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Research Article

In vitro Permeability Enhancement in Intestinal Epithelial Cells (Caco-2) Monolayer of Water Soluble Quaternary Ammonium Chitosan Derivatives

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Abstract. The aim of this study was to investigate the effects of a type of hydrophobic moiety, extent of *N*-substitution (ES), and degree of quaternization (DQ) of chitosan (CS) on the transepithelial electrical resistance and permeability of Caco-2 cells monolayer, using fluorescein isothiocyanate dextran 4,400 (FD-4) as the model compound for paracellular tight junction transport. CS was substituted with hydrophobic moiety, an aliphatic aldehyde (*n*-octyl) or aromatic aldehyde (benzyl), for the improved hydrophobic interaction with cell membrane, and they were quaternized with Quat-188 to render CS soluble. The factors affecting the epithelial permeability have been evaluated in the intestinal cell monolayers, Caco-2 cells. Cytotoxicity was evaluated by using the trypan blue and MTT viability assay. The results revealed that at pH 7.4 CSQ appeared to increase cell permeability in dose-dependent manner, and this effect was relatively reversible at the lower doses of 0.05–1.25 mM. The higher DQ and ES caused the higher permeability of FD-4. Cytotoxicity of CSQ was concentration, %DQ, and %ES dependent. Substitution with hydrophobic moiety caused decreasing in permeability of FD-4 and cytotoxicity by benzyl group had more effect than octyl group. These studies demonstrated that these novel modified chitosan derivatives had potential for using as absorption enhancers.

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

INTRODUCTION

Chitosan (CS) [(1→4)-2-amino-2-deoxy-β-D-glucan], a cationic polysaccharide consisting of *N*-acetyl glucosamine (GlcNAc) and glucosamine (GlcN), obtained from the deacetylation of chitin. CS has attracted considerable attention in the pharmaceutical and biomedical fields not only because of its unique activity properties, but also due to its biocompatibility, biodegradability and mucoadhesivity (1). The ability of CS to enhance gastrointestinal drug absorption has been of special interest (2). However, CS is a weak base, in neutral and basic environments CS molecule loses its charge and precipitates from solution. Under these conditions CS is ineffective as an absorption enhancer, limiting its use in the more basic environment of the large intestine and colon. Therefore, several CS derivatives have been synthesized in the last few years in order to obtain modified carrier with altered physicochemical characteristics (3,4).

Quaternary ammonium derivatives of CS have interesting pharmaceutical applications, since it is the polycationic properties of CS that are thought to be responsible for most of the desired activities. These derivatives have two major advantages over the parent CS: (1) they are water soluble at physiological pH and (2) they possess a permanent positive charge on the polysaccharide backbone. The most extensively evaluated quaternary CS derivative is *N*,*N*,*N*-trimethyl chitosan chloride (TMC). Various studies have been carried out on different aspects of the synthetic preparation of TMC (5–8). However, the synthetic preparation of TMC is somewhat problematic, i.e., TMC cannot be prepared without methylating the hydroxyl moieties in CS. For example, higher degrees of quaternization cannot be prepared without total *O*-methylation of the material (5).

Another alternative for introduction of a quaternary ammonium group into CS backbone has been reported. Glycidyl trimethylammonium chloride (GTMAC) was selected as quaternizing agent because it has quaternary ammonium group itself. When a primary amino group at C-2 of CS reacted with GTMAC, the chain of quaternary ammonium group which was longer than that in TMC was obtained. In 1991, Loubaki *et al.* (9) synthesized *N*-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride (HPTChC), by using GTMAC as the quaternizing agent. The structure of HPTChC was confirmed using elemental

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ABBREVIATIONS: BzCSQ, *N*-Benzyl chitosan Quat-188; CS, Chitosan; CSA, Chitosan acetate; CSQ, Chitosan Quat-188; DQ, Degree of Quat-188; ES, The extent of *N*-substitution; FD-4, Fluorescein isothiocyanate dextran 4,400; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OctCSQ, *N*-Octyl chitosan Quat-188; TEER, The transepithelial electrical resistance; TM-Bz-CS, Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan.

analysis, IR, and NMR spectroscopies. All results were consistent with *N*-monoalkylation, and that a sixfold excess of GTMAC gived rise to almost complete quaternization. In 1998, Daly *et al.* (10) developed a method for the synthesis of HPTChC by using commercially available Quat-188 salt, which was an aqueous solution of 3-chloro-2-hydroxypropyltrimethylammonium chloride and has been used to introduce a cationic moiety to starch (11) and cellulose (12). Recently, Sajomsang *et al.* (13) have successfully synthesized chitosan Quat-188 (CSQ) which exhibited antimicrobial activity. This modified chitosan is water soluble at the physiological pH and might be favored to use as carrier for oral drug delivery. In this study, the water soluble CS and its derivatives have been synthesized and evaluated for their *in vitro* absorption enhancement on the transepithelial electrical resistance (TEER) and permeability of Caco-2 cells monolayers by using FITC-dextran (FD-4) as a model drug for hydrophilic macromolecules. CS was substituted with alkyl or benzyl group to provide the hydrophobic property for the improved hydrophobic interaction with cell membrane. They were also quaternized with Quat-188 to render CS soluble. A number of variables that influenced the absorption enhancing property such as types of hydrophobic group, degree of Quat-188 (DQ), extent of ES and pH of culture medium were investigated.

MATERIALS AND METHODS

Materials

CS with an average molecular weight, Mw, of 276 kDa was purchased from Seafresh Chitosan (lab) Co., Ltd. in Thailand. The degree of deacetylation (DDA) of this material was determined to be 94% by ^{1}H-NMR according to the report by Lavertu *et al.* (14). Benzaldehyde was purchased from Merck (NJ, USA). A 65% solution of *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Quat-188) was obtained from Dow Chemical Company (Michigan, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fluorescein isothiocyanate dextran 4,400 (FD-4) and n-octyl aldehyde were purchased from Sigma-Aldrich Chemical Co. (MO, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, fetal bovine serum, glutamine, sodium pyruvate, and trypan blue stain were obtained from GIBCO-Invitrogen (NY, USA). The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Transwell (12-well plates) cell culture chambers inserted with 3.0 μm pore size were purchased from Corning Life Sciences (Massachusetts, USA). Millicell® ERS meter was purchased from Millipore (Bedford, MA, USA). Universal Microplate Analyzer was model AOPUS01 and AI53601 (Packard BioScience, CT, USA). Light microscopy for monitoring cell cultures was a Bringing Inverted Microscope (ECLIPSE, TE2000-U, Japan).

Synthesis of Quaternization Chitosan Derivatives

Synthesis of N-octyl Chitosans

N-Octyl chitosans (OctCS) was prepared as previously reported (13). Briefly, 1.00 g of CS (6.11 meq/GlcN) was dissolved in 0.2 M acetic acid (70 mL). The solution was diluted with ethanol (70 mL) prior to the addition of 0.39 g of *n*-octyl aldehyde (0.5 meq/GlcN) and then stirred at room temperature for 1 h. The pH of the solution was 4, and it was adjusted to 5 with 1N NaOH. Subsequently, 1.54 g of $NaCNBH_3$ (24.46 meq/GlcN) was added and stirred at room temperature for 24 h. The pH of the solution was adjusted to 7 with 15% (*w*/*v*) NaOH. The reaction mixture was then dialyzed in distilled water and freeze-dried to give a powder 0.93 g (87.0%) with ES of 11%. *N*-Benzyl chitosan (BzCS) was synthesized using the same procedure described above.

N-Octyl chitosan. Fourier transform infrared (FT-IR) (KBr): ν 3430 cm^{-1} (O–H and N–H stretching), 2,979 cm^{-1} (C–H stretching of CH_2), 1,155 cm^{-1} (C–O–C asymmetric stretching of GlcN), 1,081 and 1,033 cm^{-1} (C–O stretching of GlcN). ^{1}H-NMR (D_2O/CF_3COOD): δ (ppm) 3.20–3.92 (m; 7H $NHCH_2$, H3, H4, H5, H6, and H6′), 3.05 (br. s; 2H H2, H2′), 1.91 (s; 3H $NHCOCH_3$), 1.56–1.16 (m; 14H $(CH_2)_7$), 0.75 (s; 3H CH_3).

N-Benzyl chitosan. FT-IR (KBr): 3,430, 1,514, 1,452, 1,155, 1,081, 1,033, 752, and 704 cm^{-1}. ^{1}H-NMR spectrum (D_2O/CF_3COOD): δ (ppm) 7.28 (s; 5H Ph), 4.62–3.48 (br. m; 7H $NHCH_2$, H3, H4, H5, H6, and H6′), 2.93 (br. s; 2H H2 and H2′), 1.89 (s; 3H $NHCOCH_3$). ^{13}C-NMR spectrum (D_2O/CF_3COOD): δ (ppm) 131.33-130.06 (C-Ph), 97.74 (C1), 77.28 9(C40), 75.05 (C5), 69.92 (C3), 61.13 (C6), 52.89 (C2), 51.26 ($NHCH_2$).

Preparation of Regenerated Chitosan, N-octyl and N-benzyl Chitosans

Either 0.50 g of CS or OctCS and BzCS was regenerated by dissolving in 1% (*w*/*v*) aqueous acetic acid. This solution was added dropwise into 2% (*w*/*v*) of Na_2CO_3, H_2O:MeOH [40:60] (*v*/*v*). The pH of the solution was adjusted to 9 by adding 15% (*w*/*v*) NaOH. The regenerated CS and its derivatives were then recovered by filtration with Whatman filter number 1 and used while still moist for subsequent synthesis steps.

Quaternization of Chitosan, N-octyl and N-benzyl Chitosans

A 20 mL of 65% (*w*/*w*) aqueous solution of *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride (Quat-188, 80.21 mmole) was added to the reaction flask and the pH of the solution was raised to 8 by using 15% (*w*/*v*) NaOH. Then 0.25 g (0.10 mmole) of iodine was added along with regenerated chitosan. The solution was stirred for 48 h at room temperature and then distilled water was added and temperature was raised to 50°C for another 24 h. The solution was dialyzed with distilled water for 48 h. The dialyzed solution was then concentrated under vacuum using a rotary evaporator. The concentrated solution was added into acetone to precipitate the product, which was collected and dried overnight at room temperature under a stream of nitrogen. Using the same procedure described above to synthesize *N*-octyl chitosan Quat-188 (OctCSQ) and *N*-benzyl chitosan Quat-188 (BzCSQ).

Chitosan Quat. FT-IR (KBr): ν 3430 (O–H and N–H stretching), 1,644 and 1,369 (C=O and C–O stretching of amide,(1,594 (N–H deformation of amino), 1,480 cm^{-1})C–H symmetric bending of $(CH_3)_3N^+R$(, 1,155 (C–O–C asymmetric stretching of GlcN), 1,081 and 1,033 cm^{-1} (C–O stretching of GlcN) cm^{-1}. ^{1}H-NMR (D_2O): δ (ppm) 4.78–3.30 (m; 18H H1,

H3, H4, H5, H6, and H6′, s; 1H CHOH b, s; 2H $CH_2N^+(CH_3)_3$ c, s; 9H $N^+(CH_3)_3$ d), 2.87)br. s; 1H H2), 2.68 (br. s; 2H CH_2NH, a), 1.94 (s; 3H $NHCOCH_3$). ^{13}C-NMR (D_2O): δ (ppm) 102.15 (C1), 77.80 (C4), 74.74 (C5), 72.88 (C3), 68.96 ($CH_2N^+(CH_3)_3$, c), 65.07, 64.57 (CHOH, b), 63.80, 62.34 (C2), 60.25 (C6), 54.06 ($N^+(CH_3)_3$, d), 51.66 (CH_2NH, a), 23.5 ($NHCOCH_3$).

N-n-Octyl chitosan Quat. FT-IR (KBr): ν 3,430, 2,979, 1,480, 1,155, 1,081, and 1,033 cm^{-1}. ^{1}H-NMR (D_2O): δ (ppm) 4.86–3.30 (m; 18H H1, H3, H4, H5, H6, and H6′, s; 1H CHOH b, s; 2H $CH_2N^+(CH_3)_3$ c, s; 9H $N^+(CH_3)_3$ d), 2.87 (br. s; 1H H2), 2.68 (br. s; 2H CH_2NH, a), 1.94 (s; 3H $NHCOCH_3$), 1.56–1.16 (m; 14H $(CH_2)_7$), 0.75 (s; 3H CH_3).

N-Benzyl chitosan Quat. FT-IR (KBr): ν 3,430, 1,514 1,480, 1,155, 1,081, 1,033, 752, and 704 cm^{-1}. ^{1}H-NMR (D_2O): δ (ppm) 7.34 (s; 5H Ph), 4.73–3.30 (m; 18H H1, H3, H4, H5, H6, and H6′, s; 1H CHOH b, s; 2H $CH_2N^+(CH_3)_3$ c, s; 9H $N^+(CH_3)_3$ d), 2.87 (br. s; 1H H2), 2.68 (br. s; 2H CH_2NH, a), 1.94 (s; 3H $NHCOCH_3$). ^{13}C NMR (D_2O): δ (ppm) 129.4–128.7 (C-Ph), 102.12, 100.00 (C1), 77.78 (C4), 74.74 (C5), 72.91 (C3), 68.60 ($CH_2N^+(CH_3)_3$, c), 65.03, 64.56 (CHOH, b), 63.79, 62.65 (C2), 60.33 (C6), 54.07 ($N^+(CH_3)_3$, d), 51.65 (CH_2NH, a).

Characterization

IR spectra were recorded on a Nicolet Impact 410 FT-IR spectrometer and all samples were prepared as potassium bromide pellets. The ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Mercury Varian 400 MHz and Bruker DPX 250 MHz spectrometer, respectively. For ^{1}H-NMR measurements, 5 mg of CS, OctCSQ and BzCS were dissolved in 1% (*v*/*v*) D_2O/CF_3COOD whereas Quat-188 derivatives of CS were dissolved in D_2O and then recorded at 300°K, using pulse accumulating of 64 scans and the LB parameter of 0.30 Hz. The extent of ES was calculated from ^{1}H-NMR data.

Determination of Molecular Weight of Chitosan and its Derivatives

The molecular weights of CS and its derivatives were determined using a gel permeation chromatography laser light-scattering (GPC-LS) system consisting of Agilent 1100 Series generic pump and injector, three Viscotek Columns (ViscoGEL Poly-CAT™), Wyatt Optilab rEX differential refractive index detector, Wyatt Dawn Heleos laser LS detector and a Wyatt viscometer detector. The mobile phase was 5% acetic acid (pH 4) at a flow rate of 1 mL/min at 20°C. The chromatograms were collected by Astra V software, and analyzed with the Astra 5.3.1.5 program.

Cell Cultures

Caco-2 cells were maintained in DMEM at pH 7.4, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential 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 at a seeding density of 2×10^4cells/cm^2. 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 with monitoring of TEER values were more than 600 $\Omega.cm^2$ using a Millicell® ERS meter (Millipore, Bedford, MA, USA).

Measurement of the Transepithelial Electrical Resistance

Measurement of TEER was performed to evaluate possible damage of 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 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) at apical compartment. Chitosan acetate (CSA) solution was prepared by dissolving chitosan base in 1% *w*/*v* 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 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 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 CS derivatives (1.25 mM in HBSS pH 7.4) at 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 FD-4 solution (1 mg/ml) was added 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-wells 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 Eq. (1):

$$P_{app} = (dQ/dt) \times (1/A\,C_o) \quad (1)$$

where P_{app} is the apparent permeability coefficient (cm/s), dQ/dt (μg/s) 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):

$$R = P_{app}(\text{sample})/P_{app}(\text{control}) \quad (2)$$

Cytotoxicity Studies

Trypan Blue Test

After completion of FD-4 transport experiments, the polymers were removed carefully. Both sides of Caco-2 cell monolayers were rinsed with HBSS at pH 7.4. Subsequently, Caco-2 cell monolayers were incubated apically with 0.1% trypan blue solution in PBS at 37°C. The basolateral medium was replaced by PBS. After 30 min incubation, the dye was removed and monolayers were rinsed with PBS. Thereafter, monolayers were examined by light microscopy (bringing inverted microscope, ECLIPSE, TE2000-U, Japan) for dye exclusion. The intact monolayers showed no inclusion of dye, whereas the damaged cells showed dye inclusion.

MTT Assay

MTT is a tetrazolium salt which is cleaved to a dark blue product by mitochondrial dehydrogenases in living but not in dead cells (16). Caco-2 cells used for MTT assay were seeded at a density 2×10^4 cells/well in 96 well cell culture plates and pre-incubated for 24 h before CS derivatives treatment. The cells were then treated with CS derivatives at various concentrations 0.05–5 mM in serum-free medium pH 7.4 for 24 h. Dilution CS derivatives was made by using serum-free medium to ensure that the cells did not die from nutrition deficiency. After treatment, CS derivatives 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 DMSO 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 non-treated control cells was arbitrarily defined as 100% (17).

$$\text{Relative cell viability} = \frac{(OD_{550,\text{sample}} - OD_{550,\text{blank}}) \times 100}{(OD_{550,\text{control}} - OD_{550,\text{blank}})} \quad (3)$$

Statistical Analysis

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

RESULTS

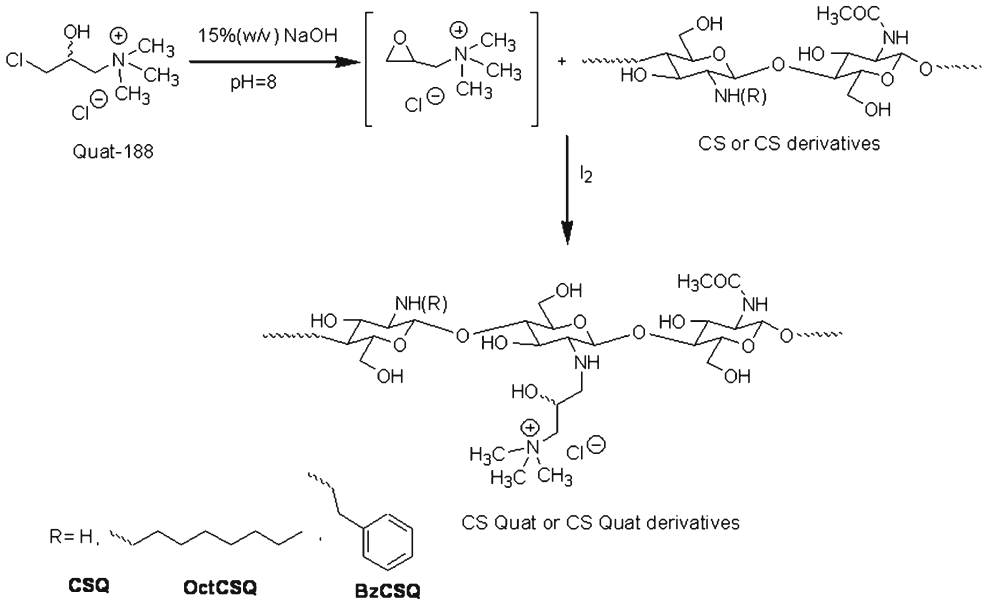
Synthesis of Quaternary Ammonium Chitosan Containing Hydrophobic Moieties

N-Octyl chitosan (OctCS) and *N*-benzyl chitosan (BzCS) were synthesized by reactive amination which previously reported by Sajomsang *et al.* (13). The formation of these chitosan derivatives occurred via the corresponding Schiff base intermediates and followed by reduction with sodium cyanoborohydride. The extent of ES was determined by ^{1}H-NMR method (18). It was found that the ES was in the range of 2–18%, depending on the mole ratios of aldehyde used. Previously, the biocidal activity of CS has been reported and it can be increased by at least an order of magnitude by treatment with Quat-188 (19). The treatment provides a quaternary ammonium function which is effective in solubilizing derivatives with hydrophobic substituents in water at all pH ranges. We have applied this treatment to each of the CS derivatives described above. Quaternization was performed using Quat-188 in a heterogeneous process under alkaline conditions. Under this condition, Quat-188 readily generates the corresponding epoxide, which reacts with the primary amino groups of CS in a nucleophilic substitution pathway (Scheme 1) to introduce the quaternary ammonium substituent (20). The quaternization process imparts water solubility to the CS, OctCS and BzCS over a wide pH range. A similar quaternization of CS using GTMAC has been reported (9,21–23). In this study, however, we selected to use commercially available Quat-188. The FT-IR spectra of the quaternized derivatives of CS were similar to those of the modified CS precursors except for the presence of an absorption band at 1,480 cm^{-1} due to C–H symmetric bending of the methyl groups on the quaternary ammonium substituents (9). Figure 1 showed ^{1}H-NMR spectra of CSQ. It exhibited characteristic resonances at δ 4.20, 3.3, 3.2, and 2.9 ppm which were attributed to methine protons (b), methylene protons (c), *N,N,N*-trimethyl protons (d), and methylene protons (a), respectively. Figures 2 and 3 exhibited the ^{1}H-NMR spectra of OctCSQ and BzCSQ. Resonances in addition to those found in OctCS and BzCS included the signals at δ 4.20, 3.3, 3.2, and 2.9 ppm, which were attributed to methine protons (b), methylene protons (c), *N,N,N*-trimethyl protons (d), and methylene protons (a), respectively. For DQ of CSQ, OctCSQ, and BzCSQ were calculated by ^{1}H-NMR spectrum (Eq. 4) and the data were summarized in Table I.

$$DQ = 1 - [(NHAc/3)/b] \times 100 \quad (4)$$

where NHAc is the integral area of GlcNAc protons and b is the integral area of methine proton of Quat-188 substituted derivatives.

It was found that the DQ obtained from Eq. 4 was higher than those of primary amino group of the OctCSQ and BzCSQ. This result indicated that *O*-alkylation could occur in this condition, but *O*-alkylation was not higher than 21% because the reaction was performed at room temperature and pH was adjusted to 8. In general, treatment of the hydro-



Scheme 1. Synthesis of CSQ and *N*-substituted derivatives of CSQ

phobically modified chitosan derivatives under the conditions described above would lead to 80–90% conversion of the residual glucosamine derivatives to *N*-2-hydroxypropyl-3-trimethylammonium chloride substituents.

Effect of Chitosan Derivatives on TEER

The effect of the different degree of Quat-188 (DQ), the extent of ES of CS derivatives and the polymer concentrations on TEER of Caco-2 cell monolayers was summarized in left side of Figs. 4, 5, and 6. 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 immediate reduction in TEER values compared with the control group. Table II showed the TEER values of CS and CS derivatives after 2 h incubation on the apical side of the monolayers. All CS derivatives showed the concentration-dependent manner on TEER values. Increasing the concentration of polymers resulted in significant decrease in TEER values compared with control. The results revealed that increasing the degree of DQ and ES tended to decrease in TEER values compared with control. All CS derivatives were dissolved at neutral pH 7.4 and showed reduced 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.

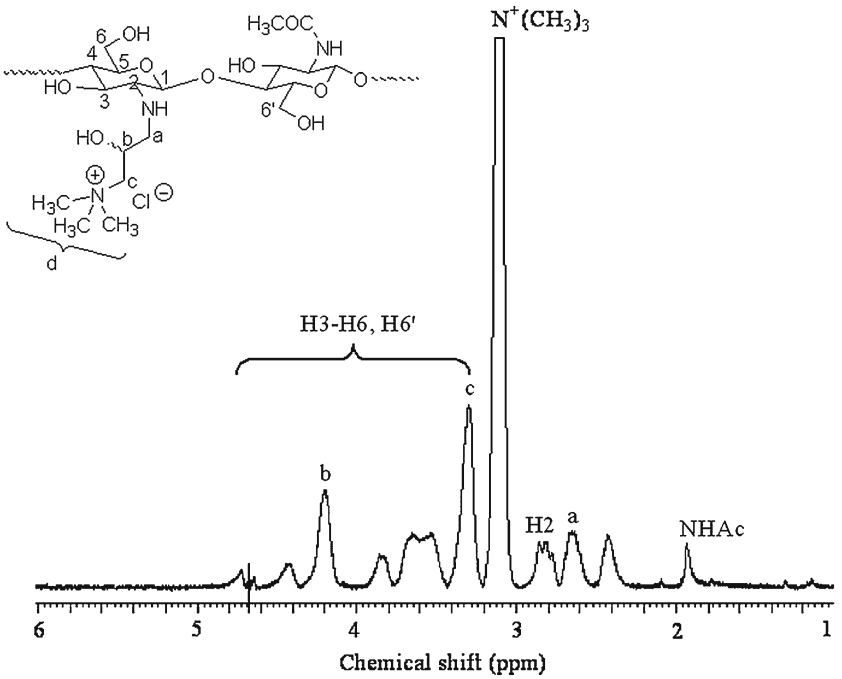


Fig. 1. ^{1}H-NMR spectrum of CSQ in D_2O

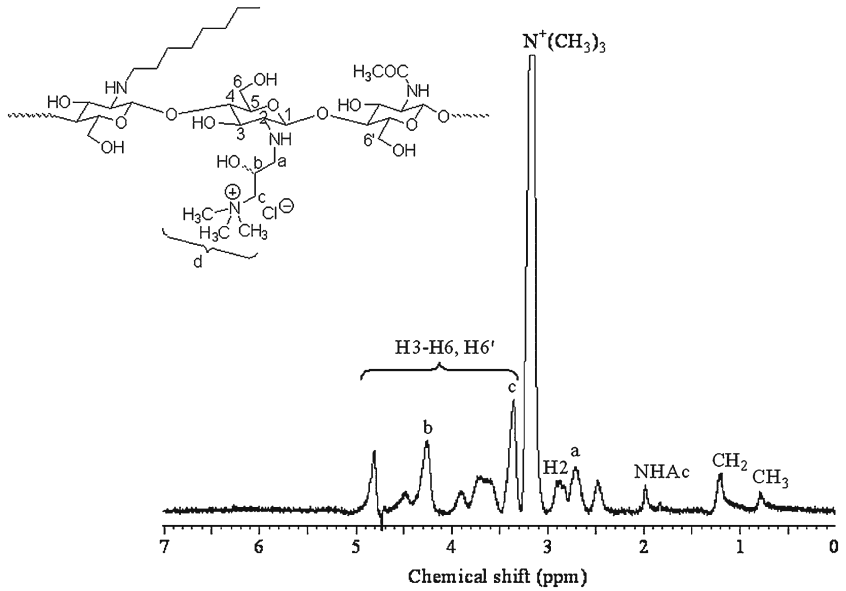


Fig. 2. ^{1}H-NMR spectrum of *N*-*n*-octyl CSQ in D_2O

After removal of the polymer solutions followed by repeated washing of the cell and medium substitution, an increase in resistance towards the initial values was found in control and CS derivatives treated with 0.05–5 mM of the polymers by 24 h (right side of Figs. 4, 5, and 6). The TEER recovered more than 60% of initial values within 24 h of CS derivatives removal in the cells treated with concentrations up to 1.25 mM, except in CSQ_{94} and $Oct_{11}CSQ_{94}$, indicating that the effects of CSQ and its derivatives of CSQ were reversible at lower concentrations. Therefore, in transport study, effects of the different %ES and %DQ of CSQ were investigated at a concentration of 1.25 mM, which showed the safest and the most effective enhancer. In comparison, the reduction in TEER prolonged incubation, up to 2 h with 1.25 mM concentrations of CSQ and *N*-substituted derivatives of CSQ were in the following order: CSQ_{94} (87.44±0.2% reduction) > CSQ_{73} (82.86±0.1% reduction) ≥ CSQ_{60} (81.31±0.3% reduction), *N*-benzyl CSQ was in the following order: $Bz_{18}CSQ_{94}$ (77.80±2.2% reduction) > $Bz_{11}CSQ_{94}$ (70.03±3.4% reduction) ≥ Bz_2CSQ_{94} (68.87±1.5% reduction) and *N*-octyl CSQ was in the following order: $Oct_{11}CSQ_{94}$ (83.89±0.1% reduction) > Oct_2CSQ_{94} (79.20± 1.9% reduction).

Effect of Chitosan Derivatives on the Transport of FD-4

To further evaluate CSQ and *N*-substituted derivatives of CSQ direct ability to increase permeability, it was important

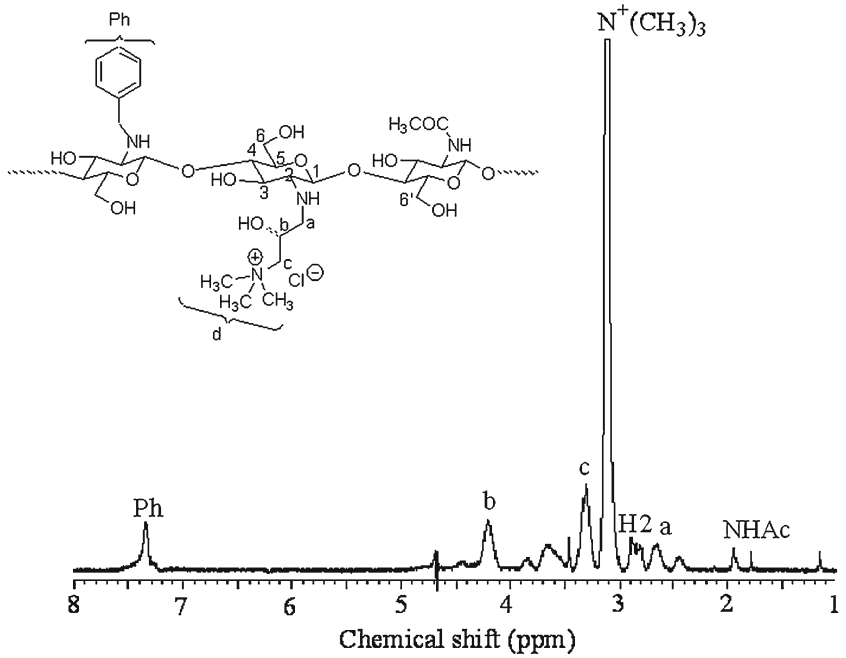


Fig. 3. ^{1}H-NMR spectrum of *N*-benzyl CSQ in D_2O

Table I. Degree of Quat-188 and the Extent of *N*-substitution of CS Derivatives

Samples	ES[a] (%)	DQ[b] (%)
CSQ_{94}	–	93.3
CSQ_{73}	–	73.0
CSQ_{60}	–	60.0
Bz_2CSQ_{94}	2.3	94.0
$Bz_{11}CSQ_{94}$	11.4	92.5
$Bz_{18}CSQ_{94}$	18.5	91.4
Oct_2CSQ_{94}	1.8	94.0
$Oct_{11}CSQ_{94}$	10.3	91.4

[a] ES is the extent of *N*-substitution
[b] DQ is degree of Quat-188

to use a hydrophilic macromolecule. The effect of the different %DQ and %ES of CSQ on the transport of FD-4 across the Caco-2 cell monolayers at pH7.4 was investigated. Since FD-4 is negatively charged polymer, the aggregation resulted from strong electrostatic interactions with the positively charged CSQ. In the present studies, Caco-2 cells were therefore pre-incubated with CS derivatives for 2 h before FD-4 were added in the apical compartments and accumulation of FD-4 in the basolateral compartments up to 4 h were measured. Figure 7 showed the transport of FD-4 across the Caco-2 cell monolayers in the presence of CS derivatives with different DQ and ES at the concentration of 1.25 mM and in the absence of CS derivatives for control. The cumulative transport of FD-4, P_{app} values and absorption enhancement ratios (R) were calculated and shown in Table III. Under the conditions described, very low baseline permeability was found. Incubation with CS derivatives 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 of CSQ and *N*-substituted derivatives of CSQ up to 4 h after incubation with FD-4 were in the following order: CSQ_{94} (14.28±1.2 μg) > CSQ_{73} (9.10±2.3 μg) ≥ CSQ_{60} (8.87±0.7 μg), *N*-benzyl CSQ was in the following order: $Bz_{18}CSQ_{94}$ (4.70±0.1 μg) ≥ $Bz_{11}CSQ_{94}$ (4.36±3.4 μg) > Bz_2CSQ_{94} (3.25±0.8 μg) and *N*-octyl CSQ was in the following order: $Oct_{11}CSQ_{94}$ (10.65±0.6 μg) > Oct_2CSQ_{94} (5.99±0.3 μg). These results were in agreement with the results of CSQ on reduction of TEER.

Cytotoxicity of Chitosan Derivatives

Cationic polymers have known to be cytotoxic materials. It is believed that the cytotoxic effect mainly originates from cationic charge. Therefore, in this study effect of the different

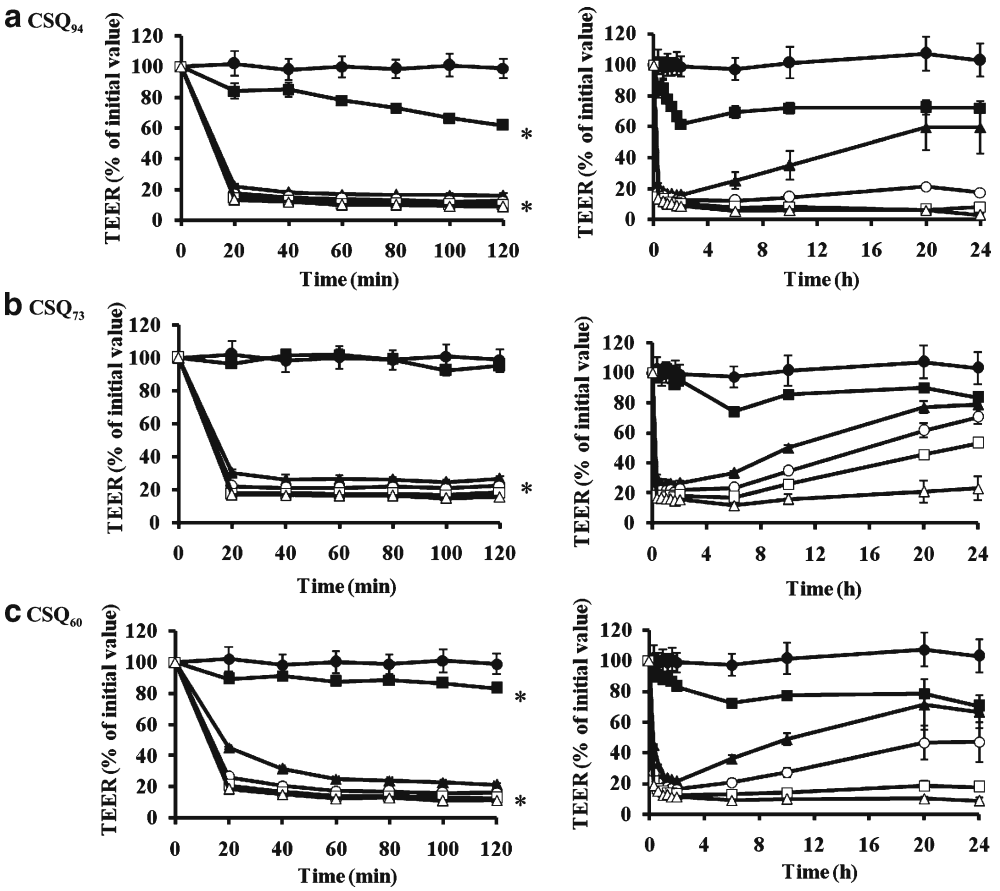


Fig. 4. Effect of CSQ_{94} (a), CSQ_{73} (b), and CSQ_{60} (c) concentrations from 0.05–5 mM, at pH 7.4 on the TEER (*left*) and TEER recovery (*right*) of Caco-2 cell monolayers. *Filled circles* control, *filled squares* 0.05 mM, *filled triangles* 0.5 mM, *empty circles* 1.25 mM, *empty squares* 2.5 mM, *empty triangles* 5 mM. Each point represents the mean of three experiments. *$p<0.05$

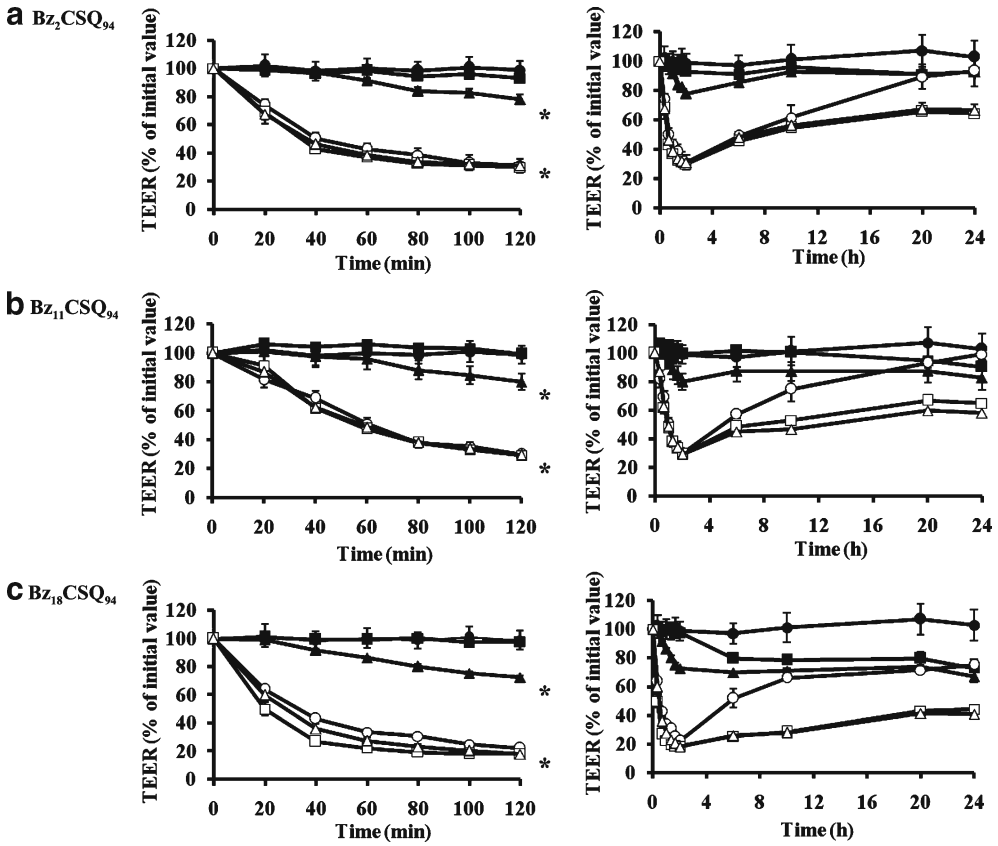


Fig. 5. Effect of Bz_2CSQ_{94} (a), $Bz_{11}CSQ_{94}$ (b), and $Bz_{18}CSQ_{94}$ (c) concentrations from 0.05–5 mM, at pH 7.4 on the TEER (*left*) and TEER recovery (*right*) of Caco-2 cell monolayers. *Filled circle* control, *filled squares* 0.05 mM, *filled triangles* 0.5 mM, *empty circles* 1.25 mM, *empty squares* 2.5 mM, *empty triangles* 5 mM. Each point represents the mean of three experiments. *$p<0.05$

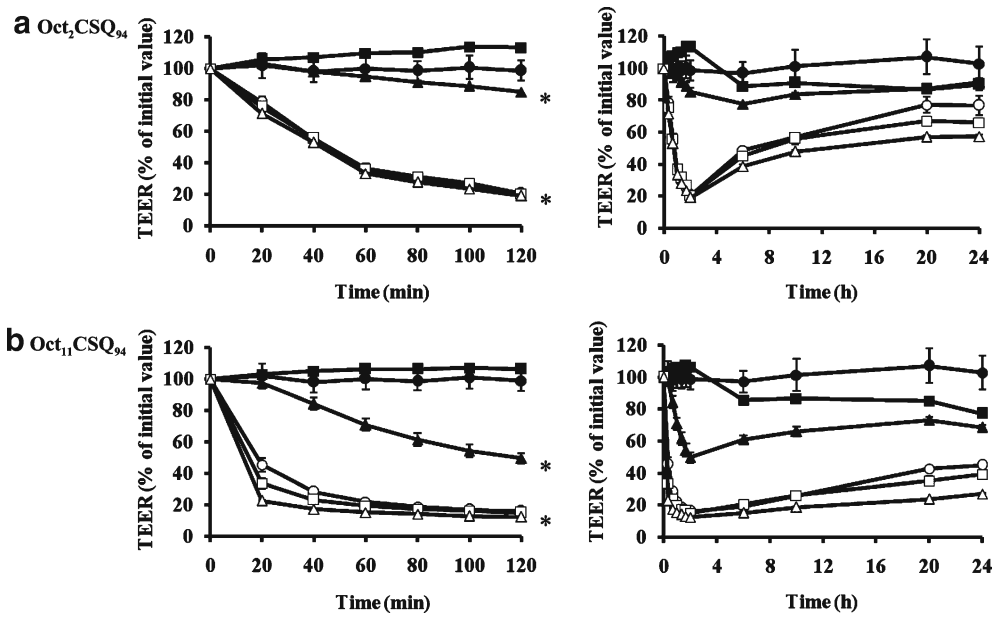


Fig. 6. Effect of Oct_2CSQ_{94} (a) and $Oct_{11}CSQ_{94}$ (b) concentrations from 0.05–5 mM, at pH 7.4 on the TEER (*left*) and TEER recovery (*right*) of Caco-2 cell monolayers. *filled circles* control, *filled squares* 0.05 mM, *filled triangles* 0.5 mM, *empty circles* 1.25 mM, *empty squares* 2.5 mM, *empty triangles* 5 mM. Each point represents the mean of three experiments. *$p<0.05$

Table II. Effect of Degree of Quat-188 (DQ), the Extent of Substitution (ES of CS Derivatives, Polymer Concentration and pH on TEER (n=3)

		TEER (% of initial value) 2 h				
		CS derivatives concentration (mM)				
CS derivatives	pH	0.05	0.5	1.25	2.5	5
CSQ_{94}	7.4	61.72±1.2	16.09±1.5	12.56±0.2	10.38±0.4	8.76±0.3
CSQ_{73}	7.4	95.22±3.9	26.64±1.7	17.14±0.1	16.21±0.2	15.74±0.8
CSQ_{60}	7.4	83.25±1.3	21.50±0.2	18.69±0.3	12.78±0.1	11.45±0.8
Bz_2CSQ_{94}	7.4	93.02±0.7	78.08±3.6	31.13±1.5	30.05±0.8	30.99±2.7
$Bz_{11}CSQ_{94}$	7.4	99.44±1.4	79.92±5.7	29.97±3.4	29.19±0.5	29.36±1.1
$Bz_{18}CSQ_{94}$	7.4	97.89±0.7	72.55±1.0	22.20±2.2	18.58±1.1	18.01±1.5
Oct_2CSQ_{94}	7.4	113.36±1.1	85.26±2.7	20.80±1.9	20.58±1.4	19.23±1.2
$Oct_{11}CSQ_{94}$	7.4	106.35±1.0	49.71±3.4	16.11±0.1	14.99±1.2	12.51±0.3
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±1.5	21.81±2.5	16.58±0.8

%ES and %DQ of CSQ on cytotoxicity was investigated. The effect of CS derivatives on mitochondrial dehydrogenase activity is shown as IC_{50} value which high value showed low toxicity. The IC_{50} values of CS and CS derivatives are shown in Table IV. The results showed that CSA at pH 7.4 incubated with Caco-2 cells for 24 h did not remarkably change the viability of Caco-2 cells, whereas at pH 6.2 showed concentration-dependent cytotoxicity with IC_{50} of 0.9±0.1 mM. All CS derivatives tested showed concentration-dependent cytotoxicity in Caco-2 at pH 7.4 incubated for 24 h. Increasing % DQ of CSQ resulted in significant increase in cytotoxicity. However, *N*-substituted derivatives of CSQ showed IC_{50} values of BzCSQ were more than CSQ, whereas IC_{50} values of OctCSQ were less than CSQ.

DISCUSSION

There is an intense search for novel, biocompatible permeability enhancers to be used in drug delivery. CS is an efficient permeability enhancer but it suffers from poor aqueous solubility in the physiological pH range. To increase the solubility at neutral physiological pH, the quaternary ammonium moiety in CS is required. The above mentioned properties of cationic polymers have been known to be cytotoxic materials (15). Because of the amphiphilic nature of the cell membrane, an increase in interaction between the cell membrane and CS derivative could be favored and safer when the macromolecule itself contained hydrophobic residues. As a result, CS derivatives containing quaternary ammonium functionality in addition to different hydrophobic substitutions were of interest for novel absorption enhancer. To investigate different water soluble chitosan, CSQ, have been evaluated for its *in vitro* absorption enhancement on the TEER and permeability of Caco-2 cells monolayers by using FD-4 as a model drug for hydrophilic macromolecules. Because of TEER, as a measure of ionic permeability of the cell monolayer, 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 (24). For these reasons both methods were utilized in the present study to

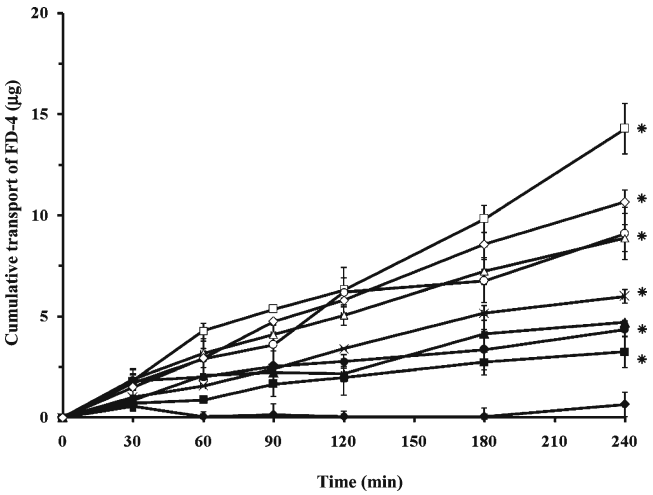


Fig. 7. Cumulative transport of FD-4 in the presence of 1.25 mM CS derivatives at pH7.4; CSQ_{94} (*empty squares*), CSQ_{73} (*empty circles*), CSQ_{60} (*empty triangles*), Bz_2CSQ_{94} (*filled squares*), $Bz_{11}CSQ_{94}$ (*filled circles*), $Bz_{18}CSQ_{94}$ (*filled triangles*), Oct_2CSQ_{94} (*multiplication symbols*), $Oct_{11}CSQ_{94}$ (*empty diamonds*), control (HBSS; *filled diamonds*). Each point represents the mean of three experiments. *p<0.05

Table III. Effect of the Different Degree of Quat-188 (DQ) and the Extent of Substitution (ES) of CS Derivatives on the Transport of FD-4 (n=3)

Samples	Cumulative transport of FD-4 at 4 h (µg)	P_{app}[a] (10^{-6}cm/s)	R[b]
Control	0.64±0.1	0.02	1.00
CSQ_{94}	14.28±1.2	0.85	51.64
CSQ_{73}	9.10±2.3	0.55	33.45
CSQ_{60}	8.87±0.7	0.53	32.45
Bz_2CSQ_{94}	3.25±0.8	0.20	12.27
$Bz_{11}CSQ_{94}$	4.36±3.4	0.23	13.82
$Bz_{18}CSQ_{94}$	4.70±0.1	0.28	17.36
Oct_2CSQ_{94}	5.99±0.3	0.38	23.45
$Oct_{11}CSQ_{94}$	10.65±0.6	0.67	40.73

[a] P_{app} is the apparent permeability coefficient
[b] R is the absorption enhancement ratio

Table IV. Cytotoxicity of CS and its Derivatives Incubated with Caco-2 Cells for 2 and 24 h (n=8)

Samples	pH	IC_{50}[a] (mM) at 2 h	IC_{50} (mM) at 24 h
CSQ_{94}	7.4	>5	0.15±0.01
CSQ_{73}	7.4	>5	0.74±0.01
CSQ_{60}	7.4	>5	1.64±0.02
Bz_2CSQ_{94}	7.4	>5	1.24±0.04
$Bz_{11}CSQ_{94}$	7.4	>5	1.91±0.07
$Bz_{18}CSQ_{94}$	7.4	>5	1.58±0.08
Oct_2CSQ_{94}	7.4	3.70±0.28	0.66±0.02
$Oct_{11}CSQ_{94}$	7.4	1.06±0.14	0.34±0.04
CSA	6.2	>5	0.90±0.01
CSA	7.4	>5	>5

[a] IC_{50} is the half maximal inhibitory concentration

assess the effect of CSQ and *N*-substituted derivatives of CSQ. CSQ were synthesized by the covalent bond formation between the primary amino groups of CS and aliphatic or aromatic aldehyde to provide hydrophobic moiety for the improvement of hydrophobic interaction with cell membrane and followed by quaternization with Quat-188 to render CS soluble. As shown in Figs. 4, 5, and 6, all of polymer was able to decrease TEER in a dose-dependent effect on tight junction permeability at pH 7.4. Treated with polymers (0.05–1.25 mM), the recovery of TEER could be observed by 24 h, except for CSQ_{94} and $Oct_{11}CSQ_{94}$, where it was not observed when treated with 2.5–5 mM. This is consistent with previous reports (15) in which complete removal of the polymers, without damaging the cells, proves 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 polymer resulted in higher cytotoxicity. This is in agreement with the findings of a previous study that the CS showed concentration-dependent cytotoxic effects on Caco-2 cells (17). Therefore, this high concentration (2.5–5 mM) of CS derivatives might damage the cells and resulted in the unrecovery of TEER. For these reasons concentration at 1.25 mM, which the cells could be recovered, was used in transport study of FD-4. TEER reduction was associated with an increase in flux of FD-4 across Caco-2 cell monolayers, indicating the capability of CS derivatives to open tight junctions and modulate the paracellular permeability of the monolayers. The results in Tables II and III revealed that both degree of DQ and ES affected the decreasing of TEER values and FD-4 transport. Increasing the degree of DQ and

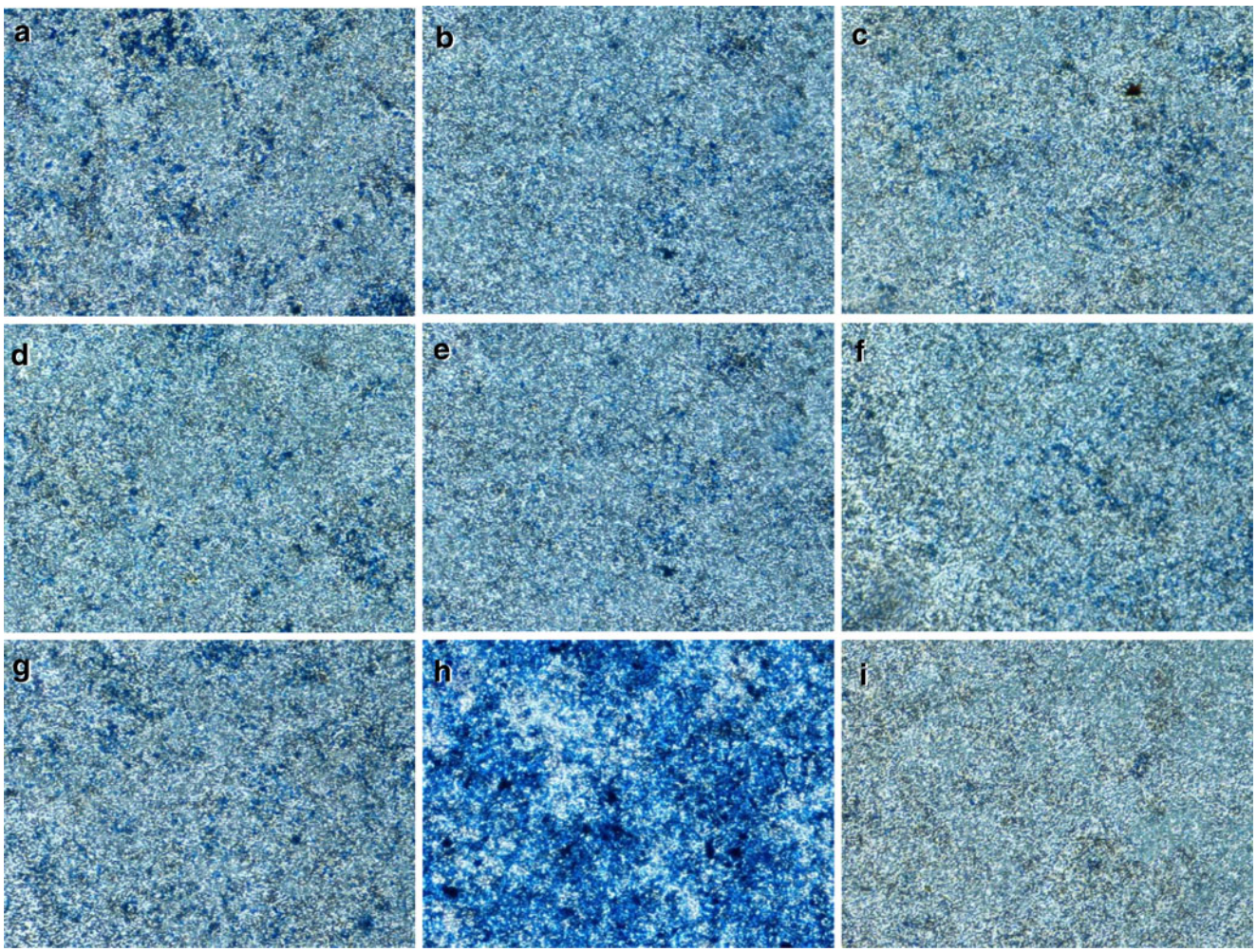


Fig. 8. Images of Caco-2 cells after completion of transport experiments following staining with Trypan blue (magnified ×4); CSQ_{94} (**a**), CSQ_{73} (**b**), CSQ_{60} (**c**), Bz_2CSQ_{94} (**d**), $Bz_{11}CSQ_{94}$ (**e**), $Bz_{18}CSQ_{94}$ (**f**), Oct_2CSQ_{94} (**g**), $Oct_{11}CSQ_{94}$ (**h**), and control (HBSS (**i**))

ES tended to decrease in the TEER values compared with control and increased 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 (25).

In this study, the results indicated that the introduction of Quat-188 group into the CS polymer backbone enhanced the solubility of CS and improved the water soluble CS to be an efficient absorption enhancer. In addition, the type of hydrophobic substitution on CS polymer backbone which improved the hydrophobic interaction between polymer and cell membrane had effected on permeability of FD-4 and cytotoxicity. Comparison in the same %DQ and %ES of each CSQ, IC_{50} values of BzCSQ were more than CSQ, whereas IC_{50} values of OctCSQ were less than CSQ. These results indicated that BzCSQ had less toxic than CSQ, whereas OctCSQ had more toxic than CSQ. These results were confirmed by trypan blue staining with the Caco-2 cells after completion of transport experiments that revealed OctCSQ had the most of intracellular dye uptake (Fig. 8). These evidences suggested that addition of aliphatic hydrocarbon (alkyl group) in the structure of hydrophobic substitution possibly induced toxicity on cells. Conversely, addition of aromatic hydrocarbon (aryl group) in the structure of hydrophobic substitution possibly reduced toxicity on cells. This is in agreement with the findings of a previous study (26) that TM-Bz-CS showed increasing %ES, leading to a slight decrease in cytotoxicity. However, an increase in the %DQ of TM-Bz-CS resulted in a significant increase in cytotoxicity. These results might be explained that CSQ_{94} and $Oct_{11}CSQ_{94}$ which high DQ and ES could not be recovered at concentration 1.25 mM as same as the other CS derivatives. These results indicated that an optimal degree of DQ and ES for CS derivatives, as in $CSQ_{60–73}$, Oct_2CSQ_{94}, $Bz_{11}CSQ_{94}$, and $Bz_{18}CSQ_{94}$, was essential to obtain efficient absorption enhancers with low cytotoxicity. It should be noted that *in vitro* Caco-2 cell monolayer tests may have some limitations, such as the absence of villi and a mucus layer. Therefore, the *in vivo* permeability of these CS derivatives could require further investigation.

CONCLUSION

CSQ is a novel polymeric permeation enhancer that is superior in solubility. It also decreased the TEER of Caco-2 cell monolayers and increased the paracellular permeability of hydrophilic markers at optimum degree of Quat-188 (60–73%DQ). The addition of hydrophobic groups, especially aromatic hydrophobic groups, on the CS polymer backbone can reduced the cytotoxicity on Caco-2 cells.

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