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Effects of *N*-trimethyl chitosan chloride, a novel absorption enhancer, on Caco-2 intestinal epithelia and the ciliary beat frequency of chicken embryo trachea

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Abstract

N-trimethyl chitosan (TMC) polymers are quaternized chitosans in different degrees of trimethylation. These polymers enhance the absorption of macromolecules through mucosal epithelia by triggering the reversible opening of tight junctions and only allow for paracellular transport. To investigate the safety of these novel absorption enhancers cytotoxicity and ciliotoxicity studies have been performed. Intestinal Caco-2 cell monolayers were chosen to study possible membrane damaging effects of these polymers, using confocal laser scanning microscopy visualization of nuclear staining by a membrane impermeable fluorescent probe during transport of the paracellular marker Texas red dextran (MW 10 000). Ciliated chicken embryo trachea tissue was used to study the effect of the polymers on the ciliary beat frequency (CBF) in vitro. In both studies the TMC polymers of different degrees of substitution (20, 40 and 60%) were tested at a concentration of 1.0% (w/v). No substantial cell membrane damage could be detected on the Caco-2 cells treated with TMCs, while the effect on the CBF in vitro was found to be marginal. TMC60 and TMC40 enhance paracellular transport of Texas red dextran in Caco-2 cell monolayers, whereas TMC20 is ineffective. In conclusion, TMCs of high degrees of substitution may be effective and safe absorption enhancers for peptide and protein drug delivery.

Keywords: *N*-trimethyl chitosan chloride; Tight junctions; Caco-2; Texas red dextran; Ciliary beat frequency

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1. Introduction

Chitosan is a β-(1,4) linked polymer of 2-amino-2-deoxy-D-glucose and is obtained by deacetylation of chitin, the second most abundant naturally occurring polymer after cellulose, in different molecular weights and degrees of deacetylation. Recently, chitosan has been demonstrated to be a potent absorption enhancer for hydrophilic macromolecular model compounds and peptide drugs such as buserelin and insulin in vitro and in vivo (Artursson et al., 1994; Illum et al., 1994; Lueßen et al., 1996, 1997).

However, chitosan (Seacure 244, 93% deacetylated) has an apparent pK_a of 5.6, as measured by potentiometric titration, and is only soluble in acidic solutions with pH values lower than 6.0. As a cationic polymer its solubility is strongly dependent on the pH of its environment. When ionized cationic polymers carry positive charges, they swell in an acidic environment and their diffusional properties are strongly affected (Hariharan and Peppas, 1996). When the pH decreases below 6.0, the concentration of ionizable groups increases in the polymer structure and also does the repulsion between the charged groups, causing higher water uptake by the polymer and higher solubility. This property of chitosan, being soluble only in acidic solutions, is disadvantageous for the biomedical applications of this polymer, because at the physiological pH values (7.4) most chitosans precipitate from the solution and consequently become ineffective as permeation enhancer (Kotzé et al., 1999).

In recent years, several derivatives of chitosan have been synthesized which are water-soluble over a wider pH range. Among these is *N*-trimethyl chitosan chloride (TMC), which recently has been synthesized in different degrees of substitution (Sieval et al., 1998). These methylated chitosan derivatives have been evaluated in vitro and in vivo as permeation enhancers in intestinal and nasal epithelia (Kotzé et al., 1997, 1998a,b; Thanou et al., 1999). A major conclusion from these studies was that the highly quaternized TMC with a degree of trimethylation of 60% (TMC60) was the most efficient permeation enhancer at neutral pH values when compared to TMC with lower degrees of quaternization of 20 and 40%. High charge density promotes the solubility and the permeation enhancing effect of this polymer. It is believed that TMC60 like chitosan (when dissolved in an acidic environment) opens the tight junctions of the epithelia, allowing for the paracellular transport of hydrophilic high MW compounds, but does not interact with mucosal membrane components and hence does not induce transcellular transport.

The mechanism of the opening of the tight junctions under the influence of either acidic chitosan or the quaternized derivatives has not yet been elucidated. Chitosan like most cationic macromolecules such as protamine and polylysine, can interact with anionic components (sialic acid) of the glycoproteins on the surface of the epithelial cells. Furthermore, the interior of the tight junction (pores) are highly hydrated and contain fixed negative charges. An alteration in the relative concentration of specific ion species in the pore volume would result in substantial alterations in tight junction resistance, which might lead to loosening or opening of the pore (Madara, 1989).

The above mentioned properties of cationic polymers can also exhibit damaging effects on the epithelial cells. It has been shown that polylysine and protamine can bind to anionic sites of gall bladder epithelial cell membranes, thereby producing morphological changes such as (a) collapse of the microvillar structure; (b) membrane folding from the apical border into the terminal web; (c) 'fused' membranes with pentalaminar substructure. These morphological changes were already observed with small concentrations of polylysine of 1.0 μg/ml (Quinton and Phillpot, 1973).

Chitosan is considered as a biocompatible, biodegradable and non-toxic polymer (Hirano et al., 1988; Chandy and Sharma, 1990). It does not have a damaging effect on nasal membranes in rats (Aspden et al., 1996) and also does not affect the mucociliary clearance rate after daily nasal application in human volunteers (Aspden et al., 1997a). Even when chitosans of different MW were applied chronically in guinea pig nasal epithelia, they had a mild effect on the nasal ciliary beat frequency of the excised tissue (Aspden et al.,

1997b). However, in one study using a murine melanoma cell line (B16F10) and rat erythrocytes it has been found that chitosan affected the cell viability and increased the haemoglobin release in a concentration-dependent way (Carreño-Gómez and Duncan, 1997).

For the evaluation of novel absorption enhancers safety studies are required in order to guarantee the absence of tissue damaging effects of the compound under investigation. In the present study both Caco-2 cell cultures and ciliated chicken embryo trachea tissue in vitro are used as models of epithelial membranes to assess the safety of the cationic polymer TMC. The Caco-2 system is one of the most established in vitro model for intestinal epithelia (Anderberg et al., 1992; Hurni et al., 1993), and this cell culture has also been used in preformulation studies of the cellular effects of various pharmaceutical excipients as co-solvents and absorption enhancers (Artursson and Borchardt, 1997). Studying the effects on the ciliary beat frequency of chicken embryo trachea in vitro has proven to be a valuable tool in the design of safe nasal drug excipients (Merkus et al., 1993).

2. Materials and methods

2.1. Materials

N-trimethyl chitosan chloride was synthesized in different degrees of substitution as described previously (Sieval et al., 1998; Thanou et al., 1999). Briefly, sieved chitosan with a particle size 200–400 μm (Seacure 244, 93% deacetylated; gift of Pronova Biopolymer A.S., Drammen, Norway) was mixed with methyliodide in an alkaline solution of *N*-methylpyrrolidinone both obtained from Acros (Geel, Belgium) at 60°C for 75 min. The product was isolated by precipitation with ethanol and subsequent centrifugation. This product consists of TMC20 (15–20% of trimethylation). After this first step the obtained product underwent a second step of reductive methylation, to yield the final products TMC40 and TMC60 iodide depending on the duration of the second step. The product was precipitated by addition of ethanol and isolated by centrifugation. The purification step of the final products included the exchange of the counter-ion iodide with chloride in a NaCl solution and extended washing with ethanol and diethylether. The products were dried in vacuo and measured in D_2O at 80°C using a 600 MHz spectrometer (Bruker, Switzerland) for the characterization of the degree of quaternization. For all experiments TMCs of different degrees of substitution were tested. TMC20 (D.S. 18%) TMC40 (D.S.39%) and TMC60 (D.S. 65%), as characterized by ^{1}H-NMR, were used in the present study.

Sodium dodecyl sulfate (SDS) was obtained from Baker (Deventer, The Netherlands) and YO-PRO™-1 iodide and Texas red dextran (MW 10 000) from Molecular Probes (Leiden, The Netherlands). All other reagents were of analytical grade. The water (MQ) used was filtered by a Milli-Q UF plus ultrapure water system from Millipore (Etten-Leur, The Netherlands).

2.1.1. Caco-2 cell cultures

Caco-2 cell cultures of passage numbers 78–83 were used for all of the experiments. The cells were seeded on tissue culture polycarbonate membrane filters (pore size 0.4 μm, area 4.7 cm^2 and 0.33 cm^2) in Costar Transwell 6- and 24-well plates (Costar Europe, Badhoevedorp, The Netherlands) at a seeding density of 10^4 cells/cm^2 (Kotzé et al., 1999). Dulbecco's Modified Eagle's Medium (DMEM, Sigma, Bornem, Belgium pH 7.4), supplemented with 1% non-essential amino acids, 10% fetal calf serum (Hyclone, Greiner, The Netherlands), benzylpenicillin G (160 U/ml) and streptomycin sulfate (100 μg/ml) (both obtained from Sigma) was used as culture medium, and added to both the donor and the acceptor compartment. The medium was changed every second day. The cell cultures were kept at a temperature of 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. For all experiments cells were used 23–25 days after seeding. The initial transepithelial electrical resistance (TEER) values ranged from 1000 to 1200 Ω*cm^2. At 2 h before the experiments the medium was replaced by Hanks Balanced Salt Solution (HBSS) containing 11 mM D-Glucose buffered to pH 7.4 with 25 mM *n*-(2-hydroxyethyl) piperazine-*N*-(2-ethanosulfonic acid) (HEPES).

Apical applications with or without (control) polymer and Texas red dextran were prepared in HBSS-HEPES. Texas red dextran was dissolved in HBSS-HEPES at a concentration of 0.06% (w/v). For co-application 1.0% (w/v) TMC60, TMC40 or TMC20 was incorporated and 1 ml of this solution was applied on Caco-2 cell monolayers. The cells were incubated for 4 h at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

Before visualization by CLSM, YO-PRO™-1 was added to the apical solution at a concentration of 1.25 μM, and the cells were incubated for 5 min more. Then, the apical solution was removed and the monolayers were detached from the culture well and carefully transferred on coverslips.

2.1.2. Confocal laser scanning microscopy visualization

The supporting filter with the monolayer was sandwiched between two coverslips and held in position with a stainless steel ring and a Teflon ring (especially designed for the stage) and then mounted on the heated microscope stage (37°C) of the confocal laser scanning microscope (Hurni et al., 1993). A MRC-600 Lasersharp system (Bio-Rad Laboratories, Richmond, CA, USA) linked to a Zeiss IM 35 inverted microscope (Carl Zeiss, Oberkochen, Germany) was used to visualize the monolayers. The YO-PRO™-1 excitation/emission wavelengths are 491/509 (nm/nm). YO-PRO™-1 was excited with the 488nm argon-ion laser. The Texas red dextran conjugates have characteristic excitation/emission wavelengths of 595/615 (nm/nm). The degree of molar substitution on the Texas red dextran was 0.58. The Texas red dextran was excited by the 643 nm HeNe laser. For dual labeling a block with a DC 580 and a LP600 filter was used for the detection of both probes (Nagelkerke and De Bont, 1996). Plan Apo 60 or 40 oil immersion objective lenses Nikon were used. All experiments were done in triplicate. Images of damaged cells were also used for comparison of the viability of the cells. In that case the cells were incubated with 0.1% SDS and 0.06% of Texas red dextran for 10 min, and during the last 5 min of the incubation 1.25 μM YO-PRO™-1 was also present in the apical solution.

2.2. Ciliary beat frequency in vitro

Ciliary beat frequency (CBF) measurements were performed with ciliated tissue of chicken embryo trachea (Merkus et al., 1993). Briefly, the chicken embryo trachea was dissected from the embryo, and sliced into small rings of approximately 1 mm thickness. The trachea slices were placed in stainless steel supporting rings, and were allowed to recover for 30 min in Locke-Ringer solution at 33°C. Then the tissue samples were put in a well containing 1 ml of the TMC solution in Locke Ringer (pH 7) and placed under an Olympus BH-2 light microscope. The microscope table was connected with a thermostat to maintain the temperature of 33°C. The CBF was subsequently monitored using a photo-electric registration device. A light beam was transmitted through the moving cilia, and after magnification by a microscope the flickering light was projected on a photocell. The electrical signal generated by this photocell, was visualized by a computer monitor and the frequency of this signal was calculated electronically by a Fast Fourier Transform algorithm and displayed as a frequency distribution. The CBF was measured every 5–10 min. Control experiments were performed on tissue samples in pure Locke-Ringer solution at pH 7. Data were calculated as the relative frequency of the initial frequency measured in the Locke-Ringer solution at the start of the experiment, the latter being expressed as 100%.

3. Results

3.1. Effects on the Caco-2 cell cultures

In the present studies Caco-2 cells were incubated with a novel permeation enhancer and fluorescent probes. In these experiments the Texas red dextran marker was chosen, because it is pH-independent probe and does not interfere with the quaternized chitosans. Furthermore, this probe especially marks the cell-to-cell contact and the paracellular pathways (Haugland, 1996). Attempts to co-administer the frequently used FITC-dextrans (MW 4400) with TMCs were not

successful, since FITC-dextrans are negatively charged polymers, which result in strong electrostatic interactions with the positively charged TMCs (aggregation). Texas red dextran, being pH-independent, proved to be suitable for the present studies with the positively charged TMCs. Propidium iodide, a nucleic acid probe which has been used to stain cell nuclei in our previous studies (Thanou et al., 1999), is unsuitable since its excitation and emission wavelengths are interfering with those of the Texas red dextran conjugates. Therefore in the present experiments YO-PRO™-1 is used, being a membrane impermeable staining probe, which is emitting only when it is bound to the nuclei of the cells. Cells, which do not take up this nuclear probe, are supposed to be viable.

For all TMC-treated cells the number of the stained nuclei was not different from those of the control (untreated cells), and nuclear staining was limited to less than 1% of the total cell population. As a positive control a 0.1% (w/v) SDS solution was used. After 10 min of incubation almost all of the cells had stained nuclei. Furthermore, tight junctions and intercellular contacts, could not be detected anymore (Fig. 1b). When the cells were incubated only with Texas red dextran (control) and YO-PRO™-1, the fluorescence of Texas red dextran was limited only at the apical side between the cells and no further than about 1 μm depth from the apical level (Fig. 1a).

In the case of TMC60 treated cells at concentration of 1.0% (w/v) horizontal cross sections were visualized also close to the basolateral level, where the nuclei of the Caco-2 cells are located. Such cross sections are shown at Fig. 2 at three different depth levels: apical, medial and basal. In this case the monolayers were incubated with TMC60, Texas red dextran and YO-PRO™-1. From the left part of the figure it is evident that Texas red dextran is mainly visualized between the cells at the basal side of the monolayers. Cells treated with 1.0% (w/v) TMC40 gave similar pictures of Texas red dextran distribution as cells treated with TMC60, while cells treated with 1.0% (w/v) TMC20 showed no paracellular fluorescence at the basal level (data not shown). The right part of Fig. 2 demonstrates that 1.0% (w/v) TMC60 does not lead to any nuclear staining, and similar results have been observed for TMC40 and TMC20 in concentrations of 1.0% (w/v) (data not shown).

In Fig. 3 vertical cross-sections of Caco-2 monolayers are shown. In the upper image, cells were incubated only with the paracellular Texas red probe and the nucleic acid staining probe, while the lower image represents monolayers treated also with 1% (w/v) TMC60. It is obvious that in the latter case (treatment with TMC60) the Texas red dextran fluorescence is also present in the intercellular space and at the basolateral side of the monolayers. The fluorescence staining of the paracellular routes is evident and rather homogenous in the whole Caco-2 monolayer. No staining of transcellular passage of the fluorescent probe could be detected.

3.2. Ciliary beat frequency in vitro

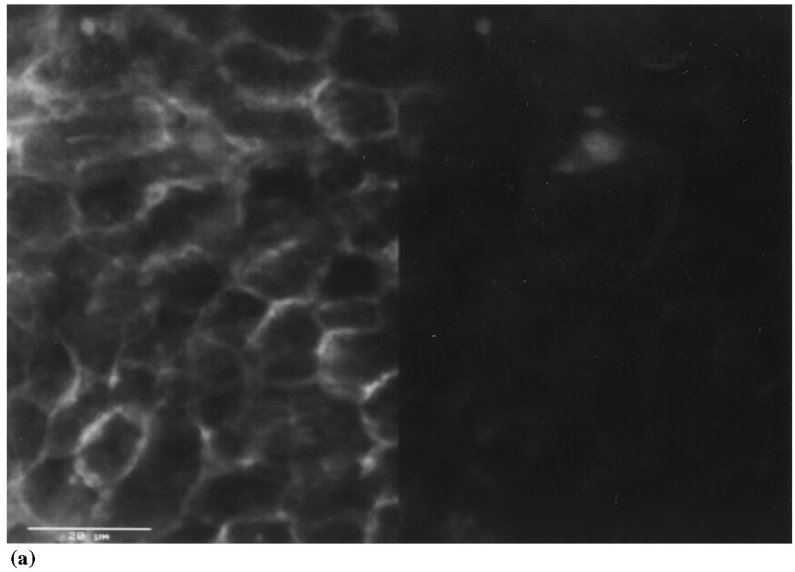
The results of the CBF measurements are depicted in Fig. 4. As apparent from this figure, the effect of all TMC polymers tested, in concentrations of 1.0% (w/v), on the ciliary activity in vitro is very mild, and even less than that of physiological saline (0.9% NaCl).

4. Discussion

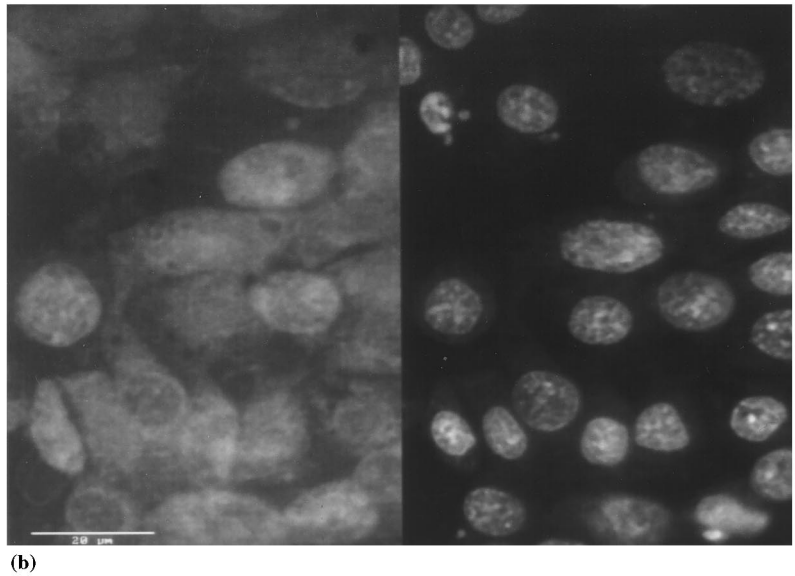
The evaluation of novel absorption enhancers for hydrophilic macromolecular drugs such as peptides and proteins should meet several requirements. The absence of cyto- and systemic toxicity is the most important. Chitosan and its quaternized derivatives are polymeric compounds of high number MW (600 000–100 000 Da). It is expected that these polymers are not absorbed from different epithelia, since their size is too large to pass across paracellular pathways or to be absorbed transcellularly. In this study the cytotoxic and the ciliostatic effects of TMCs were investigated in order to assess possible damaging effects of these polymers.

From previous studies using propidium iodide as a cell membrane impermeable nucleic acid stain, the first indications for the absence of cyto-

toxicity under the influence of TMC polymers were shown. The most potent transport enhancing TMCs (TMC40 and TMC60) were tested in the Caco-2 cell model at a neutral pH value, and their effect on the cell viability was found to be insignificant when compared to controls (Thanou et al., 1999). Nucleic acid stains are used to evaluate cytotoxicity of compounds in a number of cell



(a)



(b)

Fig. 1. CLSM horizontal cross sections of Caco-2 cell monolayers. (a) Treated with Texas red dextran 10 000 for 4 h and YO-PRO™-1 (dual labelling). Left part, image visualization of the Texas red dextran channel; paracellular fluorescence was observed only at the apical level. Right part, image visualization of the YO-PRO™-1 channel; limited number of cells showed intracellular fluorescence. (b) Treated with SDS 0.1% (w/v) and Texas red dextran (MW 10 000) for 10 min; nuclear staining was performed using YO-PRO™-1. Left part: the paracellular marker is diffused between and into the cells. Right part: all cells have stained nuclei.

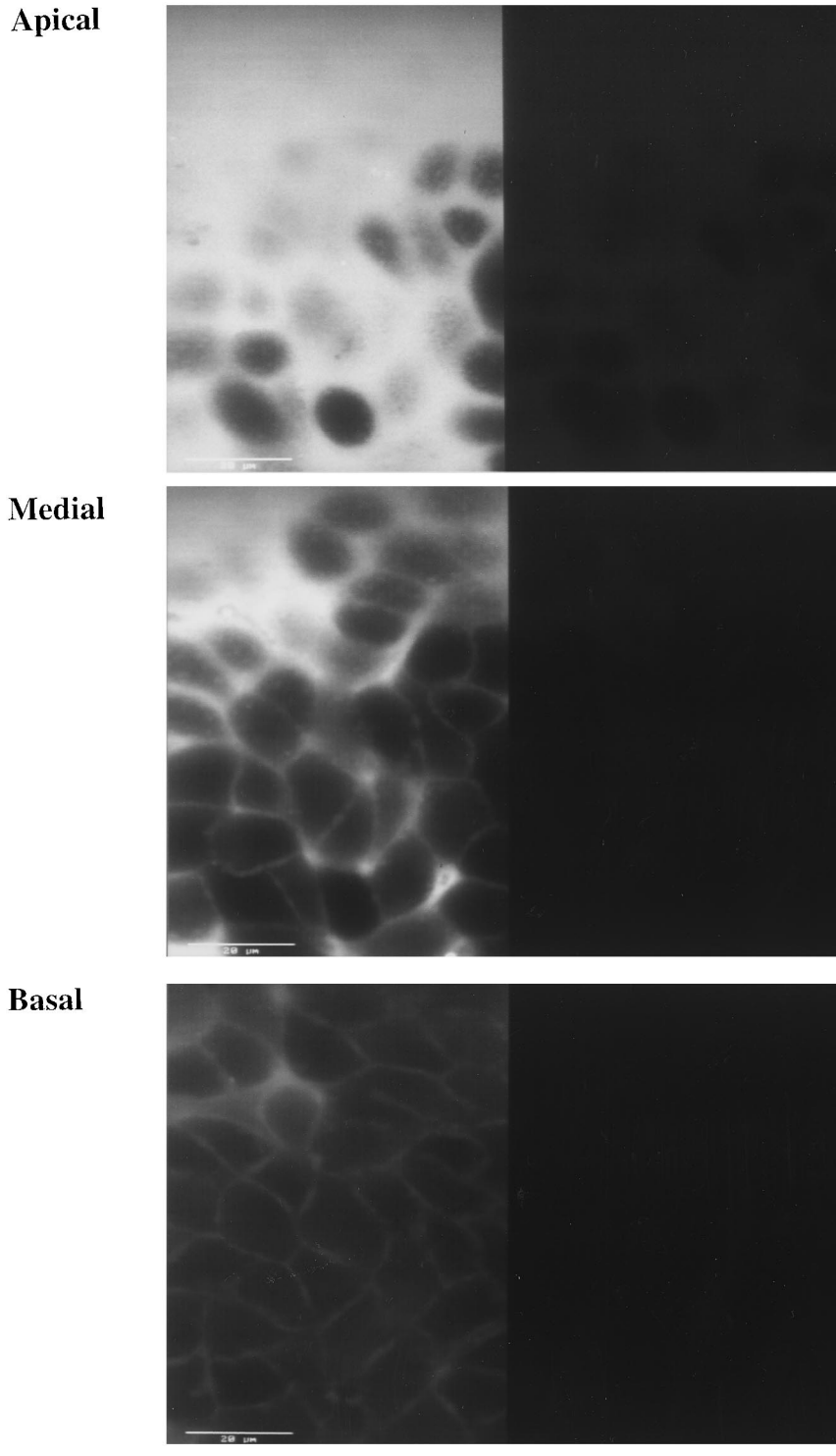


Fig. 2. CLSM horizontal cross sections of Caco-2 monolayers treated with 1% (w/v) TMC60 for 4 h. Left part visualization of Texas red dextran fluorescence channel. Right part visualization of YO-PRO™-1 fluorescence channel. Series of three horizontal cross-sections at successive lower focal levels (apical, medial, basal). Note the absence of fluorescence at the YO-PRO™-1 channel, indicating the absence of stained nuclei.

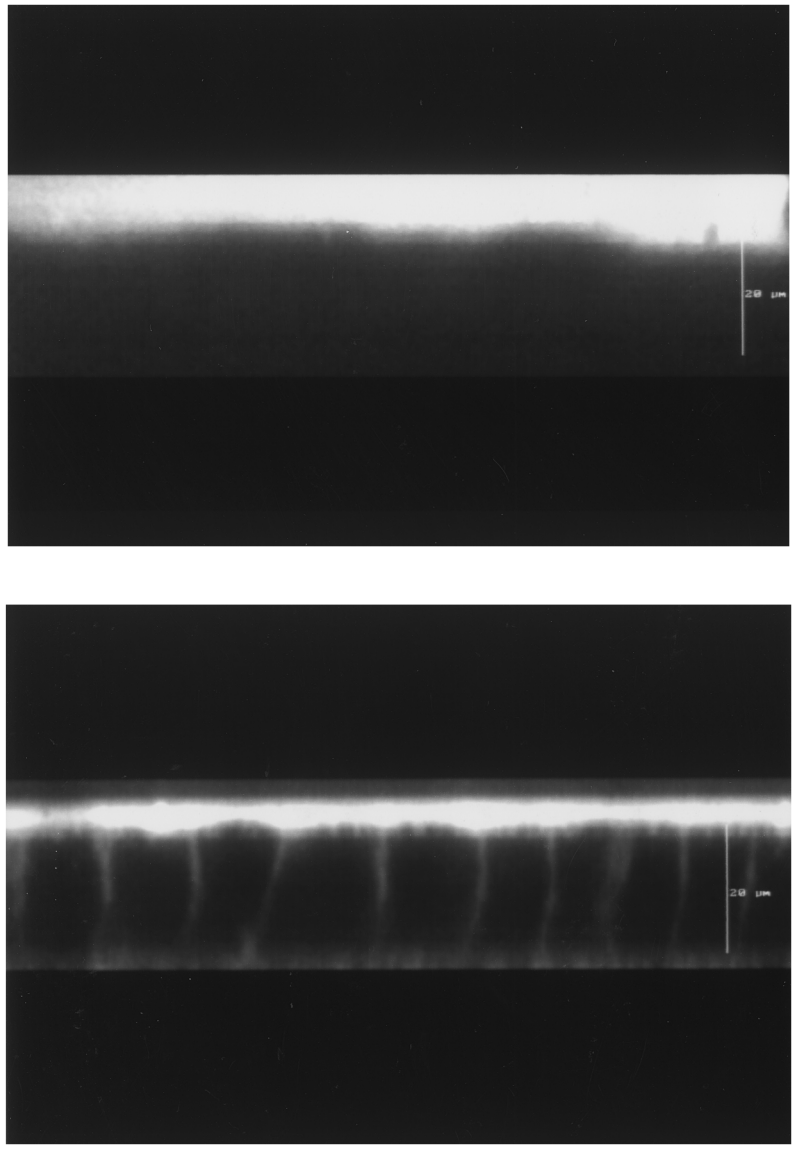


Fig. 3. CLSM vertical XZ cross sections through Caco-2 cell monolayers. Upper panel: cells were treated only with the fluorescent markers Texas red dextran for 4 h and YO-PRO™-1. Lower panel: cells were treated additionally with 1% (w/v) TMC60 for 4 h. Note the paracellular presence of Texas red dextran 10 000 and the absence of intracellular YO-PRO™-1 staining.

lines. Since these stains are not emitting fluorescence when not bound to nucleic material of the cell (Haugland, 1996), they are suitable probes to visualize cell membrane disrupture caused by different factors. In the present study the YO-PRO™-1 nucleic acid stain has been used in the presence of the novel absorption enhancer TMC and a high MW fluorescence marker dextran as a model for peptide drugs. In all Caco-2 cell monolayers tested the effect of highly substituted TMCs (1.0% w/v) on the paracellular transport of the marker dextran was found to be independent on possible cytotoxic effects of TMCs. Furthermore, a previously observed difference in efficacy between TMC20 and TMCs with higher degrees of trimethylation (Thanou et al., 1999; Kotzé et al.,

1998b) was also clear in the present study for polymers applied on cell monolayers at pH 7.2. From these CLSM visualization studies it is evident that the effect of TMCs on the paracellular permeability of the Caco-2 intestinal epithelia is not occurring by damaging effects of the polymers on the cell membranes, but because of opening of the tight junctions and thereby facilitating the paracellular transport of the marker dextran. Absorption enhancers such as bile salts, cyclodextrins, laureth-9, fusidate derivatives and phospholipids have been used to increase the absorption of peptide and protein drugs across the nasal mucosa. Ciliotioxicity studies of a number of absorption enhancers and preservatives using the chicken embryo trachea in vitro, have shown that L-*a*-lysophosphatidylcholine, sodium taurodihydrofusidate, laureth-9 and sodium deoxycholate in concentrations of 1% (w/v) induce complete and irreversible ciliostasis within 5–10 min because they directly interact with the cell membrane constituents and are absorbed by themselves as well, resulting in severe toxic cellular effects (Marttin et al., 1998). Methylated β-cyclodextrins in concentrations of 2% (w/v) were demonstrated to be relatively non-toxic for the ciliated tissue, having similar effects on ciliary beating as physiological saline (Marttin et al., 1998). Measuring of CBF in vitro is probably a too sensitive approach for studying the local toxicity of drugs and excipients, since in vivo experiments in rats and monkeys showed no deleterious effects on nasal ciliated epithelium of formulations containing the preservative benzalkonium chloride in concentrations up to 0.02% (w/v), whereas 0.02% benzalkonium chloride has strong cilio-inhibitory potency in vitro (Romeijn et al., 1996; Ainge et al., 1994).

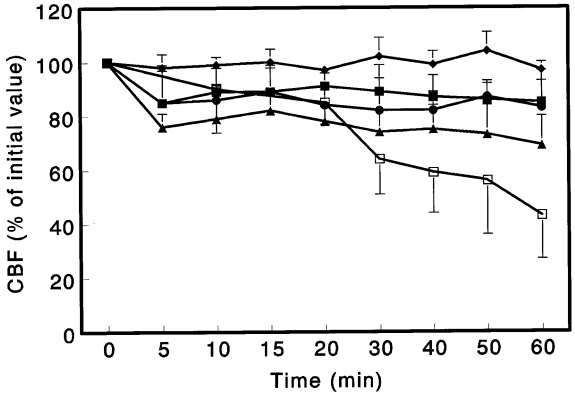


Fig. 4. The effects of 1% (w/v) TMC polymers (pH 7) and physiological saline (0.9% NaCl) on the ciliary beat frequency (CBF) of chicken embryo trachea in vitro. Control solutions: Locke-Ringer (pH 7) (◆) and physiological saline (0.9% NaCl;□). TMC polymers: TMC20 (●), TMC40 (▲), TMC60 (■). Data are expressed as the mean ± SD of 6–8 experiments.

The results of the present study show that TMCs in concentrations of 1.0% (w/v) does not substantially influence the ciliary beating in vitro. This suggests the safety of TMC as absorption enhancers for nasal and other mucosal tissues of hydrophilic macromolecules such as peptide and protein drugs.

5. Conclusions

Toxicity experiments with Caco-2 cell monolayers and ciliated embryo trachea tissue have been performed for the evaluation of the novel TMC polymers supposed to be safe absorption enhancers for intestinal and nasal epithelia. Using both approaches, it has been proven that TMC polymers are non-toxic. Furthermore, CLSM visualization studies have shown that 1.0% TMC60 and TMC40 are able to facilitate the permeation of hydrophilic Texas red dextran (MW 10 000) across Caco-2 intestinal epithelia. It is expected that these polymers are safe absorption enhancers at neutral or basic pH values for peptide and protein drugs. Further investigations on the mechanism of opening the tight junctions, enhancing the paracellular permeability and avoiding the transcellular permeability, are necessary for the complete characterization of these novel absorption enhancing polymers.

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