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Review article

Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs

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Abstract

The absorption enhancing effects of chitosan and its derivatives have been intensively studied in recent years. It has been shown that these compounds are potent absorption enhancers. Chitosan is only soluble in acidic environments and is therefore incapable of enhancing absorption in the small intestine, the main absorption area in the gastrointestinal tract. Special emphasis has been placed on the absorption enhancing properties of *N*-trimethyl chitosan chloride (TMC), a partially quaternised derivative of chitosan, due to its solubility in neutral and basic environments. TMC is prepared by the reductive methylation of chitosan. The degree of quaternisation can be altered by increasing the number of reaction steps or by increasing the reaction time. Although the molecular weight of the polymer increases with addition of the methyl groups, a net decrease in the molecular weight is observed due to a decrease in the chain length of the polymer. TMC, like chitosan, possesses mucoadhesive properties. In vitro studies performed on Caco-2 cell monolayers showed a pronounced reduction in the transepithelial electrical resistance (TEER). TMC is also able to increase the permeation of hydrophilic compounds such as [^{14}C]-mannitol and [^{14}C] polyethylene glycol 4000 ([^{14}C] PEG 4000, MW4000) across the cell monolayers. It was also shown that the degree of quaternisation of the polymer plays an important role on its absorption enhancing properties, especially in neutral environments where chitosan is ineffective as an absorption enhancer. The reduction in TEER is an indication of the opening of the tight junctions located between epithelial cells. Opening of the tight junctions will result in enhancement of absorption via the paracellular route. Confocal laser scanning microscopy confirmed transport of large hydrophilic compounds via the paracellular 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. 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 -jejunal administration. However, TMC has always been administered as a solution in these studies. 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. Recent studies have focused on the development and in vivo evaluation of solid oral dosage forms.

Keywords: Chitosan; *N*-trimethyl chitosan chloride; Absorption enhancer; Peptide drugs; Mucoadhesive; Transepithelial electrical resistance; Tight junctions

1. Introduction

In recent years, chitosan has attracted a great deal of attention as a potential absorption enhancer across mucosal epithelia. This linear polysaccharide derived by *N*-deacetylation of the natural polymer chitin, which is the second most abundant naturally occurring polymer in nature, has already been approved as a food additive in Japan and is believed to be non-toxic. Besides the food industry, chitosan has also been applied in the agricultural and the cosmetic industry and advantages of this polymer include high availability, high biocompatibility, biodegradability and ease of chemical modification. Although chitosan has been widely used by these industries it was only in recent years that its potential application in the pharmaceutical field was identified by several scientists [1–4]. Although chitosan has been extensively studied as a potential absorption enhancer, the polymer is only soluble in an acidic environment. This article will focus mainly on

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N-trimethyl chitosan chloride (TMC), a soluble derivative of chitosan, for the peroral delivery of peptide drugs.

2. Chitosan

Chitosan, $(C_6H_{11}O_4N)_n$, depicted in Fig. 1, consists of linear 1–4 linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-β-D-glucopyranose (GlcN) units which is derived from chitin, the main component of shells of crab, shrimp and krill, by alkaline *N*-deacetylation [5,6]. If the degree of *N*-acetylation of chitin is lowered to less than 50% it becomes soluble in acidic solutions and is referred to collectively as chitosans [7]. The solubility of chitosans (pK_a 5.5–6.5) is obtained only under acidic conditions and is due to the protonation of the amino group of the D-glucosamine monomeric units [8]. Chitosans are polysaccharides individually characterised by their ratio of acetylated to deacetylated units as well as their high molecular weight, both parameters being equally responsible for the properties of the polymer [1].

Different grades of chitosans are commercially available; some of which are ultrapure and even well suited for implantation. These grades depend on the manufacturing process, which is briefly outlined in Fig. 2, and are assessed by determining the levels of heavy metals and proteins present in the chitosan, as well as the pyrogenicity and cytotoxicity of the chitosan [5].

Chitosan is also known for its biological properties namely biocompatibility and degradability, wound healing acceleration, reduced blood cholesterol levels as well as its immune stimulant effect [5].

2.1. Pharmaceutical applications of chitosan

As previously mentioned chitosan has been used for a range of applications ranging from a food additive to a water purification agent as well as for numerous pharmaceutical applications [1,9] The popularity of this polymer is attributed to its high availability and ease of chemical modification due to the many functional groups available for modification. Chitosan has been used in the pharmaceutical industry as a tablet binder [2] as well as a disintegrant [10]. In the low pH range chitosan has gel-forming properties and is used as a drug carrier in hydrocolloids and gel formulations [11]. If compared to other polysaccharides, which are usually neutral or negatively charged,

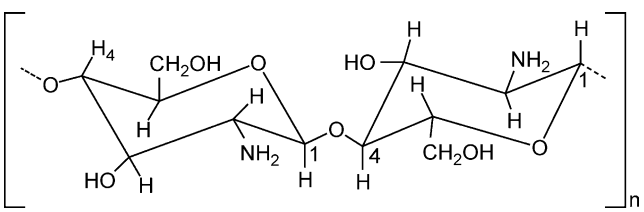

Fig. 1. The chemical structure of chitosan.

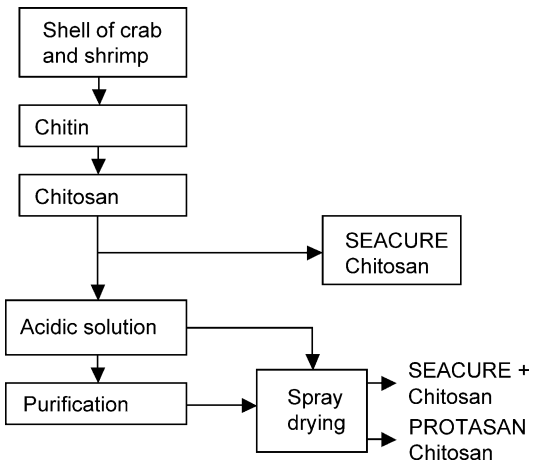

Fig. 2. Production of chitosan (adapted from Ref. [11]).

the uniqueness of this acid soluble, cationic biopolymer is apparent when used in drug delivery systems. The main areas of application for chitosan in drug delivery systems are its use as a constituent in matrix systems, as a bioadhesive—and mucoadhesive material and its use in immobilisation and encapsulation [3,12].

2.2. Chitosan as an absorption enhancer for hydrophilic drugs

In recent years, chitosan has attracted a lot of attention as a potential absorption enhancer across mucosal epithelia especially for peptide drugs [13–15]. Chitosan shows some favourable properties, namely:

- it is not absorbed, due to its high molecular weight, and therefore not expected to display systemic toxicity;
- it intensifies the contact between the dosage form and the site of absorption due to its mucoadhesive properties;
- it improves peptide transport across the epithelial barrier [16].

Lueßen et al. [17] 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. Chitosan glutamate at a pH of 5.60 proved to be able to decrease the transepithelial electrical resistance (TEER) of these human intestinal cells. The TEER was reduced to 45 ± 2% of the control value by a 1% (w/v) chitosan glutamate solution. At concentrations of 0.4 and 1% (w/v), chitosan glutamate strongly increased the transport of DGAVP (M_w = 1412 Da) across the Caco-2 cell monolayers with transport of 1.2% of the total dose applied after 4 h. The similarity in the transport between the two different chitosan glutamate concentrations indicates that at 0.4% (w/v) a maximum in transport rate is reached. Chitosan glutamate also showed a pronounced and comparable improvement of DGAVP absorption across

intestinal mucosae in the vertically perfused loop model [17].

Chitosan glutamate and chitosan hydrochloride, at a pH of 6.20, lead to a pronounced reduction in TEER across Caco-2 cell monolayers [13]. The reduction in TEER, 1 h after apical incubation with 1.5% (w/v) solutions of the polymers was in the following order: chitosan hydrochloride (71 ± 4% reduction) > chitosan glutamate (64 ± 6% reduction). Prolonging the incubation time only resulted in slight decrease in the initial TEER reduction measured after 1 h of incubation. 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 up to 4 h was in the order: chitosan hydrochloride (4.3 ± 0.3% of the total dose applied) > chitosan glutamate (3.0 ± 0.9% of the total dose applied) [13]. Similar increases in the transport of peptide drugs across the Caco-2 cell monolayer were also observed with insulin at a pH of 4.40. The highest increase in the transport of insulin was obtained with chitosan hydrochloride [13].

Lueßen et al. [14] also evaluated the in vivo absorption enhancing effects of chitosan hydrochloride, after intraduodenal administration, on the peptide drug buserelin. In this study, the following polymers were tested: carbomer 934P (C934P), its freeze-dried neutralized sodium salt (FNaC934P) and chitosan hydrochloride. Of all the polymers tested, chitosan hydrochloride (1.5% w/v) in a gel formulation resulted in the highest absolute bioavailability for intraduodenally administered buserelin in rats (5.1 ± 1.5%), even higher than the value reported for the commercial nasal formulation Suprecur® (3.3%) in men.

It is known that the absorption enhancing effect of chitosan on epithelial permeability is dependent on the pH of the solutions and it was seen that the effect of chitosan on the transport of the marker molecule, [^{14}C]-mannitol, is optimal when the pH is well below the pK_a of 6.5. 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 ionised 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 possibility for intimate contact with the epithelial membrane. This suggests that charge density might be of importance for enhancement of mucosal drug absorption [18].

Besides pH, varying the degree of acetylation also controls the charge density of chitosan, since only the GlcN-units are positively charged. The influence of these parameters on the epithelial permeability and toxicity were investigated in monolayers of the human intestinal epithelial cell line (Caco-2). Chitosan with a degree of acetylation between 1 and 49% was used, each prepared in a low and high molecular weight form. To evaluate the toxicity profiles of the chitosans, the mitochondrial dehydrogenase activity of the Caco-2 cells were measured. The effect of the chitosans on cellular morphology was also studied with transmission electron microscopy [4].

The study revealed that the structural properties of chitosan, i.e. molecular weight and degree of acetylation, dictated absorption enhancing properties and toxicity largely. Chitosans with a low molecular weight (22 kDa) and a high degree of acetylation (≥35%) lacked absorption enhancement activity whereas chitosans with a low degree of acetylation and/or high molecular weight increased intestinal epithelial permeability [4]. The correlation between molecular weight, degree of acetylation and effect on epithelial permeability is summarized in Fig. 3.

From these results, 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.

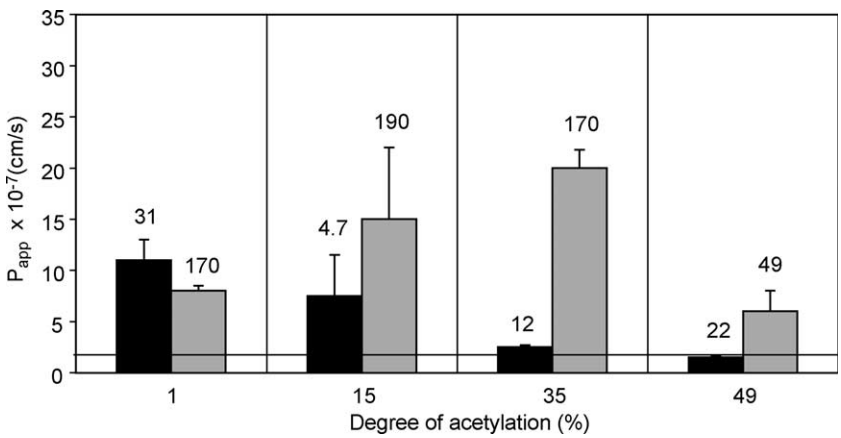

Fig. 3. The mean apparent permeability coefficient (P_{app}) of [^{14}C]-mannitol across Caco-2 cell monolayers during 60 min exposure to 50 μg/ml chitosan. The numbers associated with the bars in the graph show the molecular weight of the studied chitosans in kDa. The P_{app} of [^{14}C]-mannitol across untreated monolayers was 2.4 ± 0.2 (× 10^{-7}) cm/s and is indicated in the figure by the horizontal line. Data represents the mean of 3–4 experiments and the error bars represent SDs (adapted from Ref. [19]).

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 and very low toxicity [4].

Schipper et al. [4] 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, results showed that an unlimited increase in concentration did not lead to an unlimited increase in absorption enhancement. This saturable effect is in direct contrast to the non-saturable effect seen for surfactants and bile salts, thus suggesting a different 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 could be 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 [18]. Confocal laser scanning microscopy (CLSM) 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 visualised by staining the F-actin with the fluorescent probe, rhodamine phalloidin [19].

2.3. The need for chitosan derivatives

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 pK_a value of chitosan (5.5–6.5) As previously mentioned chitosan, a weak base, 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. At this pH, the molecule is most likely to exist in a coiled configuration [18].

In contrast to the reduction of TEER of Caco-2 cell monolayers found after the apical incubation with chitosan hydrochloride and chitosan glutamate at a pH of 6.20, no decrease in TEER, which is a good measurement of the tightness of the junctions between the cells, was observed at a pH of 7.40. At this pH, both chitosan salts did not form clear solutions. In agreement with the results of the TEER experiments, no increase in the transport of the hydrophilic model compound [^{14}C]-mannitol was found at a pH of 7.40 after incubation with these chitosan salts [20].

The peroral route is considered to be the most convenient way of drug application for the patient [6]. Most macromolecular pharmaceuticals such as peptide and protein drugs are indicated for chronic administration and therefore the peroral route will be the most suitable way of administering these drugs. The potential use of chitosans, as absorption enhancer in the more basic environments of the large intestine and colon, are limited. In this regard Kotzé et al. [21] states that chitosan derivatives with different physicochemical properties, especially water solubility at neutral and basic pH values, will be of particular interest as they might prove to be useful as absorption enhancers in these environments.

It was the hypothesis of Kotzé et al. [22] that polymers such as unmodified chitosan with a primary amino group may not be the optimal ones, but that polymers or derivatives with different substituents, different basicities, or different charged densities will have the same or even increased efficacy in opening tight junctions.

As previously mentioned, chitosan is a versatile polymer with many functional groups available for chemical modification. In the past several chitosan derivatives have been synthesised, one of which is TMC [8]. However, these derivatives have only been evaluated for their pharmaceutical applications in the last few years. TMC, a partially quaternised derivative of chitosan, has intensely been studied and described by Kotzé et al. [22] for its absorption enhancing effects. It was concluded that the potential use of TMC, in neutral and basic environments where normal chitosan salts are ineffective as absorption enhancers, could contribute significantly to the effective delivery of hydrophilic compounds such as protein and peptide drugs. Recently another derivative of chitosan was also evaluated for its absorption enhancing ability, namely mono-*N*-carboxymethyl chitosan (MCC) [23]. The absorption enhancing effects of TMC will be discussed in more detail in the following sections.

3. *N*-trimethyl chitosan chloride

3.1. Physicochemical properties of TMC

Kotzé et al. [24], based on the method of Domard et al. [8], synthesized TMC. TMC, depicted in Fig. 4, is a partially quaternised derivative of chitosan which is prepared by reductive methylation of chitosan with methyl iodide in a strong basic environment at an elevated temperature. The degree of quaternisation can be altered by increasing the number of reaction steps or by increasing the reaction time [25,26].

According to Kotzé et al. [24], the initial chitosan used to synthesize TMC was only soluble in acidic solutions, but

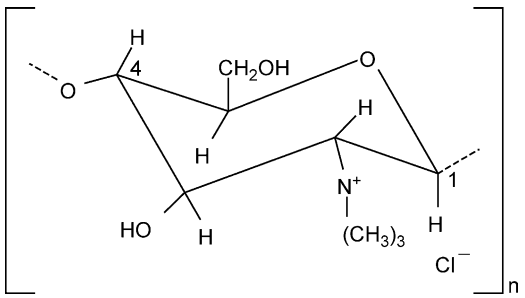

Fig. 4. The chemical structure of TMC.

after quaternisation it became perfectly soluble in water. This increased solubility, either in basic or acidic medium, was even observed for a degree of quaternisation as low as 10% as determined by ^{1}H-NMR spectra. As mentioned previously chitosan hydrochloride and chitosan glutamate are only soluble at 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. A pronounced decrease in the intrinsic viscosity of TMC, compared to the starting material, was observed which was expected, considering the strong reaction conditions under which TMC is synthesised. TMC proved to be a derivative of chitosan with superior solubility and basicity, even at low degrees of quaternisation, compared to chitosan salts. 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 quaternisation (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 degree of quaternisation. 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 specific reaction conditions during the synthesis [26].

3.2. Mucoadhesive properties of TMC

As briefly mentioned chitosan possesses mucoadhesive properties [12]. The same is true for TMC. The mucoadhesive properties of TMC with different degrees of quaternisation, ranging between 22 and 49%, were investigated by Snyman et al. [27]. TMC was found to have a lower intrinsic mucoadhesivity if compared to the chitosan salts, chitosan hydrochloride and chitosan glutamate, but if compared to the reference polymer, pectin, TMC possesses superior mucoadhesive properties. The decrease in the mucoadhesion of TMC compared to the chitosan salts was explained by a change in the conformation of the TMC polymers due to interactions between the fixed positive charges on the quaternary amino groups, which possibly also decreases the flexibility of the polymer molecules. The interpenetration into the mucus layer by the polymer is influenced by a decrease in flexibility resulting in a subsequent decrease in mucoadhesivity [27].

3.3. Effect of TMC on the transepithelial electrical resistance of human intestinal epithelial cells (Caco-2)

Tight junctions serve as barriers to paracellular diffusion and the measurement of the TEER is believed to be a good indication of the tightness of the junctions between epithelial cells [24]. It has also been suggested that measurement of TEER could be used to predict the paracellular transport of hydrophilic molecules [28].

Incubation of intestinal epithelial cells (Caco-2) with TMC with a degree of quaternisation of 12% in concentrations of 1.5, 2.0 and 2.5% (w/v) resulted in an immediate reduction in TEER values. The reduction in TEER was 9 ± 4, 52 ± 3 and 79 ± 0.3%, respectively, after 20 min. Prolonged incubation only resulted in a gradual decrease in resistance compared with the initial reduction in TEER after 20 min. The highest reduction in TEER was measured at a concentration of 2.5% (w/v) thus indicating that the reduction in TEER is concentration dependent [19].

With removal of the polymer solutions, repeated washing and substitution of the apical medium with fresh Dulbecco's Modified Eagles Medium, reversibility of the effects was noticed, especially at 1.5 and 2.0% concentrations of TMC. The monolayers started to recover slowly and a slight increase in resistance, toward the initial values was found. According to Kotzé et al. [21] complete removal of the polymer, without damaging the cells, proved to be difficult due to the high viscosity of the solutions and this might be the reason why the increase in resistance was only gradual. The viability of the monolayers was assessed, after incubation with TMC, by staining the monolayers with trypan blue. No visible uptake of the marker was observed and the authors concluded that the viability of the monolayers was not affected by incubation with TMC.

A comparison of the effect of the chitosan salts, chitosan hydrochloride and chitosan glutamate, and TMC (degree of quaternisation 12.28%) on the TEER across Caco-2 cell monolayers revealed once again that all of these compounds were able to decrease the TEER significantly. The decrease in TEER at 0.25% (w/v) concentrations, 20 min after incubation, was in the order chitosan hydrochloride (71 ± 4% reduction) > chitosan glutamate (56 ± 1% reduction) > TMC (28 ± 1% reduction), suggesting that the chitosan salts were more effective than TMC at similar weight concentrations [24].

According to the authors, the difference in effect of these polymers could be explained in terms of the equivalent weights of each repeating unit in the polymer backbone of the respective polymers (theoretically 197.62 for chitosan hydrochloride, 308.30 for chitosan glutamate and 239.80 for TMC), thus determining the amount of free chitosan base and therefore the density of the amino groups available for protonation at similar weight concentrations. About 50% of chitosan glutamate by weight is the glutamate salt whereas for chitosan hydrochloride the salt part only constitutes a small fraction (5–10%). Additionally, the attached methyl groups on the C-2 position of TMC probably causes steric effects and also partially hide the positive charge on the quaternary amino groups, thereby altering the time needed for interaction with the negatively charged cell membranes and tight junctions [24]. However, at higher concentrations of TMC (2.0–2.5% w/v) similar effects on the TEER, as seen with chitosan hydrochloride, were observed. The better solubility of TMC may therefore compensate for its lower effect at similar weight concentrations.

3.4. Effect of TMC on the absorption enhancement of hydrophilic model compounds

Mannitol and [^{14}C] PEG 4000 are metabolically inert as well as highly hydrophilic in nature and mannitol has been used previously to follow changes in the intestinal epithelial integrity of mucosal cells. Both compounds do not diffuse to a large extent into the cell membranes, but are absorbed through the alternative aqueous paracellular pathway, and are therefore ideal substances to detect changes in permeability in studies of absorption enhancement [4,18].

Table 1 shows a comparison of the effect of TMC (degree of quaternisation 12.28%), chitosan hydrochloride and chitosan glutamate on the permeability of Caco-2 cells at a pH of 6.20 for the hydrophilic marker [^{14}C]-mannitol [24].

Exposure of the apical side of the monolayers to 0.25% of the polymers resulted in a 34-fold (chitosan hydrochloride), 25-fold (chitosan glutamate) and 11-fold (TMC) increase in the absorption rate of [^{14}C]-mannitol, compared to the control group as indicated by the P_{app} values and absorption enhancement ratios (R). This changes to a 36-fold (chitosan hydrochloride), 25-fold (chitosan glutamate) and 11-fold (TMC) increase in the presence of 1.5% (w/v) concentrations of the respective polymers. Similar results were obtained for [^{14}C] PEG 4000. At higher concentrations, TMC was able to increase the P_{app} and R-values further for both [^{14}C]-mannitol and [^{14}C] PEG 4000. A 17- and 21-fold increase in R were found for [^{14}C]-mannitol at 2.0 and 2.5% (w/v) concentrations of TMC, respectively. The same tendency was also seen with [^{14}C] PEG 4000 [24]. From these results, it is evident that TMC was not as effective at similar weight concentrations as chitosan hydrochloride and chitosan glutamate. These results are also similar to the results obtained in the TEER studies referred to in the previous section. The authors concluded that additional factors play a role in the absorption enhancement mechanism of TMC. This could most likely be explained in terms of the charge density, the equivalent weight of each repeating unit in the polymer backbone and possible steric effects of the attached methyl groups and partial hiding of the positive charge on the quaternary ammonium groups [24].

Kotzé et al. [21] also showed that TMC, with a degree of quaternisation of 12%, was able to increase the transport of fluorescein isothiocyanate-labelled dextran (FD-4) across Caco-2 cell monolayers. The transport of this large hydrophilic model compound ($M_w = 4400$ Da) was increased 167-, 274- and 373-fold with 1.5, 2.0 and 2.5% (w/v) concentrations of TMC, respectively.

3.5. Effect of TMC on the absorption enhancement of peptide drugs

As mentioned in a previous section, normally poor bioavailability of peptides and proteins from oral

Table 1
Effect of TMC, chitosan glutamate and chitosan hydrochloride on the permeability of [^{14}C]-mannitol at a pH of 6.20 (adapted from Ref. [24])

Marker	Concentration (% w/v)	TMC		Chitosan glutamate		Chitosan hydrochloride	
		$P_{app} \times 10^{-7}$ (cm/s)[a]	R	$P_{app} \times 10^{-7}$ (cm/s)[a]	R	$P_{app} \times 10^{-7}$ (cm/s)[a]	R
[^{14}C]-Mannitol	Control	0.72 ± 0.08	1	0.72 ± 0.08	1	0.72 ± 0.08	1
	0.25	8.11 ± 0.21[b]	11	18.25 ± 1.10[b]	25	24.65 ± 2.13[b]	34
	0.50	9.26 ± 0.35[b]	13	14.17 ± 0.45[b,c]	20	23.28 ± 1.00[b]	32
	1.00	14.00 ± 0.40[b]	19	20.82 ± 0.30[b]	29	25.56 ± 2.95[b]	36
	1.50	7.52 ± 0.86[b]	10	18.29 ± 1.53[b]	25	26.16 ± 1.86[b]	36
	2.00	12.35 ± 0.43[b]	17	n.d.	n.d.	n.d.	n.d.
	2.50	15.21 ± 1.37[b]	21	n.d.	n.d.	n.d.	n.d.
	2.50	15.21 ± 1.37[b]	21	n.d.	n.d.	n.d.	n.d.

n.d., not determined due to insolubility of chitosan salts; R, absorption enhancement ratio.
[a] Each value represents the mean ± SD of three experiments.
[b] Significantly different from control ($P < 0.05$).
[c] Significantly different from all other treatments in group ($P < 0.05$).

and non-oral mucosal routes is a result of the interplay of poor permeability characteristics, instability towards proteolytic enzymes, cell metabolism and non-enzymatic clearance mechanisms such as the first pass effect and excretion in the bile [29]. Peptides and peptidomimetic agents are large, hydrophilic molecule pharmaceuticals that do not partition into the cell membranes, therefore they are mostly excluded from the transcellular pathway. The absorption of these compounds is for the most part limited to the alternative paracellular pathway which is primarily restricted by the tight junctions [24].

TMC, with a degree of quaternisation of 12%, was not only able to improve the transport of the hydrophilic model compounds [^{14}C]-mannitol, [^{14}C] PEG 4000 and FD-4 across intestinal epithelial membranes [21,24] but was also able, in agreement with these results, to increase the transport of several peptide drugs across Caco-2 cell monolayers.

TMC, in concentrations of 1.5 and 2.5% (w/v), was able to increase the transport of DGAVP ($M_w = 1412$ Da), at a pH of 5.60, to 0.96 ± 0.28 and 1.09 ± 0.08% of the total dose applied, respectively. The control group showed transport of 0.19 ± 0.29% of the total dose applied. TMC was also able to increase the transport of insulin ($M_w = 5778$ Da) at a pH of 4.40 compared to the control where no transport was observed. The transport of insulin was increased to 0.3 ± 0.1 and 0.8 ± 0.1% of the total dose applied at 1.5 and 2.5% (w/v) concentrations of TMC, respectively. An increase in the transport of buserelin ($M_w = 1300$ Da) was also observed at a pH of 6.20. The transport was increased to 1.4 ± 0.2 and 2.7 ± 0.3% of the total dose applied with 1.5 and 2.5% (w/v) solutions of TMC, respectively (control group: 0.04% of total dose applied) [13].

3.6. Effect of the degree of quaternisation of TMC on its absorption enhancing properties

As seen in the previous sections, TMC was not as effective at the same weight per volume concentrations in increasing transport of hydrophilic compounds as the chitosan salts chitosan glutamate and chitosan hydrochloride. Its lesser efficacy was explained by its charge density, which was determined by the degree of quaternisation, and by a partial hiding of the positive charge on the amino group by the attached methyl groups. It was proposed that TMC with higher degrees of quaternisation might be more effective as an absorption enhancer for the increased paracellular transport of hydrophilic compounds in neutral environments [30].

In a study by Kotzé et al. [31], the cumulative transport of [^{14}C]-mannitol and [^{14}C] PEG 4000 in the presence and absence of different concentrations of TMC, with degrees of quaternisation 12 and 20%, respectively, was determined at a pH of 6.20 in Caco-2 cell monolayers. Both the TMC polymers were able to increase the transport of [^{14}C]-mannitol as well as [^{14}C] PEG 4000. However, it was noticed that the permeability decreased with an increase in molecular weight resulting in a much lower transport measured for [^{14}C] PEG 4000 than for [^{14}C]-mannitol. It was suggested that the permeation of these compounds across intestinal epithelial cells depend on their molecular size and structural conformation. Overall, TMC with a degree of quaternisation of 20% was able to increase permeability across the monolayers to a much higher extent than TMC with a degree of quaternisation of 12%. The difference in effect between these two polymers was explained in terms of the charge density of each polymer, as determined by their respective degrees of quaternisation.

Kotzé et al. [30] also investigated the effect of TMC-H (degree of quaternisation 61.2%), TMC-L (degree of quaternisation 12.28%) and chitosan hydrochloride on the TEER and permeability of intestinal epithelial Caco-2 cell monolayers at pH values of 6.20 and 7.40. At a pH of 6.20 all the polymers caused a pronounced reduction (37–67% at 0.5% (w/v) concentrations) in the TEER of Caco-2 cells. On the contrary, at a pH of 7.40 only TMC-H was able to decrease the TEER values, even in a concentration as low as 0.05% (w/v) (35% reduction). Comparable results were obtained with the permeation of [^{14}C]-mannitol. Large increases in the transport rate (18–23-fold at 0.5% (w/v) concentrations) were found at pH 6.20, while only TMC-H was able to increase the permeation of [^{14}C]-mannitol at pH 7.40 (31–48-fold at 0.05–1.5% (w/v) concentrations of TMC-H).

Jonker et al. [32] used both an in vitro (everted intestinal sacs) as well as an in situ method (single pass perfusion) in rats to study the effect of the degree of quaternisation on the absorption of [^{14}C]-mannitol. TMC polymers with degree of quaternisation ranging from 22 to 48% were used in these studies. It was clearly demonstrated in this study that TMC enhances intestinal permeation in a neutral pH environment and that the extent of the absorption enhancement was dependent on the degree of quaternisation of TMC. In both models, the best permeation enhancing results were obtained with the highest degree of quaternistaion.

From the results of these studies, it is clear that TMC, in contrast to the chitosan salts, is a promising absorption enhancer in neutral and basic environments. Although a low degree of quaternisation is not sufficient to produce noticeable absorption enhancement it is easily overcome by increasing the degree of quaternisation.

The results obtained in in vivo studies performed on rats were similar to the results obtained in the in vitro and in situ studies described above. After the nasal administration of semi-synthetic human insulin (4 IU/kg body weight) at a pH of 4.40 with chitosan hydrochloride, TMC-L and TMC-H all the polymers were able to reduce blood glucose levels. Major increases in plasma insulin levels were also found after co-administration with these polymers. No major differences in effect between TMC-L and TMC-H could be demonstrated. At a pH of 7.40 only TMC-H was able to

decrease the blood glucose levels of the rats significantly. Neither chitosan hydrochloride nor TMC-L was able to produce any hypoglycemic response. A 0.5% (w/v) solution of TMC led to a reduction in blood glucose levels, 30 min after co-administration with insulin, of about 34% [22].

Octreotide, a somatostatin analogue used for the control of endocrine tumours of the gastrointestinal tract, has limited oral absorption due to its limited permeation across the intestinal epithelium. This peptide was administered (pH 7.40) with or without the polymers chitosan hydrochloride and TMC (degree of quaternisation 60%), intrajejunally in rats after which the serum peptide levels were measured by radioimmunoassay. A 1.0% (w/v) TMC solution significantly increased the absorption of the peptide analogue, resulting in a 5-fold increase in octreotide bioavailability compared to the controls (octreotide alone). No increase in bioavailability was noticed with co-administration of a 1.0% (w/v) solution of chitosan hydrochloride [33].

Octreotide was also administered to juvenile pigs with or without TMC (degree of quaternisation 60%) at a pH of 7.4. The solutions were administered intrajejunally through an in-dwelling fistula that was inserted 1 week prior to the administrations. Intrajejunal administration of 10 mg of octreotide, co-administered with 5 and 10% (w/v) TMC, resulted in a 7.7 and 14.5-fold increase in octreotide absorption with absolute bioavailabilities of 13.9 ± 1.3 and 24.8 ± 1.8%, respectively. Fig. 5 shows the effect of the polymer concentrations on the intestinal absorption of 10 mg octreotide acetate [34].

It is stated by the author that a gel was obtained with the 10% (w/v) concentration of the polymer. This high concentration of the TMC polymer was chosen to counteract the dilution of the 20 ml administration volume by the luminal fluids and mucus of the intestinal tract and to ensure that substantial amounts of both peptide and enhancer could reach the absorptive site of the intestinal mucosa [34]. Although the results show very high bioavailabilities the impracticality of administering such high concentrations in a solid oral dosage form cannot be overlooked as concentrations of 1–2 g of the polymer have to be administered in an attempt to obtain the same results.

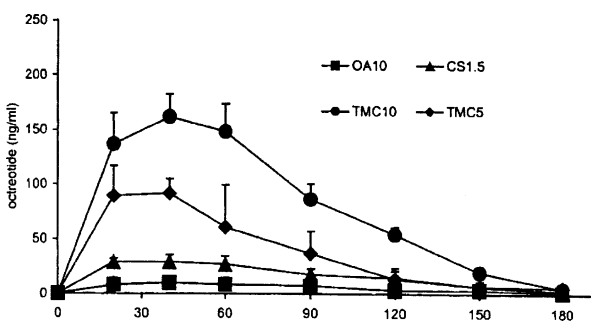

Fig. 5. Plasma octreotide concentration (mean ± SE) versus time curves after intrajejunal administration in pigs (10 mg/20 ml/pig) with the polymers chitosan HCl [CS15: 1.5% (w/v); pH 5.5; $n = 6$] and TMC [TMC10: 10% (w/v); pH 7.4; $n = 6$ and TMC: 5% (w/v); pH 7.4; $n = 3$] or without any polymer [OA10: octreotide in 0.9% NaCl; pH 7.4; $n = 5$] (adapted from Ref. [34]).

3.7. Mechanism of action of TMC and visualisation of the transport pathway

Schipper et al. [19] found that the effect of chitosan on the paracellular permeability is initiated by its direct and specific binding at the cell membrane. This binding could be inhibited by heparin, indicating that the positive charge is important for the binding properties of chitosan. TMC, at all degrees of quaternisation, bears positive charges, independently of the environmental pH. It can therefore be speculated that TMC will also bind to the cell surfaces in a similar way as chitosan.

In a study by Thanou [35], the tight junction's membrane protein occludin was visualised by immunocytochemistry staining in the presence and absence of TMC60 (degree of quaternisation 60%) using CLSM. Additionally, the effects of TMC60 on cytoskeletal F-actin were determined by visualisation using CLSM.

The transmembrane protein occludin displayed a disrupted pattern after incubation with 1.0% (w/v) TMC60, suggesting that the interaction of TMC60 with the tight junction's proteins is the major mechanism for opening the tight junctions and subsequently increased paracellular permeability. These observations were quite similar to images of Caco-2 cells with 0.1% (w/v) chitosan, but the effect appeared to be stronger than that reported for 0.1% (w/v) chitosan. Chitosan treated cells showed a thickened pattern of occludin at the cell periphery and not a disrupted one, which might be due to the 10-fold difference in concentration or to an effect exclusively related to the quaternised derivative of chitosan, TMC60. Schipper et al. [19] stained the protein ZO-1 (a protein related to occludin) to study the effects of chitosans on tight junctions. A distinctive disruption of the ZO-1 patterns similar to the ones observed after occludin staining was observed. In the study by Thanou [35], it was observed that TMC60 provoked a redistribution of the cytoskeletal F-actin, a phenomenon that appeared to correlate well with the opening of epithelial tight junctions.

In order to visualise the transport pathway of FD-4 ($M_w = 4400$ Da), a fluorescent hydrophilic compound, across Caco-2 cell monolayers Kotzé et al. [24] used CLSM. After 60 min incubation with 0.5% (w/v) concentrations of chitosan hydrochloride, chitosan glutamate and TMC (degree of quaternisation 12%) at a pH of 6.20, fluorescence was detected in the intercellular spaces. After incubation with the control solution containing only FD-4, no intracellular or intercellular fluorescence was observed. The fluorescence observed after incubation with TMC clearly demonstrated the ability of these polymers to open these intercellular (paracellular) spaces. Similar results were obtained after incubation with fluorescein

isothiocyanate-labelled dextran with a molecular weight of 19 600 Da (FD-20). A 0.5% (w/v) solution of TMC was able to open the tight junctions resulting in the transport of FD-20 through these paracellular spaces. Similar results were obtained by Thanou et al. [36] with 1.0% (w/v) TMC60 and a texas red-labelled dextran with a molecular weight of 10 000 Da. Transport of these large hydrophilic molecule suggests that other high molecular weight compounds such as peptides and proteins could pass through the paracellular transport pathway.

3.8. Cytotoxic evaluation of TMC

For the evaluation of novel absorption enhancers, safety studies are required to guarantee the absence of tissue damaging effects of the compound under investigation As previously mentioned Schipper et al. [19] observed some toxic effects for certain chitosans, although chitosan in general is considered safe, biodegradable and a non-toxic polymer. Thanou et al. [36] selected Caco-2 cell monolayers to study the possible membrane damaging effects of TMC. The fluorescent probe YO-PRO-1 was used in this cytotoxicity study, which only emits fluorescence upon binding with the nuclei of cells. Cells, which do not take up this fluorescent probe, were considered viable. CLSM horizontal cross sections of Caco-2 cell monolayers treated with 1.0% (w/v) TMC60 for 4 h showed no nuclei staining. The effect of 1.0% (w/v) TMC60 on the ciliary beat frequency of chicken embryo trachea resulted in a slight decrease in this frequency. This decrease in frequency was, however, less pronounced than the decrease observed after incubation with physiological saline (0.9% NaCl) [36]. It was therefore concluded that TMC is a safe absorption enhancer for hydrophilic macromolecules such as peptide and protein drugs across nasal and other mucosal tissues.

3.9. Solid dosage form design and in vivo evaluation

In the past TMC has only been administered as a solution. However, 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 in which TMC can be administered with peptide drugs. To optimally make use of the absorption enhancing properties of TMC in a solid dosage form, the polymer should be able to dissolve rapidly and then be allowed to spread over a wide area of the epithelium in the small intestine. The opening of the tight junctions is a time-dependent process and it is therefore necessary that most of the TMC should be released from the dosage form prior to the release of the peptide drug. The site at which the peptide is released should coincide with the site where the TMC is opening the paracellular pathway for maximum paracellular absorption of the peptide drug.

Minitablets, which are tablets with a diameter of 2–3 mm, and granule formulations were developed as solid oral dosage forms for the delivery of TMC and the peptide drug desmopressin (1-(3-mercaptopropionic acid)-8-D-arginine vasopressin monoacetate; DDAVP). Both the developed minitablet and granule formulations showed an initial burst release of TMC with a delayed release for DDAVP. Maximum release of TMC was in the order of 50% for all formulations, which is acceptable considering the high molecular weight of the polymer [37].

Domestic pigs were used as animal model for the evaluation of the developed minitablet and granule formulations. However, the somatostatin analogue, octreotide, was used as peptide drug in this study and therefore the formulations were slightly adapted. Similar release profiles, from these slightly adapted octreotide containing solid dosage forms, were obtained as described by Van der Merwe et al. [37] for DDAVP. The minitablet and granule formulations were filled into a size 000 capsule after which it was enteric-coated. Octreotide alone, in the same size enteric-coated capsule, was administered as a negative control and a solution with the same amount of TMC and octreotide, as present in the minitablet and granule formulations, was filled into specially developed capsules for the administration of aqueous solutions. A variation in the transit times of the enteric-coated capsules between the stomach and the small intestine was observed as was also noted in a study performed by Dorkoosh et al. [38]. Fig. 6 depicts the mean values obtained for this in vivo study.

Statistical analysis showed no significant difference between the absolute bioavailabilities for the different formulations administered via the peroral route. The average bioavailabilities for the negative control, minitablet formulation, granule formulation and TMC/octreotide solution were, respectively, 0.9 ± 0.5, 1.0 ± 1.5, 1.4 ± 0.5, and $0.5 \pm 0.2\%$. Delayed absorption of octreotide was observed from the minitablets, which did not correspond to the in vitro release profiles obtained for the minitablet and granule formulations where almost all of

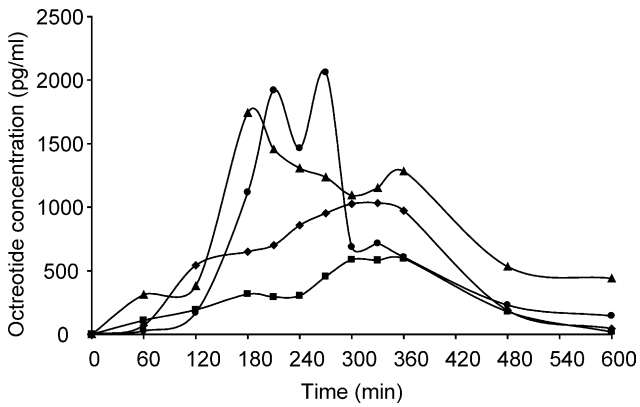

Fig. 6. Mean plasma profiles of octreotide after peroral administration in pigs (10 mg/pig): (◆) Only octreotide without TMC; (●) Minitablets; (▲) Granule formulation; (■) Octreotide and TMC solution. $N = 6$, except in the case of minitablets where $n = 5$. Error bars are omitted for reasons of clarity.

the peptide drug present in the formulations was released within an hour. Gelatin is known to form a sticky mass upon contact with aqueous solutions. The combination of the gelatin, the enteric-coating and the sticky properties of TMC might have resulted in a delay of the release of both the octreotide and the TMC, resulting in unsatisfactory absorption enhancement with the polymer. However, it seemed as if a trend existed towards an increase in absorption enhancement with the granule formulation as was concluded from the higher absolute bioavailability as well the higher peak plasma concentrations obtained with this formulation.

As previously mentioned in a study performed by Thanou et al. [34] very high concentrations of TMC in solution with octreotide were administered to pigs. The TMC solution had the consistency of a gel and was administered directly in the jejunum of the pigs by means of surgical procedures. Results from this study showed very high absolute bioavailability values. However, if a solid dosage form should be prepared containing these large amounts of TMC (2 g per dosage form) an unrealistically large dosage form will be the result, which will probably be impossible to swallow. It was concluded that the amount of TMC that should be delivered to obtain an absorption enhancing effect was not sufficient in this study and was also confirmed by the solution of TMC that was administered in the specially designed capsules. Although the intrinsic mucoadhesive properties of TMC compares well to that of Carbopol 934P [27], which is known to possess excellent mucoadhesive properties, it seems that in these small amounts it is not sufficient to localise the polymer against the intestinal wall of the small intestine for interaction with the tight junctions and cell surfaces.

Both the minitablet and granule formulations show promise in the delivery of TMC as absorption enhancer. Current studies are focusing on finding a balance between the amount of TMC needed for significant absorption enhancement of peptide drugs, a realistic size of the solid oral dosage form as well as the amount necessary of the polymer to ensure localised delivery of both TMC and the drug.

4. Conclusion

In the previous sections an overview of the diverse pharmaceutical applications of chitosan is given and special emphasis is placed on the use of chitosan as an absorption enhancer. Chitosan is able to allow paracellular transport by opening the tight junctions of epithelial cells, but this absorption enhancing effect is only possible in acidic environments due to the insolubility of this polymer in neutral and basic environments. There is, however, a need to use chitosan in more neutral and basic environments such as those found in the large intestine and colon.

The partially quaternised derivative of chitosan, TMC, shows excellent solubility over a wide pH range, suggesting that it can be used as an absorption enhancer in neutral and basic environments. These enhancing effects are demonstrated by a decrease in TEER values across epithelial cell monolayers (Caco-2) as well as the increase in transport of large hydrophilic compounds across these monolayers at neutral pH values. Similar results were also obtained in in vivo experiments, where an increase in intestinal peptide drug absorption was observed. It has also shown that the degree of quaternisation of TMC plays an important role in its absorption enhancing properties, especially in neutral environments. Cytotoxic evaluation of the polymer showed that it is safe to use as absorption enhancer in mucosal delivery of peptide drugs. Minitablet and granule formulations were the first formulations to be developed for oral delivery of TMC as absorption enhancer with a peptide drug. Both formulations showed promise, but still have to be optimised.

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