Research Article

Methylated *N*-(4-*N*,*N*-Dimethylaminobenzyl) Chitosan, a Novel Chitosan Derivative, Enhances Paracellular Permeability Across Intestinal Epithelial Cells (Caco-2)

Jariya Kowapradit,[1] Praneet Opanasopit,[1,3,4] Tanasait Ngawhiranpat,[1] Auayporn Apirakaramwong,[1] Theerasak Rojanarata,[1] Uracha Ruktanonchai,[2] and Warayuth Sajomsang[2]

Abstract. The aim of this study was to investigate the effect of methylated *N*-(4-*N*,*N*-dimethylamino-benzyl) chitosan, TM-Bz-CS, on the paracellular permeability of Caco-2 cell monolayers and its toxicity towards the cell lines. The factors affecting epithelial permeability, e.g., degree of quaternization (DQ) and extent of dimethylaminobenzyl substitution (ES), were evaluated in intestinal cell monolayers of Caco-2 cells using the transepithelial electrical resistance and permeability of Caco-2 cell monolayers, with fluorescein isothiocyanate dextran 4,400 (FD-4) as a model compound for paracellular tight-junction transport. Cytotoxicity was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide viability assay. The results revealed that, at pH 7.4, TM-Bz-CS appeared to increase cell permeability in a concentration-dependent manner, and this effect was relatively reversible at lower doses of 0.05–0.5 mM. Higher DQ and the ES caused the permeability of FD-4 to be higher. The cytotoxicity of TM-Bz-CS depended on concentration, %DQ, and %ES. These studies demonstrated that this novel modified chitosan has potential as an absorption enhancer.

KEY WORDS: absorption enhancer; Caco-2 cells; chitosan derivatives; permeability.

INTRODUCTION

Macromolecular drugs are poorly absorbed across mucosal membranes due to their hydrophilic nature and molecular mass. These compounds represent a class of valuable therapeutics that is still administered via the parenteral route. One of the greatest challenges is to deliver macromolecules orally (1). These poorly bioavailable drugs are inefficient when administered orally, however, because only a limited portion of the dose reaches the plasma in order to exert its pharmacological effect. Low oral bioavailability leads to low plasma levels and increased intersubject and intrasubject variability (2). Therefore, there is a practical need for improving the bioavailability of such compounds when applied at mucosal absorptive surfaces.

In order to increase the absorption of poorly permeable drugs, excipients, such as absorption enhancers, have been evaluated (3). These enhancers facilitate the absorption of hydrophilic macromolecules by increasing their transcellular and/or paracellular transport across mucosal epithelia (4). During the last decade, a number of structurally different compounds with absorption-enhancing properties have been investigated. Chitosan (CS) [(1→4)-2-amino-2-deoxy-β-D-glu-can] is a copolymer of *N*-acetyl glucosamine (GlcNAc) and glucosamine (GlcN). It is a deacetylated chitin that is now of great interest as a functional material of great potential in various areas, such as the biomedical field. Chitosan has already been approved as a food additive in Japan and is believed to be nontoxic (5), although a recent report indicates that the toxicity of the compound depends on its molecular size, degree of acetylation, and specific counter ion (6). Due to its mucoadhesive character and favorable toxicological properties, CS has been studied as a potential absorption enhancer across intestinal epithelia. CS is able to reduce the transepithelial electrical resistance of a cultured intestinal epithelial cell line (Caco-2) (7–9) and to increase the transport of hydrophilic molecules, such as [^{14}C]-mannitol, 9-desglycinamide, 8-arginine vasopressin (DGAVP, molecular

[1] Nanotechnology for Drug/Gene Delivery Systems Group, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand.
[2] National Nanotechnology Center, Thailand Science Park, Pathumthani, Thailand.
[3] Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, 73000, Thailand.
[4] To whom correspondence should be addressed. (e-mail: Praneet@email.pharm.su.ac.th)

ABBREVIATIONS: *A*, the surface area of the monolayers; C_0, the initial drug concentration in the donor compartment; CS, chitosan; CSA, chitosan acetate; DM-Bz-CS, *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan; DMEM, Dulbecco's modified Eagle's medium; DQ, degree of quaternization; ES, the extent of dimethylaminobenzyl substitution; FD-4, fluorescein isothiocyanate dextran 4,400; GlcN, glucosamine; GlcNAc, *N*-acetyl glucosamine; HBSS, Hank balanced salt solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; P_{app}, the apparent permeability coefficient; PBS, phosphate-buffered saline; *R*, absorption enhancement ratios; TM-Bz-CS, methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan; TM-CS, trimethylated chitosan; TEER, the transepithelial electrical resistance.

weight (MW) 1,299.5), and fluorescein dextrans, significantly in Caco-2 cells (10). Chitosan salts, like chitosan glutamate and chitosan hydrochloride, have been used *in vivo* as absorption enhancers for peptide drugs. The nasal application of insulin with chitosan glutamate at pH 4.4 led to a significant reduction in the blood glucose levels of rats and sheep (11), while the intraduodenal application of buserelin and chitosan hydrochloride in a gel formulation at pH 6.7 increased the absolute bioavailability of buserelin from $0.1\pm0.1\%$ to $5.1\pm1.5\%$ (12). The increase in the transport of these compounds is believed to be the result of an interaction between the positively charged amino groups on the C-2 position of chitosan with negatively charged sites on the cell membranes and tight junctions, thereby altering the integrity of the tight junctions to allow for paracellular transport (7). Additionally, mucoadhesion may play an important role in this process by increasing the residence time of the drugs on the cell surfaces.

However, the increased transport of all these compounds was obtained in acidic conditions, where the pH was less or on the order of the pK_a (6.0 to 6.5) of chitosan. This suggests that the charge density of chitosan is an important factor for the enhancement of mucosal transport (7). Chitosan is a weak base and, in neutral and basic environments, chitosan molecules will lose their charge and precipitate from solution. Under these conditions, chitosan will be ineffective as an absorption enhancer, limiting its use in the more basic environment of the large intestine and colon. Therefore, several chitosan derivatives have been synthesized in the last few years in order to obtain modified carriers with altered physicochemical characteristics. Recently, our research groups have successfully synthesized a modified chitosan, methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (TM-Bz-CS), that showed antibacterial activity (13) and gave high gene transfection efficiency (14). This modified chitosan is water soluble at physiological pH and, thus, it might be used as a carrier for oral drug delivery. Therefore, in this study, water-soluble CS derivatives, TM-Bz-CS, have been synthesized and evaluated for their *in vitro* absorption-enhancing properties on the transepithelial electrical resistance (TEER) and permeability of Caco-2 cell monolayers by using fluorescein isothiocyanate dextran 4,400 (FD-4) as a model drug for hydrophilic macromolecules. CS was substituted with the *N*,*N*-dimethylbenzyl group to provide the hydrophobic moiety for the improved hydrophobic interaction with the cell membrane, and it was quaternized to render CS soluble. A number of variables that influenced absorption enhancement, such as degree of quaternization (DQ), the extent of dimethylaminobenzyl substitution (ES), and pH of culture medium, were investigated.

MATERIALS AND METHODS

Materials

CS was purchased from Seafresh Chitosan Lab (Bangkok, Thailand) with a MW of 276 kDa and a 94% degree of deacetylation. Sodium cyanoborohydride ($NaCNBH_3$) was purchased from Aldrich (Milwaukee, WI, USA). 4-*N*,*N*-Dimethylaminobenzaldehyde, iodomethane, sodium iodide, and 1-methyl-2-pyrrolidone were purchased from Fluka

(Deisenhofen, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and FD-4 were purchased from Sigma-Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin–ethylenediaminetetraacetic acid, penicillin–streptomycin antibiotics, and fetal bovine serum were obtained from GIBCO-Invitrogen (Grand Island, NY, USA). The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Transwell® (12-well plates) cell culture chambers with a 3.0-μm pore size were purchased from Corning Life Sciences (MA, USA). All other chemicals were of cell culture and molecular biology quality.

Synthesis of Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) Chitosan

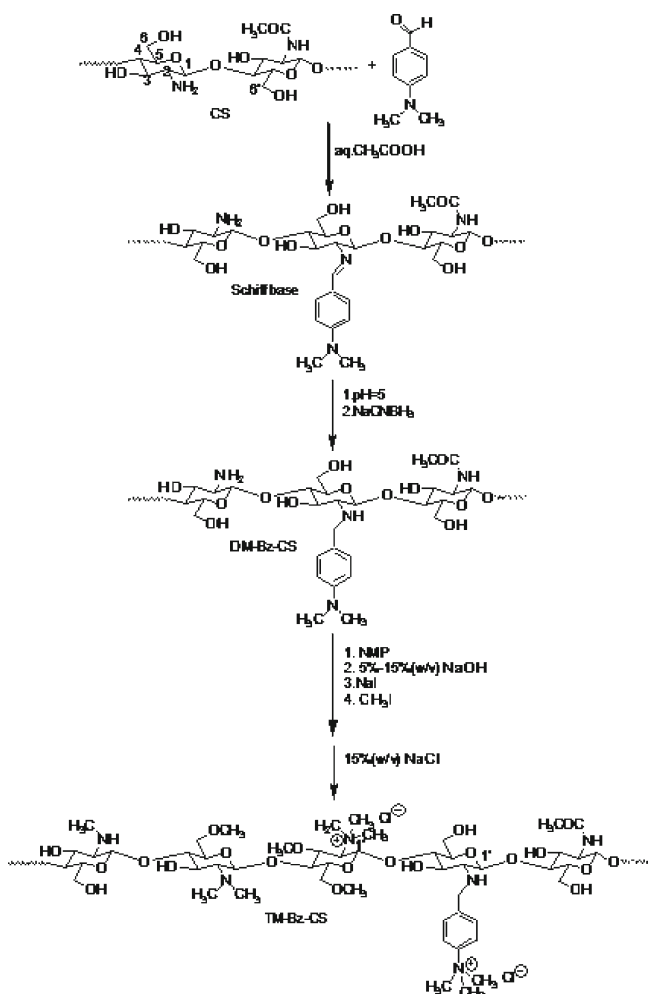#### Synthesis of N-(4-N,N-dimethylaminobenzyl) Chitosan

N-(4-*N*,*N*-Dimethylaminobenzyl) chitosan (DM-Bz-CS) was synthesized in accordance with a reported procedure (13). Briefly, 1.00 g of CS (6.11 meq/GlcN) was dissolved in 0.2 M acetic acid (pH 4, 70 mL). The solution was diluted with ethanol (70 mL), and aromatic aldehyde (4-*N*,*N*-dimethylamino benzaldehyde (1.0–3.0 meq/GlcN) was added and stirred at room temperature for 1 h. The pH of the solution was adjusted to 5 with 1 N NaOH. Subsequently, 1.54 g of $NaCNBH_3$ (24.46 meq/GlcN) was added and stirred at room temperature for 24 h, followed by pH adjustment to 7 with 15% (*w*/*v*) NaOH. The reaction mixture was then dialyzed in distilled water and freeze-dried to give powder DM-Bz-CS 1.31 g (74.3%) with ES of 42.0% (Scheme 1).

N-(4-*N*,*N*-Dimethylaminobenzyl) *Chitosan.* Fourier transform infrared (FT-IR; KBr): ν 3,430, 1,602, 1,526, 1,155, 1,081, 1,033, and 811 cm^{-1}. ^{1}H-nuclear magnetic resonance (NMR; D$_2$O/CF$_3$COOD): δ (ppm) 7.52 (s; 4H Ph), 4.93 (s; 1H H1), 4.42–3.53 (br. m; 7H NHCH_2, H3, H4, H5, H6, and H6′), 3.32 (s; 6H N(CH_3)$_2$ Ph), 2.97 (br. s; 2H H2 and H2′), 2.12 (s; 3H NHCOCH_3).

Synthesis of Methylated N-(4-N,N-dimethylaminobenzyl) Chitosan

DM-Bz-CS of 0.50 g was dispersed in 25 mL of *N*-methyl pyrrolidone for 12 h at room temperature. Then, 1.5 g of sodium iodide and 5–15% (*w*/*v*) NaOH (3.0 mL) were added and stirred at 50°C for 15 min. Subsequently, 1 mL of iodomethane was added in three portions at 4-h intervals and stirred for 12 h at 50°C. The reaction mixture appeared yellow and clear. The obtained compound was precipitated in 300 mL of acetone. The precipitate was dissolved in 15% (*w*/*v*) NaCl solution in order to replace the iodide ions with chloride ions. The suspension was dialyzed with deionized water for 3 days to remove inorganic materials and then freeze-dried.

Methylated N-(4-N,N-dimethylaminobenzyl) Chitosan. FT-IR (KBr): ν 3,442, 1,559, 1,475, 1,147, 1,104, 1,059 cm^{-1}. ^{1}H-NMR (D$_2$O): δ (ppm) 7.75–7.50 (dd; 4H Ph), 5.40, 4.96 (s; 2H H1, H1′), 4.42–3.13 (br. m; 32H-NHCH_2, H2, H3, H4, H5,

Scheme 1. Synthesis of methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (TM-Bz-CS)

H6, and H6′, br. s; N^+ (CH_3)$_3$ Ph, s; OCH_3, br. s; N^+ (CH_3)$_3$), 2.71 (br. m; 6H N(CH_3)$_2$), 2.31 (s; 3H NHCH_3), 2.12 (s; 3H NHCOCH_3). ^{13}C-NMR (D$_2$O): δ (ppm) 145.52, 141.53, 130.98, 119.56 (C-Ph), 96.55 (C1), 77.13–58.67 (C2, C3, C4, C5, and C6), 56.98 (N$^+$ (CH_3)$_3$ Ph), 53.88 (N$^+$ (CH_3)$_3$), 41.77 (N (CH_3)$_2$), 36.30 (NCH_3).

Characterizations

FT-IR spectra were recorded on a Nicolet Impact 410 FT-IR spectrometer; all samples were prepared as potassium bromide pellets. The ^{1}H and ^{13}C-NMR spectra were measured on a Mercury Varian 300-MHz spectrometer. All measurements were performed at 300 K, using pulse accumulation of 64 scans and an LB parameter of 0.30 Hz. D$_2$O/CF$_3$COOD and D$_2$O were the solvents for 5 mg chitosan, *N*-(4-*N,N*-dimethylaminobenzyl) chitosan and methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan, respectively.

The zeta potentials of the chitosan derivatives were determined by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The polymers were diluted with

distilled water that was passed through a 0.22-μm membrane filter prior to use. All samples were measured in triplicate

Cell Cultures

Caco-2 cells were maintained in DMEM at pH 7.4, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acid solution, and 0.1% penicillin–streptomycin solution in a humidified atmosphere (5% CO$_2$, 95% air, 37°C). The cells were grown under standard conditions until 60–70% confluency. Cells from passages 20–40 were used for all of the experiments. The cells were seeded on tissue culture polycarbonate membrane filters (pore size 3.0 μm) in 12-well Transwell® plates (Costar®, Corning Inc., Corning, NY, USA) at a seeding density of 2×10^4 cells per square centimeter. The culture medium was added to both the donor and the acceptor compartment. Medium was changed every second day. The cells were left to differentiate for 15–21 days after seeding while monitoring the TEER values, which were more than 600 Ω cm^2, using a Millicell® ERS meter (Millipore, Bedford, MA, USA).

Measurement of the TEER

Measurement of the TEER was performed to evaluate possible damage to the cellular monolayer during the experiments. The values of TEER were determined by measuring the potential difference between the two sides of the cell monolayer using a Millicell® ERS meter (Millipore, Bedford, MA, USA) connected to a pair of chopstick electrodes. On the day of the experiments, the cells were washed twice with phosphate-buffered saline (PBS) and pre-equilibrated for 1 h with Hank balanced salt solution (HBSS) buffered at pH 7.4. After removing the medium, the Caco-2 cell monolayers were treated with CS or CS derivative solutions (0.05–5 mM in HBSS at pH 7.4) in the apical compartment. Chitosan acetate (CSA) solution was prepared by dissolving chitosan base in 1% solution of acetic acid with gentle stirring for 12 h. The pH of CSA solutions was adjusted to pH 6.2 and 7.4 by adding 0.1 N NaOH. CS derivative solutions were prepared by dissolving in HBSS at pH 7.4 with gentle stirring. The TEER was measured every 20 min. After 2-h treatment, the cells were carefully washed twice with PBS and incubated with a fresh culture medium. The recovery of TEER values was monitored for 24 h after the treatment.

Transport Studies

The transport of FD-4 across the Caco-2 cell monolayer at pH 7.4 was studied. Caco-2 monolayers were grown in Transwell® (12-well) plates and were used for transport studies when they had differentiated and the monolayer was intact, as checked by measuring the TEER. Prior to the experiment, the cells were washed twice with PBS and pre-equilibrated for 1 h with HBSS buffered at pH 7.4. After removing the medium, the cells were treated with TM-Bz-CS solutions (1.25 mM in HBSS pH 7.4) in the apical compartment for 2 h. In the control wells, the same media without chitosan derivatives were used. In all cases, the basolateral medium used was HBSS pH 7.4. After 2 h treatment, the cells

were carefully washed twice with PBS and added with FD-4 solution (1 mg/mL) on the apical side of the monolayers. Samples (1,000 μL) were taken under sink conditions at 30, 60, 90, 120, 180, and 240 min from the basolateral side and replaced with an equal volume of fresh HBSS solution. The amount of FD-4 was determined using a fluorescence 96-well plate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). The excitation and emission wavelengths were 400 and 535 nm, respectively. Results were expressed as cumulative transport as a function of time. All experiments were done in triplicate at 37°C. The apparent permeability coefficient was calculated according to the following equation:

$$P_{app} = (dQ/dt) \times (1/AC_0)$$

where P_{app} is the apparent permeability coefficient (cm/s); dQ/dt (μg/s) is the rate of appearance of FD-4 on the basolateral side; A is the surface area of the monolayers, and C_0 (μg/mL) is the initial drug concentration in the donor compartment. All rate constants were obtained from the permeation profiles of each compound. Absorption enhancement ratios (R) were calculated from P_{app} values by (15):

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

Evaluation of Cytotoxicity

The cytotoxic effects of chitosan derivatives were investigated with Caco-2 cells with an MTT cytotoxicity assay. Cells were seeded at a density of 2×10^4 cells per well in 96-well cell culture plates and preincubated for 24 h before chitosan derivative treatment. The cells were then treated with chitosan derivatives at various concentrations of 0.05–5 mM in serum-free medium with pH 7.4 for 2 or 24 h. After treatment, chitosan derivative solutions were removed, and fresh cell culture medium was added and incubated for 4 h to stabilize the cells. Finally, the cells were incubated with 100 μL MTT-containing medium (0.1 mg/mL MTT in serum-free medium) for 4 h. Then, the medium was removed, and the formazan crystal formed in living cells was dissolved in 100 μL dimethyl sulfoxide per well. The relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of nontreated control cells was arbitrarily defined as 100% (16).

Relative cell viability
$$= \left[\left(OD_{550,sample} - OD_{550,blank} \right) / \left(OD_{550,control} - OD_{550,blank} \right) \right]$$
$$\times 100$$

Statistical Analysis

All experimental measurements were collected in triplicate. Values are expressed as mean±standard deviation (SD). Statistical significance of differences in permeability enhancer and cell viability was examined using one-way analysis of variance followed by the least-significant difference post hoc test. The significance level was set at $p < 0.05$.

RESULTS

Synthesis and Characterization of CS Derivatives

The DM-Bz-CS synthesized by reductive amination of the corresponding Schiff base intermediates (Scheme 1) have already been reported by Sajomsang *et al.* (13). The ES was determined by the ^{1}H-NMR method because the aromatic region did not overlap with the proton resonances of the GlcN. The aromatic proton signal in the ^{1}H-NMR spectrum of each DM-Bz-CS was used to determine the ES by comparison of the integral area of the aromatic protons or methyl protons to the integral area of the H2+1/3 *N*-acetyl proton signals (17). It was found that the ES was in the range of 17–60%, which depended on the mole ratios of aldehyde (18). The chemical structures of DM-Bz-CS and TM-Bz-CS were characterized by FT-IR and ^{1}H-NMR spectroscopy. Figure 1 shows the characteristic FT-IR pattern of chitosan, i.e., the absorption bands at wavenumbers 3,430 cm^{-1} are due to OH and NH$_2$ groups; those at 1,648 and 1,377 cm^{-1} correspond to the C=O and C–O stretching of amide group; the one at 1,594 cm^{-1} is due to N–H deformation of amino groups, and the bands at 1,155, 1,081, and 1,033 cm^{-1} correspond to the symmetric stretching of the C–O–C and involved skeletal vibration of the C–O stretching (19). The FT-IR spectrum of DM-Bz-CS was similar to that of CS except that additional absorption bands at wavenumbers 1,605, 1,526, and 811 cm^{-1} were observed. These bands were assigned to the C=C stretching and C–H deformation (out of plane) of the aromatic group. The TM-Bz-CS exhibited the characteristic FT-IR spectrum at wavenumber 1,475 cm^{-1} due to C–H symmetric bending of the methyl substituent of quaternary ammonium groups (Fig. 1) (20).

Figure 2 shows ^{1}H-NMR spectra of CS, DM-Bz-CS and TM-Bz-CS. All the spectra exhibit the characteristic ^{1}H-NMR pattern of chitosan, i.e., the multiplet at δ 4.4–3.3 ppm due to H3, H4, H5, H6, and H6′ and two singlets at δ 3.1 and 2.0 ppm due to the H2 proton of the GlcN and *N*-acetyl protons of GlcNAc, respectively. The ^{1}H-NMR spectrum of
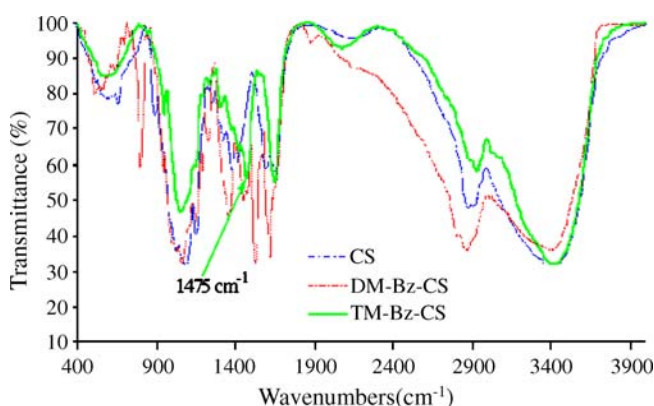
Fig. 1. FT-IR spectra of chitosan (CS), *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (DM-Bz-CS), and methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (TM-Bz-CS) with ES 42%
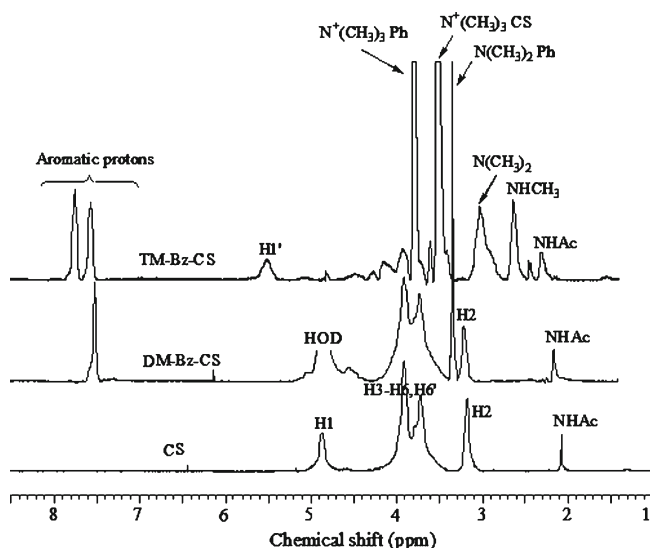
Fig. 2. ^{1}H-NMR spectra of chitosan (CS), N-(4-N,N-dimethylaminobenzyl) chitosan (DM-Bz-CS) in D_2O/CF_3COOD, and methylated N-(4-N,N-dimethylaminobenzyl) chitosan (TM-Bz-CS) in D_2O with ES 17%

Table I. Quaternization of N-(4-N,N-dimethylaminobenzyl) Chitosan

Samples	ES (%)	DQ$_T$ (%)	
		DQ$_{Ar}$ (%)	DQ$_{CS}$ (%)
TM$_{75}$Bz$_{60}$CS	60	60	15
TM$_{56}$Bz$_{42}$CS	42	42	14
TM$_{47}$Bz$_{42}$CS	42	42	4.4
TM$_{47}$Bz$_{17}$CS	17	17	30

ES extent of N-substitution, *DQ$_{Ar}$* degree of quaternization at aromatic substituents, *DQ$_{CS}$* degree of quaternization at primary amine of chitosan, *DQ$_T$* total degree quaternization on the chitosan derivative

DM-Bz-CS exhibited the broad singlet, δ 7.5 ppm, in the aromatic region, and another singlet at δ 3.3 ppm was assigned to N,N-dimethyl protons. The ^{1}H-NMR spectra of TM-Bz-CS showed the signal at δ 5.45 ppm, which is unambiguously assigned to the H1′ proton of the GlcN of TM-Bz-CS. The signals at δ 3.5, 3.2, 2.7, and 2.3 were assigned to N,N,N-trimethyl protons of benzyl substituent, N,N,N-trimethyl protons, N,N-dimethyl protons, and N-methyl protons of GlcN, respectively. In this case, O-methylation was also observed by the appearance of a small signal at δ 3.3 ppm. In this study, the DQ of TM-Bz-CS was determined by the ^{1}H-NMR method in accordance with previous reports by Sieval *et al.* (21). The DQ was generally determined by using the following equation:

$$DQ_{CS}(mol\%) = N^+(CH_3)_3/9]/H1$$

In the experimental results, DQ at the primary amino group of chitosan is denoted as DQ_{CS}; $N^+ (CH_3)_3$ is the integral area of the N,N,N-trimethyl protons at δ 3.2 ppm, and H1′ is the integral area of the proton at δ 5.45 ppm.

The results showed that the methylation of DM-Bz-CS with various values of ES using iodomethane yielded the corresponding quaternary ammonium salt, TM-Bz-CS. In this case, N-methylation could occur at both the aromatic substituent and primary amino group of chitosan. The results demonstrate that the DQ_T, the sum of DQ_{Ar} and DQ_{CS}, increased with increasing ES of DM-Bz-CS (Table I).

The zeta potential of all chitosan derivatives was found to be a positively charged value in the range of +20 to +30 mV.

Effect of TM-Bz-CS on TEER

The effect of the different DQ, the ES of TM-Bz-CS, and the polymer concentrations on the TEER of Caco-2 cell monolayers are summarized in Fig. 3. Incubation on the apical side of the monolayers with 0.05–5 mM of the polymers, at pH of 7.4, for 2 h resulted in an immediate reduction in TEER values compared with the control group. Table II shows the TEER values of CS and CS derivatives after 2-h incubation on the apical side of the monolayers. All CS derivatives show concentration dependence for TEER values. Increasing the concentration of polymers resulted in significant decrease in TEER values compared with the control. The results reveal that both the degree of DQ and ES affect the decrease in TEER values. Increasing the degree of DQ and ES tended to decrease TEER values compared with the control. All CS derivatives are dissolved at neutral pH 7.4 and show a reduction in TEER values. On the other hand, CSA could not dissolve at neutral pH 7.4; therefore, it reduced TEER values only at acidic pH of 6.2 (Fig. 4).

After removal of the polymer solutions, repeated washing of the cells and substituting the apical with medium, an increase in resistance towards the initial values was found in the control and CS derivatives treated with 0.05–5 mM of the polymers by 24 h (Fig. 5). The TEER recovered more than 60% of initial values within 24 h of CS derivative removal when treated with concentrations up to 1.25 mM, except TM$_{75}$Bz$_{60}$CS treatment with 1.25–5 mM of TM$_{75}$Bz$_{60}$CS, which did not significantly increase the resistance after 24 h (Fig. 5a), indicating that the effects of TM-Bz-CS were reversible at lower concentrations. Therefore, in the transport study, the effects of the different %ES and %DQ of TM-Bz-CS were investigated at a concentration of 1.25 mM, which proved the most safe and effective for enhancement. In comparison, the reduction in TEER prolonged incubation up to 2 h with 1.25-mM concentrations of TM-Bz-CS (Fig. 4), in the following order: TM$_{75}$Bz$_{60}$CS (87.31% reduction) > TM$_{56}$Bz$_{42}$CS (84.38% reduction) > TM$_{47}$Bz$_{42}$CS (81.35% reduction) > TM$_{47}$Bz$_{17}$CS (74.39% reduction).

Effect of TM-Bz-CS on the Transport of FD-4

To further evaluate the direct ability of TM-Bz-CS to increase permeability, it was important to use a hydrophilic macromolecule. The effect of the DQ and the ES of TM-Bz-CS on the transport of FD-4 across the Caco-2 cell monolayers at pH 7.4 was investigated. Because FD-4 is a negatively charged polymer, the aggregation resulted from strong electrostatic interactions with the positively charged TM-Bz-CS. In the present studies, Caco-2 cells were therefore preincubated with TM-Bz-CS 2 h before FD-4 was added in the acceptor compartments and accumulation of FD-4 in the basolateral compartments up to 4 h were measured. Figure 6 shows the
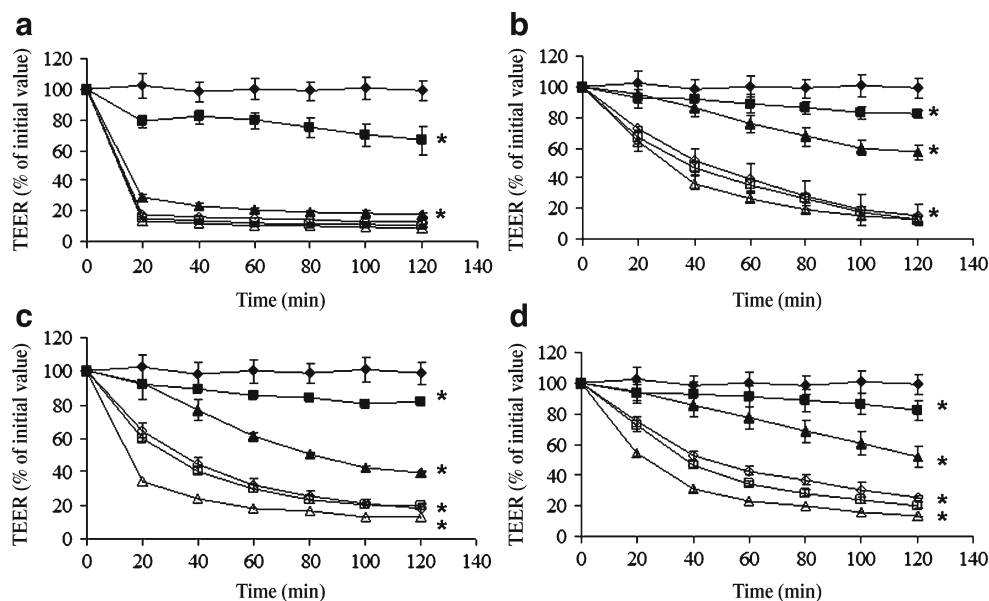
Fig. 3. Effect of TM-Bz-CS: **a** $TM_{75}Bz_{60}CS$, **b** $TM_{56}Bz_{42}CS$, **c** $TM_{47}Bz_{42}CS$, and **d** $TM_{47}Bz_{17}CS$ concentrations from 0.05 to 5 mM, at pH 7.4 on the TEER of Caco-2 cell monolayers; control (*filled diamonds*), 0.05 mM (*filled squares*), 0.5 mM (*filled triangles*), 1.25 mM (*empty diamonds*), 2.5 mM (*empty squares*), 5 mM (*empty triangles*). Each point represents the mean of three experiments. *Asterisks* indicate $p < 0.05$

transport of FD-4 across the Caco-2 cell monolayers in the presence of TM-Bz-CS with different DQ and ES at a concentration of 1.25 mM and in the absence of TM-Bz-CS for the control. The cumulative transport of FD-4, P_{app} values, and absorption enhancement ratios (R) were calculated and were shown in Table III. Under the conditions described, very low baseline permeability was found. Incubation with TM-Bz-CS resulted in an accumulation of FD-4 in the acceptor compartment. A difference in the transport of FD-4 was found at different DQ and ES. The cumulative amounts transported up to 4 h after incubation with FD-4 were in the following order: $TM_{75}Bz_{60}CS$ (35.88±2.7 μg), $TM_{56}Bz_{42}CS$ (26.86± 0.7 μg), $TM_{47}Bz_{42}CS$ (17.86±6.8 μg), and $TM_{47}Bz_{17}CS$ (10.89 ±2.0 μg). Among chitosan derivatives, $TM_{75}Bz_{60}CS$ showed the greatest enhancement effect in FD-4 transport. It is of interest that the results were in agreement with the results of TM-Bz-CS on the reduction in the TEER.

Cytotoxicity of TM-Bz-CS

Cationic polymers have been known to be cytotoxic materials. It is believed that the cytotoxic effect mainly

originates from cationic charge. Therefore, in this study, the effect of the different %ES and %DQ of TM-Bz-CS on cytotoxicity was investigated. The results showed that CSA at pH 7.4 incubated with Caco-2 cells for 2 or 24 h did not remarkably change the viability of Caco-2 cells, whereas at pH 6.2 CSA incubated with Caco-2 cells for 24 h showed concentration-dependent cytotoxicity with IC_{50} of 0.9± 0.1 mM (Table IV). All TM-Bz-CS tested showed that, at up to 5 mM, incubation with Caco-2 cells for 2 h did not alter the viability of Caco-2 cells. However, they showed the concentration-dependent cytotoxicity when incubated for 24 h. Increased %ES led to a slight decrease in cytotoxicity. However, increasing %DQ of TM-Bz-CS resulted in a significant increase in cytotoxicity. Therefore, the least cytotoxic TM-Bz-CS was $TM_{56}Bz_{42}CS$, with the highest IC_{50} value of 1.7±0.01 mM (Table IV).

DISCUSSION

Tight-junction permeability can be measured *in vitro* by different methods: TEER, as a measure of ionic permeability

Table II. Effect of the Degree of Quaternization (DQ), the Extent of Dimethylaminobenzyl Substitution (ES) of TM-Bz-CS, Polymer Concentration, and pH on TEER

|		TEER (percent of initial value) 2 h				
		CS derivatives concentration (mM)				
CS derivatives	pH	0.05	0.5	1.25	2.5	5
$TM_{75}Bz_{60}CS$	7.4	66.66±6.5	17.48±1.1	12.69±1.0	10.29±0.7	9.21±0.5
$TM_{56}Bz_{42}CS$	7.4	82.30±2.4	56.92±5.4	15.62±6.5	13.00±1.8	12.58±1.7
$TM_{47}Bz_{42}CS$	7.4	82.12±1.9	39.85±0.4	18.65±2.6	19.62±0.9	13.20±0.7
$TM_{47}Bz_{17}CS$	7.4	82.65±6.5	51.95±7.0	25.61±1.2	20.34±2.0	13.98±0.9
CSA	7.4	105.39±3.1	104.63±1.5	105.44±2.1	107.36±3.9	106.23±2.8
CSA	6.2	81.35±5.9	32.18±4.8	21.07±2.1	21.81±2.5	16.58±0.8
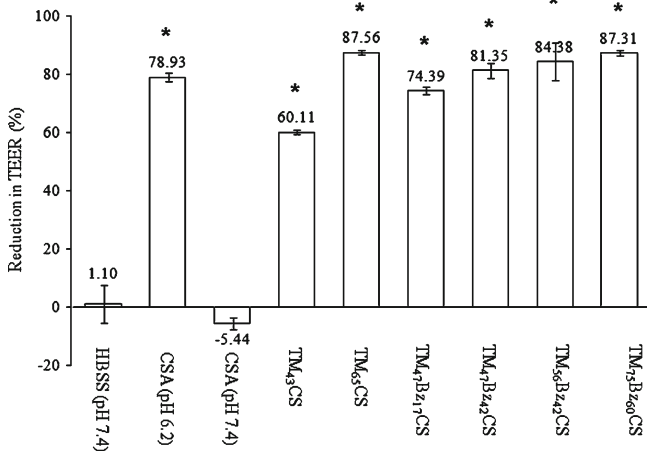
Fig. 4. The percentage of reduction in the TEER after 2-h incubation with 1.25 mM chitosan acetate (CSA) and chitosan derivatives. Each point represents the mean of three experiments. *Asterisks* indicate $p < 0.05$

of the cell monolayer, and paracellular passage of extracellular markers of different sizes that are not absorbed or metabolized by the cells (e.g., mannitol, insulin). TEER does not distinguish between membrane and paracellular conductance and is a useful indicator of early perturbations of the ionic permeability of the cell monolayer. Conversely, paracellular marker passage is a more suitable parameter to describe long-term variations in the permeability of tight junctions to larger molecules (22). For these reasons, both methods were utilized in the present study to assess the effect of TM-Bz-CS, methylated N-(4-N,N-dimethylaminobenzyl) chitosan, a novel polymeric absorption enhancer on differentiated Caco-2 cells. It is well known that CS solutions cause a significant and dose-dependent decrease in the TEER of Caco-2 cell monolayers. The first evidence of this phenomenon was reported in the early 1990s (9). It has been proposed that CS acts on the negatively charged sites at the cell surfaces and tight junctions. It has been shown that CS is able to induce changes in the F-actin distribution (7,9). Although F-actin is directly or indirectly associated with the proteins in the tight junctions (23), chitosan most probably allows for the paracellular transport of hydrophilic compounds by an indirect mechanism, whereby the integrity of the tight junctions is altered by changes in intracellular F-actin. Changes in paracellular barrier properties occur as a cellular action mediated by polycations rather than as a consequence of direct action on the junctional complex (22). This is supported by a recent study that concluded that the interaction of CS with the cell membrane results in a structural reorganization of tight-junction-associated proteins, followed by enhanced transport through the paracellular pathway. Binding of CS to the Caco-2 cells thus precedes absorption enhancement, and this is mediated by the positive charges on the polymer (7). However, CS shows its absorption enhancement activity only in acidic conditions due to solubility problems. Many attempts have thus been made in the synthesis of CS derivatives to overcome the solubility problem. To increase the solubility at neutral physiological pH, a quaternary ammonium moiety in CS is required. The above-mentioned properties of cationic polymers have been known to be cytotoxic materials (15). Several reports are available on the cytotoxic effect of cationic polymers, such as poly(L-lysine), poly(L-ornithine), polyethylenimine, histone, protamine, etc. (24–26). Because of the amphiphilic nature of the cell membrane, an increase in interaction between the cell membrane and CS derivative could be favored and more safe when the macromolecule itself contains hydrophobic residues. As a result, CS derivatives containing quaternary ammonium functionality in addition to different hydrophobic substitutions were interesting as novel absorption enhancers.
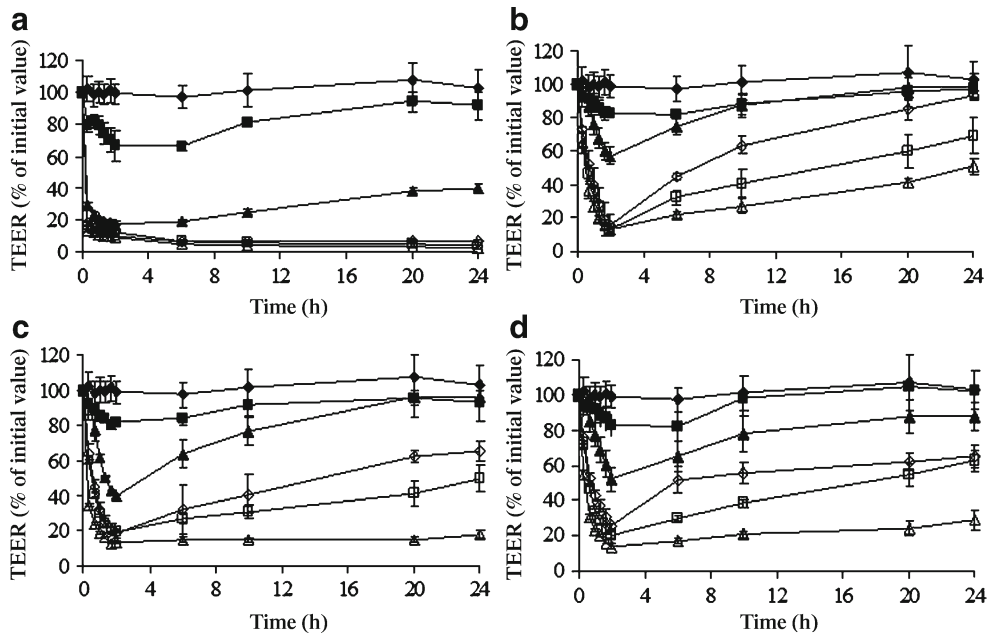
Fig. 5. Effect of TM-Bz-CS: **a** $TM_{75}Bz_{60}CS$, **b** $TM_{56}Bz_{42}CS$, **c** $TM_{47}Bz_{42}CS$, **d** $TM_{47}Bz_{17}CS$ concentrations from 0.05 to 5 mM, at pH 7.4 on the TEER recovery pattern of Caco-2 cell monolayers; control (*filled diamonds*), 0.05 mM (*filled squares*), 0.5 mM (*filled triangles*), 1.25 mM (*empty diamonds*), 2.5 mM (*empty squares*), 5 mM (*empty triangles*). Each point represents the mean of three experiments
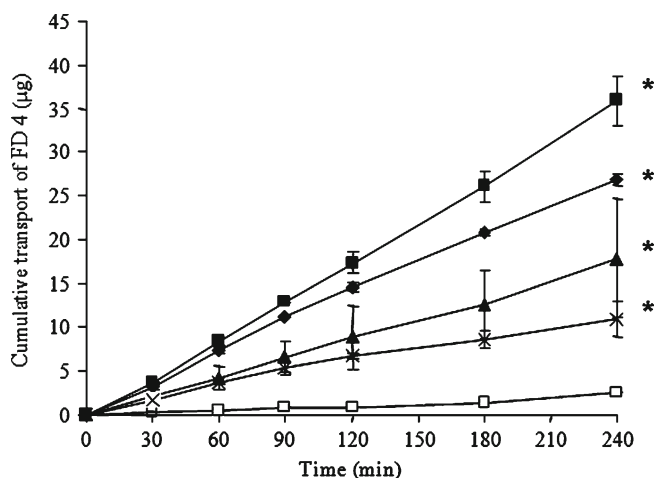
Fig. 6. Cumulative transport of FD-4 in the presence of 1.25 mM TM-Bz-CS at pH 7.4: control (*empty squares*), $TM_{75}Bz_{60}CS$ (*filled squares*), $TM_{56}Bz_{42}CS$ (*filled diamonds*), $TM_{47}Bz_{42}CS$ (*filled triangles*), $TM_{47}Bz_{17}CS$ (*x*). Each point represents the mean of three experiments. *Asterisks* indicate $p < 0.05$

Table IV. Cytotoxicity of CSA and TM-Bz-CS Incubated with Caco-2 Cells for 2 and 24 h ($N=8$)

Samples	pH	IC_{50} (mM) at 2 h	IC_{50} (mM) at 24 h
$TM_{75}Bz_{60}CS$	7.4	>5	0.40 ± 0.03
$TM_{56}Bz_{42}CS$	7.4	>5	1.70 ± 0.01
$TM_{47}Bz_{42}CS$	7.4	>5	0.90 ± 0.01
$TM_{47}Bz_{17}CS$	7.4	>5	0.35 ± 0.03
CSA	6.2	>5	$0.90 \pm 0..01$
CSA	7.4	>5	>5

The chitosan derivatives, such as *N*-trimethyl chitosan chloride, mono-carboxymethyl chitosan, triethyl chitosan, and diethyl methyl chitosan, have been synthesized and evaluated as absorption enhancers of hydrophilic drugs at pH values similar to those found in the intestine (15,27–29). *N*-sulfonato-*N*,*O*-carboxymethylchitosan showed a potential intestinal absorption enhancement for Reviparin (30). Therefore, in this study, TM-Bz-CS, a methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan, a novel polymeric absorption enhancer, was investigated.

In this study, the results showed that increasing concentrations of TM-Bz-CS (0.05–0.5 mM) resulted in a dose-dependent effect on tight-junction permeability at pH 7.4. All CS derivatives showed concentration dependence (Fig. 3). Increasing the concentration of the polymers from 1.25 to 5 mM did not result in significant decreases in TEER values compared with the reduction measured with 0.05–0.5 mM. A similar trend in TEER decrease as a function of low chitosan concentration was previously reported (8,15). The effect on TEER values seems to be saturable since no significant differences were found at higher concentrations of polymers (1.25–5 mM). This phenomenon might be due to the ability of Caco-2 cells to recover from the treatment of polymers. The complete recovery of TEER was observed during the 24-h observation period (Fig. 5). Treated with TM-Bz-CS (0.05–0.5 mM), the recovery of TEER could be observed by

24 h, whereas it was not observed when treated with 1.25–5 mM. All TM-Bz-CS-treated monolayers (0.5 mM), except for $TM_{75}Bz_{60}CS$, reached a maximum of 90% of their initial baseline value after full recovery. $TM_{75}Bz_{60}CS$ only at the lowest dose of 0.05 mM reached a maximum of 90% of its initial baseline value after full recovery. This is consistent with previous reports (15), in which complete removal of the polymers, without damaging the cells, proved to be difficult due to the high viscosity of the solution, and this may be the reason why the increase in resistance is only gradual and did not reach 100%. Our results showed that the higher concentrations of TM-Bz-CS resulted in higher cytotoxicity. This is in agreement with the findings of a previous study in which the CS showed concentration-dependent cytotoxic effects on Caco-2 cells (16). Therefore, this high concentration (2.5–5 mM) of TM-Bz-CS might damage the cells and result in the lack of recovery in the TEER. For these reasons, the concentration at 1.25 mM, in which the cells could recover, was used in transport of FD-4.

The results in Fig. 1 and Table II reveal that both the degree of DQ and ES affected the decrease in TEER values and FD-4 transport (Table III). Increasing the degree of DQ and ES tended to decrease the TEER values compared with the control and increase FD-4 transport. The effect of the degree of DQ is in agreement with the previous report that the CS showed DQ-dependent effects on Caco-2 cells; higher charge density results in higher transport enhancement (31). These results indicate that the introduction of the trimethylaminobenzyl group into the CS polymer backbone enhances the hydrophobicity, which improves the hydrophobic interaction between the polymer and the cell membrane. These help the water-soluble CS to be an efficient absorption enhancer. As reported previously, hydrophobically modified polymers have shown absorption enhancement as an optimal substitution by increasing cell membrane–polymer interactions or

Table III. Effect of the Different Degrees of Quaternization (DQ) and the Extent of Dimethylaminobenzyl Substitution (ES) of TM-Bz-CS on the Transport of FD-4

Samples	Cumulative transport of FD-4 at 4 h (µg)	Papp (10^{-6} cm/s)	R
Control	2.47 ± 0.7	0.14	1.00
$TM_{75}Bz_{60}CS$	35.88 ± 2.7	2.23	15.60
$TM_{56}Bz_{42}CS$	26.86 ± 0.7	1.68	11.75
$TM_{47}Bz_{42}CS$	17.86 ± 6.8	1.09	7.64
$TM_{47}Bz_{17}CS$	10.89 ± 2.0	0.67	4.68

destabilization of the cell membranes (32). It is likely that the mechanism of permeation enhancement by TM-Bz-CS is different from that for chitosan base and further investigation is required to delineate the exact pathway. Although chitosan is generally considered a biodegradable and safe polymer, Schipper *et al.* (6) observed some toxic effects of certain chitosans on Caco-2 cells with the MTT assay. According to the MTT assay, all the tested chitosan derivatives showed that, at up to 5 mM, incubation with Caco-2 cells for 2 h did not alter the viability of Caco-2 cells. However, they showed concentration-dependent cytotoxicity when incubated for 24 h. The cytotoxicity slightly varied with the degree of DQ and ES. Increased %ES led to a slight decrease in cytotoxicity. However, an increase in the %DQ of TM-Bz-CS resulted in a significant increase in cytotoxicity. The results reveal that the least cytotoxic of the CS derivatives was $TM_{56}Bz_{42}CS$. These results indicate that an optimal degree of DQ and ES for CS derivatives was shown to be an efficient absorption enhancer with less cytotoxicity. The *in vitro* permeation characteristics of drugs across Caco-2 cell monolayers correlate with their human intestinal mucosa permeation characteristics. However, limitations of these *in vitro* Caco-2 cell monolayer tests, such as the absence of villi and a mucus layer, have to be noted. Additionally, Caco-2 cells represent tighter junctions in comparison with human or animal small intestine cells, which is explained by the colonic origin of these cells (33). Foger *et al.* showed that the P_{app} values of hydrophilic peptides across Caco-2 monolayers are lower than the permeability across rat intestinal mucosa in an *in vitro* study and an *in vivo* study using the rat model (34). Therefore, the *in vivo* permeability of these CS derivatives could require further investigation.

CONCLUSION

TM-Bz-CS is a novel polymeric permeation enhancer that is superior in solubility and results in a decrease in the TEER of Caco-2 cell monolayers and an increase in the paracellular permeability of hydrophilic markers. At the optimum degree of quaternization, the addition of attached hydrophobic groups on the C-2 position of the backbone can reduce the cytotoxicity in Caco-2 cells.

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