ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 47 (1999) 269–274

European Journal of Pharmaceutics and Biopharmaceutics

Research paper

Effect of the degree of quaternization of *N*-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2)

Awie F. Kotzé[a,b,*], Maya M. Thanou[b], Henrik L. Lueßen[b,c], (A)Bert G. de Boer[d], J. Coos Verhoef[b], Hans E. Junginger[b]

[a]*Department of Pharmaceutics, Potchefstroom University for Christian Higher Education, Potchefstroom, South Africa*
[b]*Department of Pharmaceutical Technology, Leiden University, Leiden, The Netherlands*
[c]*LTS Lohmann Therapie-Systeme GmbH, Andernach, Germany*
[d]*Department of Pharmacology, Leiden University, Leiden, The Netherlands*

Received 12 March 1998; accepted 14 December 1998

Abstract

N-trimethyl chitosan chloride (TMC), a partially quaternized derivative of chitosan with superior water solubility, was synthesized with different degrees of quaternization [12.6% quaternized (TMC-L) and 19.9% quaternized (TMC-H)] and the effects of these novel polymers on the permeability of intestinal epithelial cells were investigated in Caco-2 cell monolayers. Transepithelial electrical resistance (TEER) measurements showed that both polymers in 1.5–2.5% w/v concentrations caused a pronounced, concentration dependent lowering in TEER values, but that TMC-H was more effective than TMC-L at similar concentrations (36 ± 3% reduction with TMC-L and 53 ± 6% reduction with TMC-H at 2.0% concentrations). Similar results were obtained in transport studies with the hydrophilic radioactive markers [^{14}C]mannitol (MW 182.2) and [^{14}C]polyethylene glycol 4000 ([^{14}C]PEG 4000, MW 4000). The transport of [^{14}C]mannitol was increased 51-fold (TMC-L) and 97-fold (TMC-H) at 2.5% concentrations. No deleterious effects to the cells could be demonstrated with trypan blue exclusion studies. The results show that TMC is able to open the tight junctions of intestinal epithelial cells to allow for paracellular transport of hydrophilic molecules. It is concluded that the charge density of TMC, as determined by the degree of quaternization, is an important factor determining its potential use as an absorption enhancer across intestinal epithelia.

Keywords: N-trimethyl chitosan chloride; Degree of quaternization; Absorption enhancer; Paracellular transport; Transepithelial electrical resistance; Caco-2 cells

1. Introduction

Previous studies have established that the mucoadhesive polymer chitosan is a potent enhancer of drug transport across mucosal surfaces. It has been shown that chitosan salts like chitosan glutamate and chitosan hydrochloride were able to reduce the transepithelial electrical resistance in vitro in a cultured human intestinal epithelial cell line (Caco-2) [1,2] and that these polymers were able to increase the transport of hydrophilic molecules such as [^{14}C]mannitol, [^{14}C]polyethylene glycol and fluorescein-dextran significantly in Caco-2 cell monolayers [1–3]. Perhaps more significant is the use of chitosan as an absorption enhancer in vivo for peptide drugs. The intraduodenal administration of buserelin with chitosan hydrochloride in a gel formulation increased the absolute bioavailability of buserelin from 0.1 to 5.1% [4], while the nasal application of insulin with chitosan glutamate led to a significant reduction in blood glucose levels in rats and sheep [5]. The increase in the transport of these 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

* Corresponding author. Department of Pharmaceutics, Potchefstroom University for Christian Higher Education, Potchefstroom, 2520, Republic of South Africa. Tel.: +27-18-299-2249; fax: +27-18-299-2251; e-mail: fmsafk@puknet.puk.ac.za

0939-6411/99/$ - see front matter
PII: S0939-6411(99)00006-5

cells. It has been reported that chitosan induces a redistribution of cytoskeletal F-actin and the tight junction protein ZO-1 [3,6], thereby altering the integrity of the tight junctions which is associated directly or indirectly with the F-actin filaments [7]. Confocal laser scanning microscopy has confirmed that chitosan is able to open the tight junctions to allow for the paracellular transport of large hydrophilic compounds [1,2].

However, chitosan is a polycation (apparent pK_a 5.5) and in neutral and basic environments, as found in the large intestine and colon, the chitosan molecules will lose their charge and precipitate from solution, rendering it unsuitable for use as an absorption enhancer in these environments. In all the above mentioned studies, absorption enhancement was only found in acidic or slightly acidic environments. We have shown recently that a partially quaternized derivative of chitosan, N-trimethyl chitosan chloride (TMC), could be prepared by a relatively easy chemical procedure and that this polymer has excellent water solubility, even at low degrees of quaternization, over a wide pH range compared with chitosan salts. TMC reduces the TEER of a cultured epithelial cell line (Caco-2) in a reversible way at concentrations of 1.5% and higher, and is able to increase the transport of several hydrophilic compounds such as [^{14}C]mannitol (MW 182.2), fluorescein-dextran (FD-4, MW 4400) and the peptide drugs buserelin (MW 1300), 9-desglycinamide, 8-arginine vasopressin (DGAVP, MW 1412) and porcine insulin (MW 5778) in Caco-2 cell monolayers (8-9). Confocal laser scanning microscopy confirmed that TMC acts in a similar way as chitosan, by opening tight junctions to allow for the paracellular transport of these compounds [2,8]. Although it was shown that TMC was not quite as effective as other chitosan salts at similar concentrations, such as chitosan glutamate and chitosan hydrochloride, the superior water solubility of TMC, especially at neutral and basic pH values, may compensate for its lesser activity in acidic mediums. The results of these investigations clearly showed that the charge, charge density and structural configuration of chitosan and the chitosan derivative, TMC, play an important role in their ability to act as absorption enhancers across intestinal epithelia.

It was concluded that the potential use of TMC could be an important contribution towards effective delivery of hydrophilic compounds, in the more neutral and basic environments of the large intestine and colon [2,8]. The present study investigates the effects of the degree of quaternization, which determines the charge density of TMC, on the permeability of intestinal epithelial cells.

2. Materials and methods

2.1. Synthesis and characterization of N-trimethyl chitosan chloride (TMC)

Two batches of chitosan, with degrees of deacetylation of 93 and 83%, respectively, were kindly provided by Pronova Biopolymer (Drammen, Norway). TMC was synthesized from sieved fractions (<500 μm) of the different grades of chitosan based on methods described previously [10,11]. Briefly, the experimental conditions are reductive methylation of the chitosan for 75 min with iodomethane in a strong basic environment at 60°C. The counterion (I^-), of the modified polymers recovered, was exchanged with Cl^- by dissolving the quaternized polymers in a small quantity of water, followed by the addition of HCl in methanol. The degree of quaternization of the polymers was calculated from ^{1}H NMR spectra (600 MHz) obtained with a Bruker DMX-600 spectrometer. Viscosity measurements on all the polymers (0.1% w/v) were performed on a Haake rotation viscosimeter (Haake CV 100/ME 30, Haake, Karlsruhe, Germany) at 23.5°C in aqueous acetic acid (0.1% v/v). The pK_a values were obtained by potentiometric titration. From the synthesis two TMC products were obtained, one with 12.6% of quaternization (TMC-L) and one with 19.9% of quaternization (TMC-H).

2.2. Cell cultures

Caco-2 cells (passages 76–79) were seeded on tissue culture treated polycarbonate filters (area 4.7 and 0.33 cm^2) in Costar Transwell 6- and 24-well plates, respectively (Costar Europe, Badhoevedorp, The Netherlands) at a seeding density of 10^4 cells/cm^{-2}. Dulbecco's Modified Eagle's Medium [DMEM, pH 7.40] (Sigma, Bornem, Belgium), supplemented with 1% non-essential amino acids, 10% foetal bovine serum, benzylpenicillin G (160 U/ml) and streptomycin sulphate (100 μg/ml) (all obtained from Sigma), was used as culture medium, and added to both the donor and acceptor compartments. The medium was changed every second day and cell cultures were kept at a temperature of 37°C in an atmosphere of 95% air and 5% CO_2. Filters were used for transepithelial electrical resistance measurements (24-well plates) and transport experiments (6-well plates) 21–23 days after seeding [8,9,12,13].

2.3. Measurement of the transepithelial electrical resistance (TEER)

A Millicell® ERS meter (Millipore, Bedford, MA), connected to a pair of chopstick electrodes, was used to measure the effect on the TEER of the filters every 20 min [8,9,12,13]. Solutions of TMC-H (1.5–2.5% w/v), synthesized from chitosan 93% deacetylated and TMC-L (1.5–2.5% w/v), synthesized from chitosan 83% deacetylated, were prepared in serum-free DMEM (pH 7.40). The pH of the resulting polymer solutions was adjusted to 6.80 since it was found that dissolving TMC in DMEM led to a slight decrease in the pH (6.70–7.00) of DMEM. This is probably caused by traces of HCl, from the synthesis procedure, in the TMC samples. Two hours before the start of each experiment, the medium in the acceptor compartments

was removed and replaced with DMEM buffered at pH 7.40 with 25 mM n-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) (HEPES) (Sigma). Measurements started 1 h prior to incubation on the apical side of the cells with the different TMC solutions. The TEER was measured for a period of 4 h. Control experiments were performed under the same conditions without dissolved polymers. Experiments were done in triplicate at 37°C in an atmosphere of 95% air and 5% CO_2. Average TEER values for untreated cell monolayers were in the range of 800–1000 Ω cm^2.

2.4. Permeability studies

2.4.1. [^{14}C]Mannitol transport

[^{14}C]Mannitol (MW 182.2; specific radioactivity 57 mCi/mmol, 200 mCi/ml) was obtained from Amersham Life Science (Little Chalfort, UK). The transport of [^{14}C]mannitol across Caco-2 cell monolayers was studied as described previously [1,2,8]. Solutions of TMC-H and TMC-L (1.5–2.5% w/v) were prepared in serum-free DMEM (pH 7.40) containing the radioactive marker. The pH of the polymer solutions were adjusted to 6.80. The medium in the acceptor compartment was DMEM buffered at pH 7.40 with HEPES. Samples of 200 μl were taken every 20 min for 4 h from the basolateral side. Samples taken from the basolateral side were replaced with an equal volume of DMEM with HEPES. Controls were run in every experiment with solutions containing the radioactive marker without the dissolved polymers. All experiments were done in triplicate in an atmosphere of 95% air and 5% CO_2 at 37°C. The total radioactivity applied to the cells was determined in 200 μl samples of the solutions tested and background radioactivity was determined in 200 μl samples of DMEM and HEPES without the radioactive marker. The radioactivity present in the samples was determined after adding 3 ml scintillation cocktail (Ultima-Gold, Packard Instruments, Meridan, USA) in a liquid scintillation counter (Tri -Carb 1500, Packard Instruments, Meridan, USA). Results were corrected for dilution and expressed as cumulative transport at time *t*.

2.4.2. [^{14}C]Polyethylene glycol 4000 ([^{14}C]PEG 4000) transport

[^{14}C]PEG 4000 (MW 4000; specific radioactivity 50 mCi/ml) was obtained from Amersham Life Science (Little Chalfort, UK). The transport of [^{14}C]PEG 4000 across Caco-2 cell monolayers was studied as described above for [^{14}C]mannitol.

2.5. Viability of Caco-2 cell monolayers

Both the apical and basolateral sides of the cell monolayers were rinsed twice with 0.01 M phosphate-buffered saline (PBS, pH 7.40) after completion of all the TEER and transport experiments. The cell monolayers were incubated apically with a solution of 0.1% trypan blue (Sigma) in PBS [1,2,8]. The basolateral medium was PBS. After 30 min, the medium was removed from both sides of the cell monolayers and the cell monolayers were examined by light microscopy for exclusion of the marker. Cells incubated for 5 min with 0.5% w/v sodium dodecyl sulphate in PBS and stained with trypan blue were used as a reference for uptake of the marker. Cells excluding trypan blue were considered to be viable.

2.6. Data analysis

Apparent permeability coefficients (P_{app}) for [^{14}C]mannitol and [^{14}C]PEG 4000 were calculated according to the following Eq. [2,3,8]:

$$P_{app} = [dc/dt].[1/(A.60.C_0)] \quad (1)$$

where P_{app} is the apparent permeability coefficient (cm/s), dc/dt is the permeability rate (concentration unit/min), A is the diffusion area of the monolayer (cm^2) and C_0 is the initial concentration of the respective radioactive compounds. All rate constants were obtained from the permeation profiles of each radioactive compound. The regression coefficients (r^2) obtained from the curve fits were generally between 0.90–1.00. Transport enhancement ratios (R) were calculated from P_{app} values by Eq. (2) [2,8]:

$$R = P_{app}\ (\text{specific polymer and concentration})/P_{app}(\text{baseline}) \quad (2)$$

3. Results and discussion

3.1. Synthesis and characterization of TMC

In Table 1, the physical and chemical properties of the two batches of TMC, synthesized from the chitosan with different degrees of deacetylation, are shown. In contrast with the parent chitosan, which is only soluble in acidic mediums, both batches of TMC are perfectly soluble in water over the whole pH range in concentrations up to 10%. The increase in solubility could be attributed to the replacement of the primary amino groups on the C-2 position of chitosan with quaternary amino groups [10,11]. In general, TMC is soluble for a degree of quaternization as low as 12.6%, as determined from ^{1}H NMR spectra. Although the pK_a values and viscosities of the two TMC batches do not differ from each other, the degree of quaternization (19.9%), calculated from ^{1}H MNR spectra, is much higher in the batch (TMC-H) synthesized from the chitosan with the higher degree of deacetylation. This could be explained in terms of the amount of amino groups available for methylation in each batch of chitosan. The decrease in the intrinsic viscosity of TMC, compared with the starting materials, correlated well with the degradative reaction conditions (temperature of 60°C) in a strong alkaline medium.

Table 1
Physical and chemical properties of chitosan and TMC*

Polymer	Degree of quaternization (%)	pK_a	Viscosity [0.1% w/v] (mPa/s)	[1]Solubility			
				pH 4	pH 6	pH 7	pH 9
Chitosan (83% deacetylated)	–	5.5 ± 0.0	19.57 ± 0.0	Soluble	Soluble	*Insoluble*	*Insoluble*
TMC-L	12.6	6.0 ± 0.1	1.48 ± 0.0	Soluble	Soluble	Soluble	Soluble
Chitosan (93% deacetylated)	–	5.5 ± 0.1	3.64 ± 0.0	Soluble	Soluble	*Insoluble*	*Insoluble*
TMC-H	19.9	6.0 ± 0.1	1.20 ± 0.0	Soluble	Soluble	Soluble	Soluble

*Data are presented as the mean ± SD of three experiments. [1]Solubility, soluble and insoluble for chitosan (1.5% w/v) and TMC (1–10% w/v). Measurements performed in water (pH adjusted with 0.1 M HCl or 0.1 M NaOH).

NMR spectra show that a high proportion of the amino groups is still dimethylated. Dimethylation is significantly decreased by repeating the basic reaction twice to obtain higher degrees of quaternization [11]. However, at higher degrees of quaternization evidence of O-methylation on the 3 and 6 hydroxyl groups of chitosan is found. In general, O-methylation led to less soluble products. These results show that the degree of quaternization of TMC can be controlled by using different deacetylation grades of chitosan or by controlling the reaction steps.

3.2. Effect of TMC on the TEER of intestinal epithelial cells

The effect of TMC-L and TMC-H on the TEER of Caco-2 cell monolayers is presented in Fig. 1. Measurement of TEER values is believed to be a good indication of the tightness of the junctions between cells. The results in Fig. 1 clearly demonstrate that TMC-L and TMC-H are able to decrease the TEER of Caco-2 monolayers. Incubation on the apical side of the monolayers with 1.5–2.5% w/v of the polymers resulted in a marked reduction in TEER values compared with the control group. Both polymers caused an immediate reduction in TEER, especially at 2.0 and 2.5% concentrations. The effect of TMC seems to be concentration dependent, with the highest reduction in TEER measured at 2.5% concentrations of the respective polymers.

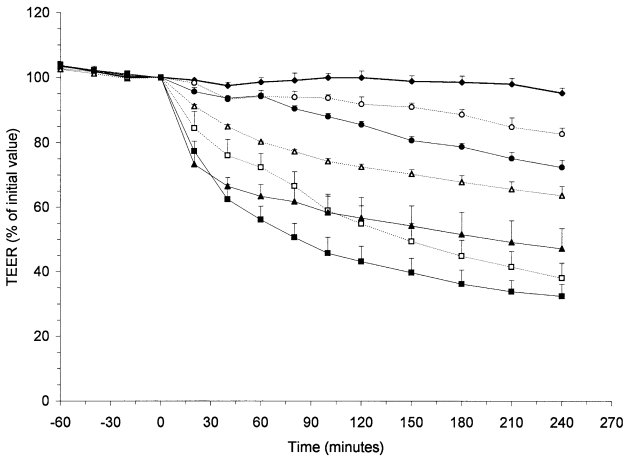

Fig. 1. Effect of TMC-L and TMC-H on the TEER of Caco-2 cell monolayers. Each point represents the mean ± S.D. of three experiments. Control (♦), TMC-L 1.5% (○), TMC-H 1.5% (●), TMC-L 2.0% (△), TMC-H 2.0% (▲), TMC-L 2.5% (□), TMC-H 2.5% (■).

The results presented here show that TMC-H, at similar concentrations, is more effective in reducing the TEER than TMC-L. The decrease in TEER at 2.0% w/v concentrations was in the order TMC-H (53 ± 6% reduction) > TMC-L (36 ± 3% reduction). At 2.5% concentrations the TEER was reduced by 68 ± 4% (TMC-H) and 62 ± 5% (TMC-L), respectively. The difference in effect at similar concentrations of TMC could be explained by the difference in the degree of quaternization of these polymers. The degree of quaternization gave an indication of the charge density on the respective polymers. TMC-H, with the highest degree of quaternization, has more positively charged (quaternary) amino groups for interaction with the negative sites on the cell membranes and is therefore more effective in reducing the TEER of the monolayers at similar concentrations of TMC-L. Several studies have shown that chitosan acts primarily by an interaction of the positively charged amino group on the C-2 position with negatively charged sites on the cell membranes and/or tight junctions [2,3,6]. In previous studies, it has been proposed that TMC most likely acts by the same mechanism as chitosan salts [2,8,9].

Previous studies have shown that it is unlikely, because of the high viscosity and mucoadhesive character of chitosan and TMC, that all the polymer solution could be removed from the monolayers without damaging the cells. Only a gradual reversibility of the effect of these polymers on the TEER was demonstrated while complete reversibility towards initial TEER values could not clearly be established [2,8]. However, staining with trypan blue, after completion of the TEER experiments, did not result in any visible intracellular uptake of this marker. The absence of intracellular trypan blue, after 4 h of incubation with both TMC-L and TMC-H, implies that the cell membranes remained undamaged. Similar results were obtained in a recent study where Caco-2 cell monolayers were able to exclude the propidium iodide nucleic stain after 4 h of incubation with

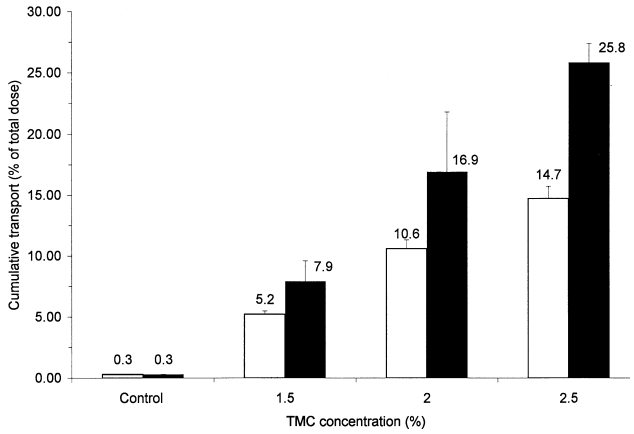

Fig. 2. Cumulative transport of [^{14}C]-mannitol by TMC-L (white bars) and TMC-H (black bars). Each point represents the mean ± S.D. of three experiments.

several TMC polymers with different degrees of quaternization [14].

3.3. Effect of TMC on the permeability of intestinal epithelial cells

Fig. 2 and Fig. 3 show the cumulative transport of [^{14}C]mannitol and [^{14}C]PEG 4000, expressed as percentage of the total dose applied, in the presence and absence of different concentrations of TMC-L and TMC-H. From the individual permeation profiles of the radioactive markers, P_{app} values and transport enhancement ratios (R) were calculated. These results are presented in Table 2. Both mannitol and PEG 4000 are metabolically inert and are highly hydrophilic in nature. Under the conditions described, very low baseline permeabilities were found for both compounds and only negligible amounts were transported in the control groups. Incubation of the monolayers with both TMC-L and TMC-H resulted in a marked accumulation of [^{14}C]mannitol and [^{14}C]PEG 4000 in the acceptor compartment. [^{14}C]Mannitol, with the lowest molecular weight, exhibited the highest permeability and the cumulative amounts transported up to 4 h, at 2.5%w/v concentrations of the polymers, were 25.8 ± 1.6% (TMC-H) and 14.7 ± 1.0% (TMC-L) of the total dose applied, respectively (control: 0.28 ± 0.02%). This represents a 97-fold (TMC-H) and a 51-fold (TMC-L) increase in the permeability compared with the control situation (Table 2). The permeability decreased with an increase in molecular weight and the cumulative amounts of [^{14}C]-PEG 4000, transported up to 4 hours, were much lower than with [^{14}C]mannitol at similar polymer concentrations. At 2.5% concentrations a 17-fold and 4-fold increase in permeability were found after incubation with TMC-H and TMC-L, respectively. This suggests that the permeation of these compounds across intestinal epithelial cells is, among other factors, dependent on their molecular size and structural conformation.

In agreement with the TEER results, higher P_{app} and R values for both radioactive marker molecules were found with TMC-H as with TMC-L at every concentration tested of the respective polymers. This could be explained in terms of the charge density of each polymer, as determined by their respective degrees of quaternization. In agreement with results obtained with the TEER experiments, no evidence of trypan blue inclusion into the intracellular spaces of the cells was found when cells were stained with this dye after completion of all transport studies, which is a good indication that the cell membranes are not affected by incubation with any concentration of these polymers.

4. Conclusion

Chitosan is a weak base which requires a certain amount of acid to transform the glucosamine units into the positively charged, water-soluble form. Therefore, it is only effective as an absorption enhancer in acidic environments. In previous studies, the prospects for derivatives of chitosan, soluble in neutral and basic environments, have been highlighted [2,8,9]. The present study confirms that TMC is a partially quaternized derivative of chitosan with superior water solubility, especially in neutral and basic environments compared to chitosan and chitosan salts [2]. Even at a degree of quaternization as low as 12.6%, TMC becomes perfectly soluble in neutral and basic pH environments and is able to increase the transport of hydrophilic compounds across intestinal epithelia in a neutral environment. These investigated derivatives of chitosan are extremely effective in enhancing the paracellular transport of small hydrophilic molecules, such as [^{14}C]mannitol, but the transport of larger molecules, such as [^{14}C]PEG 4000, is also increased substantially. TMC reduces the TEER of intestinal epithelial cells, thereby opening the tight junctions to allow for the paracellular transport of these hydrophilic molecules. The degree of quaternization of TMC plays an important role in determining the ability and effectiveness

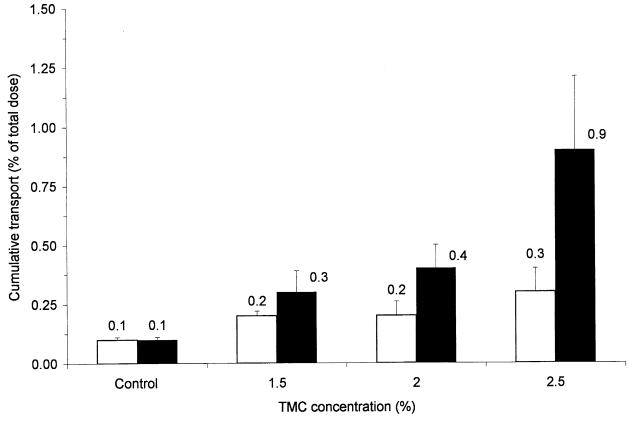

Fig. 3. Cumulative transport of [^{14}C]-PEG 4000 by TMC-L (white bars) and TMC-H (black bars). Each point represents the mean ± S.D. of three experiments.

Table 2
Effect of TMC-L and TMC-H on the permeability of [^{14}C]-mannitol and [^{14}C]-PEG 4000 across Caco-2 cell monolayers*

Marker	Polymer concentration (% w/v)	TMC-L		TMC-H	
		$P_{app} \times 10^{-7}$ (cm/s)	R	$P_{app} \times 10^{-7}$ (cm/s)	R
[^{14}C]-Mannitol	–	0.41 ± 0.03	1	0.41 ± 0.03	1
	1.5	7.97 ± 0.40	19	11.63 ± 3.14	28
	2.0	14.37 ± 0.50	35	22.95 ± 5.16	56
	2.5	21.07 ± 1.32	51	39.63 ± 2.62	97
[^{14}C]-PEG 4000	–	0.08 ± 0.01	1	0.08 ± 0.01	1
	1.5	0.28 ± 0.03	4	0.41 ± 0.14	5
	2.0	0.32 ± 0.09	4	0.59 ± 0.16	7
	2.5	0.33 ± 0.11	1	1.39 ± 0.49	17

*P_{app}, apparent permeability coefficient; R, transport enhancement ratio. Data presented as the mean ± SD of three experiments.

of TMC to open the tight junctions, which regulates permeation through the paracellular transport pathway. The degree of quaternization determines the amount and density of the positive charges on the C-2 position of this derivative, which could result in different effects on the TEER and permeability of Caco-2 cells. We conclude that the potential use of TMC can be an important contribution towards the development of effective delivery systems for hydrophilic compounds such as peptide drugs, especially in the more neutral and basic environments of the large intestine and colon, where chitosan is not effective as an absorption enhancer.

Acknowledgements

This study was supported in part by grants from The South African Druggist Group, the Foundation for Pharmaceutical Education of the Pharmaceutical Society of South Africa and the State Scholarship Foundation of Greece. The authors wish to thank John Beliën and Alex Sieval for their help in the synthesis of TMC.

References

[1] G. Borchard, H.L. Lueßen, A.G. de Boer, J.C. Verhoef, C.-M. Lehr, H.E. Junginger, The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption. III. Effects of chitosan glutamate and carbomer on epithelial tight junctions in vitro, J. Control. Rel. 39 (1996) 131–138.

[2] A.F. Kotzé, H.L. Lueßen, B.J. de Leeuw, A.G. de Boer, J.C. Verhoef, H.E. Junginger, Comparison of the effect of chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells, J. Control. Rel. 51 (1998) 35–46.

[3] P. Artursson, T. Lindmark, S.S. Davis, L. Illum, Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2), Pharm. Res. 11 (1994) 1358–1361.

[4] H.L. Lueßen, B.J. de Leeuw, M.W.E. Langemeÿer, A.G. de Boer, J.C. Verhoef, H.E. Junginger, Mucoadhesive polymers in peroral drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug Buserelin in vivo, Pharm. Res. 13 (1996) 1666–1670.

[5] L. Illum, N.F. Farraj, S.S. Davis, Chitosan as a novel nasal delivery system for peptide drugs, Pharm. Res. 11 (1994) 1186–1189.

[6] N.G.M. Schipper, S. Olsson, J.A. Hoogstraate, A.G. de Boer, K.M. Vårum, P. Artursson, Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement, Pharm. Res. 14 (1997) 923–929.

[7] J.L. Madara, Intestinal absorptive cell tight junctions are linked to cytoskeleton, Am. J. Physiol. 253 (1987) C171–C175.

[8] A.F. Kotzé, H.L. Lueßen, B.J. de Leeuw, A.G. de Boer, J.C. Verhoef, H.E. Junginger, N-trimethyl chitosan chloride as a potential absorption enhancers across mucosal surfaces: in vitro evaluation in intestinal epithelial cells, Pharm. Res. 14 (1997) 1197–1202.

[9] A.F. Kotzé, B.J. de Leeuw, H.L. Lueßen, A.G. de Boer, J.C. Verhoef, H.E. Junginger, Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelia: in vitro evaluation in Caco-2 cell monolayers, Int. J. Pharm. 159 (1997) 243–253.

[10] A. Domard, M. Rinaudo, C. Terrassin, New method for the quaternization of chitosan, Int. J. Macromol. 8 (1986) 105–107.

[11] A.B. Sieval, M. Thanou, A.F. Kotzé, J.C. Verhoef, J. Brussee, H.E. Junginger, Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride, Carbohydrate Polymers 36 (1998) 157–165.

[12] A.B.J. Noach, Y. Kurosaki, M.C.M. Blom-Roosemalen, A.G. de Boer, D.D. Breimer, Cell-polarity dependent effect of chelation on the paracellular permeability of confluent Caco-2 cell monolayers, Int. J. Pharm. 90 (1993) 229–237.

[13] M.A. Hurni, A.B.J. Noach, M.C.M. Blom-Roosemalen, A.G. de Boer, J.F. Nagelkerke, D.D. Breimer, Permeability enhancement in Caco-2 cell monolayers by sodium salicylate and sodium taurodihydrofusidate: assessment of effect reversibility and imaging of transepithelial transport routes by confocal laser scanning microscopy, J. Pharmacol. Exp. Ther. 267 (1993) 942–950.

[14] M. Thanou, A.F. Kotzé, T. Scharringhausen, H.L. Lueßen, A.G. de Boer, J.C. Verhoef, H.E. Junginger, Effect of the degree of quaternization of N-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 cell monolayers, J. Control. Rel. (Special issue) (1999) in press.