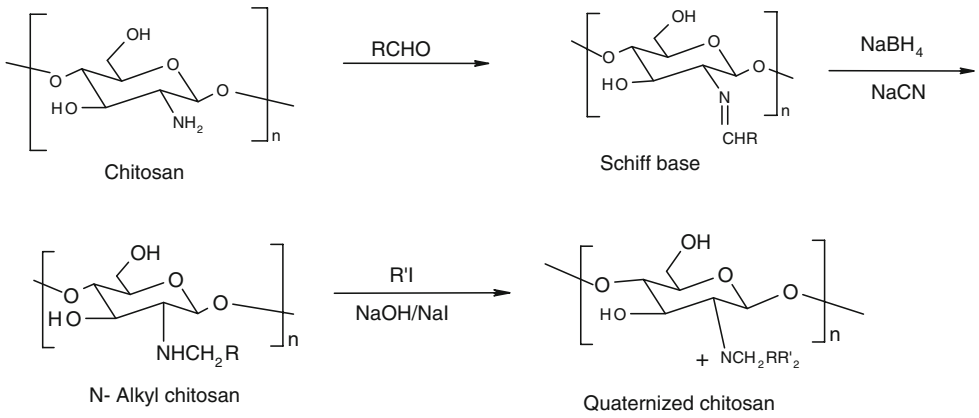

Fig. 3 Alkylation of chitosan followed by quaternization (R = H, R′ = –CH_3)

5 Physicochemical properties of trimethyl chitosan

TMC proved to be a derivative of chitosan with superior solubility and basicity, even at low degrees of quaternization, compared to chitosan and salts. The chitosan and salts are only soluble in acidic pH levels Even at these low pH levels, it was difficult to prepare 1.5% (w/v) solutions due to the high viscosity of the solutions. TMC, even with a DQ as low as 10%, on the other hand is soluble either in acidic, basic or neutral medium (pH range 1–9 up to 10% w/v concentration). The highest solubility is reported with TMC of an intermediate DQ (40%) regardless of DD and molecular weight [25]. The increase in solubility was attributed to the replacement of the primary amino group on the C-2 position of chitosan with quaternary amino groups.

The absolute molecular weights, radius and polydispersity of a range of TMC polymers with different degrees of quaternization (22.1, 36.3, 48.0 and 59.2%) were determined with size exclusion chromatography (SEC) and multi-angle laser light scattering (MALLS). The absolute molecular weight of the TMC polymers decreased with an increase in the DQ. The respective absolute molecular weights measured for each of the polymers were 2.02, 1.95, 1.66 and 1.43 g/mol $\times$ 10^5. It should be noted that the molecular weight of the polymer chain increases during the reductive methylation process due to the addition of methyl groups to the amino group of the repeating monomers. However, a net decrease in the absolute molecular weight is observed due to degradation of the polymer chain caused by exposure to the reaction conditions such as the strong alkaline environment and elevated experimental temperatures during the synthesis [22]. The intrinsic viscosity, as an indication of molecular weight, also decreases with an increase in the DQ of the polymer.

Like the native chitosan, TMC has the mucoadhesive properties [35]. The intrinsic mucoadhesivity of TMC was found to be lower than the chitosan salts, chitosan hydrochloride and glutamate, but if compared to the reference polymer, pectin, TMC possessed superior mucoadhesive properties [36]. The mucoadhesive properties of TMC with different DQ have been explored but the results are controversial. Sandri et al. reported the increase in mucoadhesive properties towards buccal mucosa with increase in DQ in the study of fluorescien isothiocyanate dextran (MW 4,400 Da) as a model drug [37]. On the other hand Synman et al., had found that the mucoadhesive properties of TMC decreased with an increase in DQ between 22.1 and 48.8% [22, 36, 38]. This may be due to the presence of fixed positive charges and their interaction with the negative sialic groups on the mucus protein structure. The decrease in mucoadhesion with an increase in the DQ may be explained by changes in the conformation of the respective TMC polymers due to interactions between the fixed positive charges on the C-2 position of each polymer. These interactions may force the polymer to change its conformation with a decrease in polymer-chain flexibility. Furthermore, steric effects caused by the attached methyl groups may also hide the positive charges on the amino groups. This decrease in flexibility and screening effect influences both the rate and amount of charge exchange between the negatively charged sialic groups of the mucus and the fixed positive charge of the TMC polymers and the interpenetration into the mucus layer with a subsequent lower mucoadhesivity.

6 Charecterization of trimethyl chitosan

6.1 FTIR

The FTIR spectrum of TMC is obtained from cut films samples of 10 $\times$ 10 $\times$ 0.01 mm prepared by dissolution of the TMC sample in deionized water and chitosan in aqueous acetic acid and casting in Petri dishes. The FTIR spectrum of TMC provides the evidence for the occurrence of methylation especially in the region 1,700–1,200 cm^{-1} (Figs. 4, 5; Table 1). The evidences are: (a) the band

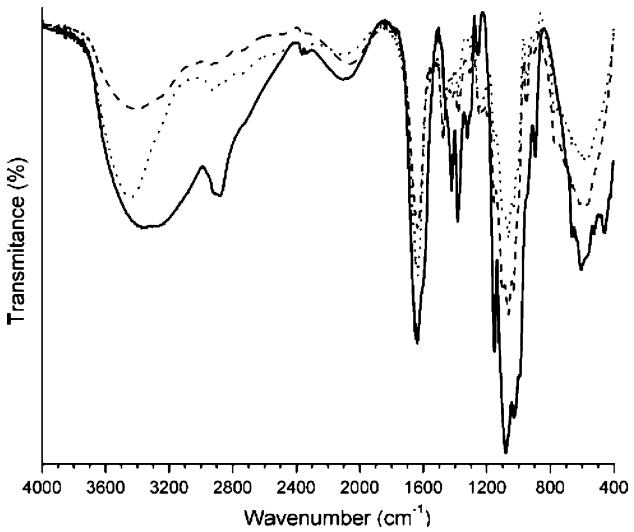

Fig. 4 FTIR spectra of the chitosan DD 96% (——), TMC DQ 52.5% (- - - -) and TMC DQ 27% (……)

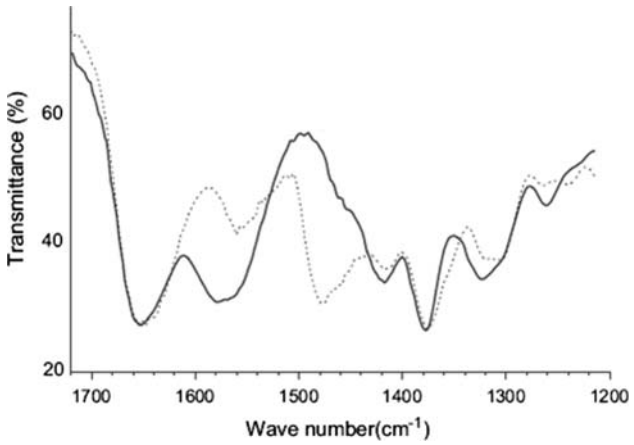

Fig. 5 Infrared spectra of chitosan (——) trimethyl chitosan (….) in the region 1,700–1,200 cm^{-1}

Table 1 Characteristic bands of chitosan by FTIR

Absorption bands of chitosan	Attributed to
3,448 cm^{-1}	Axial stretching of O–H and N–H bonds
2,877 cm^{-1}	Axial stretching of C–H bonds
1,663 cm^{-1}	Axial stretching of C=O bonds
1,561 cm^{-1}	Angular deformation of the N–H bonds of the amino groups
1,417–1,377 cm^{-1}	Coupling of C–N axial stretching and N–H angular deformation
1,153–897 cm^{-1}	Glycosidic bonds, C–O and C–O–C stretchings

centered at 1,475 cm^{-1} in the spectrum of TMC, which is attributed to the asymmetric angular deformation of C–H bonds of methyl groups, is absent in the spectrum of chitosan [28, 39, 40] and (b) the band due to the angular deformation of N–H bond of amino groups occurs in both spectra, at 1,577 cm^{-1} (1,500–1,620 cm^{-1}) for chitosan and at 1,559 cm^{-1} for TMC, but it is weaker or disappears due to the occurrence of N-methylation [20]. A new peak appears at a high wave number 1,630–1,660 cm^{-1} which are assigned to the quaternary ammonium salt [28]. There are peaks at about 1,415–1,430 cm^{-1}, which are assigned to the characteristic absorption of N–CH_3. Characteristic peaks of alcohol and second alcohol between 1,160 and 1,030 cm^{-1}, if do not change, confirms the lack of the introduction of an alkyl group at C-3 and C-6 in the chitosan.

6.2 ^{1}H NMR [21, 28]

^{1}H NMR spectra were measured with a 300 or 600 MHz spectrometer by dissolving TMC samples in D_20 at 80°C The residual water in the NMR sample does not pose a difficulty since at 80°C this peak does not interfere with the spectrum of the polymer. The signals assigned include a peak at 3.4 ppm for quaternized amino group and a peak at 2.5 ppm for dimethyl amino group, as well as at 3.36–3.56 ppm for O-methylated group. (Apart from these, the signal of native monomers are evidenced as peaks at, 4.5–5.0 ppm for hydrogen bonded to the anomeric carbon 1; at 3.4–4.0 due to hydrogen bonded to the carbon atoms 3, 4, 5 and 6 of the glycopyranose unit; 3.18 attributed to the hydrogen atom bonded to the carbon 2 of the glycopyranose ring and ~2 corresponding to the hydrogen atoms of the methyl moieties of the acetamido groups) (Fig. 6).

The NMR spectroscopy can be employed to determine the pattern of substitution as DQ, degree of dimethylation and degree of acetylation. The concept is illustrated as follows $DQ\% = \left[\frac{[(CH_3)_3]}{[H]} \times \frac{1}{9}\right] \times 100$ where DQ% is the DQ as a percentage, $[(CH_3)_3]$ is the integral of the trimethyl amino group (quaternary amino) peak at 3.1–3.4 ppm, and [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm on the ^{1}H-NMR spectrum. The later corresponds to the hydrogen bonded to C1 of the glycoside ring.

The degree of dimethylation can be calculated by equation

$$DM\% = \left[\frac{[(CH_3)_2]}{[H]} \times \frac{1}{6}\right] \times 100$$

where % DM is the degree of dimethylation as a percentage, $[(CH_3)_2]$ is the integral of the dimethyl amino peak at 3.1–3.4 ppm.

Curti and Campana-Filho [41] reported a new method of calculating TMC's DQ based on the intensities of all methyl hydrogen signals on the ^{1}H NMR spectrum that does not

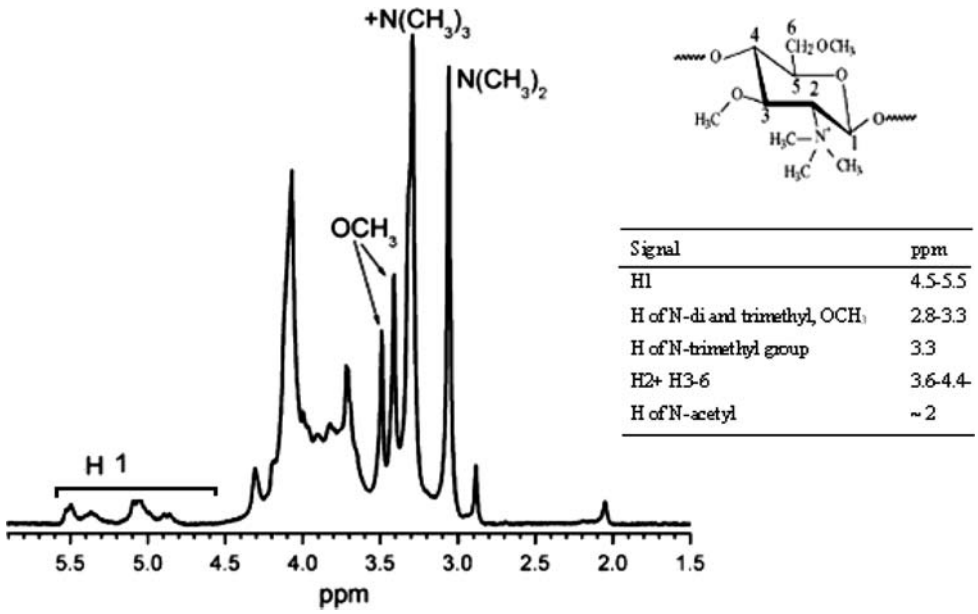

Fig. 6 ^{1}H-NMR spectrum of *N*-trimethyl chitosan chloride after two step synthesis

require the use of the C1 hydrogen signal as reference. It is important to note however, that in using this method, the intensity of the signal referent to dimethyl site is superestimated, once the signals of the C2 hydrogen in the glycoside ring and that of the dimethyl site are overlapped.

$$DQ\% = \left[\frac{[(CH_3)_3]}{9} \times \frac{1}{S}\right] \times 100$$

$$S = \frac{[(CH_3)_3]}{9} + \frac{[(CH_3)_2]}{6} + \frac{[(NHCOCH_3)]}{3}$$

$[(NHCOCH_3)]$ is the intensity of the signal due to the hydrogen of the methyl groups of the acetamido moieties.

With these parameters Curti and Campana-Filho also reported degree of acetylation of TMC with following expression [41]

$$DA\% = \left[\frac{[(NHCOCH_3)]}{3} \times \frac{1}{S}\right] \times 100$$

^{1}H NMR spectroscopy demands the use of a suitable deutered solvent. Besides, concentrated chitosan solutions results in a very viscous solutions or are even insoluble for some derivatives, requiring measurements at 80°C in order to obtain narrow spectral line widths.

6.3 Solid-state CP-MAS ^{13}C NMR [42]

The solid-state CP-MAS ^{13}C NMR technique has mostly been applied to characterize chitosan and its derivatives; the characteristic signals can be identified for TMC (Fig. 7).

The DQ can be calculated by following equation where, Ix is the intensity of the signal. $DQ = \frac{I_{CH_3}}{(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})/6}$.

6.4 ^{13}C NMR [21]

^{13}C-NMR spectra were measured in D_2O at 80°C at 150 MHz. The signals assigned include the dimethylated signal at 43.7 ppm, and the trimethylated signal, at 55.1 ppm (Fig. 8).

6.5 Determination of degree of quaternization (DQ) by titration [41]

The average DQ of the sample TMC was also determined from its titration with aqueous 0.1 M $AgNO_3$. Thus, an aliquot of the aqueous solution of TMC ($C_P = 1.2$ g/l) was transferred to a glass cell maintained at 25°C ± 0.1°C and the solution conductivity was measured upon the addition of aqueous $AgNO_3$. The average DQ was then determined by using the expression:

$$DQ\% = \left[\frac{M_{TMC} \times V \times [AgNO_3]}{m}\right] \times 100$$

where M_{TMC} is the molecular weight (g/mol) of the repeating unit of TMC containing the quaternized site, V (dm^3 or l) and $[AgNO_3]$ (mol/dm^3 or mol/l) are the equivalent volume and concentration of $AgNO_3$ aqueous solution, respectively, and m (g) is the mass of TMC.

6.6 DSC [39]

The DTG curves of chitosan and samples TMC1, TMC2 and TMC3 with increasing DQ, respectively recorded in nitrogen atmosphere from room temperature to 500 are shown in Fig. 9.

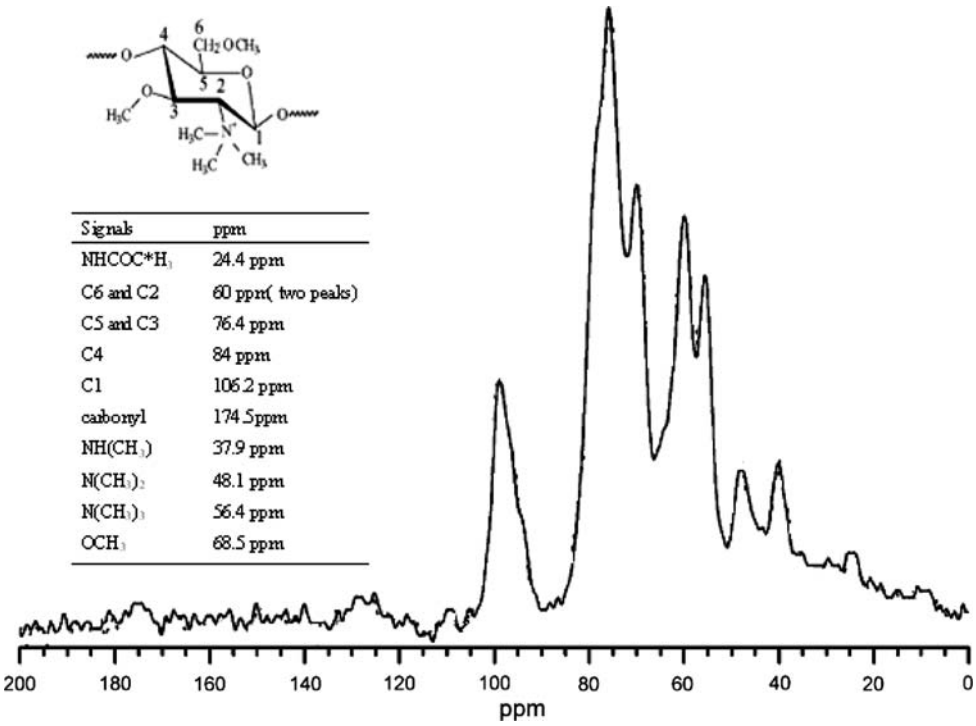

Fig. 7 Solid state CP-MAS ^{13}C NMR spectrum of TMC DQ 52.5%

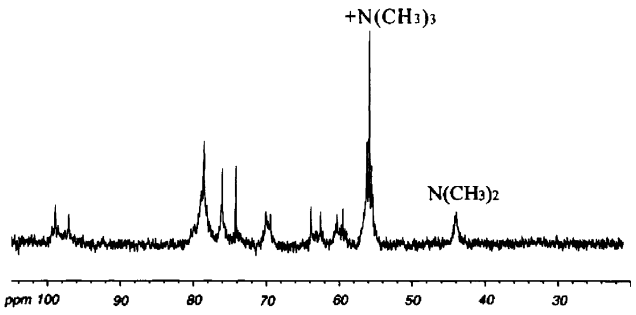

Fig. 8 ^{13}C NMR spectrum of *N*-trimethylchitosan chloride

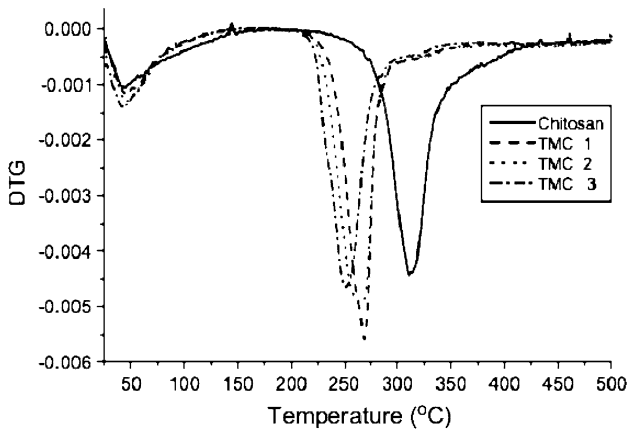

Fig. 9 DTG curves of the parent chitosan and TMC samples

The first thermal event occurs in the temperature range 25–140°C. This is attributed to the evaporation of water, whose content is a function of the morphology, crystallinity and hydrophilicity of the polymers. The study of mass loss corresponding to the evaporation of water indicated direct dependence of water content on the presence and number of charges on the polymer chains. The second thermal event occurs in the temperature range 200–400°C for chitosan and 190–350°C for the TMC samples, and it is attributed to the thermal degradation of the polymers, including the deacetylation of chitosan and the decomposition of the substituted sites in the methylated derivatives. The complete thermal decomposition of the polymers, involving the depolymerization and pyrolytic processes, is attained at temperatures higher than 350°C and 400°C for the TMC samples and chitosan, respectively. This stage of the thermal decomposition begins at lower temperatures for the TMC samples, showing that these derivatives are less thermally stable than the parent chitosan.

7 Applications of trimethyl chitosan

7.1 In pearmeation enhancement

In an attempt to prove the hypothesis of increased efficiency of charged derivatives of chitosan Kotzé and the group synthesized TMC and evaluated it for the permeation enhancement effect. [12, 43]. The studies showed that TMC was effective in enhancing the transport of small hydrophilic compounds as [^{14}C]-mannitol, large molecules as [^{14}C] PEG4000, and peptide drugs buserelin, octreotide across the cell monolayers. Similar to chitosan, TMC

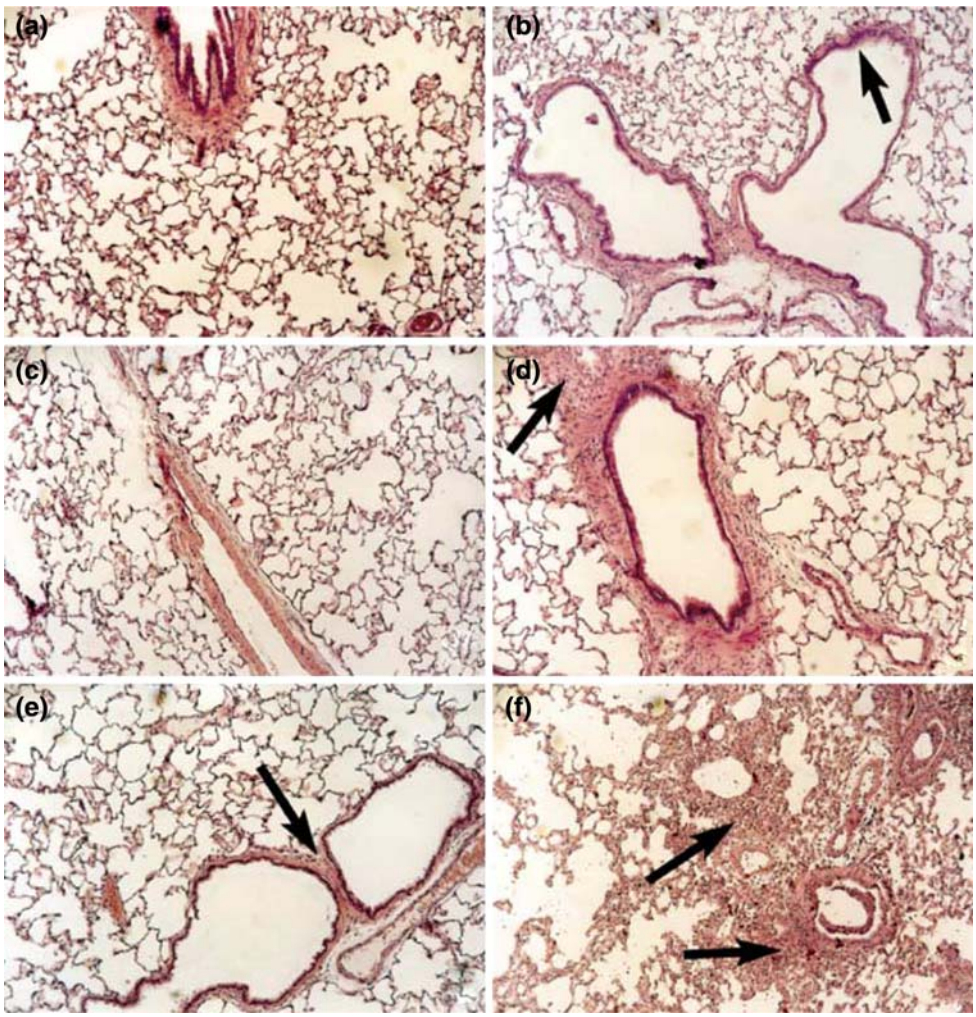

Fig. 10 Representative photographs for histopathological evaluation of rat lungs after in vivo experiments with octreotide by i.v. administration (**a**); intratracheal instillation of control solutions, 0.9% saline pH 5.5 (**b**) or 7.4 (**c**); 1.5% TMC(DQ 20%), pH 7.4 (**d**); 1.5% TMC (DQ 60%), pH 7.4 (**e**) or chitosan 1.5%, pH 5.5 (**f**). Arrows indicate regions with increased neutrophil accumulation or areas that sustained structural change to the lung tissue [45]

increased the transport via paracellular route by opening of the tight junctions located between epithelial cells as indicated by reduction in TEER. The confocal laser scanning microscopy confirmed transport of large hydrophilic compounds via this route as well as the mechanism of action of the polymer in which redistribution of the cytoskeletal F-actin is provoked which leads to the opening of the tight junctions (Fig. 10). Various in vivo studies in different animal models confirmed the ability of TMC to increase the absorption of the peptide drugs buserelin and octreotide after intraduodenal or intrajejunal administration [44]. Intratracheal instillation of octreotide as 1.5% (w/v) gel-phase formulations with chitosan (pH 5.5) and TMC of DQ 20 and 60 (pH 7.4) in rats displayed sustained release properties and enhancement in bio-availability by 2.4-, 2.5- and 3.9-fold, respectively. The permeation enhancement in vitro was observed by 21-, 16- and 30-fold across Calu-3 cell monolayers for chitosan and TMC formulations, respectively. A linear in vitro/in vivo correlation between calculated absorption rates was found suggesting that the permeation enhancement by polymers, both in vitro and in vivo, proceeded via an analogous mechanism [45].

The DQ of TMC (charge density) determines the number of positive charges available on the molecule for interactions with the negatively charged sites on the epithelial membrane and thereby influences its drug absorption-enhancing properties. The ambiguous outcomes are reported for the relationship between DQ and permeation enhancement. Transport studies with Caco-2 cell monolayers at neutral pH confirmed that the increase in [^{14}C]-mannitol permeation is dependent on the charge of TMC and the high charge density is necessary for TMC to substantially improve the paracellular permeability of intestinal epithelia where as TMC with the lowest DQ (12.3%) was ineffective at neutral pH for improvement of permeability [46]. TMC DQ 60% has been proven to be a potent enhancer of both nasal and rectal insulin absorption in rats, especially at neutral pH values where TMC of DQ 12.3% and chitosan HCl were ineffective [47]. In agreement with the results, in vivo data revealed highly

increased bioavailability of buserelin following intraduodenal co-administration with 1% w/v TMCs of DQ 40 and 60% (Mean bioavailability values of drug were between 6 and 13% as compared to 0.8% absolute bioavailability of drug alone) [48]. Similar to the buserelin studies, octreotide absorption after intrajejunal administration on co-administration of 1% w/v TMC of DQ 60% resulted in peptide bioavailability of 16% [49].

Conversely, Hamman et al. did not completely agree with the observations in view of the hypothesis that TMC with the highest possible DQ (ca. 60%) may not be the optimum DQ for absorption enhancement by TMC in a neutral environment due to the steric effects caused by the attached methyl groups and flexibility of the molecule [50]. They studied the effects of six different TMC polymers (DQ between 12 and 59%) on the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers and on the transport of hydrophilic and macromolecular model compounds ([^{14}C]-mannitol and [^{14}C] PEG 4000) across Caco-2 cells. All the TMC polymers were able to decrease the TEER markedly in a slightly acidic environment (pH 6.2). However, only TMC polymers with higher DQ (>22%) were able to reduce the TEER in a neutral environment (pH 7.4). The maximum reduction in TEER (47.3–6.0% at a concentration of 0.5% w/v and pH 7.4) was reached with TMC of DQ 48%, and this effect did not increase further with higher DQ of TMC. In agreement with the TEER results, the transport of model compounds across Caco-2 cell monolayers increased with an increase in the DQ of TMC. However, the transport reached a maximum for TMC with a DQ of 48% (25.3% of the initial dose for [^{14}C]-mannitol and 15.2% of the initial dose for [^{14}C] PEG 4000), and this effect did not increase further with higher degrees of quaternization of TMC. Therefore, the increase in the effects of TMC on intestinal epithelia did not directly correlate up to the maximum quaternization degree of this polymer, but reached an optimum value at an intermediate DQ (ca. 48%). These observations agreed with the previous results of in vitro (everted intestinal sacs experiment) and in situ (single pass intestinal perfusion method) studies where both the results showed the best permeation enhancing effect with TMC of DQ 48.8% at concentration of 0.5% w/v [51].

TMCs with DQ of 40% and 60% were used as transdermal enhancers in testosterone gels [52]. Studies of ATR-FTIR combined with the technique of deconvolution found that TMCs could change the secondary structure of keratin in stratum conium to enhance the transcutaneous permeation of drugs. As compared to 2% Azone, 5% TMC of DQ 60% had a stronger enhancement ($P < 0.05$) while 5% TMC of DQ 40% had a similar effect ($P > 0.05$). The results suggested that the enhancement of permeation by TMCs increased with the increase of DQ.

The permeation enhancement effect of chitosan has not only been observed for epithelial cells but for stratified epithelial cells also such as the buccal [53] and cornea [54].

Sandri et al. investigated chitosan and TMC as penetration enhancement on pig buccal mucosa for the hydrophilic fluorescein isothiocyanate dextran (FD4; 4,400 Da) The effects of the polymers on the mucosa with stratified epithelium and lacking in tight junctions were assessed with by means of diffusion studies, histopathological analysis coupled with microscopical evaluation with light microscopy, transmission electron microscopy, confocal laser scanning microscopy. The enhancement in permeation observed probably involves a mechanism a repackaging of epithelial cells up to the basal membrane and a partial disarrangement of desmosomes without causing cell damage.

The permeation enhancement effect of TMC was proven for transcorneal application by DiColo et al. [55]. The TMC of high MW chitosan (1,460 kDa) and low MW chitosan (580 kDa) of DQ 3%, 4%, 35%; 46%, 78%, 90% were tested and compared for their ability to enhance the permeability of ofloxacin across rabbit corneal epithelium, reconstituted in vitro. TMC polymers of intermediate DQ (35 and 46%), in the concentration of 0.001% w/v, produced significant permeability enhancement, independent of polymer MW. The enhancing effect did not increase when DQ was increased (DQ 78 and 90%), while it was not significant with low DQ values (3 and 4%). The transcorneal permeability-enhancing property of intermediate DQ (35 and 46%), were confirmed by in vivo tests on rabbit eyes. However, unlike the in vitro experiments, the in vivo ones showed a stronger effect of the TMC having higher MW. TMC MW 1,460 kDa, DQ = 35%; produced antibiotic levels in the aqueous humor higher than the minimum inhibitory concentration (MIC90%) for the more resistant ocular pathogens.

The experiment to determine the mode of transcorneal transport suggested that TMC enhances transcorneal transport via the transcellular route (marker-dexamethasone), whereas it is unable to effectively open the tight junctions between corneal cells (marker-tobramycin) [56].

Many of the experimental procedures for evaluating the effect of TMC utilized the administration of the drug in the solution form along with TMC. The impracticality of administering a solution, as well as the fact that most peptides are unstable in the presence of water, have led to the need for a solid oral dosage form with which TMC can be administered together with peptide drugs. This has directed the development of various formulations as minitablets, granules, nanoparticles, microspheres etc. The minitablet and granule formulations were developed as solid oral dosage forms for the delivery of peptide drugs with TMC. Such formulations of desmopressin with TMC

as absorption enhancer are reported. The TMC granules provided burst release too (granules with tetraglycerol pentastearate provided delayed release in optimized formulation). Both the optimized minitablet formulation and the granule formulation show suitable release profiles for the delivery of peptide drugs with TMC as absorption enhancer [57].

The nanoparticles were prepared by the polyelectrolyte complexation (PEC) method with different degrees of quaternization which exhibited differential loading efficiency for the bovine serum albumin and bovine hemoglobin (95% and 30%, respectively) [58]. The polydispersity index (PDI) of nanoparticles (indicative of particle size distribution, stability and polydispersion homogeneity if value lies between 0 and 0.3) was found to be 0.193–0.259. The particle size, zeta potential and PDI of nanoparticles were significantly affected by the bovine serum albumin concentration but not by the bovine hemoglobin concentration. Nanoparticles of TMC with a lower DQ showed an increase in particle size, a decrease in zeta potential and a slower drug-release profile. These parameters could be further modified by addition of sodium alginate. Alginate modification led to an increase in loading efficiency and decrease in particle size and zeta potential and increase in PDI (0.13–0.31) for its negative charge and a strong inter-chain reaction with TMC. However the modification barely had any effect on TMC-nanoparticles' property of decreasing TEER or enhancing drug paracellular transport pathway [59]. Probably smaller particle size (100–200 nm) enhanced transport through transcellular pathway (transcytosis) since particles up to about 100–200 nm can be internalized by receptor-mediate endocytosis, while larger particles (such as formed by aggregation of nanoparticles when resuspended in physiological buffers) have to be taken up by phagocytosis.

However the alginate modification barely had any effect on TMC-nanoparticles' property of decreasing TEER or enhancing drug paracellular transport [59]. Oral administration of such nanoparticles loaded with urease showed much higher antibody titers of IgG (systematic immune response) and secretory IgA (mucosal response) than those on administration of urease loaded TMC nanoparticles subcutaneous, urease solution or urease coadministrated with TMC solution.

One of the reasons for permeation enhancement observed with chitosan is mucoadhesion. Nanoparticles with TMC were prepared expecting mucoadhesion in addition to advantage offered by nanoparticulate systems as protection of peptidic drugs from intestinal degradations due to internalisation behaviour. Evaluation of TMC nanoparticles loaded with FD4 (MW4400 Da) on Caco-2 cell layer and excised rat jejunum model showed that the mucoadhesive properties delayed the absorption of nanoparticles; however they produced an increase in the contact time with intestinal epithelium, offering a better chance for internalization [60].

Many other examples with different drugs and TMC as enhancer have been reported (Table 2).

7.2 In colonic delivery

The absorption enhancing and the mucoadhesive properties have been put to assessment for the assistance of mucoadhesive properties of pectinate beads and colonic delivery [71]. Pectinate beads showed mucoadhesion towards the

Table 2 Some more examples of applications of TMC

Use	Result
Nanoparticles [61]	Self-assembled nanoparticles prepared by TMC and poly(gamma-glutamic acid) for oral delivery of insulin. Superior stability of nanoparticles in a broader pH range and sustained release profile of insulin with permeation enhancement observed
Micropartilces [62, 63]	Microparticles of TMC prepared by a supercritical fluid drying technique as a carrier for pulmonary delivery of insulin. Enhanced bioavailabilty of insulin observed without local adverse reactions after single administration of insulin powders
Micropartilces [64]	Microparticles of TMC prepared by a supercritical fluid drying technique as a carrier for pulmonary delivery of diphtheria toxoid. The induction of immune response observed was equivalent to subcutaneous toxoid administration and superior for IgG_2/IgG_1 ratioand IgA level
Nanoparticles [65]	Thymopentin-loaded TMC nanoparticles on oral administration show higher efficiency for ratio of lymphocyte CD4+/CD8 than thymopentin alone
Nanocomplexes, nanoparticles [66]	PEC and nanoparticles prepared with TMC and PEG-graft-TMC copolymer for peroral insulin delivery. Comparision of their properties suggest PEC as a potentially useful technique to achieve the objective
Absorption enhancer [67, 68]	Superporous hydrogel based systems developed for desmopressin with TMC which increased absorption of the drug across intestinal cells
Absorption enhancer [69]	The combinations of TMC DQ 48 and 64%, dicarboxymethyl chitosan oligosaccharide, and chitosan lactate oligomer with monocaprin and melittin showed synergistic performance in terms of absorption across Caco-2, intestinal epithelial cells and TEER as compared to the individual absorption enhancers
Film for food application [70]	Biocide against *Listeria monocytogenes* and *Salmonella typhimurium*

gastrointestinal tissues which was in some cases comparable to Carbomer 934P granules with the ranking: duodenum = jejunum = ileum > cecum > colon > stomach. In the dry state, the beads containing TMC were more mucoadhesive; while in the moist state simple pectinate beads were found to be more mucoadhesive as compared to hydrated TMC-containing beads. Over-hydration of TMC-containing beads may be the reason. The results of this study suggest that in cases where prehydration can be avoided, such as when the beads are protected in a site-specific oral capsule, prior to reaching the target tissue, the incorporation of TMC into beads might be useful as a means of increasing the mucoadhesive properties. Such beads were prepared using Coomassie Brilliant Blue G250 as a relatively high molecular weight water-soluble model drug [72]. TMC was found to form complex with this anionic dye when investigated by Job's method. In vitro studies confirmed that this multiparticulate system was capable of delivering the model drug to colon which was both, time- and pH controlled and possibly microbially triggered with provision of protection from the gastric acid environment by coating with high-methoxy pectin or Eudragit L30-D 55.

Sadeghi et al. when synthesized the TMC- and diethylmethyl-chitosan nanoparticles loaded with insulin by PEC technique and ionic gelation technique, the results showed that nanoparticles prepared by the PEC method had higher insulin loading efficiency and zeta potential than those made by the ionotropic gelation method [73]. The PEC method of nanoparticle preparation gave particles in the range of 170–270 nm with spherical and smooth surface morphology and PDI below 0.3. In vitro release studies showed a small burst effect at the beginning and then a sustained release characteristic for 5 h. Ex vivo investigations revealed better insulin transport across the colon membrane of rats for nanoparticles made with quaternized derivatives than those made of chitosan. In vivo studies in rats have shown enhanced colonic absorption of insulin by using these nanoparticles compared to free insulin in diabetic rats [74].

7.3 In nasal delivery

The nanoparticles as a nasal delivery system were prepared by ionic gelation technique with TMC and tripolyphosphate. The nanoparticles prepared had an average size of about 350 nm and a positive zeta-potential, a loading efficiency up to 95% and a loading capacity up to 50% (w/w) for model compound ovalbumin with preservation of its integrity. Release studies showed that more than 70% of the protein remained associated with the TMC nanoparticles for at least 3 h on incubation in phosphate buffer solution (pH 7.4) at 37°C. In vivo uptake studies indicated the transport of fluorescin isothiocyanate-albumin-associated TMC nanoparticles across the nasal mucosa. Cytotoxicity tests with Calu-3 cells showed no toxic effects of the nanoparticles, whereas a partially reversible cilio-inhibiting effect on the ciliary beat frequency (CBF) of chicken trachea was observed [75]. The procedure of nanoparticle preparation was extended for monovalent influenza subunit H3N2 antigen TMC nanoparticle. The intranasal administered of antigen-TMC nanoparticles induced higher immune responses induced significant IgA levels in nasal washes of all mice as compared to other tested antigen formulations [76]. The protective immune responses in terms of bactericidal activity to meningococcal C conjugate vaccine was observed after intranasal immunization of mice with the LTK63 mutant plus chitosan or TMC as novel delivery platform (microparticles or powder suspensions) [77].

The analogous results were obtained for entrapment of tetanus toxoid in nanoparticles formulated with TMC by tripolyphosphate (ionic) gelation technique (loading efficacy >90%, particle size within the range of 40–400 nm), ex-vivo studies for cellular uptake of nanoparticles by J774A.1 cells and in vivo studies for adjuvant effect on nasal application [78].

The influence of DQ of TMC on level of immune induction was demonstrated with use of TMC DQ 40% and TMC DQ 60 the level of immune induction It has been demonstrated that responses as adjuvants for inducing immune respons relies upon DQ of TMC. In demonstration of such influence on the level of immune induction, TMC DQ 40% appeared to be the most potent adjuvant for intranasal administration with ovalbumin [79].

7.4 In delivery of small drug molecules

N-Methylated chitosan with hydrophilic groups $N^+(CH_3)_3$ and hydrophobic groups $N(CH_3)_2$ is amphiphilic and water-soluble in character at physiological pH which can be self assembled to vesicles. The hollow microspheres were fabricated using the cyclohexane droplets as a template and N-methylated chitosan cross-linked with glutaraldehyde as the shell followed by the removal of core cyclohexane to form a cavity [80]. The microspheres exhibited a very smooth and hollow structure with sizes between 2 and 5 μm. The drug encapsulated in the microspheres displayed pH sensitive sustained release accompanying the permeation of solvent. Ofloxacin encapsulated in these microspheres was more rapidly released to reach 90 wt% at pH 7.4 within 8 h than at pH 1.2. In continuation of the studies a series of microspheres encapsulated with ofloxacin were prepared from N-methylated chitosan having different molecular weight and DQ [81]. The degree of swelling of microspheres in phosphate buffer solution, pH 7.4, was much higher than that in 0.1 M

HCl (pH 1.2), and decreased with an increase of MW of the N-methylated chitosan. The release speed of ofloxacin in the microspheres having N-methylated chitosan of high DQ was greater than that with low DQ. The ofloxacin release mechanism was a kind of non-Fickan diffusion through the swollen macrostructure of microspheres, and was controlled by the MW, the cross-linking density of shell and the DQ of N-methylated chitosan. Such microspheres may find use in stomach specific drug delivery.

TMC was evaluated as assisting polymer to native chitosan to formulate microparticles with sodium citrate as the ionic cross linker. The addition of TMC to the chitosan microparticles resulted in an increase in particle size of the microparticles and an increase in ibuprofen release rate as compared to the microparticles containing chitosan alone. However permeation of ibuprofen across Caco-2 cell monolayers did not show significant difference, the reason probably was the release of TMC molecules from the microparticles not sufficient to interact with the intestinal epithelial cells in order to change the permeation of the released drug [82].

The TMC was introduced into preparation of nanoparticles consisting of alginate complexed with cisplatin, to impart positive charge to particles. The cytotoxic activities of the positive nanoparticles was similar to or lower than that of cisplatin, probably depending on the combination of sizes and zeta potential values, on P388 murine and A2780 human cells. On A549 human cells, the nanoparticles with the smallest size and the lowest positive zeta potential were more active than cisplatin and showed a similar capability in inducing apoptosis in A2780 human cells [83].

7.5 In DNA delivery

Chitosan being a cation forms polyelectrolyte complex with negatively charged DNA and offers a means of DNA delivery. TMC with its added positive charges should be superior to chitosan in this respect. The use of TMC oligomers (<20 monomers) with RSV-α3 luciferase plasmid in COS-1 and Caco-2 cell lines has shown that as gene transfecting agents they outperform native chitosan [84]. The trasfection efficiency and cytotoxicity was investigated with respect to DQ in chitosans using pGL3 luciferase reporter gene in COS-7 and MCF-7 cell lines. DQ of 44% on the chitosan backbone gave an optimum derivative for transfection. In cytotoxicity a general trend of increasing toxicity with increasing DQ was seen. However, all derivatives with different DQ were significantly less toxic than cationic linear polyethylenimine and higher toxicity was seen in polymeric chitosan derivatives over oligomeric chitosan derivatives at similar DQ [85]. It appears that quaternization of 44% on the chitosan backbone gave an optimum derivative for transfection. TMC has been employed as a stabilizer for preparation of nanoparticles of DNA with poly-(ε-caprolactone) by an emulsion-diffusion-evaporation along with poly-(vinyl alcohol). The cytotoxicity was found to be moderate and virtually independent of the stabilizer's concentration (TMC DQ 4%, 10%, 18%, 66%) with the exception of the highly quaternized TMC (DQ 66%) being significantly more toxic [86].

8 Derivatives of TMC and their applications

The quaternization of not all but only some of the nitrogens in chitosan molecule also renders it cationic and water soluble. This also offers a possibility of modulating the solubility as per requisite. The substitution of nitrogens from the rest of unsubstituted ones is one way of such modulation. Alkylation of TMC therefore produces the amphiphilic polymeric molecules since it possesses both charged groups and non-polar linear hydrocarbon branches into chitosan molecule. These chitosan derivatives may self-assemble in aqueous environment and form polymeric micelles, entrap hydrophobic drugs and enhance their water solubility. Zang et al. synthesized such trimethyl chitosan derivative by quaternizing *N*-alkyl chitosan as *N*-octyl chitosan obtained by reductive alkylation process of Schiff's base formed by aldehydes [87] (Fig. 11).

Other amphiphilic chitosan derivatives, *N*-octyl, *N*-decanal, and *N*-lauryl-*N*-trimethyl chitosan derivatives were obtained according to the same procedure. They further investigated utilization of these amphiphilic *N*-alkyl-*N*-trimethyl chitosan derivatives in solublization and controlled release of 10-hydroxycamptothecin (10-HCPT), a hydrophobic anticancer drug. The results showed that *N*-alkyl-*N*-trimethyl chitosan derivatives especially OTMCS (*N*-octyl TMC) derivatives were able to self-assemble and form spherical shape polymeric micelles with an average particle size range of 24–280 nm and a drug loading content of 4.1–32.5%, depending on the modified structures and loading procedures. The solubility of 10-HCPT in aqueous fluid was increased about 80,000-fold from 2 ng/ml in water to 1.9 mg/ml in OTMCS micellar (degree of octyl and trimethyl substitution is 8% and 54%, respectively) solution. OTMCS was able to modulate the in vitro release of 10-HCPT and improve its pharmacokinetic properties and lactone ring stability in vivo. The significant impact of hydrophobic tail length on micellar forming properties of *N*-alkyl-*N*-trimethyl chitosan derivatives was suggested. The incorporation of alkyl chain along with trimethylation improves the mechanical properties of the film formed as observed with 4, 8 and 12 carbons *N*-alkylated TMC [88].

The *N*-alkyl TMCs were found to be endowed with antibacterial activities also. The antibacterial activity

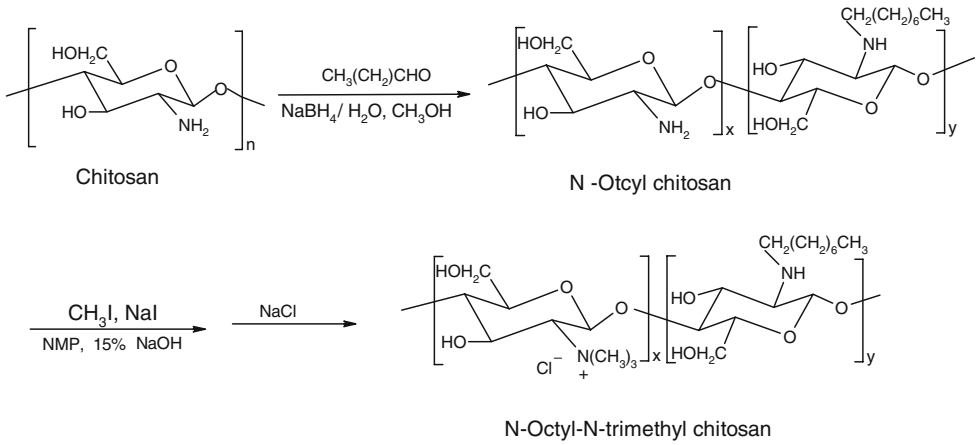

Fig. 11 Preparation of *N*-octyl-*N*-trimethyl chitosan derivatives

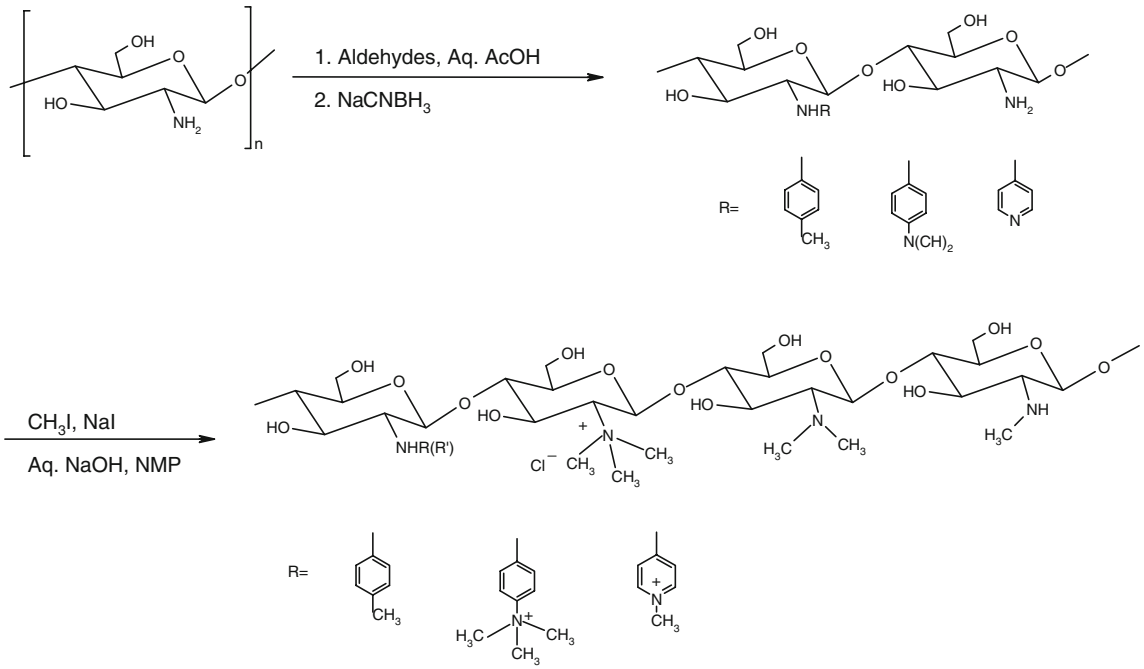

Fig. 12 Synthesis of methylated chitosan and *N*-aryl chitosans

increased with increase of the alkyl chain length [40] and of molecular weight of the derivative [31].

The *N*-aryl substituted TMC obtained by the reductive alkylation and quaternization sequence such as quaternized *N*,(4-methylbenzyl)l chitosan, *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan and quaternized *N*-(4-pyridylmethyl) chitosan have also been prepared and tested for antibacterial activity (Fig. 12). These substituents did not impart increase in the antibacterial activity of chitosan backbone [89]. Of these derivatives, quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan was investigated for the transfection efficiency using the plasmid DNA encoding green fluorescent protein pEGFP-C2 on human hepatoma cell lines (Huh7 cells),in comparison to TMC and chitosan [90]. The results revealed that quaternized *N*-(4-*N*, *N*-dimethylaminobenzyl) chitosan was able to condense with pDNA with the complete complexe formation at a weight ratio of polymer/DNA above 0.5 whereas TMC-CS/DNA and chitosan/DNA were formed at a ratio of above 1. The highest transfection efficiency was found at a weight ratio of 8. The results indicated that the improved gene transfection was due to the hydrophobic group (*N*,*N*-dimethylaminobenzyl) substitution on chitosan, which promoted the interaction and condensation with DNA, as well as N-quaternization which increased the water solubility.

The substitution of nitrogens from the rest of unsubstituted ones in TMC can be done with acyl group. For example reaction of TMC with 3-[(4-hydroxy-3,5-diter-tbutyl)phenyl]propionic acid in DMSO in presence of

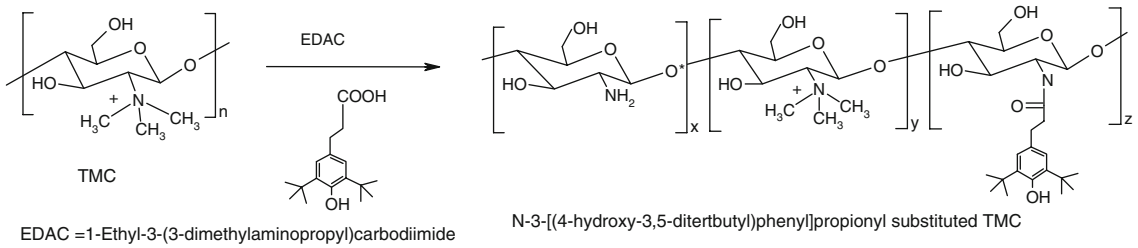

Fig. 13 Synthesis of *N*-3-[(4-hydroxy-3,5-ditertbutyl)phenyl]propionoyl substituted TMC

condensing agent 1-ethyl-3(3-dimethylaminopropyl)carbodiimide gave *N*-3 [(4-hydroxy-3,5-ditertbutyl)phenyl] propionoyl substituted TMC (Fig. 13). This polymer molecule has displayed antimutagenic activity in the barley seeds test with pronounced increase (89–93%) as compared to native polymer [91].

Quaternization of chitosan has been achieved by other alkyl groups as ethyl groups alone or ethyl and methyl groups to get triethyl chitosan TEC or diethylmethyl chitosan DEMC, respectively. The TEC and DEMC, like TMC, can be used for enhancing absorption and to form nanoparticles [73, 92, 93]. DEMC have exhibited the absorption enhancer effects for insulin in normal and diabetic rats [30].

9 Grafts of TMC and their applications

Although TMCs do possess outstanding properties for pharmaceutical and biomedical applications, it possesses the problem of toxicity. Using intestinal Caco-2 cell monolayers and ciliated chicken embryo trachea, the cytotoxicity and ciliotoxicity of TMC polymers with different DQ were studied. No substantial cell membrane damage could be detected on the Caco-2 cells, while the effect on the ciliary beat frequency of chicken in vitro was found to be marginal at a concentration of 1.0% (w/v) [94]. Amidi et al. [75] and Florea et al. [45] also indicated that TMC was nontoxic even at high DQ.

No acute toxicity was found with TMC and TMC oligomers by means of the CBF tests. But the TMC oligomers exhibited lower inhibition of the CBF of human nasal epithelial cells compared to that of the TMC polymers may be due to the lower viscosity and molecular weight of TMO [95].

However, Mao et al. found that TMC with DQ of 40% exhibited time- and dose-dependent cytotoxic responses by methyltetrazolium assay in L929 mouse fibroblast cells [96] and in HEK293 cells [97]. which increased with increasing molecular weight. It was also found that reversibility of transepithelial resistance at 0.5% concentrations of TMC with different DQ could not be demonstrated at pH 6.2 and 7.4 in Caco-2 cells [98]. The results of cytotoxicity of TMC had been conveyed by Kean et al. [85] who showed that the cytotoxicity increased with increasing DQ. The effect of dimethylation on cytotoxicity is observed when proportion of dimethylation to DQ is higher than 1. TMC with relatively high dimethylation showed reduction in both solubility and mucoadhesion and hence decreased cytotoxicity. However, the influence of dimethylation was insignificant when DQ of TMC was higher than 40% at which physicochemical properties and cytotoxicity were mainly dependent upon DQ [25].

Besides; in the applications that involve blood-contact such as a hemodialysis membrane, chitosan promotes hemolysis, surface induced thrombosis and embolization [99]. Therefore further improvement of the biocompatibility of TMC is desirable.

The toxicity of cationic polymers is suggested to arise from their effect to the plasma membranes and interaction with negatively charged cell components and proteins [100, 101].

The grafting of TMC with different polymers provides an attractive option for such improvement. For example PEGylation of TMC has been sought because PEG has recognized biocompatibility and ability to reduce the interaction between cationic polymers and cell membranes. The PEGylation was achieved by reaction of activatead PEG and TMC. The monohydroxy-terminated PEG (methoxy PEG) was converted with cyclic aliphatic anhydride (*N*-hydroxy succinimide NHS) to a carboxyl-terminated intermediate (NHS-mPEG) by esterification and then grafted onto TMC at primary amino groups [96] (Fig. 14). PEG-g-TMC copolymers were completely water-soluble over the entire pH range of 1–14 regardless of the PEG MW, even when the graft density was as low as 10%. A series of PEGylated TMC copolymers exhibited a time-and dose-dependent cytotoxic response that increased with molecular weight. PEGylation can decrease the cytotoxicity of TMC to a great extent in the case of low molecular weight TMCs. According to the cytotoxicity results, PEG 5 kDa is superior for PEGylation when compared to PEG 550 Da at similar graft ratios. Complexation with insulin further increased cell viability. The

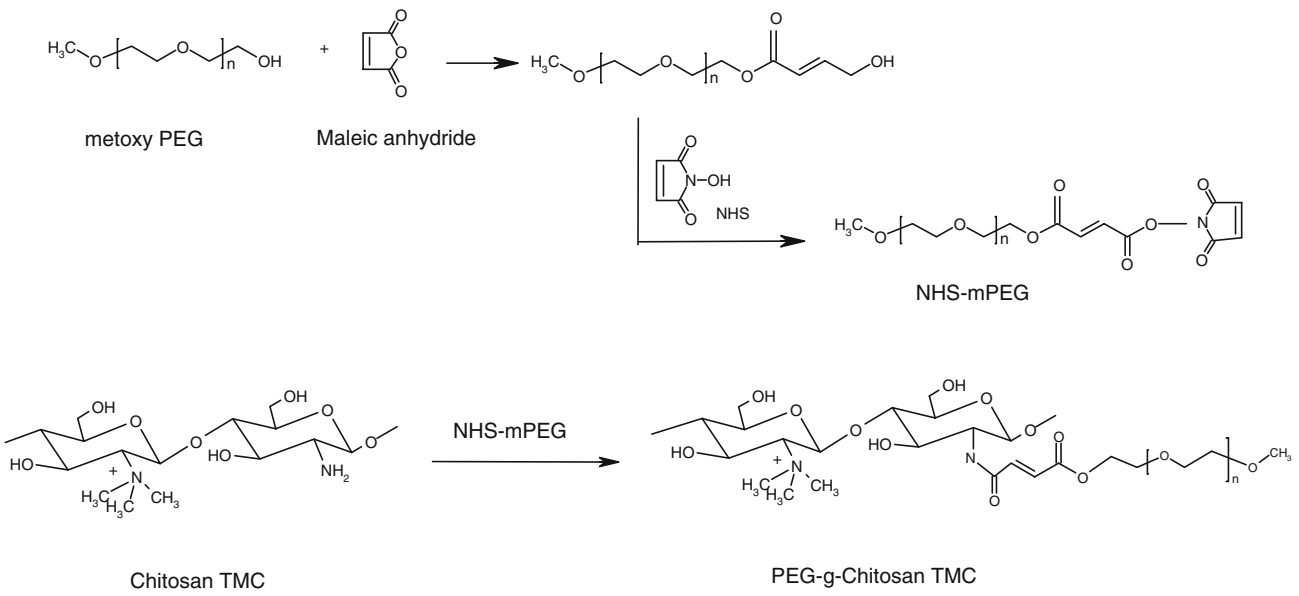

Fig. 14 PEGylation of TMC

nanocompelex of insulin-PEGylated trimethyl chitosan significantly enhanced the uptake of insulin in Caco-2 cells by adsorptive endocytosis. However, nanocomplexation did not seem to enhance transcellular insulin transport across cell monolayers, which is in line with animal data in rats [102].

Apart from cytotoxicity problem, TMC due to its strong electrostatic interaction between TMC and DNA deters gene dissociation from its carrier inside cells, impeding the access of RNA polymerase to DNA so that the gene expression level is limited. Therefore to improve the gene delivery efficiency and biocompatibility of TMC, modification is needed. The PEGylation is one such modification. The complex of PEGylated TMC with DNA along with that of chitosan and TMC were studied or characterized concerning physicochemical properties such as hydrodynamic diameter, condensation efficiency and DNA release [103]. The superiority of TMC over native chitosan was confirmed. Under conditions found in cell culture, formation of aggregates of $\sim$1,000 nm and strongly decreased DNA condensation efficiency was observed in the case of chitosan polyplexes. These characteristics resulted in only 7% cellular uptake in NIH/3T3 cells and low transfection efficiencies. By contrast, quaternization of chitosan strongly reduced aggregation tendency and pH dependency of DNA complexation. Accordingly, cellular uptake was increased 8.5-fold compared to chitosan polyplexes resulting in upto 678-fold increased transfection efficiency in NIH/3T3 cells. Apart from reduction of the cytotoxicity, PEGylation led to improved colloidal stability of polyplexes and significantly increased cellular uptake compared to unmodified TMC. These improvements resulted in a significant, up to 10-fold increase of transfection efficiency in NIH/3T3, L929 and MeWo cells compared to trimethyl chitosan. Poly (*N*-isopropylacrylamide) (PNIPAAm) is a typical paradigm of thermosensitive polymers that undergo a coil-to-globule phase transition at 34°C (lower critical solution temperature LCST). The affinity of the PNIPAAm copolymers to DNA and the transfection efficiency of delivered DNA can be then controlled by temperature change. When the temperature is below LCST, the gene will be unpacked from its carrier, which is beneficial to improve the gene transfection level. Such graft polymer was synthesized by condensing carboxylic end-capped PNIPAAm (PNIPAAm-COOH) onto TMC chain by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) as shown in Fig. 15 [104]. The carboxylic end-capped PNIPAAm was synthesized by reacting *N*-isopropylacrylamide (NIPAAm) and 4,4′-azobis(4-cyanovaleric acid) which is then incubated with TMC and EDCA.

The grafting of palmitoyl pendant on glycol chitosan followed by trimethylation provides a polysoap (potential drug solubiliser) which has been described as polymer non-haemolytic when present as the liquid solution and relatively noncytotoxic (Fig. 16). It increased the solubility of pyrene [105].

The modification of TMC with sugars provides means of achieving cellular recognition ability. TMC DQ 80%, bearing antennary galactose residues through 1,6-hexanediamine spacer attached to 6-*O*-carboxymethyl group were synthesized (Fig. 17) and TMC–DNA complexes were tested for specific targeting to HepG2 cells, which carries the galactose receptor with expression of pSVβGa1. The

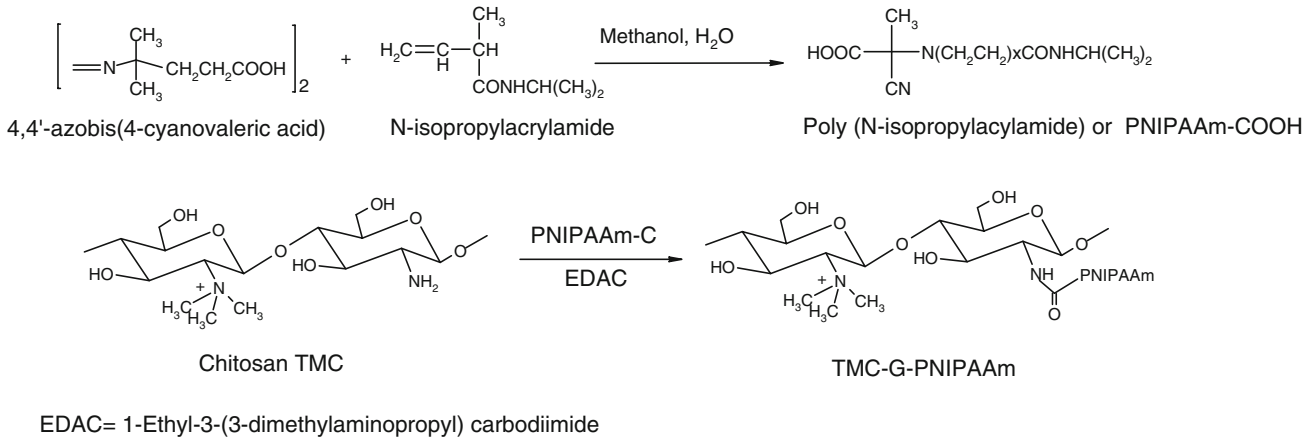

Fig. 15 Synthetic route for: PNIPAAm-COOH and TMC-g-PNIPAAm

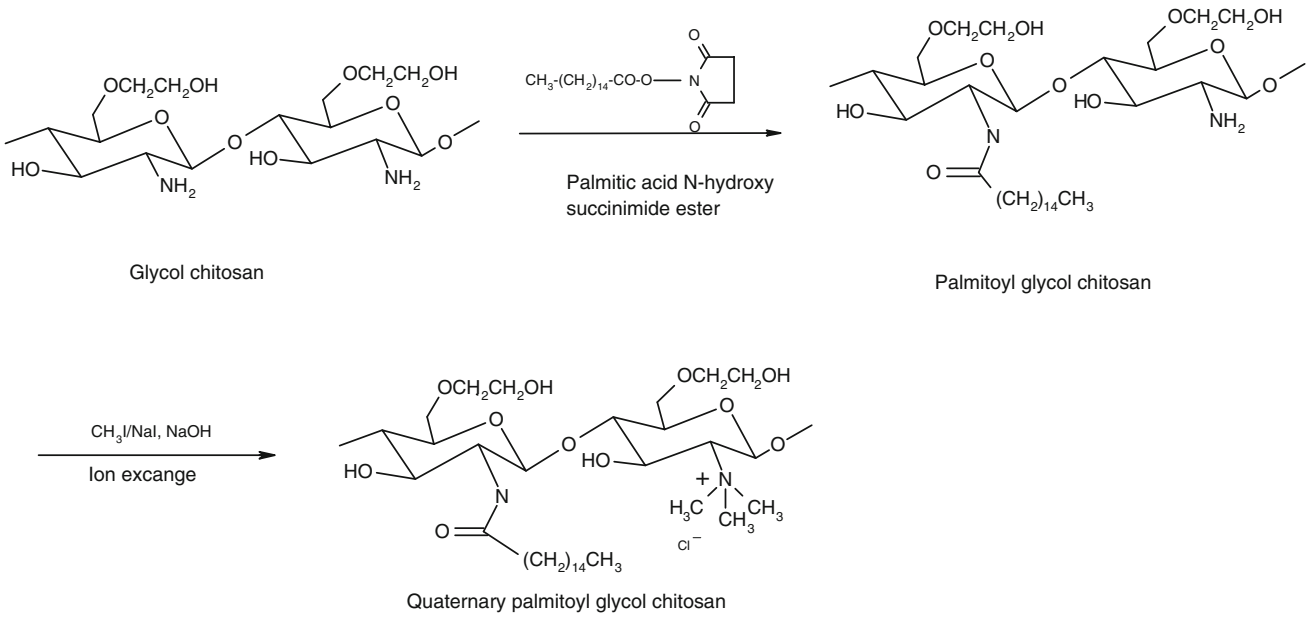

Fig. 16 Synthesis of palmitoyl glycol chitosan

complexes efficiently transfected the HepG2 cells and the transfection efficiency was significantly inhibited in the presence of an inhibitor indicating that the conjugates were specifically internalized via the galactose receptor present on the cellular surface of HepG2 cells [106]. The TMC designed with tetragalactose antenna also (Fig. 18) [107].

10 Other uses of TMC and derivatives

TMCs are reported to possess antimicrobial (antifungal, antibacterial) activities of their own. Antifungal activity of TMC was demonstrated to be higher than chitosan against *Botrytis cinerea Pers. and Colletotrichum lagenarium (Pass) Ell.ethalsttosan*. Quaternized chitosan derivatives with high molecular weight appeared to have ever stronger antifungal activities than those with low molecular weight [108]. Other quaternized derivatives like *N*-(substituted phenyl)-*N*,*N*-dimethyl chitosans such as with 2-hydroxyl-phenyl; 5-chloro-2-hydroxyl-phenyl; 2-hydroxyl-5-nitro-phenyl; 5-bromo-2-hydroxyl-phenyl when investigated for antifungal activities against same fungal species were found to posses better inhibitory effects than chitosan. This improvement in antifungal activity is probably consequence of the cationic charge in the quaternized macromolecules [109].

The antibacterial activities of quaternized chitosan like TMC, *N*-*N*-propyl-*N*,*N*-dimethyl chitosan and *N*-furfuryl-*N*,*N*-dimethyl chitosan, diethylmethylchitosan against *Escherichia coli* were explored. Results showed that antibacterial activity of quaternized chitosan was stronger than chitosan and it increased with acidic condition provided by

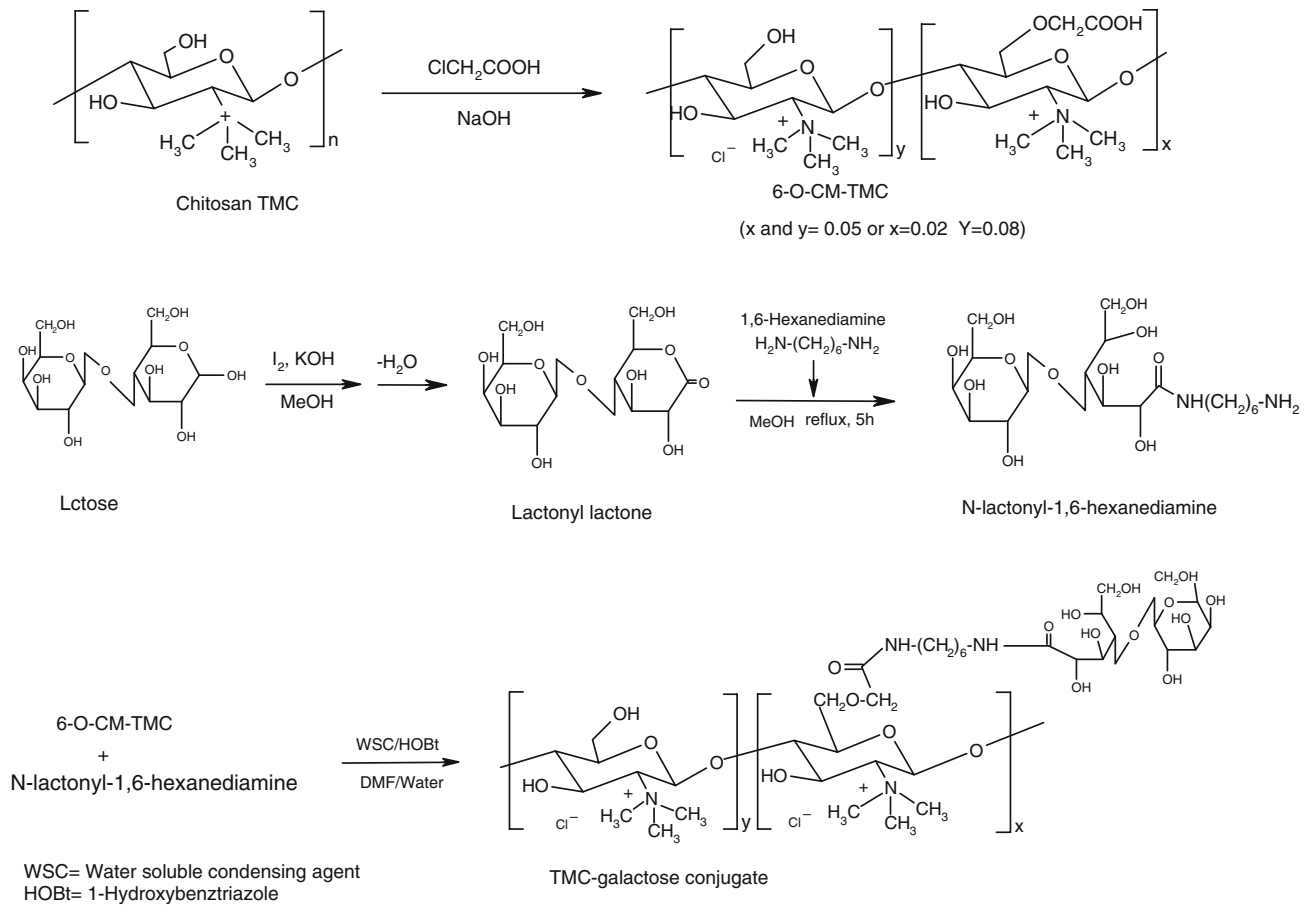

Fig. 17 Synthesis of 6-O-Carboxymethyl TMC and TMC-Galactose conjugate

acetic acid as well as molecular weight [31, 110]. The parallel results were obtained for triethyl chitosan's antimicrobial activity against *Staphylococcus aureus.* One of the quaternary derivatives, *N*-butyl-*N*,*N*-dimethyl chitosan was used to prepare antibacterial fiber and mat by electrospining with polyvinylpyrrolidone followed by photocrosslinking which exhibited high antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [32].

Quaternization (triethylation or trimethylation) of modified chitosan as 6-amino-6-deoxy-chitosan also offers antibacterial compounds [111] (Fig. 19). Where as trimethylation of *O*-carboxymethyl chitosan gave compounds with hydroxyl radical scavenging activity [112]. The free radical scavenging activity is exhibited by TMC also where it is implied that the positive charges of TMC assist in theses actions [113].

The derivitization of chitosan for introduction of positive charge as quaternized nitrogen can also be achieved by placing a quaternary methyl group on *N* of glucosamine with a spacer of 2-hydroxy propyl group between these two nitrogens. Such derivative *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) can be synthesized by reaction of chitosan with glycidyl-trimethyl-ammonium chloride [114] (Fig. 20).

HTCC, the water soluble derivative, can be harnessed to develop several different formulations, such as hydrogel [115], microsphere [116]] microgels [117], coating of beads [118] nanaoparticles [114]. It has been evaluated as antioxidant [119], and antimicrobial too [120–122]. The other uses of HTCC include formation of nanofilrtation memberanes [123, 124] adsorbent for removal of dyes [125], catalyst [126], support for catalysts [127]. Such derivative may bear alkyl groups other than methyl on N. For example, amphipathic octadecyl-quaternized carboxymethyl chitosan obtained by reaction of glycidyl octadecyl dimethylammonium chloride with carboxymethyl chitosan and studied for its potential for delivery vector for lipophilic drugs [128]. HTCC with carboxymethyl groups on N and O of the monomer has been studied for its flocculating properties [129]. Analogous quaternized derivatives have been patented exclusively for various applications (Table 3). Another quaternary chitosan derivative with comparable physicochemical, biological, and pharmaceutical properties comparable to *N*-trimethylchitosan is *N*-betainate synthesized by acylating chitosan or its suitable

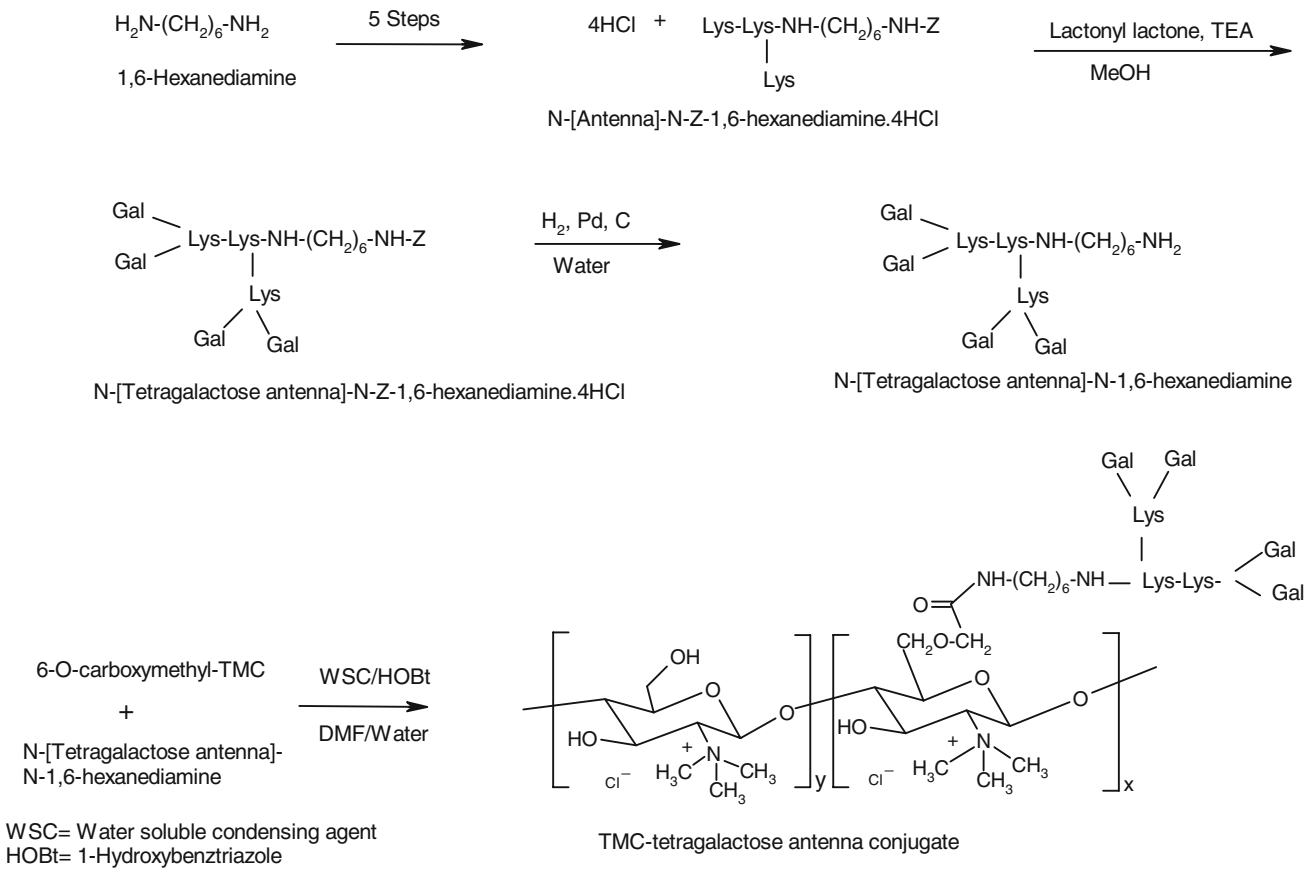

Fig. 18 Synthesis of tetragalactose antenna conjugate

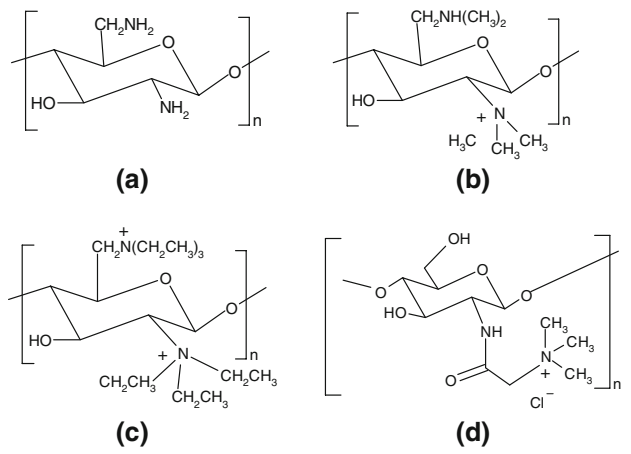

Fig. 19 Chemical structures of **a** 6-NH_2-6-deoxy chitosan, **b** C2–C6 methylated chitosan, **c** C2–C6 ethylated chitosan, **d** Chitosan –*N*-betainate. These units are present in backbone of chitosan

derivative with betaine in aqueous acidic solutions in presence or absence of coupling reagents [140–142].

Novel Quaternary Chitosan derivatives as *N*,*N*,*O*-[*N*,*N*-diethylaminomethyl(diethyldimethylene ammonium) methyl] chitosans are also reported and will be of interest to further applications of chitosan [143].

11 Intellectual properties, regulatory issues and commercial exploitation

Chitosan and its derivatives are the subject of investigation since long. Despite of significant academic exploration of TMC, significant scope exists in creating IPR wealth in the area of applications of TMC. It has been used in designing gastroretantive systems due to its bioadhesive properties. An interesting application has been mentioned in using TMC as tissue permeability enhancer in fabricating implantable patch. More recently in a collobrative project of industry and academics, nanoparticle fabrication of peptide drugs using TMC is found to deliver the drug in transocular and transnasal delivery of drug (Table 3). Significant information on regulatory aspects of chitosan is still needed.

Commercial exploitation of chitosan native faces significant barriers as difficulties in preparing uniformly reproducible charges in bulk quantities from various marine organisms and the high prices of the polymers. Trimethylation and quaternization by various methods further add up to cost and may forbid the wider usage of such derivatives. High cost of TMC and unavailability of vendors thus is still a constrain in developing applications

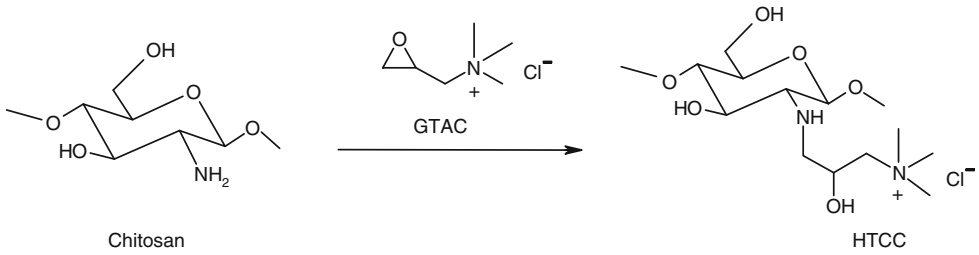

Fig. 20 Cationic derivatives of chitosan, GTAC = Glycidyltrimethylammonium chloride, HTCC = *N*-(2-hydroxyl) propyl-3-trimethylammonium chitosan chloride

Table 3 US patents of quaternized chitosan

Patent	Description	Refs.
US Patent 4,921,949	Process for making quaternary chitosan derivatives for cosmetic agents	[130]
US Patent 5,770,712	Crosslinked hydrogel beads from chitosan	[131]
US Patent 6,306,835	Biocidal chitosan derivatives	[132]
US Patent 7,381,716	Nanoparticles for protein drug delivery	[133]
US Patent 7,291,598	Nanoparticles for protein drug delivery	[134]
US Patent 7,282,194	Nanoparticles for protein drug delivery	[135]
US Patent 6,726,920	Implantable drug delivery patch	[136]
US Patent 6,410,046	Administering pharmaceuticals to the mammalian central nervous system	[137]
US Patent 6,328,967	Delivery system to modulate immune response	[138]
US Patent 6,207,197	Gastroretentive controlled release microspheres for improved drug delivery	[139]

of TMC. Moreover particulate matters as nanoparticles of chitosan may or may not cause proinflammatory effects [144, 145]. Such a parameter should be attended for TMC and quaternized derivatives since particulate drug delivery is main mode of application.

12 Conclusion and perspective

The unique characteristics of chitosan make it a polymer of interest but its limited solubility is major obstacle in its applicability. Hence it is widely explored for the introduction of new functionalities and properties such as water solubility among others. The TMC can be synthesized by quaternization of the amino groups of the parent polymer. The quaternization methods include use of methylating agent as methyl iodide and dimethylsulfate or the sequence of reductive alkylation via Schiff base formation. Trimethylation of chitosan generates the *N*,*N*,*N*-trimethyl derivative with permanent positive charges and water solubility. This derivative has shown promising results as drug delivery agent as well as a DNA delivery agent. The derivatives and grafts of TMC can be developed and utilized in the same manner along with advantages of modulation of solubility properties, cell specificity, reduced cytotoxicity, etc. The permanent positive charge on chitosan backbone can be introduced by covalent addition of a substituent containing a quaternary ammonium group too. Such derivatives may also assure to advance the applications of chitosan.

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