N-Trimethyl Chitosan Chloride as a Potential Absorption Enhancer Across Mucosal Surfaces: *In Vitro* Evaluation in Intestinal Epithelial Cells (Caco-2)

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Purpose. Previous studies have established that chitosan hydrochloride and glutamate are potent absorption enhancers for large hydrophilic compounds across mucosal surfaces. However, these compounds lack solubility at neutral pH values. A partially quaternized and well-soluble derivative of chitosan, N-trimethyl chitosan chloride, was synthesized and the effects of this polymer on the transepithelial electrical resistance and permeability of intestinal epithelial cells were investigated *in vitro*.
Methods. N-trimethyl chitosan chloride was synthesized by reductive methylation and characterized with NMR. The effect of this polymer (1.0–2.5% w/v) on the transepithelial electrical resistance of intestinal epithelial cells, using Caco-2 cell monolayers, was investigated. Permeation of the hydrophilic model compounds [^{14}C]-mannitol (MW 182.2), FITC-Dextran (MW 4400) and the peptide drug buserelin (MW 1299.5), in the presence of N-trimethyl chitosan chloride (1.5–2.5% w/v), was followed for 3 hours. The transport process of the fluorescent marker, FITC-Dextran 4400, across the cell monolayers was visualised with confocal laser scanning microscopy. Viability of the cells was checked with the trypan blue exclusion technique.
Results. N-trimethyl chitosan chloride was found to be a perfectly water-soluble, partially quaternized (about 12%) derivative of chitosan. This polymer (1.5–2.5% w/v) caused a pronounced and immediate reduction (25–85%) in the transepithelial electrical resistance of Caco-2 cells. Large increases in the transport rate of [^{14}C]-mannitol (32–60 fold), FITC-Dextran 4400 (167–373 fold) and buserelin (28–73 fold) were demonstrated. Confocal laser scanning microscopy confirmed that N-trimethyl chitosan chloride opens the tight junctions of intestinal epithelial cells to allow increased transport of hydrophilic compounds through the paracellular transport pathway. No deleterious effects to the cells could be demonstrated with trypan blue.
Conclusions. The potential use of N-trimethyl chitosan chloride as an absorption enhancer across mucosal surfaces could be an important contribution towards the development of effective delivery systems for hydrophilic drugs.

KEY WORDS: N-trimethyl chitosan chloride; absorption enhancer; paracellular transport; transepithelial electrical resistance; Caco-2 cell monolayers.

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INTRODUCTION

Chitosan is a polycationic polymer with numerous applications in the food, agricultural and cosmetic industries. Advantages of this polymer include high availability, low cost, high biocompatibility, biodegradability and ease of chemical modification (1–4). Pharmaceutical applications include use as a tablet binder (5) and a disintegrant (6). Chitosan has gel forming properties in the low pH range and is used as a drug carrier in hydrocolloids and gel formulations (7–8). Chitosan is also used as a constituent in polymeric matrix systems, microspheres and microcapsules for the sustained release of water soluble drugs (9–12). The mucoadhesive properties of chitosan (13) and ability to act as an absorption enhancer has lead to its use as a coating material for multilamellar liposomes (14) and use in formulations aimed at controlled drug delivery at specific sections in the gastro-intestinal tract (15) and across other mucosal surfaces such as the nasal, buccal and vaginal epithelia (16–17).

Chitosan glutamate was able to improve the transport of the peptide drug 9-desglycinamide, 8-L-arginine vasopressin (DGAVP), *in vitro* across intestinal epithelial cells (Caco-2 cell monolayers) (18). Similar results were obtained with the peptide drug buserelin after intraduodenal administration with chitosan hydrochloride in *in vivo* experiments in rats (19). The nasal administration of insulin with chitosan glutamate leads to a pronounced reduction in blood glucose levels in rats and sheep (20). These increases in absorption could be attributed to the effect of chitosan on the integrity of the epithelial tight junctions. Chitosan opens the tight junctions in a concentration and pH dependent way to allow paracellular transport of large hydrophilic compounds. This interaction with the tight junctions is believed to be an interaction of the positively charged amino group on the C-2 position of chitosan, with negatively charged sites on the cell surfaces and tight junctions (21).

Chitosan is a weak base and a certain amount of acid is required to transform the glucosamine units into the positively charged, water-soluble form. At neutral pH values, most chitosan molecules will loose their charge and precipitate from solution. Most macromolecular pharmaceuticals such as peptide drugs are indicated for chronic administration, and therefore possibilities for the potential use of chitosan in the more basic environment of the large intestine and colon, are limited. In this regard chitosan derivatives with different physico-chemical properties, especially water solubility at neutral and basic pH values, will be of particular interest. Our hypothesis is, that polymers like chitosan with a primary amino group may not be the best polymer, but that polymers or derivatives with different substituents, basicities or charged densities, will have the same or even increased efficiency in opening tight junctions.

The aim of this investigation was to synthesize a water-soluble chitosan derivative, N-trimethyl chitosan chloride, and to evaluate the effect of this polymer on the permeability of intestinal epithelial cells *in vitro* for increased transport of large hydrophilic compounds such as peptide drugs.

MATERIALS AND METHODS

Synthesis and Characterization of N-Trimethyl Chitosan Chloride (TMC)

TMC was synthesized from sieved fractions (<500 μm) of chitosan (degree of acetylation ca. 25%) (Pronova Biopolymer,

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Drammen, Norway) based on the method of Domard *et al.* (22). Briefly, the experimental conditions are reductive methylation of chitosan for 60 minutes with iodomethane in a strong basic environment at 60°C. The counterion (I^-) was exchanged to Cl^- by dissolving the quaternized polymer in a small quantity of water, followed by the addition of HCl in methanol. The final product obtained was characterized by NMR spectroscopy, viscosity, solubility and pK_a measurements. The degree of quaternization of the polymer was calculated from H^1 NMR spectra (600 MHz) obtained with a Bruker DMX-600 spectrometer. Viscosity measurements on the polymers (0.1% w/v) were performed on a Haake rotation viscosimeter (Haake CV 100/ME 30, Haake GmbH & Co., Karlsruhe, Germany) at 23.5°C in aqueous acetic acid (0.1% v/v). The pK_a values were obtained by potentiometric titration.

Cell Cultures

Caco-2 cells (passages 79–81) 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 7.4] (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. 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), transport experiments (6-well plates) and confocal laser scanning microscopy experiments (24-well plates), 21–23 days after seeding (23–25).

Measurement of the Transepithelial Electrical Resistance (TEER)

A Millicell® ERS meter (Millipore Corp., Bedford, MA, USA), connected to a pair of chopstick electrodes, was used to measure the effect on the TEER of the filters every 20 minutes (23–25). Measurements started 1 hour prior to incubation on the apical side of the cells with solutions of TMC (1.5–2.5% w/v) in serum-free DMEM. After 2 hours the polymer solutions were removed and cells were washed three times with, and replaced by DMEM. The TEER was measured for an additional hour to study the reversibility of the effect of the polymer solutions. 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 1000–1200 Ωcm^2.

Permeability Studies

[^{14}C]-Mannitol Transport

[^{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 (23). Solutions containing the radioactive marker, with and without TMC (1.5–2.5% w/v), were added on the apical side of the monolayers and samples of 200 μl were taken every 20 minutes for 3 hours from the basolateral side. Samples taken from the basolateral side were replaced with an equal volume of clean medium. The medium in the acceptor compartment was DMEM buffered at pH 7.4 with HEPES (40 mmol/l) (Sigma). TMC was dissolved in serum-free DMEM + HEPES. 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. The radioactivity present in the samples was determined in a liquid scintillation counter (Tri-Carb 1500, Packard Instrument Co.). Results were corrected for dilution and expressed as cumulative transport at time *t*.

FITC-Dextran 4400 (FD-4) Transport

Fluorescein isothiocyanate-labeled dextran (FD-4, MW 4400) was purchased from Sigma. Solutions containing FD-4 (1 mg/ml), with and without TMC (1.5–2.5% w/v) in serum-free DMEM + HEPES, were added on the apical side of the monolayers. Transport experiments were performed as described for [^{14}C]-mannitol. Samples of 100 μl from the basolateral side were diluted with 300 μl water and analysed for FD-4 content by Size Exclusion Chromatography as described previously (26). Results were corrected for dilution and expressed as cumulative transport at time *t*.

Buserelin Transport

Buserelin acetate (MW 1299.5) was a gift from Hoechst AG (Frankfurt, Germany). Solutions of buserelin (198.15 μg/ml), with and without TMC (1.5–2.5% w/v) in serum-free DMEM + HEPES, were incubated apically on Caco-2 cell monolayers. DMEM + HEPES was used as basolateral medium. Transport studies were performed as described for [^{14}C]-mannitol. Samples of 200 μl from the acceptor compartment were diluted with 800 μl phosphoric acid (pH 3.0) and analysed with HPLC-UV. The system used was a Spectraseries UV 150 detector (wavelength: 210 nm), a Spectraseries P 200 gradient pump, a Spectraseries AS 100 autosampler and a Datajet integrator (all Spectra-Physics). A Chrompak 250 mm/4.6 mm reversed phase C8 column was used. The eluent system was 35% acetonitrile/65% buffer system: 0.12 M KH_2PO_4, (pH 6.2). Results were corrected for dilution and expressed as cumulative transport at time *t*.

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 7.4) 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 (23). The basolateral medium was PBS. After 30 minutes 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.

Confocal Laser Scanning Microscopy (CLSM)

The cell monolayers were incubated apically for 60 minutes with a TMC solution (1.5% w/v) containing FD-4 (1 mg/ml) in serum-free DMEM + HEPES. A MRC-600

Lasersharp system (Bio-Rad Laboratories, Richmond, CA, USA), linked to a Zeiss IM 35 inverted microscope equipped with a Zeiss Neofluar × 63, NA 1.25 oil objective (Carl Zeiss, Oberkochen, Germany), was used for confocal imaging. FD-4 was excited at a wavelength of 488 nm. Data were processed with a desk-top computer linked to an optical disk drive (Reflection Systems, Melbourn, UK) with Comos software (Bio-Rad Laboratories). The filters were prepared for imaging according to a method described previously (25). Pictures were taken within 5–10 minutes after removal from the transwell system to ascertain viability of the cells. Pictures were printed with a CP50E video printer (Mitsubishi, Tokyo, Japan).

Analysis

Apparent permeability coefficients (P_{app}) were calculated according to the following equation:

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

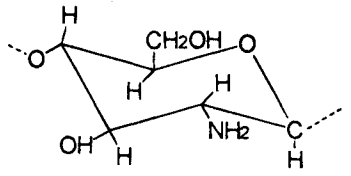
where P_{app} is the apparent permeability coefficient (cm/s), dc/dt is the permeability rate (concentration unit/min.), A is the diffusion area of the monolayer (cm^2) and C_0 is the initial concentration of the respective compounds. The regression coefficients (r^2) obtained from the curve fits were generally between 0.90–1.00. Absorption enhancement ratios (R) were calculated from P_{app} values.

RESULTS

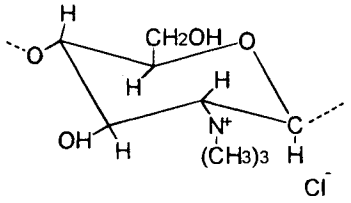
Synthesis and Characterization of TMC

Fig. 1 shows the chemical structure of chitosan and TMC and Table I shows the physical and chemical properties of quaternized chitosan. The initial chitosan used in the reaction was only soluble in acidic solutions, but after quaternization it became highly soluble in water at every pH. In general these polymers are soluble for a degree of quaternization as low as 10%. A decrease in the intrinsic viscosity is observed and this correlated with the degradative reaction conditions in alkaline medium. The reaction was stopped at certain times and polymers recovered after reaction times of 6 hours did not differ from polymers recovered after reaction times of 75 minutes.

Repeating the reaction several times, under the same conditions, with the polymer recovered after an initial 60 minutes, gave polymers with degrees of quaternization of 40–60% (data not shown). However, at these degrees of quaternization ^{13}C and ^{1}H NMR spectra show a high extend of methylation on the hydroxyl groups of chitosan. TMC with a degree of quaternization of 12.28% was used in this study.



(A) Chitosan



(B) N-trimethyl chitosan chloride

Fig. 1. Chemical structure of (A) chitosan and (B) N-trimethyl chitosan chloride)

Effect of TMC on the TEER of Intestinal Epithelial Cells

The effect of TMC on the TEER of Caco-2 cell monolayers is shown in Fig. 2. Incubation with TMC (1.5%, 2.0% and 2.5%) resulted in a pronounced and immediate reduction (9 ± 4%, 52 ± 3% and 79 ± 0.3% respectively after 20 minutes) in TEER values in a concentration dependant way, compared to the control group. In concentrations of 1% and less no significant reduction in the resistance of the cells were measured. Prolonged incubation, up to 2 hours, only results in a gradual decrease in resistance, compared with the initial reduction in TEER after 20 minutes.

Reversibility of the effect can be seen from Fig. 2, especially at 1.5 and 2.0% concentrations of TMC. With removal of the polymer solutions, repeated washing and substituting the apical medium again with fresh DMEM, monolayers started to recover slowly and a slight increase in resistance, towards the initial values, were found. Complete removal of the polymers, without damaging the cells, prove to be difficult due to the high viscosity of the solutions and this may be the reason why the increase in resistance is only gradual. Staining with trypan blue after completion of the experiments did not result in any visible intracellular uptake of the marker and we concluded

Table I. Physical and Chemical Properties of Chitosan and TMC

Polymer	Reaction time (min)	Degree of quaternization (%)	pK_a	Viscosity[a] (mPa/s)	Solubility[b] pH 4	Solubility[b] pH 9
Chitosan	–	–	5.5	19.570 ± 0	√	x
TMC	45	10.73	5.9	1.484 ± 0.003	√	√
TMC	60	12.28	6.0	1.269 ± 0.009	√	√
TMC	360	13.06	6.0	1.208 ± 0.007	√	√

[a] Each value represents the mean ± S.D. of 3 experiments.
[b] Solubility: √ = soluble and x = insoluble.

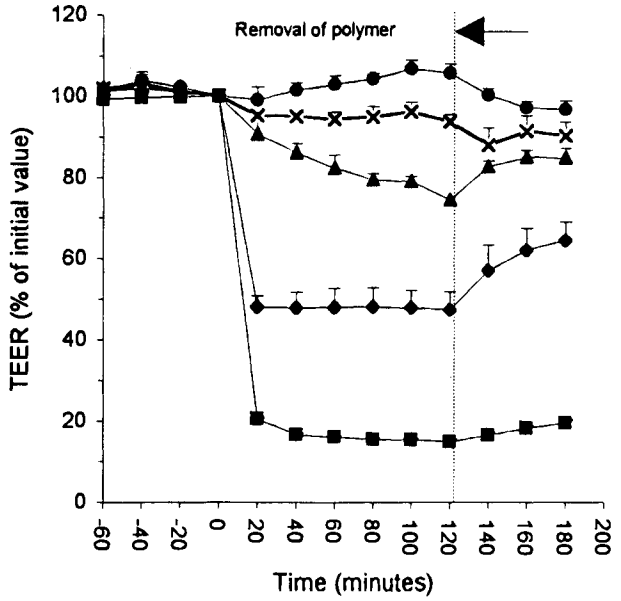


Fig. 2. Effect of TMC on the TEER of Caco-2 cell monolayers. Each point represents the mean ± S.D. of 3 experiments. Keys: Control (X), TMC 1.0% (●), TMC 1.5% (▲), TMC 2.0% (♦), TMC 2.5% (■), dotted line (-----) represents start of reversibilty experiment.

that the viability of the monolayers was not affected by incubation with TMC.

Effect of TMC on the Permeability of Intestinal Epithelial Cells

The permeation profiles of the three compounds are given in Fig. 3A–C and the P_{app} values and absorption enhancement ratios (R) are give in Table II. Under the conditions described, a very low baseline permeability was found and only negligible amounts were transported in the control groups. Incubation with TMC resulted in a marked accumulation of all the compounds in the acceptor compartments. [^{14}C]-Mannitol, with the lowest molecular weight, exhibited the highest permeability and the cumulative amount transported up to 3 hours was 5.3 ± 1.1% at a 1.5% concentration of TMC (Fig. 3A). The permeability decreased with an increase in molecular weight and the cumulative amounts of buserelin and FD-4, transported up to 3 hours, were 0.9 ± 0.1% (Fig. 3C) and 0.4 ± 0.1% (Fig. 3B) respectively. This suggests that the permeation of these compounds across intestinal epithelial cells, is among other factors, dependent on molecular size. In general an increase in TMC concentration resulted in an increase in transport and P_{app}. With all three substances P_{app} was the highest for 2.5% concentrations of TMC.

These results also suggest that a higher baseline permeability, although already very low, does exist for the smaller mannitol and buserelin molecules and that a certain amount can diffuse through aqueous pores in the cell membranes and/or tight junctions, compared to the larger FD-4 molecule. Further proof of this is evident in comparing the P_{app} of the control groups of each compound (Table II).

In all the concentration curves a short lag time (0–30 minutes) is observed. The lag time is indicative of the time each molecule needs to diffuse through the viscous polymer

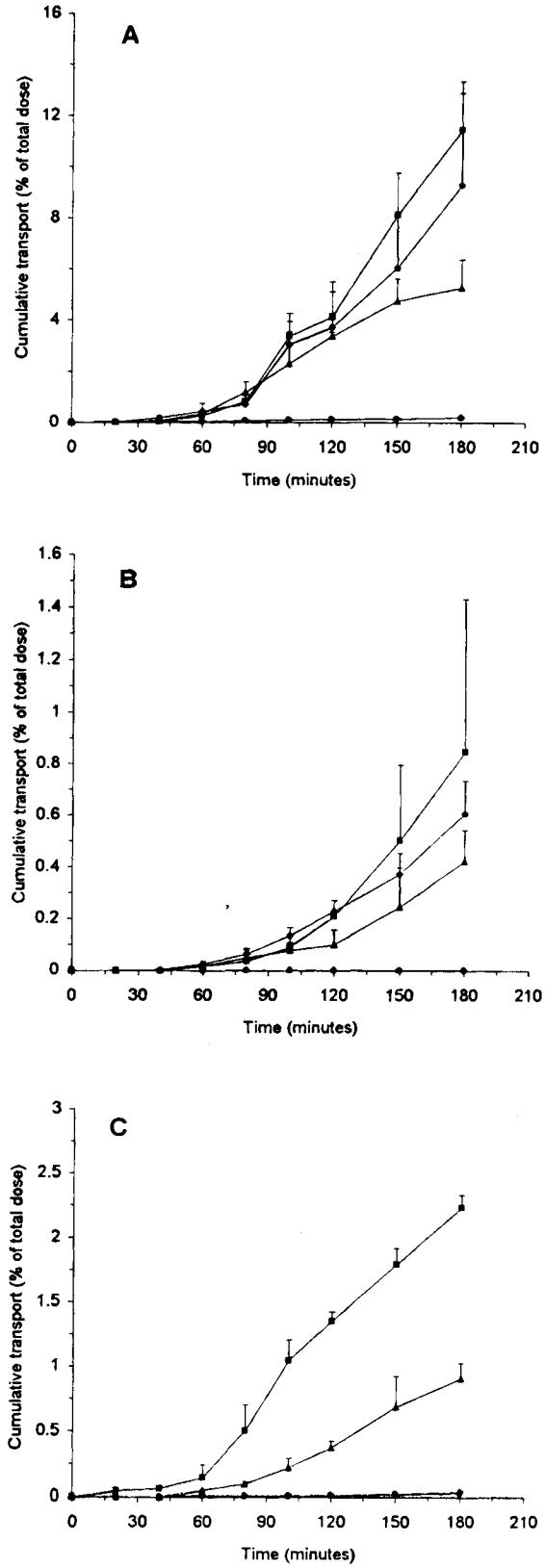


Fig. 3. Each point represents the mean ± S.D. of three experiments. [A] Effect of TMC on the cumulative transport of [^{14}C]-mannitol in Caco-2 cell monolayers. Keys: Control (●), TMC 1.5% (▲), TMC 2.0% (♦), TMC 2.5% (■). [B] Effect of TMC on the cumulative transport of FD-4 in Caco-2 cell monolayers. Keys: Control (●), TMC 1.5% (▲), TMC 2.0% (♦), TMC 2.5% (■). [C] Effect of TMC on the cumulative transport of buserelin in Caco-2 cell monolayers. Keys: Control (●), TMC 1.5% (▲), TMC 2.5% (■).

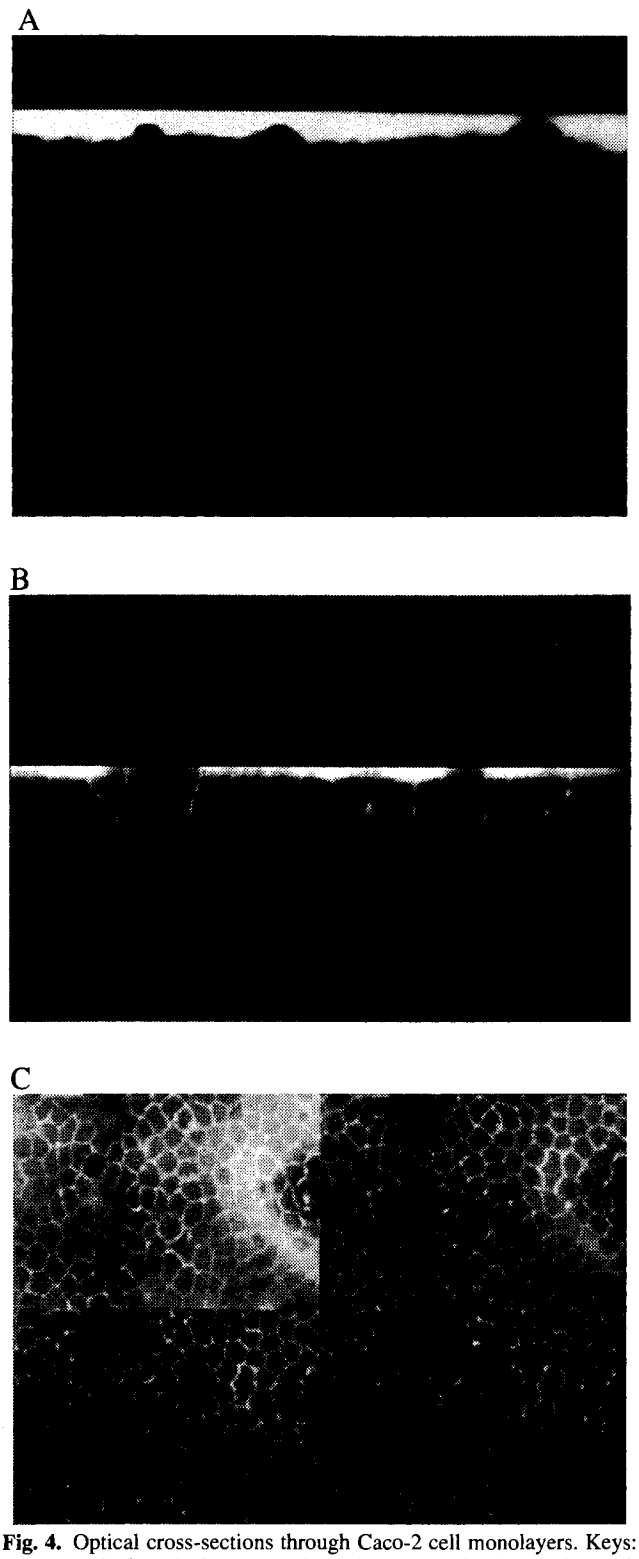


Fig. 4. Optical cross-sections through Caco-2 cell monolayers. Keys: (A) An optical vertical cross-section (XZ image) after incubation with FD-4 (1 mg/ml) alone (control). Top is apical and bottom is basolateral. (B) An optical vertical cross-section (XZ image) after incubation with FD-4 (1 mg/ml) and TMC (1.5%). Top is apical and bottom is basolateral. (C) An optical horizontal cross-section (XY image, step size 3 μm) after incubation with FD-4 (1 mg/ml) and TMC (1.5%).

network into the intercellular spaces and through the tight junctions, before it could reach the acceptor compartments. Additionally the configuration of each molecule may influence this lag time. After this lag time the transport of the compounds from the donor to the acceptor sides was relatively steady, as evident from the slope of the individual concentration curves, indicating unhindered paracellular diffusion of this hydrophilic compounds through opened tight junctions.

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 the transport studies, which is an indication that the cells were still viable and not affected by incubation with TMC.

Visualisation of the Transport Pathway

An optical cross-section of a cell monolayer after incubation with the control solution, containing only FD-4, is depicted in Fig. 4A. Fluorescence was only detected on top of the monolayer. After 60 minutes incubation with a 1.5% concentration of TMC, fluorescence was also detected in the intercellular spaces as evident from Fig. 4B and 4C which represent a vertical and horizontal scan through a monolayer. This confocal images clearly show that the tight junctions are open and that FD-4 is able to permeate into the paracellular spaces. No fluorescence could be found within the cells, which is further prove that incubation with TMC does not damage the cell membranes.

DISCUSSION

TMC is a derivative of chitosan with superior solubility and basicity, compared with other chitosan salts, and can be prepared by a relatively easy chemical procedure. The increase in solubility and basicity could be attributed to the replacement of the primary amino groups on the C-2 position of chitosan with quaternary amino groups (22). Complete quaternization of chitosan will probably be difficult due the presence of some acetyl groups (from chitin) and possible steric effects of the attached methyl groups on adjacent quaternary amino groups. Further prove of this is evident from ^{1}H NMR spectra that shows that a high proportion of the amino groups is dimethylated and can still be protonated in acidic environments. However, already at very low degrees of quaternization this polymer is soluble at every pH and an increase in basicity has been found.

In this investigation the effect of TMC was studied with respect to its potential use as an absorption enhancer for the peroral delivery of hydrophilic drugs such as peptides. As the results show, TMC is able to decrease the TEER of Caco-2 cell monolayers in concentrations of 1.5% and above. Measurement of TEER are believed to be a good indication of the tightness of the junctions between cells. At all concentrations tested, reversibility of the achieved effect could be demonstrated after removal of the polymer solutions. Because of the high viscosity and adhesive character of TMC it is unlikely that all the polymer solution can be removed without damaging the cells, therefore the reversibility observed was only gradual. The absence of intracellular trypan blue, after prolonged incubation with TMC, also implies that the Caco-2 cells remained undamaged and functionally intact.

In agreement with the reduction in TEER, large increases in the permeability rate of the two hydrophilic markers, [^{14}C]-

Table II. Effect of TMC on the Permeability of Various Compounds

TMC Concentration (% w/v)	[^{14}C]-Mannitol $P_{app} \times 10^{-7}$ (cm/s)[a]	R[b]	Buserelin $P_{app} \times 10^{-7}$ (cm/s)	R	FD-4 $P_{app} \times 10^{-7}$ (cm/s)	R
Control	0.38 ± 0.05	1	0.067 ± 0.004	1	0.0040 ± 0.0005	1
1.5	12.30 ± 2.48	32	1.86 ± 0.38	28	0.67 ± 0.25	167
2.0	18.04 ± 7.03	48	n.d.	n.d.	1.09 ± 0.23	274
2.5	22.68 ± 3.63	60	4.86 ± 0.12	73	1.49 ± 0.96	373

[a] Each value represents the mean ± S.D. of 3 experiments.
[b] Absorption enhancement ratios (R) calculated by P_{app}: (Sample)/P_{app}: (Control). n.d. = not determined.

mannitol and FD-4, and the peptide drug buserelin were found. All three compounds are highly hydrophilic in nature. The increase in the transport of these compounds is also in good agreement with a decrease in the TEER which was measured directly in the filters at several time points during the transport studies (data not shown). In all the control groups no change in the resistance was observed, while incubation with TMC resulted in reduction of the TEER which correlated well with the values of the TEER study.

It has been reported recently that the intraduodenal application of buserelin in rats resulted in an absolute bioavailability of 0.1 ± 0.1% and that a chitosan hydrochloride (1.5%) gel formulation was able to increase the bioavailablility to 5.1 ± 1.5% (19). We have also found that chitosan glutamate was able to increase the permeation of the peptide drug DGAVP across Caco-2 cell monolayers. Incubation with this polymer (1.0%, pH = 5.6) for 4 hours resulted in cumulative transport of 1.2 ± 0.1% of the total dose applied (18). In this study, incubation with TMC (2.5%) resulted in cumulative transport of 2.2 ± 0.1% of the total dose applied of buserelin. From these results it is concluded that TMC is as effective as chitosan salts to act as an absorption enhancer across intestinal epithelial cells.

Confocal laser scanning microscopy studies show that TMC is able to open the tight junctions of epithelial cells to allow for paracellular transport of large hydrophilic compounds. The TEER results suggests that this is a reversible effect. The effect of chitosan on the integrity of the tight junctions is probably due to an interaction of the positively charged amino groups with negatively charged sites on the cell surfaces and tight junctions (21). TMC most likely also acts in the same manner, and the charge density will have an important effect on the ability of TMC to open tight junctions.

In summary, our study shows that TMC is a derivative with superior solubility and basicity, compared with chitosan. TMC is especially effective in enhancing the transport of small hydrophilic compounds such as [^{14}C]-mannitol, but also improve the transport of larger compounds such as the peptide drug buserelin. The potential use of this derivative can be an important contribution towards the development of selective and effective delivery systems for protein and peptide drugs. Present studies are directed at comparing this polymer with chitosan salts and to determine the effect of the degree of quaternization (charge density) on the permeability of intestinal epithelial cells.

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