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Intestinal paracellular permeation enhancement with quaternised chitosan: in situ and in vitro evaluation

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Abstract

Previous studies have shown that *N*-trimethyl chitosan chloride (TMC) is a potent absorption enhancer for hydrophilic and macromolecular compounds across mucosal surfaces. TMC proved to be effective in neutral and basic pH environments where the absorption enhancing ability of chitosan is severely hampered by its insolubility in these environments. The absorption enhancing characteristics of TMC polymers with different degrees of quaternisation were investigated in vitro and in situ to identify the most effective polymer in a neutral pH environment. Different degrees of quaternisation were obtained by varying the number and duration of the reaction steps in the synthesis process of TMC. The TMC polymers were characterised with ^{1}H-NMR spectroscopy and the degrees of quaternisation were between 22.1 and 48.8%. Everted intestinal sacs (rats) were used to determine the effect of the polymers (0.0625–0.5% w/v) on the permeation of the hydrophilic model compound, [^{14}C]mannitol, at a pH value of 7.4. A single pass intestinal perfusion method was also used to evaluate the permeation enhancing properties of the TMC polymers under the same conditions. The results obtained from both methods clearly showed a pronounced enhancement of [^{14}C]mannitol permeation when administered with the different TMC polymers. It was shown that the permeation enhancing effects depend on the degree of quaternisation of TMC. In both models the best permeation enhancing results were obtained with the highest degree of quaternisation of TMC (48.8%) at a concentration of 0.5% w/v. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Permeation enhancement; Chitosan; *N*-Trimethyl chitosan chloride (TMC); Degree of quaternisation; Everted intestinal sac; Single pass intestinal perfusion

1. Introduction

In recent years significant progress has been made in identifying substances which increase the absorption of drugs through the paracellular pathway (Muranishi, 1990; Hochman and Artursson, 1994; Luessen et al., 1997). In this regard

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chitosan, a linear polysaccharide derived by *N*-deacetylation of the natural polymer chitin, is of special interest. Chitosan acts as an absorption enhancer by opening the tight junctions between epithelial cells to allow for the paracellular transport of hydrophilic and macromolecular compounds such as peptide drugs. The absorption enhancing ability of chitosan, a mucoadhesive polymer, is mediated by protonated amino groups on the C-2 position of the molecules that induce interaction with the anionic sites on the cell membranes to subsequently alter tight junction integrity (Artursson et al., 1994; Schipper et al., 1997; Kotzé et al., 1998a).

However, chitosan is a polycation and precipitates from solution in neutral and basic pH environments, such as those found in the small intestine, colon and rectum, thereby limiting its potential use as an absorption enhancer in these environments (Kotzé et al., 1998a). It has been shown that *N*-trimethyl chitosan chloride (TMC), a partially quaternised derivative of chitosan with superior solubility can also increase the absorption of hydrophilic and macromolecular drugs (Kotzé et al., 1998b; Thanou et al., 1999). TMC is especially effective in enhancing the transport of small hydrophilic compounds, but also improves the transport of large molecules such as the peptide drugs insulin, buserelin and 9-desglycinamide, 8-arginine vasopressin (DGAVP) in vitro in Caco-2 cell monolayers (Kotzé et al., 1997a). In this regard, Thanou et al. (2000a) concluded that in vitro and in vivo results suggests that TMC is a potent mucosal permeation enhancer of the peptide drug buserelin, especially at neutral pH values. In vitro transport studies in Caco-2 cell monolayers and in vivo studies also revealed an increased permeation of the somatostatin analogue, octreotide, in the presence of TMC (Thanou et al., 2000c).

The degree of quaternisation of TMC has been shown to play an important role on the absorption enhancing properties of this polymer, especially in neutral and basic pH environments (Kotzé et al., 1999; Thanou et al., 2000b). TMC with a degree of quaternisation of 61.2% and TMC with a degree of quaternisation of 12.3% were evaluated in Caco-2 cell monolayers with the hydrophilic model compound [^{14}C]mannitol and it has been shown that the higher quaternised chitosan is a potent absorption enhancer at a pH of 7.4 where the lower quaternised chitosan was ineffective as an absorption enhancer. The results were explained by the proportion of quaternary amino groups, that seems to be sufficient to interact with the cell membranes or the negative sites within the tight junctions, on the polymer with the higher degree of quaternisation (Kotzé et al., 1999). Further support of this was also recently obtained with the nasal administration of [^{14}C]mannitol in rats. This particular investigation also suggests that an optimum degree of quaternisation around 48% of TMC gives the best absorption enhancing results across nasal epithelia (Hamman et al., 2002). The aim of our current investigation was to evaluate the effect of the degree of quaternisation of TMC on the intestinal absorption of [^{14}C]mannitol at a pH of 7.4. Polymers with a degree of quaternisation above 49% were not investigated based on the results obtained by Hamman et al. (2002) in nasal epithelial cells.

2. Materials and methods

2.1. Synthesis and characterisation of TMC polymers

2.1.1. Synthesis

TMC was synthesised from milled chitosan (Pronova Biopolymer, Norway, degree of deacetylation ca. 93%) based on the method of Domard et al. (1986), Sieval et al. (1998). Briefly, the experimental conditions are reductive methylation of chitosan with iodomethane in a strong basic environment at 60 °C for 45 min. The degree of quaternisation of the polymers was controlled by varying the number and duration of the reaction steps involved in the synthesis process (Hamman and Kotzé, 2001). TMC with the lowest degree of quaternisation was prepared with a single-step reaction of 45 min. TMC polymers with higher degrees of quaternisation were prepared with multiple steps where the basic reaction step was repeated a few times. A later adding step involved

an additional 2 ml methyl iodide and 0.6 g sodium hydroxide pellets added to the mixture of some polymers at the end of the basic reaction step (Table 1). The counter iodide-ions were exchanged to chloride-ions by dissolving the quaternised polymers in an aqueous solution of NaCl. The polymer was precipitated from solution using ethanol and isolated by centrifugation. The product was dried in a vacuum oven at 40 °C.

2.1.2. Characterisation

The synthesised TMC polymers were characterised with ^{1}H-NMR spectroscopy as described by Sieval et al. (1998), Hamman and Kotzé (2001). The ^{1}H-NMR spectra (600 MHz) were recorded in D_2O with a DMX Brucker 600MHz spectrometer (Karlsrühe, Germany) at 80 °C. The degree of quaternisation of the synthesised TMC polymers were calculated with the following equation (Hamman and Kotzé, 2001):

$$\mathrm{DQ}\ (\%) = \left[\left(\frac{\int \mathrm{TM}}{\int \mathrm{H}} \right) \times \frac{1}{9} \right] \times 100$$

where: DQ (%) is the degree of quaternisation of TMC expressed as a percentage, ∫TM is the integral of the trimethyl amino group (quaternary amino) peak at 3.3 ppm, and ∫H is the integral of the ^{1}H peaks from 4.7 to 5.7 ppm.

2.2. Intestinal administration of TMC

2.2.1. In vitro: everted intestinal sacs

Everted intestinal sac experiments were performed based on the method described by Barr and Riegelman (1970). According to the requirements of the local ethical committee, healthy male Sprague Dawley rats (250–300 g) were fasted for 12 h prior to surgery, but water was supplied ad libitum. Rats were anaesthetised with intraperitoneal Euthapent® (Kyron Laboratories (Pty) Ltd., South Africa) injections containing 200 mg sodium pentobarbitone per millilitre. Laparotomy (a midline abdominal incision) was performed after verification of loss of the pain reflex and a 20 cm piece of jejunal intestine was quickly removed. Rats were sacrificed with an overdose of Euthapent® by injection into the heart before recovering from anaesthesia. The excised piece of jejenum was immediately flushed with ice-cold phosphate buffered saline (PBS) (Bio Whittaker, USA) to clean it from intestinal contents. Segments of approximately 5 cm in length (when stretched by a 2 g weight) were used for the everted sac experiments. The intestinal jejunal segments were tied at one end, everted on a thin plastic rod and blotted dry to remove most of the mucus present. Each intestinal segment was mounted in the perfusion apparatus and submerged in 60 ml of the mucosal fluid saturated with 5% CO_2 and 95% O_2 (carbogen) gas (AFROX, South Africa). The perfusion apparatus consisted of a 100 ml glass tube (35 mm diameter) fitted with a conical shaped rubber stopper which housed the mucosal fluid for the experiment. The mucosal fluid (outside compartment) into which the everted intestinal sac was introduced, consisted of TMC and [^{14}C]mannitol in Krebs–Ringer bicarbonate buffer (Sigma, St. Louis, USA). Each TMC polymer was dissolved in 500 ml Krebs–Ringer bicarbonate buffer in four concentrations ranging from 0.0625 to 0.5% w/v and the pH value was adjusted to 7.4 with 1 N NaOH (Merck, South Africa) or 1 N HCl (Merck, South Africa). [^{14}C]mannitol (10 μl) (MW 182.2, specific

Table 1
Number of reaction steps and degree of quaternisation of the synthesised TMC polymers

TMC polymer	Number of reaction steps (45 min)	Later adding step (30 min)	Degree of quaternisation (%)
TMC-22	1	–	22.15
TMC-38	2	–	38.14
TMC-43	2	1	42.75
TMC-49	3	1	48.75

radioactivity 57 mCi/mmol, 200 mCi/ml, Amersham Life Sciences, UK) was added to each of these test solutions. Control solutions were prepared in a similar way as the experimental test solutions, but without the dissolved TMC polymers. Experiments were performed at 37 ± 0.1 °C in a waterbath (Stuart Scientific, England).

After introduction of the everted intestine to the mucosal solution, 1 ml of clean Krebs–Ringer Bicarbonate buffer was injected into the intestinal segment, thereby generating a serosal fluid or an inside compartment. All the fluid from the inside compartment was drawn into a syringe with a blunt needle after a 15 min perfusion period of the intestinal segment and 1 ml of clean Krebs–Ringer bicarbonate buffer was again introduced to serve as the serosal solution for the next 15 min time interval. Samples from the inside compartment were taken every 15 min for a 3 h period. All the samples were stored in marked mini scintillation vials (6 ml) (Beckman Instrument Inc., USA) until analysis. Each experiment was performed six times. After each experiment the mucosal integrity of the everted intestinal sac was inspected microscopically.

2.2.2. *In situ: single pass intestinal perfusion*

Healthy male Sprague Dawley rats were used for the single pass intestinal perfusion experimental setup previously described by Stewart et al. (1995). The rats weighing 250–300 g and maintained on Epol® mice cubes (Epol Pty (Ltd)., South Africa) were fasted for 12 h prior to surgery but supplied with water ad libitum. The rats were anaesthetised with inhalation anaesthesia consisting of 4% v/v halothane (Fluothane®, Zebeca SA (Pty.) Ltd., South Africa) in medicinal oxygen. A 2% v/v halothane in medicinal oxygen mixture was used for maintenance of anaesthesia. Anaesthesia lasted for ± 3 h and rats were sacrificed at the end of the experiments before recovering from anaesthesia as described in the previous section. Laparotomy was performed after verification of loss of the pain reflex. The upper to middle part (5–8 cm) of rat jejunum was cannulated at both ends with PVC tubing (Labchem, South Africa) about 1 cm below the ligament of Treitz (a peritoneal fold marking the border between the duodenum and the jejunum; muscularis suspensorius duodeni). Both the cannulae were secured with surgical silk sutures (Sutures Research Laboratories, South Africa). The exposed intestinal segment was moistened with saline maintained at 37 °C and covered with parafilm (American National Can, USA) to decrease moisture loss. The initial delivery end was attached to a peristaltic pump (Watson Marlow Ltd., England) and the inlet tubing together with the experimental test solution was water-jacketed in a waterbath (Stuart Scientific, England) at 37 ± 0.1 °C.

At the beginning of each experiment the cannulated segment was infused with Hanks Balanced Salt Solution (HBSS) (Bio Whittaker, USA) at 37 °C until the perfusate was clean (± 15 min). Samples from the perfusate outflow were collected every 15 min from the exit tubing for 2 h. TMC was dissolved in 300 ml of HBSS to produce 0.25 and 0.5% w/v solutions, respectively. The pH value of the solutions were adjusted to 7.4 with 1 N NaOH or 1 N HCl. [^{14}C]mannitol (10 μl) was added to each of the solutions. The control solutions were prepared in a similar way as the experimental test solutions without the dissolved TMC polymers. The warmed intestinal test solution was pumped through the jejunal segment at a flow rate of ± 0.1 ml/min. Care was taken to maintain the inlet and outlet cannulae at the same height to avoid gravitational flow, which would have influenced the outflow of the perfusate. The animals were placed on a warming pad during the full period of time of the experiment to maintain body temperature at 37 °C. During the first few experiments scrapings after each experiment were made from the PVC tubing to determine the extent of non-specific binding of [^{14}C]mannitol to the tubing.

2.3. Determination of [^{14}C]mannitol concentration

A volume of 500 μl of the samples withdrawn from both experimental setups were used for analysis of [^{14}C]mannitol content. Samples were transferred into mini scintillation vials and 5 ml scintillation cocktail (Ready Gel®) (Beckman Instrument Inc., USA) was added to each vial. The

[^{14}C]mannitol content was determined by scintillation counting in a Beckman LS 3801 liquid scintillator (Beckman Coulter, USA). Each sample was counted for 5 min to keep the counting precision below 2%.

2.4. Data analysis and statistical evaluation

The amount of [^{14}C]mannitol transported in each experiment was calculated from single labelled dpm-values. The single labelled dpm-values obtained by the scintillation counter were used for conversions and averages, and standard deviations were calculated for each treatment that the rats received. Results were corrected for dilution and plotted as cumulative [^{14}C]mannitol transport (as a percentage of the total dose) at time t. Apparent permeability coefficients (P_{app}) were calculated according to the following equation (Kotzé et al., 1997b):

$$P_{app} = \left(\frac{dc}{dt}\right)\left(\frac{1}{A.60.C_0}\right)$$

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 intestine (cm^2), and C_0 is the initial concentration of [^{14}C]mannitol.

The diffusion area of the intestine (cm^2) was calculated after each experiment. The excised intestinal segment was cut along the length of the sac and spread on a flat surface. The length and width was electronically measured with a Vernier Caliper and the diffusion area was determined mathematically. The regression coefficients (r^2) obtained from the linear curve fits ([^{14}C]mannitol transport (% of the total dose) versus time) were generally between 0.90 and 1.00. Permeation enhancement ratios (R) were calculated from the P_{app} values with the following equation (Kotzé et al., 1997b):

$$R = \frac{P_{app}\ \text{test}}{P_{app}\ \text{control}}$$

where R is the permeation enhancement ratio, P_{app} test is the apparent permeability coefficient (cm/s) for the test solution, and P_{app} control is the apparent permeability coefficient (cm/s) for the control solution.

Statistical analysis of the data obtained was performed with the Tukey HSD test of Statsoft® Statistica 99 for Windows (Statsoft® Incorporated, USA). P values of 0.1 or less were considered to indicate statistically significant differences between treatments.

3. Results

3.1. Synthesis and characterisation of TMC

The degrees of quaternisation of the different TMC polymers, calculated from the ^{1}H-NMR spectra, and number of reaction steps used in the synthesis process of each polymer are given in Table 1. The degrees of quaternisation of the synthesised TMC polymers varied between 22.15 and 48.75%. The degree of quaternisation of the TMC polymers increased as the number and duration of the reaction steps in the synthesis process increased. A degree of quaternisation of 22% was obtained with a one step reaction and 49% with a four step reaction. Intermediate degrees of quaternisation (38 and 34%) were obtained with a two and three step reaction.

3.2. In vitro: everted intestinal sacs

The apparent permeability coefficient values (P_{app}) and the permeation enhancement ratios (R) obtained for [^{14}C]mannitol when administered with TMC at pH 7.4 in everted intestinal sacs are presented in Table 2. The cumulative transport of [^{14}C]mannitol at 0.5% w/v concentrations of the TMC polymers are graphically presented in Fig. 1. In general the permeation of [^{14}C]mannitol increased with an increase in the degree of quaternisation of TMC. It seems that TMC-49 was the most effective permeation enhancer at pH 7.4. TMC-43 and TMC-49 were effective in concentrations as low as 0.125% w/v. Similar effects with lower degrees of quaternisation of TMC could only be obtained at higher concentrations (TMC-22 at 0.5% w/v and TMC-38 at 0.25% and 0.5% w/v). TMC-22 did not prove to be effective as a permeation enhancer at pH 7.4 and only mild permeation enhancing effects were found at a

Table 2
Apparent permeability values (P_{app}) and permeability enhancement ratios (R) obtained for [^{14}C]mannitol administered with TMC in everted intestinal sacs

Concentration (% w/v)	$P_{app} \times 10^{-7}$ (cm/s)				R			
	TMC-22	TMC-38	TMC-43	TMC-49	TMC-22	TMC-38	TMC-43	TMC-49
Control	0.63 ± 0.03	0.63 ± 0.03	0.63 ± 0.03	0.63 ± 0.03	1.00	1.00	1.00	1.00
0.0625	0.73 ± 0.14	0.92 ± 0.03	1.06 ± 0.07	0.82 ± 0.29	1.17	1.47	1.70	1.30
0.125	0.79 ± 0.28	1.08 ± 0.13	1.29 ± 0.27	1.28 ± 0.18	1.27	1.72	2.06	2.04
0.25	0.90 ± 0.38	1.67 ± 0.16	1.78 ± 0.40	2.14 ± 0.21	1.44	2.67	2.84	3.41
0.5	1.11 ± 0.10	1.61 ± 0.42	2.25 ± 0.61	2.63 ± 0.15	1.78	2.67	3.52	4.20

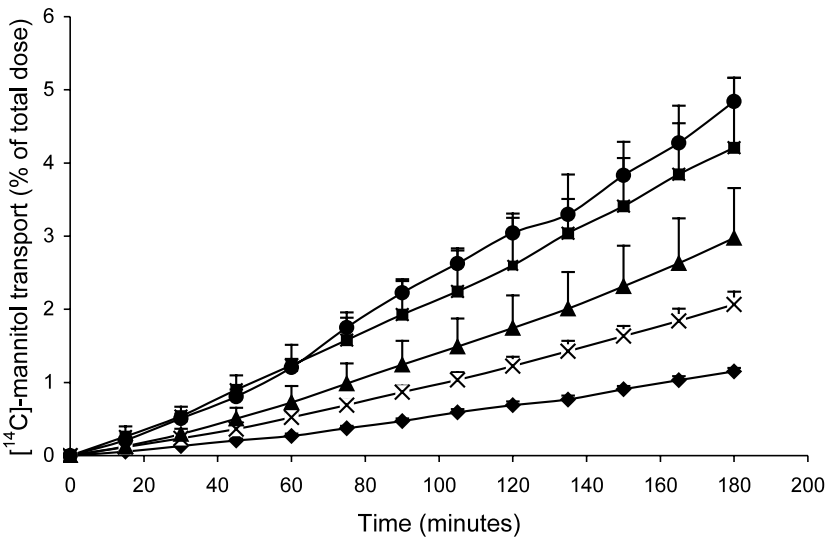

Fig. 1. Effect of TMC polymers (0.5% w/v) on the cumulative transport of [^{14}C]mannitol in everted intestinal sacs at pH 7.4. Each point represents the mean $\pm$ SD of six experiments. Control (◆), TMC-22 (×), TMC-38 (▲), TMC-43 (■), TMC-49 (●).

0.5% w/v concentration of this polymer. None of the TMC polymers proved to be effective in enhancing the transport of [^{14}C]mannitol in a concentration of 0.0625% w/v. Statistical analysis confirmed that the transport of [^{14}C]mannitol with TMC-38, TMC-43 and TMC-49 (0.125, 0.25 and 0.5% w/v) differ significantly from the control group ($P < 0.05$). However, none of the TMC-polymers at a 0.0625% w/v concentration induced significant transport compared to the control group. With TMC-22 (at a concentration of 0.5% w/v) significant transport compared to the control group was found ($P < 0.05$) while concentrations of 0.25, 0.125 and 0.0625% w/v did not show any significant effect on the cumulative transport of [^{14}C]mannitol. Microscopic inspection of the everted intestinal sacs showed no damage and mucus production during the experiment indicated normal function.

3.3. In situ: single pass intestinal perfusion

The apparent permeability coefficient values (P_{app}) and the permeation enhancement ratios (R) obtained for [^{14}C]mannitol when administered with TMC at pH 7.4 in the intestinal perfusion studies are presented in Table 3. The cumulative transport of [^{14}C]mannitol after administration

with the TMC polymers (0.5% w/v) at a pH of 7.4 are graphically presented in Fig. 2. In accordance with the in vitro results, the transport of [^{14}C]mannitol increased with an increase in the degree of quaternisation of TMC in the single pass perfusion experiments. TMC-49 was the most effective permeation enhancer of [^{14}C]mannitol across intestinal epithelia at pH 7.4. TMC-43 and TMC-49 were both very effective as permeation enhancers in a concentration of 0.5% w/v. TMC-22 and TMC-38 only showed moderate permeation enhancing effects even at a concentration as high as 0.5% w/v compared to the effects exhibited by TMC-43 and TMC-49 at a similar concentration. The results obtained with these in situ single pass intestinal perfusion experiments suggest that TMC with a degree of quaternisation of 49% in a concentration of 0.5% w/v seems to produce the most effective permeation enhancing effects in a neutral pH environment. Statistical analysis confirmed that the transport of [^{14}C]mannitol with all the TMC polymers (0.25% and 0.5% w/v) differ significantly from the control group ($P < 0.05$).

4. Discussion

The results of the studies described above indicate that the degree of quaternisation of TMC played an important role in the permeation enhancing properties of this polymer across intesti-

Table 3
Apparent permeability values (P_{app}) and permeability enhancement ratios (R) obtained for [^{14}C]mannitol administered with TMC in the intestinal perfusion studies

Concentration (% w/v)	$P_{app} \times 10^{-7}$ (cm/s)				R			
	TMC-22	TMC-38	TMC-43	TMC-49	TMC-22	TMC-38	TMC-43	TMC-49
Control	1.38 ± 0.64	1.38 ± 0.64	1.38 ± 0.64	1.38 ± 0.64	1.00	1.00	1.00	1.00
0.25	3.45 ± 1.29	3.63 ± 0.91	4.08 ± 0.68	3.92 ± 1.28	2.50	2.63	2.96	2.84
0.5	5.21 ± 0.96	5.64 ± 1.77	9.09 ± 2.10	10.19 ± 2.36	3.77	4.08	6.58	7.39

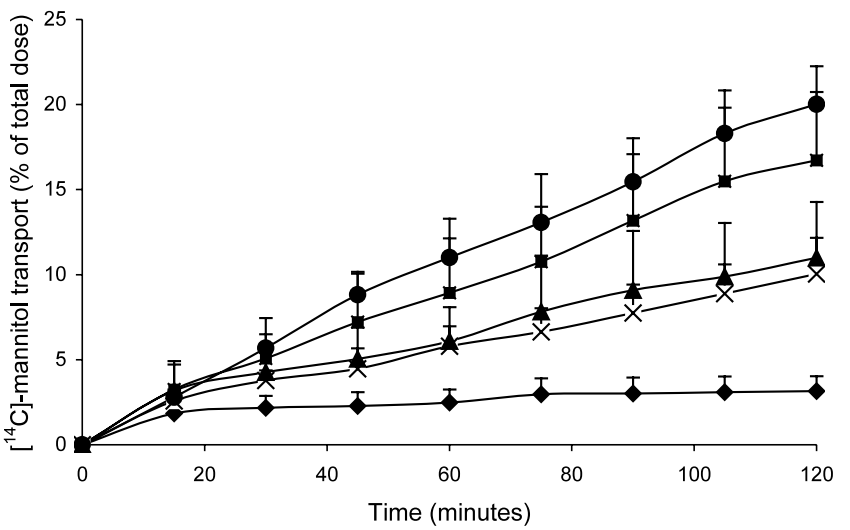

Fig. 2. Effect of TMC polymers (0.5% w/v) on the cumulative transport of [^{14}C]mannitol after intestinal administration (single pass perfusion) at pH 7.4. Each point represents the mean ± SD of six experiments. Control (◆), TMC-22 (×), TMC-38 (▲), TMC-43 (■), TMC-49 (●).

nal epithelia in a neutral pH environment. The transport of [^{14}C]mannitol across intestinal epithelia increased with an increase in the degree of quaternisation of TMC and TMC with a relative high degree of quaternisation (48.8%) was the most effective permeation enhancer in a neutral pH environment. This may be explained by the charge density on the TMC molecules, determined by the degree of quaternisation, that influences the interaction of this polymer with the negatively charged sites on the cell membranes and/or within the tight junctions.

From the everted intestinal sac experiments it can be concluded that highly quaternised polymers (TMC-43 and TMC-49 at 0.125, 0.25 and 0.5% w/v concentrations) are more efficient in increasing the transport of [^{14}C]mannitol across the intestinal membrane at a pH of 7.4 compared to lower quaternised polymers. At a lower concentration of 0.0625% w/v, TMC-43 and TMC-49 did not increase the transport of [^{14}C]mannitol effectively. A possible explanation is that the number of fixed quaternary amino groups on the C-2 position of these polymers were still too low at a weight concentration of 0.0625% to cause a substantial alteration in the tight junction's resistance and subsequent opening of the tight junctions. Although the transport of [^{14}C]mannitol was increased by TMC-38 (0.125, 0.25 and 0.5% w/v), it was not to the same extent as with TMC-43 and TMC-49. TMC-38 was also not effective in a concentration of 0.0625% w/v, while TMC-22 was ineffective at concentrations of 0.0625, 0.125 and 0.25% w/v. More positive charges are available on the TMC polymers with higher degrees of quaternisation for interaction with the negatively charged groups on the cell membranes and within the tight junctions to induce the opening of tight junctions. This can be explained by the higher number of positively charged quaternary amino groups found on the C-2 position of TMC polymers with higher degrees of quaternisation which cause a substantial opening of the tight junctions. A certain threshold value for the degree of quaternisation is probably needed for effective permeation enhancement and the amount of fixed positive charges on TMC with lower degrees of quaternisation (TMC-22 and TMC-38) is not sufficient to induce effective permeation enhancement across the intestinal epithelia at a pH of 7.4.

The permeation enhancement effects on the intestinal permeation of [^{14}C]mannitol with the TMC polymers in the in situ experiment are overall more remarkable when compared with the in vitro experiment (Tables 2 and 3 and Figs. 1 and 2). Since the duration of these in situ experiments were only 2 h whereas the duration of the in vitro experiments were 3 h, the difference in results may be explained by the following reasons: (1) 5–8 cm segments of the small intestine was used in the in situ experiment, while smaller segments (approximately 5 cm in length, when stretched by a 2 g weight) was used in the in vitro experiment; and (2) in the in vitro experiments the intestinal segments lack blood supply, while this was not the case with the in situ experiments. It is likely that no single experimental method will be ideal to study permeation enhancement and that maximum information will often require corroborative evidence from more than one method.

Kotzé et al. (1999), Thanou et al. (2000b) recorded absorption enhancement values as high as 43 for [^{14}C]mannitol with TMC with a high degree of quaternisation (60% quaternised, 0.5% w/v) in Caco-2 cell monolayers. Woodley et al. (2000) noted that values obtained in Caco-2 cell monolayers might exaggerate the potential of polymers for realistic absorption enhancement, but that the everted intestinal sac model may give more realistic values for small intestinal function. The results obtained in our study are further support for the findings of Woodley et al. (2000). Intestinal everted sacs or intestinal perfusion may give a better indication of absorption enhancement.

In conclusion, this study clearly demonstrates the ability of TMC to enhance intestinal permeation in a neutral pH environment. Furthermore it is clear that the degree of quaternisation of TMC played a major role on its permeation enhancing properties across intestinal epithelia. Whether a degree of quaternisation of TMC around 48% is the optimum degree of quaternisation, as was found in nasal epithelia (Hamman et al., 2002), is still uncertain. It may however also be assumed that conformational effects will play a

role in the permeation enhancing ability of highly quaternised (around 60%) chitosan polymers in the intestinal route.

References

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

Barr, W.H., Riegelman, S., 1970. Intestinal drug absorption and metabolism I: Comparison of methods and models to study physiological factors of in vitro and in vivo intestinal absorption. J. Pharm. Sci. 59, 154–163.

Domard, A., Rinaudo, M., Terrassin, C., 1986. New method for the quaternisation of chitosan. Int. J. Biol. Macromol. 8, 105–107.

Hamman, J.H., Kotzé, A.F., 2001. Effect of the type of base and number of reaction steps on the degree of quaternization and molecular weight of *N*-trimethyl chitosan chloride. Drug Dev. Ind. Pharm. 27, 373–380.

Hamman, J.H., Stander, M., Kotzé, A.F., 2002. Effect of the degree of quaternisation of *N*-trimethyl chitosan chloride on the absorption enhancement: In vivo evaluation in rat nasal epithelia. Int. J. Pharm. 232, 235–242.

Hochman, J., Artursson, P., 1994. Mechanisms of absorption enhancement and tight junction regulation. J. Contr. Rel. 29, 253–267.

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

Kotzé, A.F., Luessen, H.L., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 1998a. Chitosan for enhanced intestinal permeability: Prospects for derivatives soluble in neutral and basic environments. Eur. J. Pharm. Sci. 7, 145–151.

Kotzé, A.F., Luessen, H.L., De Leeuw, B.J., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 1997b. *N*-Trimethyl chitosan chloride as a potential absorption enhancer across mucosal surfaces: in vitro evaluation in intestinal epithelial cells (Caco-2). Pharm. Res. 14, 1197–1202.

Kotzé, A.F., Luessen, H.L., De Leeuw, B.J., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 1998b. Comparison of the effect of different chitosan salts and *N*-trimethyl chitosan chloride on the permeability of intestinal cells (Caco-2). J. Contr. Rel. 51, 35–46.

Kotzé, A.F., Thanou, M., Luessen, H.L., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 1999. Enhancement of paracellular drug transport with highly quaternized *N*-trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2). J. Pharm. Sci. 88, 253–257.

Luessen, H.L., Lehr, C.-M., Rentel, C.-O., Noach, A.B.J., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 1997. Bioadhesive polymers for peroral delivery of peptide drugs. J. Contr. Rel. 29, 329–338.

Muranishi, S., 1990. Absorption enhancers. Crit. Rev. Ther. Drug Carrier Syst. 7, 1–27.

Schipper, N.G.M., Olsson, S., Hoogstraate, J.A., De Boer, A.G., Vårum, K.M., Artursson, P., 1997. Chitosans as absorption enhancers for poorly absorbable drugs. 2. Mechanism of absorption enhancement. Pharm. Res. 14, 923–929.

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

Stewart, B.H., Chan, O.H., Lu, R.H., Reyner, E.L., Schmid, H.L., Hamilton, H.W., Steinbaugh, B.A., Taylor, M.D., 1995. Comparison of intestinal permeabilities determined in multiple in vitro and in situ models: relationship absorption in humans. Pharm. Res. 12, 693–699.

Thanou, M., Florea, B.I., Langemeÿer, M.W.E., Verhoef, J.C., Junginger, H.E., 2000a. *N*-trimethylated chitosan chloride (TMC) improves the intestinal permeation of the peptide drug buserelin in vitro (Caco-2 cells) and in vivo (rats). Pharm. Res. 17, 27–31.

Thanou, M., Kotzë, A.F., Scharringhausen, T., Luessen, H.L., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 2000b. Effect of the degree of quaternisation of *N*-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 monolayers. J. Contr. Rel. 64, 15–25.

Thanou, M., Verhoef, J.C., Marbach, P., Junginger, H.E., 2000c. Intestinal absorption of octreotide: *N*-trimethyl chitosan chloride (TMC) ameliorates the permeability and absorption properties of the somatostatin analogue in vitro and in vivo. J. Pharm. Sci. 7, 951–957.

Thanou, M., Verhoef, J.C., Romeijn, S.G., Nagelkerke, J.F., Merkus, F.W.H.M., Junginger, H.E., 1999. Effects of *N*-trimethyl chitosan chloride, a novel absorption enhancer, on Caco-2 intestinal epithelia and the ciliary beat frequency of chicken embryo trachea. Int. J. Pharm. 185, 73–82.

Woodley, J.F., Levillain, F., Barthe, L., Houin, G., 2000. Realistic assessment in vitro of the enhancement of drug absorption by bioadhesive polymers. Proc. Int. Symp. Control. Rel. Bioact. Mater. 27, 6205.