Carbohydrate Polymers 83 (2011) 430–437

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structure–activity relationships of methylated *N*-aryl chitosan derivatives for enhancing paracellular permeability across Caco-2 cells

Jariya Kowapradit[a], Praneet Opanasopit[a,*], Tanasait Ngawhirunpat[a], Theerasak Rojanarata[a], Warayuth Sajomsang[b]

[a] *Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand*
[b] *National Nanotechnology Center, Thailand Science Park, Pathumthani, Thailand*

ARTICLE INFO

Article history:
Received 19 May 2010
Received in revised form 30 July 2010
Accepted 2 August 2010
Available online 11 August 2010

Keywords:
Chitosan
Methylated *N*-aryl chitosan derivatives
Permeability
Absorption enhancer
Caco-2 cells

ABSTRACT

The aim of this study was to investigate three kinds of methylated chitosan containing different aromatic moieties; methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (TM-Bz-CS), methylated *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan (TM-CM-CS) and methylated *N*-(4-pyridylmethyl) chitosan (TM-Py-CS), on the paracellular permeability of Caco-2 cell monolayers and their toxicity towards the cell lines. The factors affecting epithelial permeability were evaluated in intestinal cell monolayers of Caco-2 cells using the transepithelial electrical resistance (TEER) and permeability of Caco-2 cell monolayers, with fluorescein isothiocyanate dextran 4400 (FD-4) as a model compound for paracellular tight junction transport. The results revealed that methylated chitosan containing different aromatic moieties showed the different absorption enhancing ability. The rank of enhancing paracellular permeability was $TM_{65}CM_{50}CS > TM_{56}Bz_{42}CS > TM_{65}CS > TM_{53}Py_{40}CS$. The cytotoxicity of these modified chitosans on Caco-2 cells was also studied by MTT assay where the $TM_{53}Py_{40}CS$ exhibited less toxicity than other derivatives. These studies demonstrated that the chemical structure and the positive charge location play an important role for absorption enhancement and cytotoxicity.

1. Introduction

Many researchers have attempted to search for new and safer absorption enhancers especially with respect to improving absorption of hydrophilic compounds with high molecular weight such as peptides, insulin and calcitonin. These have become available in the last two decades in sufficient and affordable amounts due to the progress in biotechnology and which are used for chronic therapy mostly by injection. The development of suitable alternative delivery systems (for the nasal, buccal, rectal, vaginal, ocular and peroral route) have not kept pace with the availability of endogenous peptides because of the lack of suitable absorption enhancers for this class of substances. However, it turned out rather surprisingly that special polymers, which show mucoadhesive properties, also are able to act as safe penetration enhancers for improved drug absorption of especially hydrophilic (peptide) drug substances. Chitosan is a natural cationic polysaccharide derived from chitin by partial deacetylation with strong alkaline solutions. It consists of β-(1,4)-2-amino-2-deoxy-D-glucopyranose units (GlcN) and a small amount of 2-acetamido-2-deoxy-D-glucopyranose or *N*-acetyl-D-glucosamine (GlcNAc) residues. It is generally regarded as biocompatible, slowly biodegradable natural origin polymer (Chandy & Sharma, 1990; Hirano & Noishiki, 1985). Chitosan is widely used in the food industry as a food additive and as a weight loss product. It is also used as a safe excipient for a number of pharmaceutical applications (e.g., excipient in granules and tablets, gels and microspheres) (Baldrick, 2000). Due to its mucoadhesive character and favorable toxicological properties, chitosan has been studied as a potential absorption enhancer across intestinal epithelia. 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 (Schipper et al., 1997).

However, chitosan is a weak base; therefore, in neutral and basic environments, chitosan molecules lose their charge and precipitate from solution. Under these conditions, chitosan will be ineffective as an absorption enhancer, which limits 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 to obtain a modified carrier with altered physicochemical characteristics. Generally, chitosan becomes soluble in water when primary amino groups are protonated. Strategies to increase neutral-solubility of chitosan include chemical derivatisation, e.g., carboxymethylation (Hjerde, Varum, Grasdalen, Tokura,

* Corresponding author. Tel.: +66 34 255800; fax: +66 34 255801.
E-mail addresses: praneet@su.ac.th, opraneet@hotmail.com (P. Opanasopit).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.08.005

& Smidsrot, 1997), sulfation (Baumann & Faust, 2001), alkylation (Sashiwa & Shigemasa, 1999) and acylation (Sorlier, Denuziere, Viton, & Domard, 2001). Alternatively, various branches may be introduced, which enhances solubility. Based on the literature search, it can be hypothesized that the transmucosal drug absorption enhancing properties of quaternized chitosan depend on molecular weight (MW), degree of quaternization (DQ) and other structural features (Colo, Burgalassi, Zambito, Monti, & Chetoni, 2004; Sandri et al., 2004). However, less attention has been paid on an effect of polymer structures that may influence its physicochemical properties. Therefore, the water-soluble chitosan derivatives, methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (TM-Bz-CS), methylated *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan (TM-CM-CS) and methylated *N*-(4-pyridylmethyl) chitosan (TM-Py-CS), containing different chain lengths and aromatic moieties were synthesized. We have found that TM-Py-CS exhibited highest transfection efficiency in Huh 7 cells compared to other methylated chitosan derivatives (Sajomsang, Ruktanonchai, Gonil, Mayen, & Opanasopit, 2009b). We also studied the effect of DQ and extent of *N*-substitution (ES) of TM-Bz-CS (Kowapradit et al., 2008, 2010a) and TM-CM-CS (Kowapradit et al., 2010b) on the paracellular permeability of Caco-2 cell monolayers. Therefore, the effect of hydrophobic chain lengths and aromatic moieties on chitosan derivatives for *in vitro* absorption enhancement, transepithelial electrical resistance (TEER) and the permeability of Caco-2 cells monolayers, and their cytotoxicities were determined and compared with *N*,*N*,*N*-trimethyl chitosan chloride (TM-CS).

2. Experimental

2.1. Materials and reagents

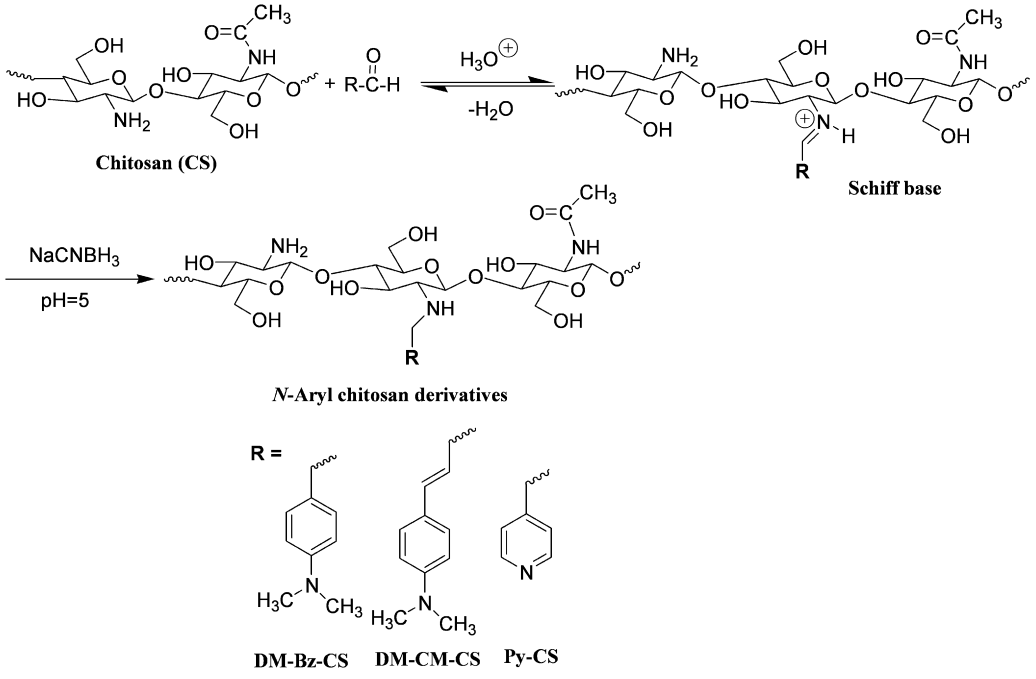
Chitosan with an average molecular weight (MW) of 276 kDa was purchased from Seafresh Chitosan (Lab) Co., Ltd. in Thailand. The degree of deacetylation (DDA) of chitosan was determined to be 94% by ^{1}H NMR spectroscopy (Lavertu et al., 2003). A dialysis tubing with MW cut-off of 12,000–14,000 g/mol from Cellu Sep T4, Membrane Filtration Products, Inc. (Segiun, TX, USA), was used to purify all modified chitosan derivatives. 4-Dimethylaminobenzaldehyde, 4-dimethylaminocinnamaldehyde and 4-pyridine carboxaldehyde were purchased from Fluka (Deisenhofen, Germany). Sodium cyanoborohydride, iodomethane and 1-methyl-2-pyrrolidone were purchased from Acros Organics (Geel, Belgium). Sodium iodide was purchased from Carlo Erba Reagent (Italy). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and fluorescein isothiocyanate dextran 4400 (FD-4) were purchased from Sigma-Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin–EDTA, penicillin–streptomycin antibiotics and fetal bovine serum (FBS) 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.

2.2. Synthesis of the methylated N-aryl chitosan derivatives

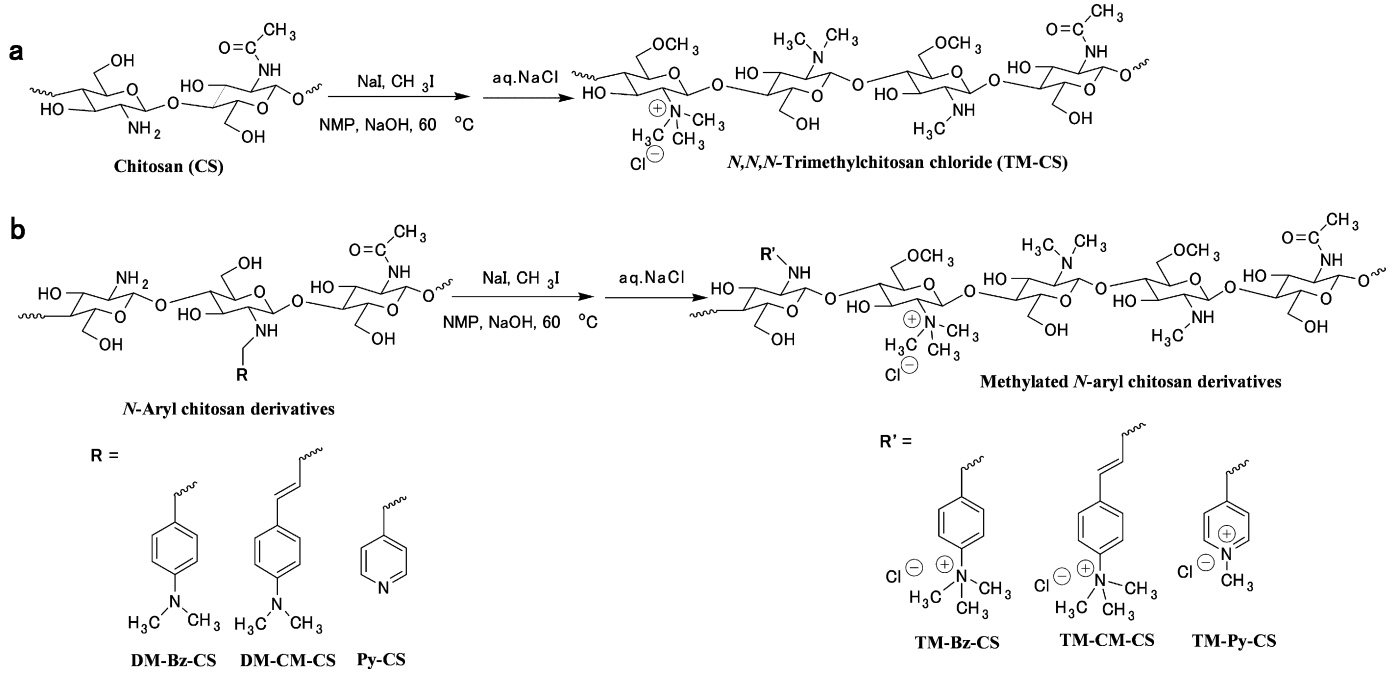
The *N*-aryl chitosan derivatives were carried out in accordance with the previous reported procedure (Scheme 1) (Sajomsang, Gonil, & Saesoo, 2009a), whereas the methylation of chitosan and *N*-aryl chitosan derivatives have been carried out by a single treatment with iodomethane in the presence of 1-methyl-2-pyrrolidone (NMP) and sodium hydroxide (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008) as shown in Scheme 2. The ES and DQ were generally determined by using ^{1}H NMR spectroscopy (Crini et al., 1997; Polnok, Borchard, Verhoef, Sarisuta, & Junginger, 2004; Sieval et al., 1998).

2.3. Cell cultures

Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) at a pH of 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



Scheme 1. Synthesis of *N*-aryl chitosan derivatives.



Scheme 2. Synthesis of methylated chitosan derivatives (a) *N,N,N*-trimethyl chitosan chloride (TM-CS) and (b) methylated *N*-aryl chitosan derivatives.

tissue culture polycarbonate membrane filters (pore size 3.0 μm) in 12-well Transwell® plates (Costar®, corning Inc., Corning, NY) at a seeding density of 2×10^4 cells/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 transepithelial electrical resistance (TEER) values were more than 600 Ω cm^2 using a Millicell® ERS meter (Millipore, Bedford, MA, USA).

2.4. Measurement of the transepithelial electrical resistance (TEER)

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 chitosan derivative solutions (0.05–5 mM in HBSS at pH 7.4) in the apical compartment. Chitosan derivative solutions were prepared by dissolving chitosan derivative in HBSS at pH 7.4 with gentle stirring. The TEER was measured every 20 min. After 2 h of treatment, the cells were carefully washed twice with PBS and incubated with fresh culture medium. The recovery of TEER values was monitored for 24 h after the treatment.

2.5. Transport studies

The transport of FD-4 across the Caco-2 cell monolayers at pH 7.4 was studied. Caco-2 monolayers grown in Transwell® (12-well) plates were used for the 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 chitosan derivative solutions at the recoverable cell concentration in the apical compartment for 2 h. In the control wells, the same media without chitosan derivatives were used. In all cases, HBSS pH 7.4 was used as the basolateral medium. After 2 h of 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 mL) 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 Eq. (1):

$$P_{app} = \left(\frac{dQ}{dt}\right) \times \left(\frac{1}{A}C_0\right) \quad (1)$$

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 using Eq. (2) (Kotze et al., 1998):

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

2.6. Evaluation of cytotoxicity

The cytotoxic effects of chitosan derivatives were investigated with Caco-2 cells using the MTT cytotoxicity assay. Cells were seeded at a density of 2×10^4 cells/well in 96-well cell culture plates. After pre-incubation for 24 h, cells were then treated with chitosan derivatives at various concentrations ranging from 0.05 to 5 mM in serum-free medium (pH 7.4) and incubated for 24 h. Dilutions of chitosan derivatives were made using serum-free medium to ensure that the cells did not die from nutrition deficiency.

Table 1
Methylation of chitosan and *N*-aryl chitosan derivatives.

Samples	ES (%)	DQ_T (%)		$N(CH_3)_2$ (%)	$NHCH_3$ (%)	Total *O*-CH_3 (%)
		DQ_{Ar} (%)	DQ_{CS} (%)			
$TM_{65}CS$	–	–	65	23	Trace	35
$TM_{65}CM_{50}CS$	50	50	15	24	Trace	15
$TM_{56}Bz_{42}CS$	42	42	14	2	17	5
$TM_{53}Py_{40}CS$	40	40	13	2	7	5

ES is the extent of *N*-substitution; DQ_{Ar} is the degree of quaternization at aromatic substituents; DQ_{CS} is the degree of quaternization at the primary amino groups of chitosan; DQ_T is the total degree of quaternization ($DQ_{Ar} + DQ_{CS}$); $N(CH_3)_2$ is *N*,*N*-dimethylation; $NHCH_3$ is *N*-methylation; total *O*-CH_3 is the total degree of *O*-methylation of 3-*O* and 6-*O* at 3-hydroxyl and 6-hydroxyl positions of GlcN of chitosan, respectively.

After treatment, chitosan derivative solutions were removed, fresh medium was added and the cells were incubated for 4 h. Finally, the cells were incubated with 100 μL MTT-containing medium (0.1 mg/mL MTT in serum-free medium) for 4 h. Next, the medium was removed, and the formazan crystal that formed in the 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). The viability of non-treated control cells was arbitrarily defined as 100% (Chae, Jang, & Nah, 2005). The relative cell viability was calculated according to the following Eq. (3), and the IC_{50} was calculated as a chitosan concentration that inhibited the growth of 50% of the cells relative to non-treated control cells:

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

2.7. 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 were examined using one-way analysis of variance (ANOVA) followed by a LSD post hoc test. The significance level was set at $p < 0.05$.

3. Results

3.1. Synthesis of the methylated N-aryl chitosan derivatives

Synthesis of *N*-aryl chitosan derivatives were successfully obtained by reductive amination of the corresponding Schiff base intermediates. It is a versatile and specific method for creating a covalent bond between a substrate and the primary amino group of the chitosan. The extent of *N*-substitution (ES) was determined by ^{1}H NMR method (Crini et al., 1997). It was found that the ES was in the range of 40-50% (Table 1). Methylation (quaternization) of either chitosan or *N*-aryl chitosan derivatives was carried out by single treatment with iodomethane under basic condition which yielded the corresponding quaternary ammonium chitosan derivatives. The methylation of chitosan was occurred mainly at primary amino group, while *N*-aryl chitosan derivatives was occurred either aromatic substituents or the primary amino groups of chitosan backbone (Sajomsang et al., 2008). Total degree of quaternization (DQ_T) was found in the range of 53–65% which was calculated by ^{1}H NMR spectroscopy (Sieval et al., 1998). Basically, methylation was based on nucleophilic substitution reaction and occurred randomly either the primary amino groups or hydroxyl groups of the chitosan backbone (Domard, Rinaudo, & Terrassin, 1986). Therefore, *N*,*N*-dimethylation, *N*-methylation, and also *O*-methylation at the primary amino groups and hydroxyl groups of the chitosan were found in this condition (Table 1). The results revealed that the methylated chitosan showed higher percentage of *N*,*N*-dimethylation and *O*-methylation than those of methylated *N*-aryl chitosan derivatives. This could be explained that the numbers of free primary amino groups in the methylated *N*-aryl chitosan derivatives were less than the chitosan due to *N*-arylation. *N*-methylation of the *N*-aryl chitosan derivatives led to quaternization at *N*-pyridyl or *N*,*N*-dimethyl groups on the aromatic moieties, and the primary amino groups of the polymer. This is the reason why the *N*,*N*-dimethylation of the methylated *N*-aryl chitosan derivatives was lower than those of the methylated chitosan at the same condition. In addition, the steric hindrance of the aromatic moieties shielded the hydroxyl positions, leading to low percentage of *O*-methylation.

3.2. Effect of chitosan derivatives on TEER

The effect of methylated *N*-aryl chitosan derivatives containing various aromatic moieties and the polymer concentration on TEER of Caco-2 cell monolayers are summarized in Fig. 1. The incubation of the monolayers on the apical side with 0.05–5 mM polymers at pH of 7.4 for 2 h resulted in an immediate reduction in TEER values compared to the control group (Table 2). All chitosan derivatives (excepted for $TM_{53}Py_{40}CS$) affected the TEER values in a concentration-dependent manner as shown by the significant decrease in TEER values when the concentration of polymers increased. After the polymer solutions were removed, the cells were repeatedly washed and subsequently supplied with fresh medium, and an increase in resistance towards the initial values was found in the control and in cells treated with 0.05–5 mM chitosan derivatives by 24 h (Fig. 2). Nevertheless, the TEER recovery was obvious within 24 h if the cells were treated with $TM_{65}CS$ and $TM_{65}CM_{50}CS$ were reversible at low concentrations of 0.05-0.5 mM. In the other hand, $TM_{56}Bz_{42}CS$ were reversible at concentrations of 0.05–5 mM but completely recovered at concentrations of 0.05–1.25 mM. For the subsequent transport study, $TM_{65}CS$, $TM_{65}CM_{50}CS$ and $TM_{56}Bz_{42}CS$ at concentration of 0.5 mM were chosen because they showed the TEER recovery and the most effective dose.

3.3. Effect of chitosan derivatives on the transport of FD-4

To further evaluate the direct ability of chitosan derivatives to increase the permeability of hydrophilic macromolecules, we studied the effect of different structure of these derivatives on the transport of hydrophilic macromolecules such as FD-4 across Caco-2 cell monolayers. FD-4 is a negatively charged polymer; therefore, its aggregation resulted from strong electrostatic interactions with the positively charged chitosan derivatives. In the present study, Caco-2 cells were pre-incubated with chitosan derivatives for 2 h before the addition of FD-4 in the acceptor compartments, and the accumulation of FD-4 in the basolateral compartments was measured. Fig. 3 shows the transport of FD-4 across the Caco-2 cell monolayers in the presence of chitosan derivatives with different chemical structure at concentration of 0.5 mM of all chitosan derivatives and in the absence of chitosan

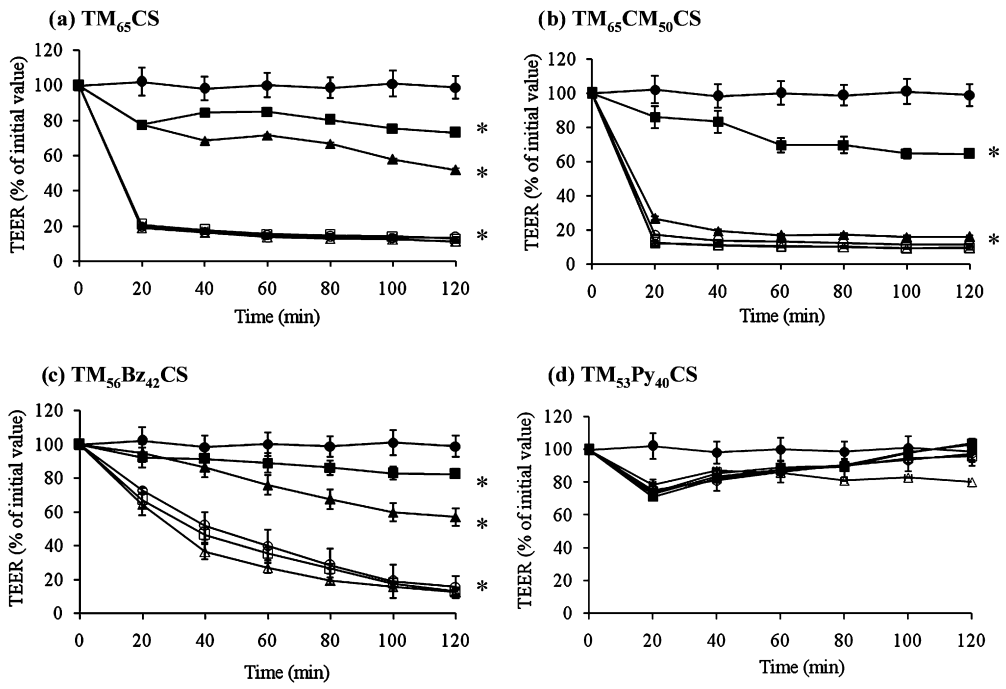


Fig. 1. The effect of chitosan derivatives on the TEER of Caco-2 cell monolayers: (a) $TM_{65}CS$, (b) $TM_{65}CM_{50}CS$, (c) $TM_{56}Bz_{42}CS$, (d) $TM_{53}Py_{40}CS$ concentrations ranging from 0.05 to 5 mM, at pH 7.4 (●) control, (■) 0.05 mM, (▲) 0.5 mM, (○) 1.25 mM, (□) 2.5 mM, (△) 5 mM. Each point represents the mean of three experiments. (*) indicates $p < 0.05$.

Table 2
The effect of chitosan derivatives and the polymer concentration on TEER ($n = 3$).

Samples	pH	TEER (% of initial value) 2 h				
		Polymer concentrations (mM)				
		0.05	0.5	1.25	2.5	5
$TM_{65}CS$	7.4	73.06 ± 2.1	51.90 ± 1.1	13.44 ± 0.8	12.69 ± 0.6	11.23 ± 0.4
$TM_{65}CM_{50}CS$	7.4	64.58 ± 1.5	23.53 ± 2.7	11.35 ± 0.2	9.57 ± 1.1	9.33 ± 0.8
$TM_{56}Bz_{42}CS$	7.4	82.30 ± 2.4	52.81 ± 4.0	15.62 ± 6.5	13.00 ± 1.8	12.58 ± 1.7
$TM_{53}Py_{40}CS$	7.4	103.72 ± 1.2	95.38 ± 4.0	97.55 ± 3.4	96.05 ± 2.4	80.28 ± 0.1

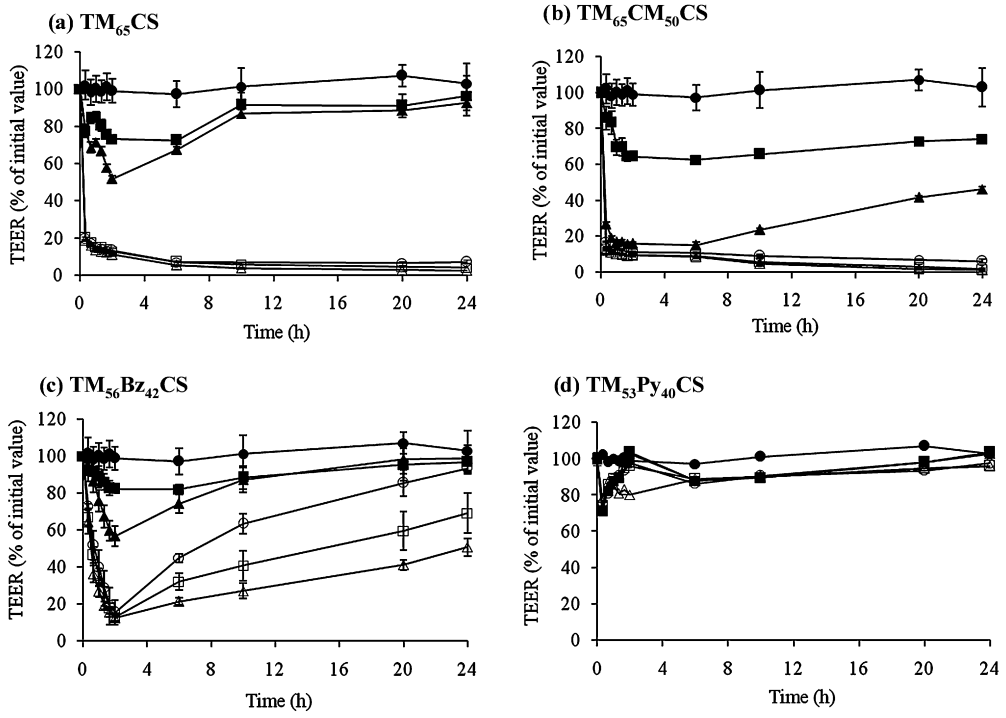


Fig. 2. The TEER recovery pattern of the Caco-2 cell monolayers: (a) $TM_{65}CS$, (b) $TM_{65}CM_{50}CS$, (c) $TM_{56}Bz_{42}CS$, (d) $TM_{53}Py_{40}CS$ concentrations ranging from 0.05 to 5 mM, at pH 7.4 (●) control, (■) 0.05 mM, (▲) 0.5 mM, (○) 1.25 mM, (□) 2.5 mM, (△) 5 mM. Each point represents the mean of three experiments.

Table 3
The effect of chitosan derivatives on the transport of FD-4 ($n = 3$).

Samples	Concentrations (mM)	Cumulative transport of FD-4 at 4 h (μg)	P_{app} (10^{-6} cm/s)	R
Control	–	2.64 ± 1.4	0.16	1.00
$TM_{65}CS$	0.5	3.29 ± 1.2	0.18	1.09
$TM_{65}CM_{50}CS$	0.5	20.22 ± 1.6	1.29	7.91
$TM_{56}Bz_{42}CS$	0.5	15.75 ± 0.7	1.01	6.18

P_{app} is the apparent permeability coefficient; R is the absorption enhancement ratio.

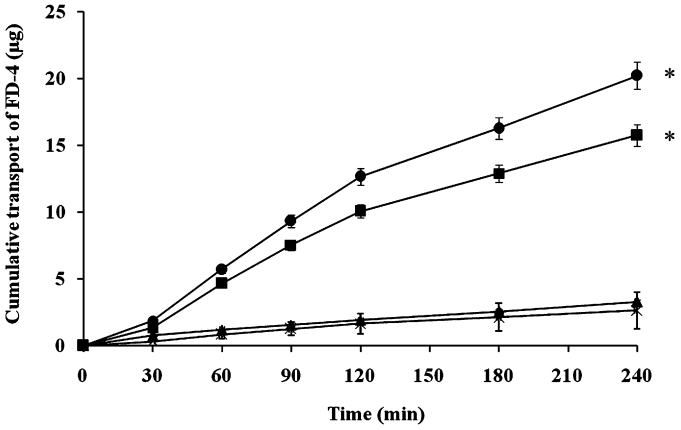


Fig. 3. Cumulative transport of FD-4 in the presence of chitosan derivatives at pH 7.4 for 4 h: (●) $TM_{65}CM_{50}CS$, (■) $TM_{56}Bz_{42}CS$, (▲)$TM_{65}CS$, (x) control. Each point represents the mean of three experiments. (*) indicates $p < 0.05$.

derivatives as a control. The cumulative transport of FD-4, P_{app} values and absorption enhancement ratios (R) were calculated and shown in Table 3. Under the conditions described, very low baseline permeability was determined. Incubation with chitosan derivatives resulted in an accumulation of FD-4 in the acceptor compartment. The chitosan with additional trimethyl groups on the aromatic moieties had a greater effect on increasing FD-4 values than the chitosan containing only trimethyl functionality, as shown by the comparison at the similar % DQ ($TM_{65}CS$). The cumulative amounts transported up to 4 h after incubation with FD-4 are in the following order: $TM_{65}CM_{50}CS$ (20.22 ± 1.6 μg) > $TM_{56}Bz_{42}CS$ (15.75 ± 0.7 μg) > $TM_{65}CS$ (3.29 ± 1.2 μg).

3.4. Cytotoxicity of chitosan derivatives

Cationic polymers are known to be cytotoxic materials, which are most likely due to their positive charges. Therefore, in this study, the effect of positively charged location in the chitosan derivatives on cytotoxicity was determined. The results showed that all chitosan derivatives tested showed concentration-dependent cytotoxicity in Caco-2 incubated for 24 h. IC_{50} value of the chitosan derivatives as shown in Table 4, suggesting that additional trimethyl and methyl groups on the benzyl and pyridyl moieties in the polymer structure can reduce the cytotoxicity in Caco-2 cells as shown by the increase IC_{50} value at 24 h. On the other hand, addition of trimethyl groups on the cinnamyl moiety showed high cytotoxicity as well as the methylated chitosan.

Table 4
Cytotoxicity of chitosan derivatives 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
$TM_{65}CS$	7.4	3.30 ± 1.02	0.14 ± 0.01
$TM_{65}CM_{50}CS$	7.4	>5	0.14 ± 0.01
$TM_{56}Bz_{42}CS$	7.4	>5	1.70 ± 0.01
$TM_{53}Py_{40}CS$	7.4	>5	3.45 ± 0.08

4. Discussion

Due to the amphiphilic nature of the cell membrane, an increase in the interaction between the cell membrane and the chitosan derivative could be favored when the macromolecule contains hydrophobic residues. As a result, chitosan derivatives containing quaternary ammonium functionality in addition to different hydrophobic substitutions were excellent candidates for novel absorption enhancers. Recently, our research group successfully synthesized modified chitosans, methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (TM-Bz-CS), methylated *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan (TM-CM-CS) and methylated *N*-(4-pyridylmethyl) chitosan (TM-Py-CS), that showed *in vitro* transfection efficiencies (Opanasopit et al., 2008, 2009; Rojanarata et al., 2008; Sajomsang et al., 2009b), mucoadhesive properties (Sajomsang, Rungsardthong Ruktanonchai, Gonil, & Nuchuchua, 2009c) and antibacterial activities (Sajomsang et al., 2008). In part of absorption enhancing properties, it is well known that chitosan solutions cause a significant and dose-dependent decrease of TEER of the Caco-2 cell monolayers by action on negatively charged sites at the cell surfaces and tight junctions, and it has been shown that chitosan is able to induce changes in F-actin distribution (Artursson, Lindmark, Davis, & Illum, 1994). Although F-actin is directly or indirectly associated with proteins in the tight junctions (Madara, 1987), chitosan most likely promotes 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 (McEwan, Jepson, Hirst, & Simmons, 1993). This hypothesis is supported by a recent study concluded that the interaction of chitosan with the cell membrane results in a structural reorganization of tight junction-associated proteins, followed by enhanced transport through the paracellular pathway. Therefore, binding of chitosan to the Caco-2 cells precedes absorption enhancement, and this increase in absorption is mediated by the positive charges on the polymer (Schipper et al., 1997).

To investigate different structure of modified chitosan containing different chain lengths and aromatic moieties, three kinds of chitosan derivatives: TM-Bz-CS, TM-CM-CS and TM-Py-CS, were synthesized and evaluated for their *in vitro* absorption enhancement on the transepithelial electrical resistance (TEER) and permeability of Caco-2 cell monolayers using FD-4 as the model drug of hydrophilic macromolecules. These modified chitosans were synthesized by the covalent bond formation between the primary amino groups of chitosan and aromatic groups of aldehyde material to provide a hydrophobic moieties, which improve the hydrophobic interaction with the cell membrane. The single methylation of the chitosan molecule containing hydrophobic moieties was carried out using iodomethane as the methylating agent to render chitosan soluble in water. The results showed that increasing concentrations of these polymer solutions (0.05–0.5 mM) resulted in a dose-dependent effect on tight junction permeability at pH 7.4, excepted for $TM_{53}Py_{40}CS$. Increasing the concentration of the polymers from 0.5 to 5 mM did not result

in significant decreases in TEER values compared with the reduction measured with 0.05–0.5 mM (Fig. 1). A similar trend in the decrease of TEER as a function of low chitosan concentration was previously reported by Kotze et al. (1998) and Kowapradit et al. (2008, 2010a,b). The effect on TEER values appears to be plateau because no significant differences were found when higher concentrations of polymers (0.5–5 mM) were used. Increasing concentrations of $TM_{53}Py_{40}CS$ was not observed on tight junction permeability. This could be explained in term of polymer action on negatively charged sites at the cell surfaces and tight junctions. The chemical structures of the TM-Bz-CS and TM-CM-CS were similar but with different chain length between chitosan backbone and quaternary ammonium moieties. It was postulated that TM-CM-CS would tightly bind to negatively charged sites more than those of TM-Bz-CS at the cell surfaces and tight junctions followed by enhanced transport through the paracellular pathway. The substitution of 4-pyridylmethyl moiety in chitosan results in no effect on tight junctions in Coco 2 cells monolayers. This finding could be possible that the steric hindrance of the *N*-pyridylmethyl group shielded the positive charges of the quaternary ammonium group on the GlcN of chitosan resulted in hinder the binding of the polymers to negatively charged sites at the cell surfaces and tight junctions. Moreover, the positive charge in the pyridine ring could be delocalized by the resonance effect, while other the positive charges in methylated chitosan derivatives were fixed. Although the exact mechanism of *N*-pyridylmethyl group hinder the binding remains to be further studied, our study showed that TM-CM-CS and TM-Bz-CS could be a potential candidate for absorption enhancer.

After the polymer solutions were removed, the recovery of TEER of $TM_{56}Bz_{42}CS$ could be gradually observed by 24 h at concentrations of 0.05–5 mM, whereas it was not observed when the cells were treated with 1.25–5 mM of $TM_{65}CM_{50}CS$ and $TM_{65}CS$ (Fig. 2). Our results showed that higher concentrations of $TM_{65}CM_{50}CS$ and $TM_{65}CS$ resulted in higher cytotoxicity. This finding is in agreement with the observations of a previous study showed that chitosan had a concentration-dependent cytotoxic effect on Caco-2 cells (Chae et al., 2005). Therefore, these high concentrations (1.25–5 mM) of these polymers may damage the cells, which resulted in the lack of TEER recovery. The results in Fig. 3 demonstrate that chitosan containing quaternary ammonium groups on the aromatic moieties, TM-CM-CS and TM-Bz-CS, affected the decrease of TEER values and FD-4 transport (Table 3). These results indicated that the introduction of the *N*-aryl group into the chitosan polymer backbone enhanced the hydrophobicity, which improved the hydrophobic interaction between the polymer and the cell membrane. These improvements helped the water-soluble chitosan to be an efficient absorption enhancer. However, it should be noted that introduction of the *N*-aryl group with long chain length could be affect on cytotoxicity observed on Caco-2 cells using MTT assay. The effect of chitosan derivatives on mitochondrial dehydrogenase activity is shown as IC_{50} value, which high value showed low toxicity. According to IC_{50} at 24 h of these modified chitosan molecules, $TM_{65}CM_{50}CS$ and $TM_{65}CS$ which had no *N*-aryl group appeared to be more toxic than $TM_{56}Bz_{42}CS$, whereas $TM_{53}Py_{40}CS$ showed less toxic.

5. Conclusion

The water-soluble chitosan derivatives, TM-Bz-CS, TM-CM-CS and TM-Py-CS, were successfully synthesized. As the similar degree of hydrophobic substitution, TM-CM-CS exhibited the highest paracellular permeability enhancer. TM-Bz-CS exhibited lower paracellular permeability enhancer and cytotoxicity than TM-CM-CS, whereas TM-Py-CS had no effect on paracellular permeability enhancer but showed less cytotoxicity. Our study indicated that the chemical structure and the positive charge location play an important role for absorption enhancement and cell cytotoxicity.

Acknowledgments

The authors wish to thank The Commission of Higher Education (Thailand), The Thailand Research Funds through the Golden Jubilee Ph.D. Program (Grant No. PHD/0114/2550), the National Research Council of Thailand and the Silpakorn University Research and Development Institute for financial support.

References

Artursson, P., Lindmark, T., Davis, S. S., & Illum, L. (1994). Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharmaceutical Research*, *11*, 1358–1361.

Baldrick, P. (2000). Pharmaceutical excipient development: The need for preclinical guidance. *Regulatory Toxicology and Pharmacology*, *32*, 210–218.

Baumann, H., & Faust, V. (2001). Concepts for improved regioselective placement of *O*-sulfo, *N*-sulfo, *N*-acetyl, and *N*-carboxymethyl groups in chitosan derivatives. *Carbohydrate Research*, *331*, 43–57.

Chae, Y., Jang, M. K., & Nah, J. W. (2005). Influence of molecular weight on oral absorption of water soluble chitosans. *Journal of Controlled Release*, *102*, 383–394.

Chandy, T., & Sharma, C. P. (1990). Chitosan as a biomaterial. *Biomaterials, Artificial Cells, and Artificial Organs*, *18*, 1–24.

Colo, D. G., Burgalassi, S., Zambito, Y., Monti, D., & Chetoni, P. (2004). Effects of different *N*-trimethylchitosans on *in vitro*/*in vivo* ofloxacin transcorneal permeation. *Journal of Pharmaceutical Science*, *93*, 2851–2862.

Crini, G., Torri, G., Guerrini, M., Morcellet, M., Weltrowski, M., & Martel, B. (1997). NMR characterization of *N*-benzyl sulfonated derivatives of chitosan. *Carbohydrate Polymers*, *33*, 145–151.

Domard, A., Rinaudo, M., & Terrassin, C. (1986). New method for the quaternization of chitosan. *International Journal of Biological Macromolecules*, *8*, 105–107.

Hirano, S., & Noishiki, Y. (1985). The blood compatibility of chitosan and *N*-acetylchitosans. *Journal of Biomedical Materials Research*, *19*, 413–417.

Hjerde, R. J. N., Varum, K. M., Grasdalen, H., Tokura, S., & Smidsrot, O. (1997). Chemical composition of *O*-(carboxymethyl)-chitins in relation to lysozyme degradation rates. *Carbohydrate Polymers*, *34*, 131–139.

Kotze, A. F., Lueben, H. L., Leeuw, B. J. D., Boer, B. G. D., Verhoef, J. C., & Junginger, H. E. (1998). Comparison of the effect of different chitosan salts and *N*-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). *Journal of Controlled Release*, *51*, 35–46.

Kowapradit, J., Opanasopit, P., Ngawhiranpat, T., Apirakaramwong, A., Rojanarata, T., Ruktanonchai, U., et al. (2008). Methylated *N*-(4-*N*, N-dimethylaminobenzyl) chitosan, a novel chitosan derivative, enhances paracellular permeability across intestinal epithelial cells (Caco-2). *AAPS PharmSciTech*, *9*, 1143–1152.

Kowapradit, J., Opanasopit, P., Ngawhiranpat, T., Apirakaramwong, A., Rojanarata, T., Ruktanonchai, U., et al. (2010). *In vitro* permeability enhancement in intestinal epithelial cells (Caco-2) monolayer of water soluble quaternary ammonium chitosan derivatives. *AAPS PharmSciTech*, *11*, 497–508.

Kowapradit, J., Opanasopit, P., Ngawhiranpat, T., Rojanarata, T., Ruktanonchai, U., & Sajomsang, W. (2010). Methylated *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan enhances paracellular permeability across Caco-2 cells. *Drug Delivery*, *17*, 301–312.

Lavertu, M., Xia, Z., Serreqi, A. N., Berrada, M., Rodrigues, A., Wang, D., et al. (2003). A validated ^{1}H NMR method for the determination of the degree of deacetylation of chitosan. *Journal of Pharmaceutical and Biomedical Analysis, 32*, 1149–1158.

Madara, J. L. (1987). Intestinal absorptive cell tight junction are linked to cytoskeleton. *American Journal of Physiology*, *253*, C171–C175.

McEwan, G., Jepson, M., Hirst, B., & Simmons, N. (1993). Polycation-induced enhancement of epithelial paracellular permeability is independent of tight junctional characteristics. *Biochimica et Biophysica Acta*, *1148*, 51–60.

Opanasopit, P., Sajomsang, W., Ruktanonchai, U., Mayen, V., Rojanarata, T., & Ngawhirunpat, T. (2008). Methylated N-(4-pyridinylmethyl) chitosan as a novel effective safe gene carrier. *International Journal of Pharmaceutics, 364*, 127–134.

Opanasopit, P., Petchsangsai, M., Rojanarata, T., Ngawhirunpat, T., Sajomsang, W., & Ruktanonchai, U. (2009). Methylated N-(4-N,N-dimethylaminobenzyl) chitosan as effective gene carriers: effect of degree of Substitution. *Carbohydrate Polymers*, *75*, 143–149.

Polnok, A., Borchard, G., Verhoef, J. C., Sarisuta, N., & Junginger, H. E. (2004). Influence of methylation process on the degree of quaternization of *N*-trimethyl chitosan chloride. *European Journal of Pharmaceutics and Biopharmaceutics*, *57*, 77–83.

Rojanarata, T., Petchsangsai, M., Opanasopit, P., Ngawhirunpat, T., Ruktanonchai, U., Sajomsang, W., & Tantayanon, S. (2008). Methylated N-(4-N,N-dimethylaminobenzyl) chitosan for novel effective gene carriers. *European Journal of Pharmaceutical biopharmaceutics*, *70*, 207–214.

Sajomsang, W., Gonil, P., & Saesoo, S. (2009). Synthesis and antibacterial activity of methylated *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan chloride. *European Polymer Journal*, *45*, 2319–2328.

Sajomsang, W., Ruktanonchai, U., Gonil, P., Mayen, V., & Opanasopit, P. (2009). Methylated N-aryl chitosan derivative/DNA complex nanoparticles for gene delivery: Synthesis and structure–activity relationships. *Carbohydrate Polymers*, *78*, 743–752.

Sajomsang, W., Rungsardthong Ruktanonchai, U., Gonil, P., & Nuchuchua, O. (2009). Mucoadhesive property and biocompatibility of methylated *N*-aryl chitosan derivatives. *Carbohydrate Polymers*, *78*(4), 945–952.

Sajomsang, W., Tantayanon, S., Tangpasuthadol, V., & Daly, W. H. (2008). Synthesis of methylated chitosan containing aromatic moieties: Chemoselectivity and effect on molecular weight. *Carbohydrate Polymers*, *72*, 740–750.

Sandri, G., Rossi, S., Ferrari, F., Bonferoni, M. C., Muzzarelli, C., & Caramella, C. (2004). Assessment of chitosan derivatives as buccal and vaginal penetration enhancers. *European Journal of Pharmaceutical Sciences*, *21*, 351–359.

Sashiwa, H., & Shigemasa, Y. (1999). Chemical modification of chitin and chitosan 2: Preparation and water soluble property of *N*-acylated or *N*-alkylated partially deacetylated chitins. *Carbohydrate Polymers*, *39*, 127–138.

Schipper, N. G. M., Olsson, S., Hoogstraate, J. A., Boer, A. G. D., Varum, K. M., & Artursson, P. (1997). Chitosans as absorption enhancers for poorly absorbable drugs II: Mechanism of absorption enhancement. *Pharmaceutical Research*, *14*, 923–929.

Sieval, A. B., Thanou, M., Kotze, A. F., Verhoef, J. C., Brussee, J., & Junginger, H. E. (1998). Preparation and NMR characterization of highly substituted trimethyl chitosan chloride. *Carbohydrate Polymers*, *36*, 157–165.

Sorlier, P., Denuziere, A., Viton, C., & Domard, A. (2001). Relation between the degree of acetylation and the electrostatic properties of chitin and chitosan. *Biomacromolecules*, *2*, 765–772.