Enhancement of Paracellular Drug Transport with Highly Quaternized *N*-Trimethyl Chitosan Chloride in Neutral Environments: In Vitro Evaluation in Intestinal Epithelial Cells (Caco-2)

AWIE F. KOTZÉ,*,†,‡ MAYA M. THANOU,‡ HENRIK L. LUEBEN,‡,§ A. G. DE BOER,¶ J. COOS VERHOEF,‡ AND HANS E. JUNGINGER‡

Contribution from *Department of Pharmaceutics, Potchefstroom University for Christian Higher Education, Potchefstroom, 2520, Republic of South Africa, Department of Pharmaceutical Technology, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands, LTS Lohmann Therapie-Systeme GmbH, D-56605 Andernach, Germany, and Department of Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9503, 2300 RA Leiden, The Netherlands.*

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Abstract □ Previous studies have established that a partially quaternized derivative of chitosan, *N*-trimethyl chitosan chloride (TMC), can be used as an absorption enhancer for large hydrophilic compounds across mucosal surfaces. This study evaluates and compares the effects of the degree of quaternization of TMC, in a neutral environment, on the permeability of intestinal epithelial cells in vitro, where normal chitosan salts are ineffective as absorption enhancers. The effects of TMC-H [61.2% quaternized, (0.05–1.5% w/v)], TMC-L [12.3% quaternized, (0.5–1.5% w/v)], and chitosan hydrochloride [0.5–1.5% w/v] on the transepithelial electrical resistance (TEER) and permeability, for the hydrophilic model compound [^{14}C]-mannitol, of intestinal epithelial Caco-2 cell monolayers, were investigated at pH values of 6.20 and 7.40. The viability of the monolayers was checked with the trypan blue exclusion technique. 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, whereas 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). For all the polymers studied, no deleterious effects to the cells could be demonstrated with the trypan blue exclusion technique. It is concluded that highly quaternized TMC is a potent absorption enhancer and the potential use of this polymer, especially in neutral and basic environments where normal chitosan salts are not effective, is expected to be an important contribution to the development of effective delivery systems for hydrophilic compounds such as peptide drugs.

Introduction

The potential use of chitosan as an absorption enhancer across mucosal surfaces has been well documented in recent years. Chitosan salts such as chitosan glutamate and chitosan hydrochloride have been shown to increase the absorption of a number of hydrophilic compounds and peptide drugs both in vitro and in vivo.[1−7] Apart from its mucoadhesive properties,[8] chitosan acts mainly by opening the tight junctions between epithelial cells to allow for the paracellular transport of these large hydrophilic molecules. Such an action is believed to be due to an interaction of a positively charged amino group on the C-2 position of chitosan with negatively charged sites on the cell membranes, which results in a structural reorganization of the tight junction-associated proteins.[1,6]

Chitosan has an apparent pK_a value between 5.5 and 6.5, and a certain amount of acid is required to transform the glucosamine units into the positively charged, water-soluble form. At neutral and basic pH values, the chitosan molecules will lose their charge and therefore the potential use of this polymer, especially in more neutral and basic environments such as those found in the large intestine and colon, is limited. It has been shown recently that a partially quaternized (12%) derivative of chitosan, *N*-trimethyl chitosan chloride (TMC), was also able to significantly increase the transport of hydrophilic compounds, such as [^{14}C]mannitol (MW 182.2), fluorescein isothiocyanate-labeled dextran (MW 4400), and [^{14}C]poly(ethylene glycol) (MW 4000), and the peptide drugs buserelin (MW 1300), 9-desglycinamide, 8-L-arginine vasopressin (MW 1412), and insulin (MW 5778) in Caco-2 cell monolayers at acidic pH values (4.40−6.20).[9−11] It was suggested that TMC most likely has the same mechanism of action on the junctional complex as other chitosan salts.[10] The derivative TMC (12% quaternized) was not as effective as other chitosan salts, such as chitosan glutamate and chitosan hydrochloride. This lesser efficacy was explained by its charge density, which was determined by the degree of quaternization, and by a partial hiding of the positive charge on the amino group by the attached methyl groups.[10,11] However, the much higher aqueous solubility of TMC may compensate for its lesser efficacy. TMC has proved to be very soluble over a wide pH range (pH 1−9) up to 10% w/v concentrations, even at degrees of quaternization as low as 10%.[9−10]

Our hypothesis is that TMC with higher degrees of quaternization may be more effective as an absorption enhancer to increase the paracellular transport of hydrophilic compounds at neutral pH values. The aim of the present study was to synthesize TMC with different degrees of quaternization (low and high) and to evaluate and compare the effect of these polymers with the effect of chitosan hydrochloride on the permeability of intestinal

* Corresponding author. Telephone: 27 18 2992249. Fax.: 27 18 2992251. E-mail: FMSAFK@PUKNET.PUK.AC.ZA.

† Department of Pharmaceutics, Potchefstroom University for Christian Higher Education.

‡ Department of Pharmaceutical Technology, Leiden/Amsterdam Center for Drug Research.

§ LTS Lohmann Therapie-Systeme GmbH.

¶ Department of Pharmacology, Leiden/Amsterdam Center for Drug Research.

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epithelial cells in vitro for increased transport of a hydrophilic compound at slightly acidic and neutral pH values (6.20 and 7.40).

Experimental Section

Synthesis and Characterization of *N*-Trimethyl Chitosan Chloride (TMC)—Two batches of TMC, one with a low and one with a high degree of quaternization, were synthesized from sieved fractions (<500 μm) of chitosan (degree of acetylation ca. 25%; Pronova Biopolymer, Drammen, Norway) based on the method of Domard et al.[12] and Sieval et al.[13] Briefly, the experimental conditions are reductive methylation of chitosan with iodomethane in a strong basic environment at 60 °C. The batch with the lower degree of quaternization (TMC-L) was prepared in a single-step reaction of 60 min. To obtain TMC with the higher degree of quaternization (TMC-H), the basic reaction was repeated twice with the product obtained after the first step.[13] The counterion (I^-) was exchanged to Cl^- by dissolving the quaternized polymers in a aqueous solution of NaCl. The final products were obtained by precipitation and washing with ethanol. The polymers were characterized by nuclear magnetic resonance (NMR) spectroscopy, and the degree of quaternization of the respective polymers was calculated from ^{1}H NMR spectra (600 MHz) obtained with a Bruker DMX-600 spectrometer.[13]

Cell Cultures—Caco-2 cells (passages 88 and 93−94) were seeded on tissue-culture-treated polycarbonate filters (area 4.7 cm^2 and 0.33 cm^2) in Costar Transwell 6- and 24-well plates (Costar Europe Ltd., 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% nonessential amino acids, 10% foetal bovine serum, benzylpenicillin G (160 U/mL), and streptomycin sulfate (100 μg/mL) (all obtained from Sigma), was used as culture medium. The medium was changed every second day. Cell cultures were kept at 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.[3,14,15]

Measurement of the Transepithelial Electrical Resistance (TEER)—The effect of TMC-H (0.05−1.5% w/v), TMC-L (0.5−1.5% w/v) and chitosan hydrochloride (0.5−1.5% w/v; SEACURE CL 210 from Pronova Biopolymer, Drammen, Norway) on the TEER of the Caco-2 cell monolayers was measured every 20 min with a Millicell ERS meter (Millipore Corp., Bedford, MA) connected to a pair of thin, side-by-side electrodes suitable for use in the small 24-well culture plates.[3,14,15] The effect of these polymers on the TEER was measured at both pH 6.20 and 7.40. The polymer solutions were prepared in serum-free DMEM, and the pH was adjusted to 6.20 with 0.1 M HCl or to 7.40 with 0.1 M NaOH. Because of the insolubility of chitosan hydrochloride at a pH of 7.40, the resulting polymer preparation was used as a dispersion of chitosan hydrochloride in DMEM. Two hours before the start of each experiment, the medium in the acceptor compartment was removed and replaced with DMEM buffered at pH 7.40 with 25 mM HEPES [*n*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid; Sigma]. Measurements started 1 h prior to incubation on the apical side of the cells with the respective polymer solutions. After 2 h, the polymer solutions were carefully removed, and cells were washed three times with and replaced by serum-free DMEM (pH 6.20 or 7.40). The TEER was measured for an additional hour to study the reversibility of the effect of the polymer solutions. Control experiments were done under the same conditions without polymers. Average TEER values for untreated cell monolayers were in the range 200−300 Ω·cm^2 at both pH 6.20 and 7.40.[6] At the end of each experiment, the solutions in both the apical and basolateral sides of the cell compartments were checked for any changes in pH. Experiments were done in triplicate at 37 °C in an atmosphere of 95% air and 5% CO_2.

Permeability Studies with [^{14}C]Mannitol—[^{14}C]Mannitol (MW 182.2; specific radioactivity 57 mCi/mmol) 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.[3,9,10] The polymers were dissolved in serum-free DMEM containing the radioactive marker and the pH was adjusted to 6.20 with 0.1 M HCL or to 7.40 with 0.1 M NaOH. Chitosan hydrochloride was used as a dispersion in DMEM at pH 7.40. Solutions with TMC-H (0.05−1.5% w/v), TMC-L (0.5−1.5% w/v), and chitosan hydrochloride (0.5−1.5% w/v) were added on the apical side of the monolayers. The medium in the acceptor compartment was DMEM buffered at pH 7.40 with 40 mmol/L 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. Control experiments were run in every experiment with solutions containing the radioactive markers without the dissolved polymers. At the end of each experiment the pH of the solutions from both the apical and basolateral sides were checked for any changes in pH. All experiments were done in triplicate in an atmosphere of 95% air and 5% CO_2 at 37 °C. The 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 of scintillation cocktail (Ultima Gold) in a liquid scintillation counter (Tri −Carb 1500, Packard Instrument Company, Meridan, CT). Results were corrected for dilution and expressed as cumulative transport at time t.[3,9−11]

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.[3,9,10] The basolateral medium was PBS. After 30 min, the medium was removed from both sides of the cell monolayers and examined by light microscopy for exclusion of the marker. Cells excluding trypan blue were considered to be viable. Cells incubated for 5 min with 0.5% w/v sodium dodecyl sulfate in PBS and stained with trypan blue were used as a reference for uptake of the marker.

Data Analysis and Statistical Evaluation—From the permeation profiles of [^{14}C]mannitol, apparent permeability coefficients (P_{app}) were calculated according to the following equation:

$$P_{app} = dQ/dt/(A \cdot 60 \cdot C_0)$$

where P_{app} is the apparent permeability coefficient (cm s^{-1}), dQ/dt is the permeability rate (amount permeated per min), A is the diffusion area of the monolayer (cm^2), and C_0 is the initial concentration of the marker molecule. The regression coefficients (r^2) obtained from the curve fits were generally between 0.9 and 1.00. Transport enhancement ratios (R) were calculated from P_{app} values by:

$$R = P_{app}(\text{sample})/P_{app}(\text{control})$$

Statistical differences were determined using one-way analysis of variance (ANOVA) and Sheffe's F-test for multiple comparisons. Differences between groups were considered to be significant at $p < 0.05$.

Results

Synthesis and Characterization of TMC—The initial chitosan used to prepare TMC was only soluble in acidic solutions, but after quaternization it became highly soluble in water at every pH.[9,10,13] By repeating the reaction steps, polymers with different degrees of quaternization were obtained. The degree of quaternization was calculated from ^{1}H NMR spectra.[12,13] After an initial 60 min, a polymer (TMC-L) with a degree of quaternization of 12.3% was recovered. Longer reaction times (up to 6 h) do not yield polymers with higher degrees of quaternization.[9] Repeating the basic reaction twice, under the same conditions, with the polymer recovered after an initial 60 min, gave a highly quaternized product (TMC-H) with a degree of quaternization of 61.2%. At this degree of quaternization, the^{13}C and ^{1}H NMR spectra showed additionally some extent of methylation on the 3- and 6-hydroxyl groups of chitosan, but the polymer recovered was still perfectly soluble in water at pH values between 1 and 9.[13]

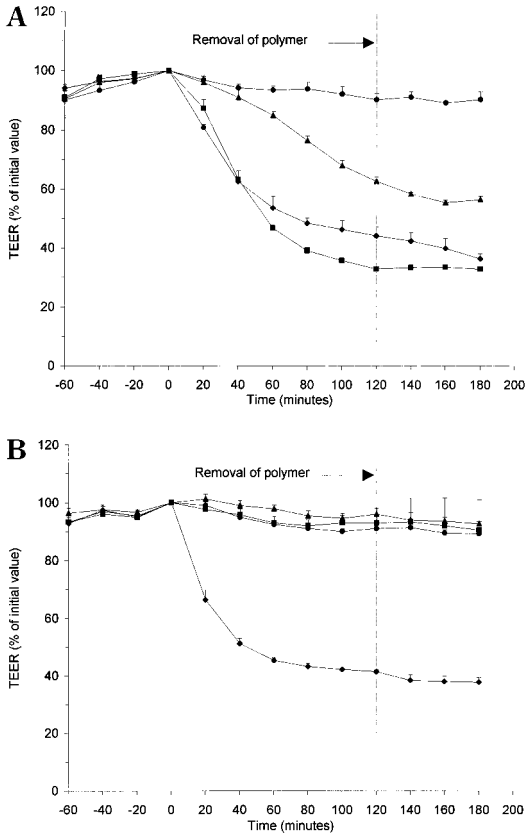

Figure 1—(A) The effects of 0.5% w/v concentrations of TMC-L, TMC-H, and chitosan hydrochloride on the TEER of Caco-2 cell monolayers at a pH of 6.20. (B) The effects of 0.5% w/v concentrations of TMC-L, TMC-H, and chitosan hydrochloride on the TEER of Caco-2 cell monolayers at a pH of 7.40. Each point represents the mean ± SD of three experiments. Key: (●) control; (▲) TMC-L; (◆) TMC-H; (■) chitosan hydrochloride; dotted line (- - -) represents start of reversibility experiment.

Effect on the TEER of Intestinal Epithelial Cells—The effects of 0.5% w/v concentrations of TMC-H, TMC-L, and chitosan hydrochloride on the TEER of the Caco-2 cell monolayers at pH 6.20 and 7.40 are shown in Figures 1A and 1B, respectively. At pH 6.20, incubation with these polymers resulted in a pronounced reduction in TEER values compared with the control group. After 2 h incubation, the reduction in TEER was in the following order: chitosan hydrochloride (67.1 ± 0.6%) > TMC-H (55.9 ± 3.1%) > TMC-L (37.3 ± 1.4%). Higher polymer concentrations (1.0 and 1.5% w/v) did not cause any further significant decreases in the TEER.

At pH 7.40, only TMC-H was able to decrease the TEER of the cell monolayers (Figure 1B). Neither TMC-L nor chitosan hydrochloride, even in concentrations of 1.5% w/v, was able to cause any significant decreases in TEER values compared with the control group. Incubation of the monolayers with TMC-H resulted in a pronounced decrease in the TEER of the cell monolayers. After 2 h incubation, the reduction in TEER was as followed: 43.1 ± 2.5% at 0.1% w/v TMC-H, 45.6 ± 3.7% at 0.25% w/v TMC-H, 58.6 ± 0.7% at 0.5% w/v TMC-H, 60.1 ± 3.1% at 1% w/v TMC-H, and 63.1 ± 2.1% at 1.5% w/v TMC-H. Even in a concentration as low as 0.05% w/v, TMC-H was able to reduce the TEER by 34.8 ± 4.1%.

Reversibility of the effect of 0.5% w/v concentrations of these polymers on TEER could not be demonstrated at any pH value (Figures 1A and 1B). Complete removal of the polymers, without damaging the cells, proved to be problematic due to the high viscosity and mucoadhesivity of the polymer solutions. Furthermore, the repeated washing of the cells and the short time allowed for recovery may also be reasons why significant reversibility of the effect could not be demonstrated. However, in concentrations <0.5% w/v of TMC-H, a clearer tendency to recover to initial values was observed. Staining with trypan blue after completion of all the TEER experiments did not result in any visible intracellular uptake of the marker, indicating that the cells were still viable after incubation with these polymers.

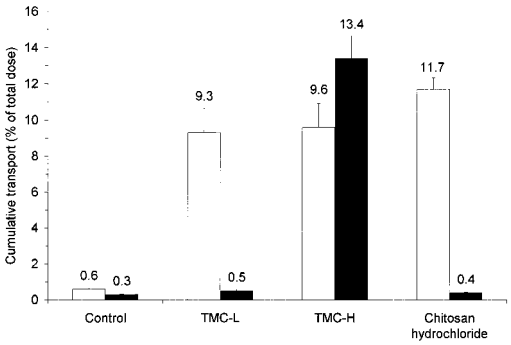

Figure 2—The effect of 0.5% w/v concentrations of TMC-L, TMC-H, and chitosan hydrochloride on the cumulative transport of [^{14}C]mannitol in Caco-2 cell monolayers at pH 6.20 (white bars) and pH 7.40 (black bars). Each point represents the mean ± SD of three experiments.

Effect on [^{14}C]Mannitol Transport—In Figure 2 the cumulative amounts of [^{14}C]mannitol transported after 4 h in the presence of the respective polymers (0.5% w/v) are given at both pH 6.20 and 7.40. From the permeation profiles of [^{14}C]mannitol at the different polymer concentrations and the two pH values, apparent permeability coefficients (P_{app}) values and transport enhancement ratios (R) were calculated (Table 1). Under the conditions described, only negligible amounts of [^{14}C]mannitol were transported in the control groups. At pH 6.20, all the polymers caused a marked accumulation of the marker molecule in the acceptor compartments. As evident from Figure 2, no major differences in the permeability of [^{14}C]-mannitol were found between TMC-L, TMC-H, and chitosan hydrochloride at 0.5% w/v concentrations. At this concentration, the permeability of [^{14}C]mannitol was increased 20-fold (TMC-H), 18-fold (TMC-L), and 23-fold (chitosan hydrochloride) compared with the control group (Table 1). Similar results were obtained at higher concentrations of TMC-L and TMC-H (1.0 and 1.5% w/v). With chitosan hydrochloride, a concentration-dependent increase in [^{14}C]mannitol transport was observed (14.6 ± 1.2% and 19.8 ± 3.8%, of the total dose applied, at 1.0 and 1.5% w/v concentrations, respectively). Apparently, chitosan hydrochloride is more effective than TMC-L and TMC-H at a pH of 6.20.

In agreement with the TEER results, only TMC-H was able to increase the transport of [^{14}C]mannitol at a pH of 7.40. The cumulative amounts of [^{14}C]mannitol transported at 0.05−1.5% w/v concentrations of TMC-H are depicted in Figure 3. These values represent a 31- to 48-fold increase in the permeation of [^{14}C]mannitol (Table 1). The efficiency of TMC-H at this pH value is evident from these results. Even in a concentration as low as 0.05% w/v, the permeability of [^{14}C]mannitol was increased 31-fold.

In all the permeation curves, both at pH 6.20 and 7.40, the transport of [^{14}C]mannitol from the donor to the acceptor compartment was relative steady, as evident from the slope of the individual concentration curves. This result indicates unhindered paracellular diffusion of this hydrophilic compound through opened tight junctions. No evidence of trypan blue inclusion into the intracellular spaces

Table 1—Effect of TMC-H, TMC-L, and Chitosan Hydrochloride on the Permeability of [^{14}C]Mannitol

		TMC-H		TMC-L		chitosan hydrochloride	
pH	polymer concentration (% w/v)	$P_{app} \times 10^{-7}$ (cm s^{-1})[a]	R	$P_{app} \times 10^{-7}$ (cm s^{-1})	R	$P_{app} \times 10^{-7}$ (cm s^{-1})	R
6.20	control	0.72 ± 0.09	1	0.72 ± 0.09	1	0.72 ± 0.09	1
	0.10	12.81 ± 2.56[b]	18	n.d.[c]		n.d.	
	0.25	13.32 ± 0.58[b]	19	n.d.		n.d.	
	0.50	14.28 ± 2.14[b]	20	13.15 ± 1.54[b]	18	16.38 ± 0.68[b,d]	23
	1.00	16.14 ± 0.72[b]	22	10.73 ± 1.87[b]	15	20.82 ± 1.07[b,d]	29
	1.50	14.44 ± 1.80[b]	20	11.32 ± 0.80[b]	16	31.03 ± 3.16[b,d]	43
7.40	control	0.47 ± 0.04	1	0.47 ± 0.04	1	0.47 ± 0.04	1
	0.05	14.69 ± 2.90[b]	31	n.d.		n.d.	
	0.10	14.06 ± 2.85[b]	30	n.d.		n.d.	
	0.25	15.10 ± 2.20[b]	32	n.d.		n.d.	
	0.50	20.14 ± 1.44[b]	43	0.63 ± 0.08	1	0.56 ± 0.09	1
	1.00	22.57 ± 1.89[b]	48	0.85 ± 0.08	2	0.51 ± 0.02	1
	1.50	15.13 ± 1.76[b]	32	1.07 ± 0.89	2	0.67 ± 0.04	1

[a] P_{app} = apparent permeability coeficient; each value represents the mean ± SD of 3 experiments. [b] Significantly different from control ($p < 0.05$). [c] n.d., Not determined. [d] Significantly different from all other treatmeants in group ($p < 0.05$).

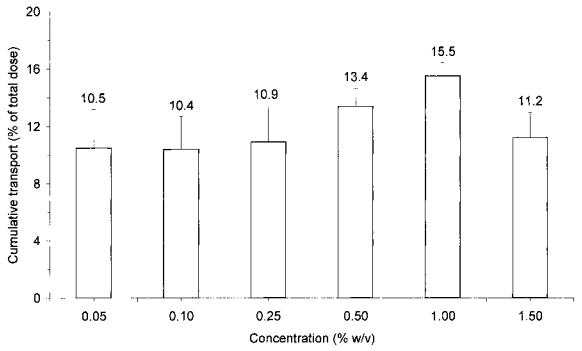

Figure 3—The effect of different concentrations of TMC-H on the cumulative transport of [^{14}C]mannitol in Caco-2 cell monolayers at a pH of 7.40. Each point represents the mean ± SD of three experiments.

of the cells was found when cells were stained with this dye after completion of all the transport studies.

Discussion

The results of this study show that both chitosan hydrochloride and TMC, with different degrees of quaternization, are potent absorption enhancers at a pH of 6.20. These polymers were able to decrease the TEER of the Caco-2 cell monolayers markedly. Measurement of TEER is believed to be a good indication of the tightness of the junctions between epithelial cells. The apparent difference in effect between chitosan hydrochloride and TMC could be explained in terms of their respective charge densities. The amino group on the C-2 position of TMC is a much larger unit, due to the attached methyl groups, compared with the amino group on the C-2 position of chitosan hydrochloride. With chitosan hydrochloride, about 10 wt % constitute the salt part and therefore the charge density is much higher as for TMC. Additionally, the attached methyl groups on TMC may partially shield the positive charge and steric effects may also influence the configuration of the TMC molecule where chitosan hydrochloride probably exists in a linear configuration at acidic pH values when the amino group is protonated. A clear comparison between these polymers is not possible because the equivalent amounts of free chitosan base could not be calculated, especially for TMC because NMR spectra showed that some amino groups are still dimethylated, depending on the degree of quaternization of the respective TMC sample.

In agreement with the reduction in TEER at a pH of 6.20, the permeation of the hydrophilic model compound [^{14}C]mannitol was also increased markedly, indicating enhanced paracellular transport. Because of their positive charge, cationic macromolecules such as chitosan and TMC can interact with the anionic components of the glycoproteins on the surface of epithelial cells. Cationic macromolecules can displace cations from electronegative sites on cell membranes, which require coordination with cations for dimensional stability.[16] It is also known that the interior of the tight junction channel are hydrated and contained fixed negative sites. Changes in the concentration of certain ions in the pore could result in alteration in tight junction resistance leading to opening of the pore with increased paracellular permeability.[17] It has been reported that chitosan is able to induce a redistribution in cytoskeletal F-action, thereby resulting in a structural reorganization of tight junction associated proteins such as ZO-1.[1,6] TMC probably acts in a similar way to open the tight junctions.[10]

At a pH of 7.40 only TMC-H was able to decrease the TEER of the cell monolayers. In agreement with this reduction in measured TEER, the permeation of [^{14}C]-mannitol was substantially increased even in concentrations as low as 0.05% w/v. Chitosan hydrochloride has an apparent pK_a value of 5.5−6.5 and, at a pH of 7.40, it probably exists in a more coiled configuration with no protonated amino groups that can interact with the cell surfaces or tight junctions. With TMC-L, the charged density has not reach the threshold concentration to induce interaction with the anionic components of the glycoproteins at the surface of the cells or with the fixed negative charges within the aqueous tight junctions. Additionally, the attached methyl groups may partially shield the positive charge from significant interaction with the cell membranes or tight junctions. TMC-H, on the other hand, has a much higher proportion of quaternary amino groups that seems to be sufficient to interact with anionic components on the cell membranes or the negative sites within the tight junctions.

Because of the high viscosity and mucoadhesive character of chitosan hydrochloride and TMC, it is unlikely that all the polymer solution could be removed without damaging the cells. Therefore, reversibility of the effect of these polymers on the TEER could not be established. Nevertheless, the absence of intracellular trypan blue staining, after prolonged incubation with these polymers, implies that the Caco-2 cells remained undamaged and functionally intact. This implication is in agreement with results obtained in a recent study where Caco-2 cell monolayers were tested with the propidium iodide nucleic stain for their viability after 4 h of incubation with TMC (40 and 60% quaternized) at different concentrations up to 1.0% w/v. In all cases, the monolayers were able to exlude propidium iodide.[18]

In summary, our study shows that the insolubility of chitosan and chitosan salts prevents these polymers from being effective as absorption enhancers at neutral pH values. It has been shown that TMC, already at very low degrees of quaternization, is superior in water solubility compared with chitosan and chitosan salts.[9,10] The degree of quaternization of TMC is demonstrated to play an important role in its ability to open the tight junctions of intestinal epithelial cells. Highly quaternized TMC proves to be a very potent absorption enhancer, especially at neutral pH values. The use of this chitosan derivative may be a valuable contribution to the development of selective and effective delivery systems for hydrophilic compounds such as peptide drugs, especially in neutral and basic environments where normal chitosan salts are ineffective.

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