JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 1111–1119
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Received March 7, 2008
Accepted April 21, 2008
DOI 10.1211/jpp.60.9.0001
ISSN 0022-3573

Potential prospects of chitosan derivative trimethyl chitosan chloride (TMC) as a polymeric absorption enhancer: synthesis, characterization and applications

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Abstract

In recent years, researchers have been working extensively on various novel properties of polymers to develop increased efficiency of drug delivery and improve bioavailability of various drug molecules, especially macromolecules. Chitosan, a naturally occurring polysaccharide, because of its protonated/polymeric nature, provides effective and safe absorption of peptide and protein drugs. Its transmucosal absorption is, however, limited to acidic media because of its strong intermolecular hydrogen bonds. A new partially quaternized chitosan derivative, N-trimethyl chitosan chloride (TMC), has been synthesized with improved solubility, safety and effectiveness as an absorption enhancer at neutral pH and in aqueous environment. It enhances the absorption, especially of peptide drugs, by reversible opening of tight junctions in between epithelial cells, thereby facilitating the paracellular diffusion of peptide drugs. This derivative thus opens new perspectives as a biomaterial for various pharmaceutical applications/drug delivery systems. This review deals with the potential use of the quaternized chitosan derivative as a permeation enhancer for the mucosal delivery of macromolecular drugs along with its other biomedical applications.

Introduction

Scientists in recent years have been working extensively on one of the greatest challenges of delivering large molecular compounds across the mucosal absorptive surfaces. These macromolecular drugs, like peptides, are hydrophilic in nature and have low and incomplete transport across mucosal epithelia (i.e. low bioavailability as a result of poor absorption at mucosal sites) (Junginger & Verhoef 1998; Polnok et al 2004) and their absorption is limited to the paracellular pathway, which, in turn, is restricted by tight junctions (Fix 1987; Gumbiner 1987; Hochman & Artursson 1994). Chitosan is a natural polysaccharide that, because of its favourable characteristics like hydrophilicity, polycationic and nontoxic nature, biocompatibility and biodegradable nature, is rendered as a promising polymer for a number of applications in drug delivery especially as an absorption enhancer for hydrophilic macromolecule drugs (Chandy & Sharma 1991; Bernkop-Schnurch & Kast 2001; Illum et al 2001; Sinha & Kumria 2001). The term chitosan was coined in 1894. It is a copolymer of glucosamine and *N*-acetyl glucosamine (Hopper Seyler 1894; Kas 1997; Singla & Chawala 2001; Kato et al 2003). It is derived by alkaline deacetylation of chitin, which is isolated from hard shells of marine-living creatures (fishes, crustaceans) or synthesised by natural organisms (fungi-like yeasts) (Sinha et al 2004).

Protonated chitosan ($pH < 6.5$) is able to increase the paracellular permeability of peptide drugs across mucosal epithelia by inducing a transient opening of the tight junctions of the cell membrane (Artursson et al 1994; Borchard et al 1996; Kotze et al 1999; Schipper et al 1999; Thanou et al 2001a; Fini & Orienti 2003). The transient opening of the tight junctions occurs due to the interaction of positively charged amino groups at the C-2 position of chitosan with the negatively charged sites on the cell surfaces and tight junctions (Artursson et al 1994; Borchard et al 1996; Schipper et al 1996). This results in a decrease in ZO-1 proteins and the change in the cytoskeleton protein F-actin from a filamentous to a globular structure (Illum et al 1994; Schipper et al 1996; Illum 1998).

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Although chitosan is soluble in aqueous acidic medium below pH 6.5, it precipitates from solution at neutral pH. Thus, application of chitosan is limited owing to its insolubility at physiological pH. In recent years various strategies have been examined for eliminating this drawback. A promising strategy in this regard is chemical modification of chitosan (i.e. its derivatization). Quaternization of chitosan has resulted in the formation of quaternized derivatives, like trimethyl chitosan chloride, that have increased solubility in water. These polymers are being extensively investigated due to their cheap production costs, biocompatibility and very low toxicity (Kotze et al 1997, 1999a; Muzzarelli 1992; Sieval et al 1998; Van der Lubben et al 2001).

Synthesis of *N*-trimethyl chitosan chloride (TMC)

N-Trimethyl chitosan chloride (TMC), a partially quaternized chitosan derivative, is synthesized by chemical modification via removal of the one or two hydrogen atoms of the amino group of chitosan and introduction of some hydrophilic substitute (Heras et al 2001). The reaction involves reductive methylation of the polymer in the presence of indometacin for 1 h in a strong basic media at a temperature of 60°C (Thanou et al 2001b). Hydrochloric acid in methanol is added to a solution of quaternized polymer in water to replace I^- ions with Cl^- ions.

N-Trimethylated chitosan chloride (TMC) has also been synthesized from chitosan via a two-step methylation procedure described by Sieval et al (Sieval et al 1998; Atyabi et al 2005; Amidi et al 2006). A weighed quantity of chitosan is dissolved in *N*-methyl-2-pyrrolidone (NMP) and stirred at 60°C. Sodium hydroxide (NaOH) 15% solution is added, followed by addition of methylation agent methyl iodide and NaI. The mixture is stirred, precipitated with ethanol, centrifuged, washed with acetone and finally dried. This is followed by addition of NMP to precipitate dimethyl chitosan iodide. Further 15% NaOH, methyl iodide and NaI are added successively and the mixture is stirred for 30 min. After addition of methyl iodide and NaOH stirring is continued for 1 h. This mixture is precipitated using ethanol, centrifuged, filtered and the resultant precipitate washed with acetone to obtain trimethyl chitosan iodide. To exchange iodide with chloride, sodium chloride is added. The solution is precipitated with ethanol and filtered to obtain TMC as a precipitate. The degree of quaternization (% DQ) is determined by ^1H NMR.

TMC is positively charged and soluble in a neutral and basic environment (Kotze et al 1997; Thanou et al 2001b). This phenomenon of enhanced solubility in both neutral and basic media occurs due to the substitution reaction, where the primary amine is replaced with a methyl group, thereby inhibiting hydrogen bond formation between amine and hydrolytic groups of the cationic polymer. Unlike chitosan, the derivative formed was found to enhance absorption above pH 6.5. It also increased the permeation and transport of hydrophilic compounds along the paracellular transport pathway by opening the tight junctions of intestinal epithelia. The process was reported to be reversible and resealing of the tight junctions was observed to occur once the polymer was removed (Thanou et al 2001c).

Factors affecting properties of TMC

Degree of substitution

The degree of substitution of TMC plays an essential role in its ability to open the tight junctions of intestinal mucosa. The charge density of TMC, as determined by the degree of quaternization, has an important effect on the absorption-enhancing property of this polymer (Kotze et al 1999b). During the synthesis of TMC, the number of positive charges on the polymer chain is increased, causing the polymeric molecule to expand in solution due to repelling force between the functional groups (Domard et al 1986). At pH 7.2, the TMC derivative with a higher degree of substitution shows higher macromolecular absorption enhancement ratios across Caco-2 cells as compared with TMC derivatives with a lower degree of substitution. Maximum absorption is achieved at optimum substitution, which, however, decreased with further substitution because of the steric effects due to the presence of attached methyl groups and an alternation in the flexibility of TMC (Hamman & Kotze 2004). The results indicate that a high charge density is necessary for TMC to substantially improve the paracellular permeability of mucosal epithelia.

The safety of quaternized chitosan, TMC and influence of substitution (20%, 40% and 60%) on absorption enhancement of marker Texas red dextran was investigated by Thanou et al (1999). The safety of TMC was shown by conducting cytotoxicity and ciliotoxicity studies on Caco-2 cell monolayers treated with TMC. Enhanced permeation was observed with 40% and 60% substituted TMC whereas TMC with 20% substitution was ineffective. Based on the observations of the study, it was concluded that TMC with a higher degree of substitution was a better and a safer absorption enhancer, especially for peptide drug delivery.

TMC with 40% and 60% degree of substitution was synthesized by Thanou et al (2000a). The effect of the prepared derivatives on the permeability of peptide drug ^{14}C mannitol across the tight junctions of the intestinal Caco-2 monolayers was studied. At pH 7.2, TMC with 60% degree of substitution produced a pronounced enhancement of ^{14}C mannitol permeation in comparison with TMC with 40% degree of substitution. It was concluded that the extent of the opening of the tight junctions was strongly dependent on the degree of quaternization and consequently on the charge density of the polymer (Thanou et al 2000a).

In another study the intestinal absorption of octreotide, a somatostatin analogue, after co-administration with the polycationic absorption enhancer TMC was investigated. The in-vitro drug release studies conducted using Caco-2 cells showed an enhanced absorption of octreotide containing TMC. In-vivo studies in rats suggested a 5-fold increase in peptide drug bioavailability in comparison with control (octreotide alone) that showed no increase. These results, in combination with the nontoxic character of TMC, suggested that the polymer was a promising excipient in the development of solid dosage forms for the peroral delivery and intestinal absorption of octreotide (Thanou et al 2000b).

Another study (Kotze et al 1999c) also evaluated the effect of degree of substitution of TMC on opening of the tight junctions of intestinal mucosa. The effects of TMC-H (61.2% quaternized), TMC-L (12.3% quaternized) and chitosan HCl

on the intestinal permeability of model drug ^{14}C mannitol were studied. A decrease in transepithelial electrical resistance (TEER) values with a consequent increase in the paracellular transport of the hydrophilic drug across intestinal epithelial Caco-2 cell monolayers was observed for both the derivatives at pH 6.2. It was further observed that TMC-H caused a decrease in TEER and an enhanced paracellular transport at pH 7.2 unlike TMC-L and chitosan HCl, which were ineffective. These results again indicated that a high degree of substitution and high charge density were necessary for TMC to enhance absorption of hydrophilic compounds at neutral pH.

Further, enhanced absorption of macromolecules such as peptides through mucosal epithelia was observed in rats and juvenile pigs, without causing toxicity and cell damage, which also suggested that TMC triggered reversible opening of tight junctions (Thanou et al 2001b).

Besides oral delivery, administration of ^{14}C mannitol for improved nasal delivery in rats was investigated by Hamman et al (2002). Chitosan derivatives with different degrees of substitution (12–59%) were synthesized. It was observed that all quaternized derivatives of chitosan showed a significant enhancement in drug absorption at pH 6.2. Superior drug absorption at neutral pH was achieved only with TMC quaternized in the range 36–40%. Forty-eight per cent quaternized TMC exhibited maximum absorption value. It was further observed that increase in degree of quaternization to 59% did not increase absorption of drug, which was attributed to the steric effects, due to the presence of attached methyl groups and an alternation in the flexibility of TMC with an increase in the degree of substitution above 48% at pH 7.4. It was thus concluded that the degree of quaternization of TMC plays an essential role in the absorption of hydrophilic molecules through the nasal epithelium. It was also observed that the mucoadhesive properties of TMC reduced with an increase in the degree of substitution. This was attributed to excessive polymer hydration associated with an increase in the degree of substitution (Snyman et al 2003).

In some cases, an increase in substitution of TMC resulted in enhanced permeation and increased bioavailability of drugs, rather than the enhanced mucoadhesive properties of the polymer (Kotze et al 1997; Thanou et al 2000a, b). Sandri et al (2005) observed and reported that an increase in quaternization degree of N-TMC produced an increase in the penetration of the protein drug fluorescein isothiocyanate dextran across the buccal mucosa. The TMCs (TMChH3 and TMChL3) with the maximum trimethylation degree presented the highest reduction in TEER values and consequently maximum permeation enhancing effects.

From the results of the above studies, it can be concluded that TMC derivatives are especially effective in enhancing mucosal permeation of small hydrophilic compounds (e.g. mannitol), though they also improve the mucosal transport of large molecules such as octreotide acetate. The permeation-enhancing effect can be attributed to TMC-triggered reversible opening of the tight junctions of the intestinal mucosa. Besides this, it was observed that the degree of quaternization also played an important role in the absorption-enhancing properties of these polymers. This may be explained by the positive charge density on the TMC molecules, determined by the degree of quaternization. TMC polymers with a higher degree of quaternization have higher positive charge density responsible for inducing opening of the tight junctions. Thus, there is general agreement that the permeation-enhancing effect of TMC derivatives increases with an increase in their degree of quaternization. However, it has also been observed by some researchers that mucoadhesion decreases with increasing degree of quaternization due to the excessive polymer hydration.

Number of methylation process steps and the base used in the process

TMC is synthesized by reductive methylation of chitosan in an alkaline environment at elevated temperature. The number of methylation process steps and the base used in the process also affect the degree of quaternization of the primary amino group and methylation of 3- and 6-hydroxyl groups. The methylation of the primary amine to the quaternary stage is achieved by using a base that is generated during the reaction and binds the acid, thereby avoiding protonation of the unreacted primary amino groups. Although a high degree of substitution of the amino group of chitosan is difficult due to the presence of some acetyl groups, the hydroxyl groups of chitosan can be substituted in the methylation process. A decrease in the solubility of the cationic polymer occurs with an increase in the high degree of *O*-methylation on the 3- and 6-hydroxyl groups (Snyman et al 2002).

Jonker et al (2002) prepared quaternized chitosan with different degrees of trimethylation (22.1–48.8% quaternized) by varying the number and duration of reaction steps. In-vitro and in-situ studies were conducted at neutral pH for evaluating the permeation-enhancing capability of the prepared polymers by using everted intestinal sacs of rats and a single-pass intestinal perfusion method, respectively. ^{14}C mannitol was used as the model drug. Based on the results of the study it was concluded that all the prepared polymers enhanced permeation of ^{14}C mannitol, though TMC with 48.8% quaternization at 0.5% w/v concentration showed maximum permeation.

The effect of two bases, sodium hydroxide and dimethyl amino pyridine, separately and in combination, along with varying number and duration of reaction steps, on the degradation and degree of quaternization of TMC polymers was studied by Hamman & Kotze (2004). It was observed that when sodium hydroxide was used as the base, the degree of quaternization increased with an increase in the number of reaction steps. With dimethyl aminopyridine the extent of degradation was lower, though quaternization remained low even with an increase in number of reaction steps. A combination of these bases showed no reduction in polymer degradation and degree of quaternization was relatively low.

Molecular mass of quaternized TMC

Quaternized *N*-trimethyl chitosan chloride was prepared by Snyman et al (2002). Absolute molecular weight, radius and polydispersity of TMC polymers were determined by using size-exclusion chromatography (SEC) and multi-angle laser light scattering (MALLS). The viscosity of the TMC polymer solutions was measured and the calculated intrinsic viscosity

values were used as a further indication of the molecular weight of each polymer. The decrease in absolute molecular weight correlated well with the intrinsic viscosity of the TMC polymers and was related to the increase in their degrees of quaternization. It was concluded that an increase in the degree of quaternization leads to a decrease in the absolute molecular weight of the TMC polymers.

Investigations regarding the molecular mass of quaternized TMC and the effect of substitution on the properties of the polymer were conducted using SEC/MALLS and Well helming plate method, respectively. Studies revealed that the prepared polymers had molecular mass above 100 000 g/mole and exhibited 22.1–48.8% substitution (Snyman et al 2003).

In another study, TMC with differing quaternization degrees (QD) and molecular weights (MW) (i.e. higher molecular weights (TMCH) and lower molecular weight (TMCL)) were prepared using the model drug ofloxacin. In-vitro studies were conducted to compare the ability of various derivatives to enhance the permeability of the drug across rabbit corneal epithelium. TMC polymers with intermediate QD showed the best permeability enhancement, independent of polymer MW (i.e. TMC H2, QD 35%, TMC L2, QD 46%). In-vivo studies conducted on the rabbit eye confirmed the transcorneal permeability-enhancing property of the polymer. Higher MW TMC H2 produced higher antibiotic levels in aqueous humour. Thus, it was concluded that TMC H2 exhibited a good potential for increasing the absorption of ofloxacin in the treatment of endophthalmitis (Colo et al 2004).

Applications of TMC

Oral delivery

Enhanced per-oral absorption of various macromolecular drugs like marker Texas red dextran, ^{14}C mannitol, octreotide and buserelin via mucosal epithelia, using TMC, has already been mentioned (Sieval et al 1998; Thanou et al 1999, 2000a, b, c). Table 1 summarizes the various applications of TMC.

TMC also forms complexes with anionic macromolecules and gels and solutions with cationic/neutral compounds at aqueous and neutral pH (Thanou et al 2001b).

TMC was used as the oral absorption enhancer for peptide drugs desmopressin and octreotide (Dorkoosh et al 2002; Van der Merwe et al 2004).

Chen et al (2007) prepared TMC nanoparticles by ionic cross-linking of TMC with tripolyphosphate (TPP). Bovine serum albumin (BSA) and bovine haemoglobin (BHb) were used as model proteins to investigate the loading and release features of the TMC nanoparticles. TMC samples with different degrees of quaternization were synthesized. The influence of different degrees of quaternization on the physicochemical properties and release profiles of the nanoparticles was evaluated. Sodium alginate was used to modify the TMC nanoparticles to reduce burst release. TMC nanoparticles exhibited a high loading efficiency (95%) for BSA. However, a lower loading efficiency (30%) was observed for TMC nanoparticles containing BHb. It was also observed that the particle size and zeta-potential were significantly affected by the BSA concentration, although BHb concentration had no effect. Nanoparticles of TMC with a lower degree of quaternization

Table 1 Various applications of *N*-trimethyl chitosan in drug delivery

Application	Route of administration	Drug candidate	Model	Reference
Absorption enhancement	Oral	Texas red dextran	Chicken embryo trachea tissue	Thanou et al (1999)
Absorption enhancement	Oral	^{14}C Mannitol	Intestinal epithelial Caco-2 cell monolayers	Thanou et al (2000a)
Increased bioavailability	Oral	Octreotide	Intestinal epithelial Caco-2 cell monolayers	Thanou et al (2000b)
Absorption enhancement	Oral	^{14}C Mannitol	Intestinal epithelial Caco-2 cell monolayers	Kotze et al (1999c)
Absorption enhancement	Oral	Desmopressin	—	Van der Merwe et al (2004)
Increased bioavailability	Peroral	Octreotide	Pig	Dorkoosh et al (2002)
Absorption enhancement	Oral	Cationic/neutral macromolecules	Rat and juvenile pig	Thanou et al (2001b)
Increased permeation	Oral	^{14}C Mannitol	Rat everted intestinal sac	Jonker et al (2002)
Absorption enhancement	Nasal	^{14}C Mannitol	Rat nasal epithelium	Hamman et al (2002)
Increased bioavailability	Pulmonary	Octreotide	Reconstituted Calu-3 cell monolayers	Florea et al (2006)
Increased permeation	Ocular	Ofloxacin	Rabbit corneal epithelium	Colo et al (2004)
Increased permeation	Ocular	Dexamethasone	Rabbit corneal epithelium	Zambito et al (2006)
Increased permeation and increased mucoadhesion	Buccal	Fluorescein isothiocyanate dextran	Bovine submaxillary mucin dispersion and porcine buccal mucosa	Sandri et al (2005)
Increased absorption and increased bioavailability	Intestinal delivery	Octreotide	Pig	Thanou et al (2004)
Increased permeation	Intestinal delivery	Fluorescein isothiocyanate dextran	In-vitro Caco-2 cell model and ex-vivo rat jejunum model	Sandri et al (2007)
Increased transfection efficacy as a gene carrier	—	Plasmid DNA	COS-1 and Caco-2 cell lines	Thanou et al (2002)
Antimicrobial activity	—	—	*E. coli*	Avadi et al (2004)

showed an increase in particle size, a decrease in zeta-potential and a slower drug-release profile. In contrast, the alginate-modified nanoparticles exhibited a smaller size and lower zeta-potential. A reduced burst release was also observed in alginate-modified nanoparticles. The results of the study demonstrated the potential of TMC nanoparticles as protein carriers.

FITC-BSA loaded TMC nanoparticles were prepared by Chen et al (2008) for oral protein delivery. The effect of alginate modification on absorption properties of FITC-BSA loaded TMC nanoparticles was investigated using Caco-2 cells. A higher FITC-BSA permeation efficiency was observed for alginate-modified TMC nanoparticles than for the non-modified TMC nanoparticles. Further, the feasibility of applying TMC nanoparticles loaded with a model vaccine urease in oral vaccination was also studied. Mice immunized with urease-loaded TMC nanoparticles showed much higher antibody titres of both immunoglobulin G (IgG) and secretory immunoglobulin A (IgA) as compared with those mice who were immunized with either urease solution or urease co-administered with TMC solution. These results indicated that TMC nanoparticles are potential carriers for oral protein as well as vaccine delivery.

Thus, all the studies mentioned above demonstrate the potential of TMC as an oral absorption enhancer, especially for peptides and proteins.

Nasal delivery

Besides oral delivery, administration of ^{14}C mannitol using TMC for improved nasal delivery in rats has been investigated (Hamman et al 2002). Forty-eight per cent quaternized TMC exhibited the maximum absorption value, thus indicating that it could play an essential role in the absorption of hydrophilic molecules through the nasal epithelium.

In another study, the potential of TMC nanoparticles as a carrier system for the nasal delivery of proteins was investigated (Amidi et al 2006). TMC nanoparticles were prepared by ionic cross-linking of TMC solution (with or without ovalbumin) with tripolyphosphate, at ambient temperature while stirring. The size, zeta-potential, morphology, protein loading, protein integrity and protein release of the nanoparticles were also evaluated. The nanoparticles had an average size of about 350 nm, a positive zeta-potential, a loading efficiency of up to 95% and a loading capacity of up to 50% (w/w). The integrity of the entrapped ovalbumin was preserved. Release studies showed that more than 70% of the protein remained associated with the TMC nanoparticles for at least 3 h on incubation in PBS (pH 7.4) at 37°C. Ciliary beat frequency measurements of chicken embryo trachea and in-vitro cytotoxicity assays were used to test the toxicity of the TMC nanoparticles. No toxic effects of the nanoparticles were observed in Calu-3 cells, whereas a partially reversible cilio-inhibiting effect on the ciliary beat frequency of chicken trachea was seen. Further, confocal laser scanning microscopy was used to study the in-vivo uptake of FITC-albumin-loaded TMC nanoparticles by nasal epithelial tissue in rats. In-vivo uptake studies indicated the transport of FITC-albumin-associated TMC nanoparticles across the nasal mucosa. The study demonstrated the potential of TMC nanoparticles as a new delivery system for transport of proteins across the nasal mucosa.

The potential of TMC nanoparticles as a carrier system for the nasal delivery of a monovalent influenza subunit vaccine was investigated by Amidi et al (2007a). The antigen-loaded nanoparticles were prepared by mixing a solution containing TMC and monovalent influenza A subunit H3N2 with a tripolyphosphate (TPP) solution at ambient temperature and pH 7.4 while stirring. The nanoparticles had an average size of about 800 nm with a narrow size distribution, a positive surface charge, a loading efficiency of 78% and a loading capacity of 13% (w/w). Single intranasal immunization with antigen-loaded TMC nanoparticles resulted in significantly higher haemagglutination inhibition and total IgG responses as compared with intramuscular administration of the subunit antigen. From the results of the study it was concluded that TMC nanoparticles were a potent new delivery system for intranasally administered influenza antigens.

Chitosan of different molecular weights (Chi-P, $MW = 2.7 \times 10^5\,g\,mol^{-1}$ and Chi-A, $MW = 5.0 \times 10^5\,g\,mol^{-1}$) and TMC with 20%, 40% and 60% degree of quaternization (DQ) (TMC-20, TMC-40 and TMC-60) were evaluated as adjuvants for inducing the immune response to ovalbumin (OVA) by Boonyo et al (2007). OVA in solution and in alum were used as controls. On day 0, 7 and 14 mice were immunized and the IgG and IgA titres were examined on day 0, 13 and 21. It was found that on day 13 and day 21, Chi-A produced a higher IgG response to OVA than did Chi-P. On day 13, OVA in TMC-40 induced IgG responses significantly higher than OVA in solution, Chi-P and TMC-60. Besides this, it was also observed that OVA in TMC-40 induced a higher IgG response than OVA in alum. At day 21, OVA in TMC-40 induced a higher IgG response than OVA in TMC-20, TMC-60 and solution. Further, it was observed that on day 21 Chi-A elicited a higher IgA response than Chi-P. However, TMC-40 induced the highest IgA response. From the findings of the study it was demonstrated that both the MW of chitosan and DQ of TMC influenced the level of immune induction. It was also concluded that TMC-40 was the most potent adjuvant for intranasal administration among those evaluated.

From the results of the above studies it can be concluded that *N*-trimethylated chitosan derivatives play an essential role in the absorption of hydrophilic molecules through the nasal epithelium. They can thus be used as potential excipients for the development of intranasal drug delivery systems.

Pulmonary delivery

Florea et al (2006) investigated the polysaccharide-mediated absorption kinetics of the peptide drug octreotide across mammalian airway epithelium besides assessing the mechanism of permeation enhancement. The 20% and 60% TMC derivatives (TMC20 and TMC60) were synthesized by alkaline methylation of chitosan. For in-vitro studies, reconstituted Calu-3 cell monolayers were used and the TEER and transport were estimated. Chitosan, TMC20 and TMC60 were found to significantly decrease TEER and enhance in-vitro octreotide permeation by 21, 16 and 30 fold, respectively. In-vivo studies conducted in male Wistar rats revealed that the octreotide bioavailability was enhanced by 2.4, 2.5 and 3.9 fold for chitosan, TMC20 and TMC60, respectively. A linear in-vitro/in-vivo correlation was found between calculated absorption rates ($R^2 = 0.93$), suggesting that the

permeation enhancement by polysaccharides, both in-vitro and in-vivo, proceeded via an analogous mechanism.

The potential of TMC (degree of quaternization 50%) and dextran microparticles for pulmonary delivery of diphtheria toxoid (DT) was investigated by Amidi et al (2007b). The antigen-containing microparticles were prepared by drying an aqueous solution of polymer and DT through a supercritical fluid (SCF) spraying process. Pulmonary immunization with DT-TMC microparticles resulted in a strong immunological response as reflected by the induction of IgM, IgG and IgG subclass (IgG1 and IgG2) antibodies, as well as neutralizing antibody titres comparable with, or significantly higher than, those achieved after subcutaneous administration of an equivalent amount of alum-adsorbed DT. Moreover, the IgG2/IgG1 ratio after pulmonary immunization with DT-TMC microparticles was substantially higher as compared with subcutaneously administered alum-adsorbed DT. In contrast, pulmonary-administered DT-dextran particles were poorly immunogenic. Among the tested formulations only pulmonary-administered DT-containing TMC microparticles induced detectable pulmonary secretory IgA levels. From the results of the study it was concluded that TMC microparticles could be used as a new delivery system for pulmonary-administered DT antigen.

Buccal delivery

Sandri et al (2005) evaluated the mucoadhesive and penetration enhancement properties of TMCs with different degrees of quaternization towards buccal mucosa. Fluorescein isothiocyanate dextran (MW 4400 Da) (FD4) was used as a model molecule. The polymer solutions were subjected to mucoadhesion measurements towards bovine submaxillary mucin dispersion and porcine buccal mucosa and to FD4 permeation tests through porcine cheek epithelium. The mucoadhesive properties of the polymers were observed to increase on increasing the degree of quaternization. At pH 6.4 the chitosan trimethylation was observed to produce an improvement in polymer solubility, which in turn resulted in better penetration enhancement. It was also postulated that the polymer–mucin interactions responsible for the formation of the mucoadhesive joint were likely to be involved in penetration enhancement mechanism. The polymer–mucin interpenetration probably weakened the epithelial barrier resulting in partial dismantling of the structure of the extracellular matrix and the intercellular joint. TMC was concluded to be a most promising polymer for the development of a drug delivery system intended for enhancing the buccal absorption of hydrophilic macromolecules such as peptides with MW lower or close to that of FD4, like insulin, calcitonin, buserelin and leuprolide.

Sandri et al (2006) compared the buccal penetration enhancement properties of chitosan hydrochloride (HCS), both as a polymeric solution and as a nanoparticulate system, with that of TMC. FD4 was used as a macromolecule model. The mechanism of penetration enhancement was investigated on pig buccal mucosa. Morphological analysis, performed by light microscopy and transmission electron microscopy, suggested that the mechanism of penetration enhancement by HCS and TMC solutions, as well as HCS nanoparticles, was similar. It involved the repackaging of the epithelial cells up to the basal membrane and a partial disarrangement of desmosomes. TMC and chitosan nanoparticulate systems increased FD4 permeation across the buccal epithelium to a greater extent than the chitosan solution, thus again confirming the potential of TMC as a buccal penetration enhancer.

Ocular delivery

Transcorneal permeability enhancing property of TMC was confirmed after it was observed to increase the absorption of ofloxacin, a drug used in the treatment of endophthalmitis (Colo et al 2004).

The effects of TMC on the transcorneal transport of dexamethasone, taken as a marker of the transcellular penetration route, and on tobramycin, a marker of the paracellular route, were studied by Zambito et al (2006) in a rabbit model. The drugs were topically applied via erodible inserts (weight 20 mg, diameter 6 mm, drug dose 0.3 mg) based on poly (ethylene oxide), containing 10% w/w medicated TMC microspheres (diameter $< 2.5\ \mu$m). Ocular pharmacokinetics were determined in the rabbit model. TMC produced significant increases of dexamethasone Cmax (5.69 ± 0.49 vs $3.07 \pm 0.31\ \mu g\,mL^{-1}$l) and AUC ($619.3 \pm 32.5$ vs $380.5 \pm 32.0\ \mu g\,min\,mL^{-1}$) in the aqueous humour with respect to the reference TMC-free insert. On the other hand, TMC was unable to yield tobramycin concentrations in the aqueous humour exceeding the determination limit ($0.5\ \mu g\ mL^{-1}$). It was concluded that TMC enhanced the transcorneal transport via the transcellular route, whereas it was unable to effectively open the tight junctions between corneal cells.

Intestinal delivery

Kotze et al (1997) synthesized TMC by reductive methylation and evaluated the effect of this polymer (1.0–2.5% w/v) on the TEER of intestinal epithelial cells, using Caco-2 cell monolayers. Further, permeation of the hydrophilic model compounds ^{14}C mannitol, FITC-dextran and the peptide drug buserelin in the presence of TMC was studied for 3 h. Confocal laser scanning microscopy was used to visualize the transport process of the fluorescent marker, FITC-dextran 4400, across the cell monolayers. Viability of the cells was checked with the trypan blue exclusion technique. It was observed that TMC (1.5–2.5% w/v) caused a pronounced and immediate reduction (25–85%) in the TEER of Caco-2 cells. A significant increase in the transport rate of ^{14}C mannitol (32–60 fold), FITC-dextran 4400 (167–373 fold) and buserelin (28–73 fold) were observed. It was confirmed by confocal laser scanning microscopy that TMC facilitated the increased transport of hydrophilic compounds through the paracellular transport pathway by opening the tight junctions of intestinal epithelial cells. No deleterious effects to the cells were observed. The study demonstrated the potential use of TMC as an effective absorption enhancer for the delivery of hydrophilic drugs across mucosal surfaces.

Thanou et al (1999) demonstrated that TMCs of high degrees of substitution (TMC60 and TMC40) enhanced the paracellular transport of Texas red dextran in intestinal Caco-2 cell monolayers. Besides this, no substantial cell membrane damage was detected in the Caco-2 cells treated with TMCs as visualized by confocal laser scanning microscopy. The

researchers thus concluded that TMC derivatives were safe absorption enhancers for peptide and protein drug delivery.

Thanou et al (2001c) investigated the enhancing effect of TMC on the enteral absorption of octreotide in juvenile pigs. A bioavailability (F) value of 1.7 ± 1.1% was observed after intrajejunal administration of 10 mg octreotide without any polymer (control solution). However, co-administration of octreotide with 5 and 10% (w/v) TMC at pH 7.4 increased the octreotide absorption by 7.7 and 14.5 fold, respectively. Similarly, a low F value (0.5 ± 0.6%) was observed after intrajejunal administration of 5 mg octreotide solutions. However, co-administration with 5% (w/v) TMC increased the intestinal octreotide bioavailability to 8.2 ± 1.5%. From the results of the study it was concluded that TMC was able to enhance the intestinal absorption of octreotide in pigs.

Jonker et al (2002) reported that TMC enhanced the intestinal paracellular permeation of ^{14}C mannitol. To evaluate the permeation-enhancing properties of the TMC polymers, rat everted intestinal sacs and a single-pass intestinal perfusion method were used. It was shown that the permeation-enhancing effects depend on the degree of quaternization of TMC. In both models, TMC with the highest degree of quaternization (48.8%) exhibited the best permeation-enhancing results.

Thanou et al (2004) investigated the enhancing effect of TMC on the enteral absorption of octreotide in pigs. Intrajejunal administration of 5 mg octreotide solution resulted in low bioavailability (0.5 ± 0.6%), whereas co-administration with 5% (w/v) TMC increased the intestinal octreotide bioavailability to 8.2 ± 1.5%.

Sandri et al (2007) prepared TMC-based nanoparticulate systems intended for the intestinal administration of macromolecules (peptides) and evaluated them for the mucoadhesive and absorption-enhancement properties. TMC with different quaternization degree (QD) was used. The nanoparticles were loaded with model macromolecule fluorescein isothiocyanate dextran (FD4, MW 4400 Da). The intestinal penetration-enhancement properties of nanoparticles were investigated in an in-vitro Caco-2 cell model and an ex-vivo rat jejunum model. The mucoadhesion of the nanosystems was evaluated using excised rat jejunum. All the nanoparticulate systems interacted with the Caco-2 cells, decreasing TEER. The increase in QD of TMC was seen to favour the mucoadhesion, resulting in a prolonged residence time on intestinal mucosa. The nanoparticle penetration into excised rat jejunum tissue, observed by means of confocal laser scanning microscopy, suggested 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. The improvement of mucoadhesion and of nanoparticle internalization with respect to chitosan nanosystems makes the TMC nanosystems suitable carriers for the intestinal absorption of peptides.

Gene delivery

To achieve efficient gene delivery via receptor-mediated endocytosis, a novel polycationic polysaccharide derivative having recognizable branched saccharide residues, *N,N,N*-trimethyl (TM)-chitosan/tetragalactose antenna conjugate (TC-Gal4A20), was synthesized by Murata et al (1997). Besides testing the cellular recognition ability of TC-Gal4A20 conjugate, the possibility of its application as a gene delivery tool was also investigated. The TC-Gal4A20 conjugate showed high affinity to RCA_{120} lectin and its polycation–DNA complex had the capability of specific gene delivery to hepatocytes.

Kean et al (2005) investigated the effect of increasing quaternization of TMC and, therefore, positive charge on cell viability and transfection. Oligomeric and polymeric chitosans were trimethylated and the toxicity and transfection efficiency of these derivatives were tested with respect to increasing degree of trimethylation. The cytotoxicity of polymer and oligomer derivatives alone and of their complexes with plasmid DNA were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on COS-7 (monkey kidney fibroblasts) and MCF-7 (epithelial breast cancer) cells. Transfection efficiency was investigated using the pGL3 luciferase reporter gene on the same cell lines. Higher toxicity was observed in polymeric chitosan derivatives as compared with oligomeric chitosan derivatives at similar degrees of trimethylation. All derivatives complexed pGL3 luc plasmid DNA efficiently at a 10:1 ratio. TMC57 and TMC93 were able to enhance the transfection efficiency of MCF-7 cells by 23 and 50 fold, respectively. Besides this, TMC57 and TMC93 also gave appreciable transfection of COS-7 cells.

Quaternized chitosan vector (i.e. trimethyl derivative of chitosan (TMO)) DNA complexes have been derived forming soluble protonated chitosan to be used as gene carriers. These quaternized chitosan plasmid DNA complexes were prepared with 40% (TMO-40) and 50% (TMO-50) degree of quaternization. Studies regarding their ability as gene carriers were conducted by using COS-1 and Caco-2 cell lines. The transfection efficiency of trimethylated chitosan oligomer and *N*-{1-(2,3-dioleoyoxy)propyl}-*N,N,N* trimethyl ammonium sulfate (DOTAP) in COS-1 as compared with controls (naked DNA) showed that TMO-40 exhibited the highest transfection efficacy. However, no prominent increase in transfection efficacy was observed in the Caco-2 cell line. It was concluded that TMO had the potential to be used as a non-toxic gene carrier (Thanou et al 2002).

Antimicrobial activity

Avadi et al (2004) prepared a quaternized chitosan (i.e. *N*-diethyl methyl chitosan (DEMC)) for antimicrobial activity against *Escherichia coli*. It involved a two-step process via a 2^2 factorial design. Microbial experiments revealed that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were higher for DEMC than for chitosan in acetic acid medium. Further, pH dependence was observed for both DEMC and chitosan. DEMC displayed good antimicrobial activity and was more effective against *E. coli* than was native chitosan (Avadi et al 2004).

Conclusion

TMC, a partially modified polycationic quaternized chitosan derivative, shows excellent solubility over wide pH range (i.e. in neutral and basic environments) besides exhibiting safety and effectiveness as a permeation enhancer especially

for the mucosal delivery of macromolecules like peptides and proteins. Its physicochemical properties surpass native chitosan's inability to solubilize at physiological pH. Thus, it opens new perspectives for use as a permeation enhancer for developing various selective and effective delivery systems for mucosal delivery of large hydrophilic compounds, besides having other biomedical applications.

However, TMC is only suitable for improved delivery and absorption of neutral or basic macromolecular drugs. Further development of new derivatives and the utilization of their unique physical and biologic properties needs to be explored.

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