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Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan as effective gene carriers: Effect of degree of substitution

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ABSTRACT

The objective of this study was to investigate the transfection efficiency of quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan; TM-Bz-CS, using the plasmid DNA encoding green fluorescent protein (pEGFP-C2) on human hepatoma cell lines (Huh7 cells). The factors affecting the transfection efficiency e.g. degree of quaternization (DQ), the degree of dimethylaminobenzyl substitution (DS) and polymer/DNA weight ratio, have been evaluated. The results revealed that all TM-Bz-CS derivatives were able to condense with DNA. Illustrated by agarose gel electrophoresis, complete complexes of TM_{57}-Bz_{42}-CS/DNA were formed at weight ratio of above 0.5, whereas those of TM_{47}-Bz_{42}-CS/DNA and TM_{57}- Bz_{17}-CS/DNA were above 1. The rank of transfection efficiency of the chitosan derivatives were TM_{57}-Bz_{42}-CS > TM_{47}-Bz_{42}-CS > TM_{57}-Bz_{18}-CS. The pH of culture medium did not affect the transfection efficiency of TM_{57}-Bz_{42}-CS/DNA complex, whereas it affected the transfection efficiency of chitosan/DNA complex. The results indicated that the improved gene transfection was due to the hydrophobic group (*N*,*N*-dimethylaminobenzyl) substitution on chitosan which promoted the interaction and condensation with DNA as well as *N*-quaternization which increased chitosan water solubility and enhance gene expression. For cytotoxicity studies, TM-Bz-CS was safe at the concentration of the highest transfection. In conclusion, this novel chitosan derivative, TM_{57}-Bz_{42}-CS showed elevated potential as gene carrier by efficient DNA condensation and mediated highest level of gene transfection with negligible cytotoxicity in Huh7 cells.

1. Introduction

As a non-viral vector for gene delivery, chitosan has several advantages over viral vectors since it does not cause virally-induced inflammatory responses, immunological reactions and oncogenic effects (Simon et al., 1993). In addition, chitosan is biocompatible, biodegradable and non-toxic; therefore, it has been proposed as a safer alternative to other non-viral vectors such as cationic lipids and cationic polymers (Kumar et al., 2003; Lee et al., 2001; Thanou, Florea, Geldof, Junginger, & Borchard, 2002; Weecharangsan, Opanasopit, Ngawhirunpat, Rojanarata, & Apirakaramwong, 2006). At acidic pH, below pK_a, the primary amines in the chitosan backbone become positively charged (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). These protonated amines enable chitosan to bind to negatively charged DNA and condense it into particles. Chitosan has shown promise to protect DNA from DNase I and II degradation (Huang, Fong, Khorc, & Lim, 2005; Richardson, Kolbe, & Duncan, 1999) and transfect into different cell types (Ishii, Okahata, & Sato, 2001; Maclaughlin et al., 1998). Formulation parameters such as molecular weight (MW), degree of deacetylation (DD), N/P ratio (molar ratio of chitosan nitrogen (N) per DNA phosphate (P)), and pH of transfection medium were found to affect the transfection efficiency of chitosan/DNA complexes (Ishii et al., 2001; Kiang, Wen, Lim, & Leong, 2004; Lavertu, Méthot, Tran-Khanh, & Buschmann, 2006; Romóren, Pedersen, Smistad, Evensen, & Thu, 2003; Sato, Ishii, & Okahata, 2001; Weecharangsan et al., 2008).

The main drawback of chitosan would be the poor water solubility at physiological pH and low transfection efficiency. But several chitosan derivatives have been synthesized in the last few years in order to obtain modified carrier with altered physicochemical characteristics (Rinaudo, 2006). Modified chitosan such as glycol chitosan or PEGylated chitosan (Yoo, Lee, Chung, Kwon, & Jeong, 2005; Zhang et al., 2007), low molecular weight soluble chitosan (Lee et al., 2001), quaternized CS (Thanou et al., 2002), and polyethylenglycol-graft-trimethyl chitosan (Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008) could be possible ways to circumvent the solubility issues. To improve gene transfection, chemically modified chitosan, such as quaternized chitosan

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(Thanou et al., 2002), urocanic acid-modified chitosan (Kim, Ihm, Choi, Nah, & Cho, 2003), galactosylated chitosan (Gao et al., 2005), deoxycholic acid chitosan oligosaccharide nanoparticle (Chae, Son, Lee, Jang, & Nah, 2005) and thiolated chitosan (Lee et al., 2007) were proposed.

Although many researches synthesized chitosan derivatives as alternative for gene carrier, a few were successful in increased transfection efficiency. Recently, our research groups have successfully synthesized the novel water soluble chitosan derivatives namely methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan. This chitosan derivatives showed elevated potential as gene carrier by efficient DNA condensation and mediated higher level of gene transfection with negligible cytotoxicity in Huh7 cells (Rojanarata et al., 2008). In this study, methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan with various substitutions was synthesized. A number of variables that influenced transfection efficiency such as the degree of dimethylaminobenzyl substitution (DS), degree of quaternization (DQ) and weight ratio were determined. The physical properties of the complexes were investigated. Their transfection efficiencies and cytotoxicity in human hepatocellular carcinoma cells (Huh7 cells) were evaluated.

2. Experimental

2.1. Materials

Chitosan was purchased from Seafresh Chitosan Laboratory, Thailand, with MW of 267 kDa and 94% degree of deacetylation. Sodium cyanoborohydride and polyethylenimine (PEI), MW 25 kDa, were purchased from Aldrich, Germany. 4-*N*,*N*-Dimethylaminobenzaldehyde, iodomethane, sodium iodide and 1-methyl-2-pyrrolidone were purchased from Fluka, Germany. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Chemical Co., USA. Dulbecco's modified Eagle's medium (DMEM), Trypsin–EDTA, penicillin–streptomycin antibiotics and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen, USA. The pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech, USA. The λ*Hin*dIII was obtained from Promega, USA. Huh7 (Human hepatocellular carcinoma) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

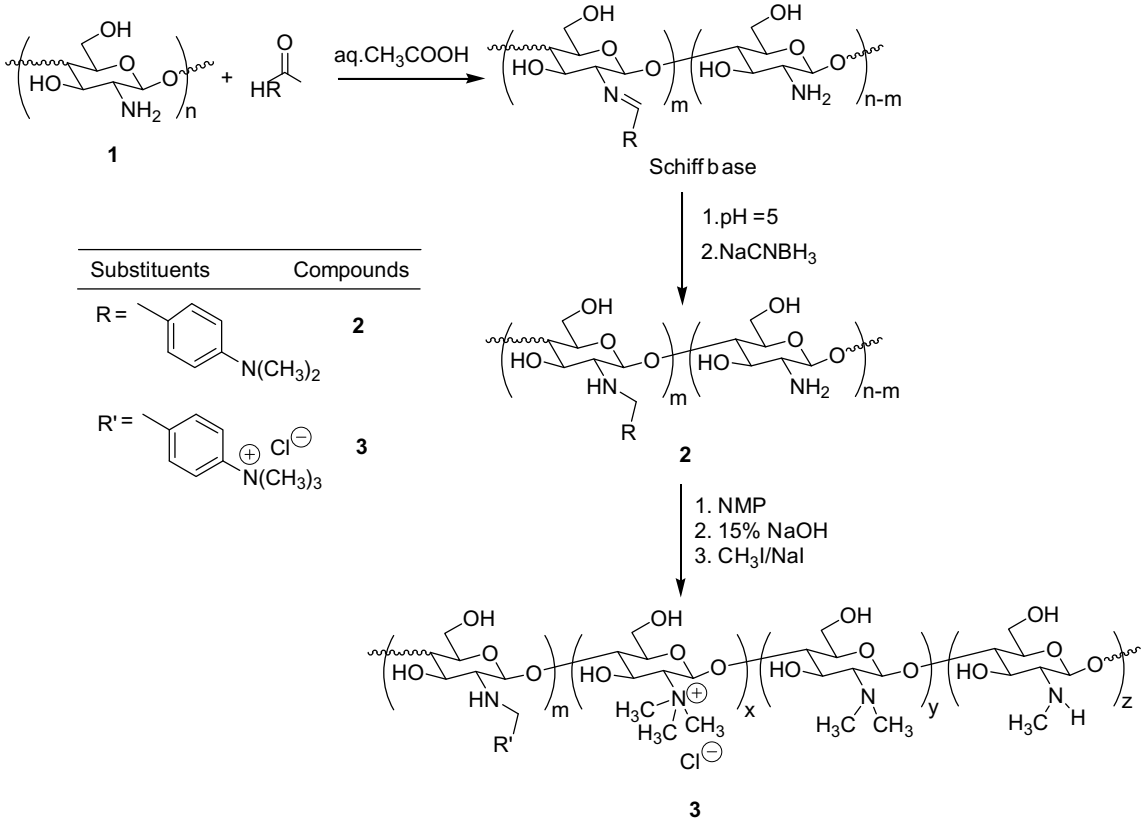
2.2. Synthesis of methylated N-(4-N,N-dimethylaminobenzyl) chitosan (TM-Bz-CS)

2.2.1. Synthesis of N-(4-N,N-dimethylaminobenzyl) chitosan (DM-Bz-CS)

DM-Bz-CS **2** was prepared as previously reported (Sajomsang, Tantayanon, Tangpasuthadol, & William, 2008). Briefly, 1.00 g of chitosan was dissolved in 70 mL of 1% acetic acid solution. The solution was diluted with ethanol (70 mL). 4-*N*,*N*-Dimethylamino benzaldehyde (3.0 mmol) was then added and the solution was 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 $NaCNBH_3$ was added and stirred at room temperature for 24 h, followed by the pH adjustment to 7 with 15% (w/v) NaOH. The reaction mixture was then dialyzed in distilled water and freeze-dried to give powder **2** (Scheme 1).

2.2.2. Synthesis of TM-Bz-CS

0.50 g of compound **2** was dispersed in 25 ml of *N*-methyl pyrrolidone (NMP) for 12 h at room temperature. Then 1.5 g of sodium



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

iodide 15% and 5% w/v NaOH (3.0 ml) were added and stirred at 50 °C for 15 min. Subsequently, 1 mL of methyl iodide 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 compounds were purified by precipitation in 300 mL of acetone. The precipitate was dissolved in 15% (w/v) NaCl solution in order to replace the iodide ion by chloride ion. The suspension was dialyzed with deionized water for 3 days to remove inorganic materials. The dialyzed solution was then concentrated under vacuum on a rotary evaporator and then precipitated in acetone (100 mL). The pure compound (TM-Bz-CS **3**) was collected and dried overnight at room temperature under a stream of nitrogen (Scheme 1).

2.3. Characterizations

FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared (FT-IR) spectrometer. All samples were prepared as potassium bromide pellets. The ^{1}H, ^{13}C NMR spectra were measured on a Mercury Varian 300 MHz spectrometer. All measurements were performed at 300 K, using pulse accumulating of 64 scans and the LB parameter of 0.30 Hz. One percent (v/v) D_2O/CF_3COOD and D_2O was a solvent for 10 mg chitosan and its derivatives, respectively.

2.4. Plasmid preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA, USA). DNA concentration was quantified by the measurement of UV absorbance at 260 nm using a GeneRay UV Photometer (Biometra). The purity of the plasmid was verified by gel electrophoresis (0.8% agarose gel) in Tris acetate–EDTA (TAE) buffer, pH 8.0, using λDNA/*Hin*dIII as a DNA marker.

2.5. Preparation and characterization of chitosan derivatives /DNA complexes

The chitosan derivatives/DNA complexes were prepared at various weight ratios by adding the DNA solution to the chitosan derivative solution. The mixture was gently mixed using pipette for 3–5 s to initiate complex formation and left for 15 min at room temperature. The complex formation was confirmed by electrophoresis. Agarose gels were prepared with 1% agarose solution in TAE buffer with ethidium bromide (0.5 μg/ml). The electrophoresis was carried out for 60 min at 100 V. The volume of the sample loaded in the well was 15 μl of chitosan derivatives/DNA complex containing 1 μg of DNA.

2.6. Size and zeta potential measurements

The particle size and surface charge of chitosan derivatives/DNA complexes were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water which was passed through 0.22 μm membrane filter prior to use. All samples were measured in triplicate.

2.7. In vitro transfection chitosan derivatives/DNA complexes in Hun7cells

Huh7 cells were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 in 1 ml of growth medium (DMEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% non-essential amino acid solution, 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown under humidified atmosphere (5% CO_2, 95% air, 37 °C) for 24 h. Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4). The cells were incubated with 0.5 ml of the chitosan derivatives/DNA complexes at various weight ratios containing 1 μg of pDNA for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid and PEI/DNA complexes were used as controls. After transfection, the cells were washed twice with PBS and grown in culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

2.8. Evaluation of cell viability

Evaluation of cytotoxicity was performed by the MTT assay. Huh7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/cm^2 in 200 μl of growth medium and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Prior to transfection, the medium was removed and the cells were rinsed with PBS. The cells were then treated with the chitosan derivatives/DNA complexes in the same concentrations as in vitro transfection experiment and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells were used as controls incubated for the same duration of time. After treatment, chitosan derivatives/DNA complexes solutions were removed. Finally, the cells were incubated with 100 μl MTT containing medium (1 mg/ml) for 4 h. Then the medium was removed, the cells were rinsed with PBS, pH 7.4, and formazan crystals formed in living cells were dissolved in 100 μl DMSO per well. Relative viability (%) was calculated based on the 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%.

2.9. Statistical analysis

Statistical significance of differences in transfection efficiency and cell viability were examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at $p < .05$.

3. Results and discussion

3.1. Synthesis and characterization of chitosan derivatives

As previously reported, DM-Bz-CS **2** was synthesized by reductive amination of the corresponding Schiff base intermediates (Scheme 1) (Sajomsang et al., 2008). The degree of *N*-substitution (DS), determined by ^{1}H NMR varied due to the different ratio of aldehydes to D-glucosamine (GlcN) of chitosan. The FT-IR spectra of DM-Bz-CS were similar to that of chitosan except that the additional absorption bands at wave numbers 1605, 1526, and 811 cm^{-1} were observed. These bands were assigned to C=C stretching and C—H deformation (out of plane) of the aromatic group. These results are in agreement with those already published for benzylated chitosans (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982). The ^{1}H NMR spectrum of DM-Bz-CS exhibited the broad singlet, δ 7.5 ppm, in the aromatic region and another singlet at δ 3.1 ppm assigned to *N*,*N*-dimethyl protons (Fig. 1). The ^{1}H NMR spectra of TM-Bz-CS were similar to that of the corresponding TM-CS except the additional signals at δ 3.5 ppm which was of *N*,*N*,*N*-trimethyl protons on benzyl substituent.

In this study, the quaternization of DM-Bz-CS **2** was based on a nucleophilic substitution of the primary amino group on the C-2 position of chitosan, using the procedures which was slightly modified from the method previously described (Curti, de Britto, & Campana-Filho, 2003). The quaternization of DM-Bz-CS **2,** with methyl iodide yielded TM-Bz-CS **3**, could occur at both the

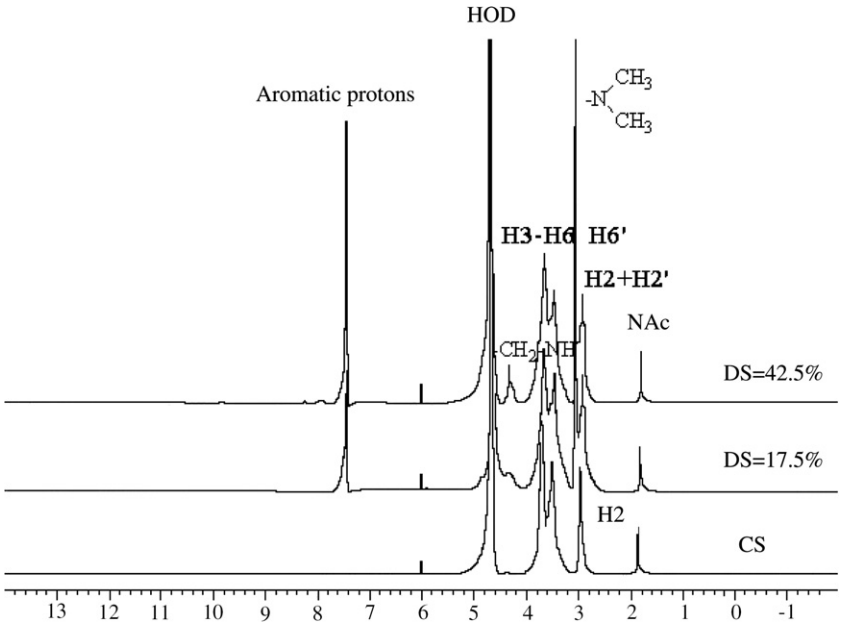


Fig. 1. ^{1}H NMR spectra of chitosan (CS) and *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (DM-Bz-CS).

aromatic substituent and primary amino group of GlcN of chitosan. The results clearly demonstrated that *N*,*N*-dimethylamino groups of DM-Bz-CS **2** were more reactive than the primary amino groups of chitosan which were completely quaternized giving DQ_{Ar} values equal to the corresponding DSs (Table 1). Besides *N*,*N*,*N*-trimethylation, *N*,*N*-dimethylation and *N*-monomethylation at the primary amino group of GlcN of chitosan were also observed.

3.2. Characterization of chitosan derivatives/DNA complexes

In order to determine the optimal complexation conditions, it was necessary to evaluate the formation of complexes between chitosan derivatives and DNA at different chitosan derivatives/DNA weight ratios. The formation of complexes was visualized by agarose gel electrophoresis. Fig. 2a shows the naked DNA (Lane 2) and $TM_{57}Bz_{18}CS$ /DNA complexes at weight ratios of 0.5, 1, 2, 4, 6 and 8 (Lanes 3–8). The naked DNA lane showed the DNA band, whereas complexed DNA was completely retained within the gel-loading well for $TM_{57}Bz_{18}CS$ at weight ratio above 1, illustrating that complete $TM_{57}Bz_{18}CS$/DNA complexes were formed. Fig. 2b shows that complexes were completely formed at weight ratio above 1for TM_{47}-Bz_{42}-CS, whereas complete complexes for TM_{57}-Bz_{42}-CS were formed at weight ratio above 0.5 (Fig. 2c). These results revealed that the hydrophobic interactions between dimethylaminobenzyl moieties of chitosan derivatives and charge-neutralized DNA segments were possibly accountable for the enhanced gene condensation.

Particle size and the zeta potential were plotted against weight ratios of chitosan derivatives/DNA complexes formulated (Fig. 3). The particle size of the TM_{57}-Bz_{42}-CS/DNA complexes increased with the increasing weight ratio from 0.5 to 1 and decreased to constant value in the range of 200 to 300 nm after a weight ratio of 1 (Fig. 3c). At the weight ratio of 1, TM_{57}-Bz_{42}-CS/DNA complexes had the largest particle size. An initial negative value of the zeta potential was observed at a low weight ratio of 1. At the weight ratio between 0.5 and 1, the zeta potential was approximately neutral. On the other hand, the particle size of the TM_{57}-Bz_{18}-CS/DNA and TM_{47}-Bz_{42}-CS/DNA complexes increased with the increasing weight ratio from 1 to 2 and decreased to constant value in the range of 176–300 nm after a weight ratio of 2 (Fig. 3a and b). The zeta potential of the complexes was found to increase with the increase in weight ratios of chitosan derivatives due to their higher density of protonated amines in the chitosan backbone. These results clearly showed that the complete formation of complex occurred at lower weight ratio if the chitosan with higher degree of dimethylaminobenzyl substitution were used. The heterocyclic bases present in DNA probably associated hydrophobically with dimethylaminobenzyl groups and promoted enhanced interaction of DNA with the modified chitosan in agreement with previous results (Chae et al., 2005).

3.3. In vitro transfection

The achievement of high gene transfection efficiency is a final goal for the development of novel gene carriers. To investigate

Table 1
Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan

Chitosan derivatives	DS (%)	DQ (%)			DMCS (%)	MCS (%)	Yield (%)
		DQ_{Ar} (%)	DQ_{CS} (%)	DQ_T (%)			
TM_{57}-Bz_{42}-CS	42.5	42.5	14.4	56.9	Trace	11.7	84.0
TM_{47}-Bz_{42}-CS	42.5	42.5	4.4	46.9	1.7	6.7	78.0
TM_{57}-Bz_{18}-CS	17.5	17.5	40.0	57.5	16.7	16.7	68.0

DS is the degree of dimethylaminobenzylation; DQ_{Ar} is the degree of quaternization at the aromatic amines; DQ_{CS} is the degree of quaternization of the primary amine of chitosan; DMCS is the degree of *N*,*N*-dimethylation of the primary amine of chitosan; MCS is *N*-monomethylation at the primary amine of chitosan; Yield (%) is (weight of product (g)/weight of initial reactant (g)) × 100, at the end of the preparation.

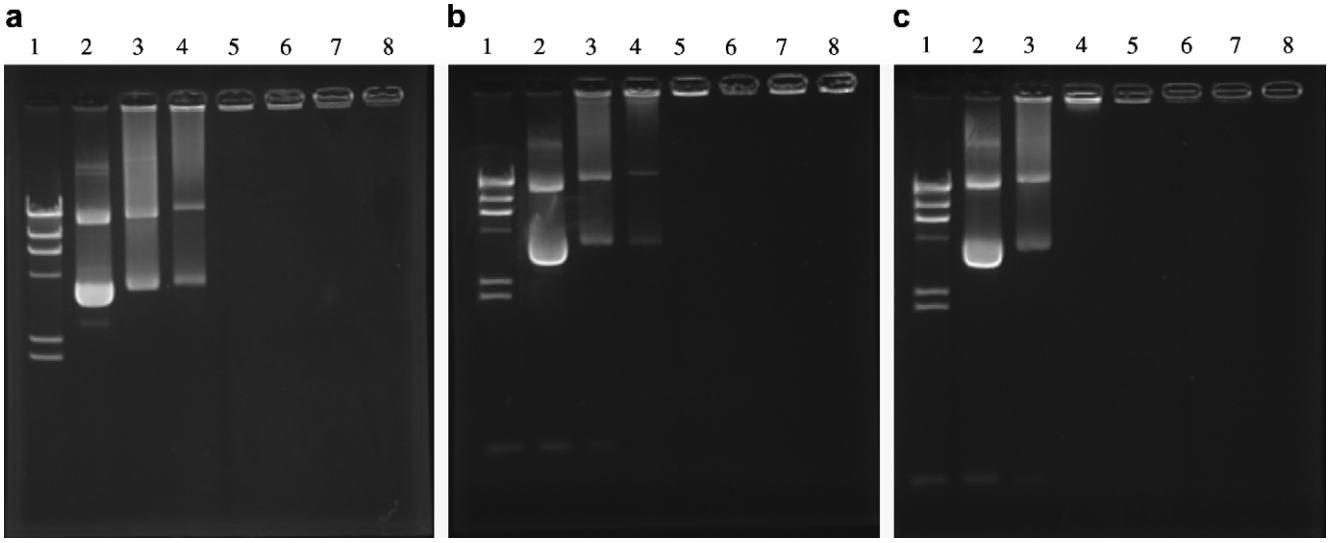


Fig. 2. Gel retarding analysis of TM-Bz-CS/DNA complexes formulated with (a) TM_{57}-Bz_{18}-CS, (b) TM_{47}-Bz_{42}-CS and (c) TM_{57}-Bz_{42}-CS. Lane 1, DNA marker and Lane 2 pEGFP-C2 plasmid; lanes 3-8, TM-Bz-CS/DNA complexes at weight ratios of 0.5, 1, 2, 4, 6, and 8, respectively.

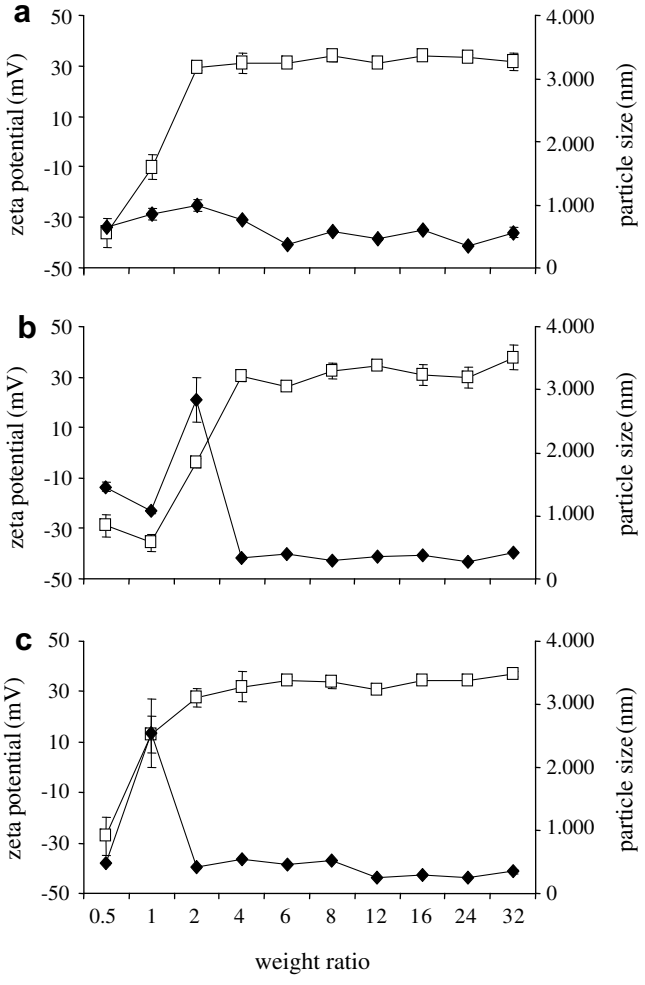


Fig. 3. Zeta potential (□) and particle size (♦) at varying weight ratios of TM-Bz-CS/DNA complexes formulated with (a) TM_{57}-Bz_{18}-CS, (b) TM_{47}-Bz_{42}-CS and (c) TM_{57}-Bz_{42}-CS. Each value represents the mean ± SD of three measurements.

the chitosan derivative mediated gene transfection efficiencies, in vitro gene transfection assay was performed with human hepatoma cell lines (Huh7 cells) using pEGFP-C2 plasmid encoding green fluorescent protein (GFP). Chitosan derivatives/DNA complexes were formulated with various weight ratios (0.5, 1, 2, 4, 6, 8, 12, 16, 24 and 32) in order to investigate the optimal conditions for gene transfection. Polyethylenimine (PEI, 25 kDa) complexed with DNA at the weight ratio of 1 was used as a positive control. In all studies, there were no transfection in control (cells without complexes) and naked DNA. As shown in Fig. 4 (at pH 7.4), the gene transfection efficiencies were significantly influenced by the weight ratios, the degree of dimethylaminobenzyl substitution (DS) and degree of quaternization (DQ). By increasing the weight ratios, the transfection efficiencies reached the highest values with a decrease by further increment of the ratios. Among chitosan derivatives, TM_{57}-Bz_{42}-CS showed the highest transfection efficiency (Fig. 4c). Its highest transfection efficiency at weight ratio of 16, was 8.3 and 12 times higher in gene transfection than that of the highest transfection efficiency of TM_{43}-CS at weight ratio of 8 (Rojanarata et al., 2008) and chitosan at weight ratio of 2 (Rojanarata et al., 2008), respectively. These results revealed that not only the trimethyl groups but also the hydrophobic groups (trimethylaminobenzyl moieties) affected the gene transfection efficiency. Increasing the hydrophobic groups (from Bz-18 to Bz-42) increased the gene transfection efficiency (Bz-18; Fig. 4a and Bz-42; Fig. 4b and c). The highest transfection efficiency of TM_{57}-Bz_{42}-CS was 580 ± 56 cells/cm^2 at weight ratio of 16, whereas the highest transfection efficiency of TM_{47}-Bz_{42}-CS and TM_{57}-Bz_{18}-CS were 435 ± 40 cells/cm^2 at weight ratio of 8 and 46 ± 7 cells/cm^2 at weight ratio of 12, respectively.

Previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH values below 6.5 (Weecharangsan et al., 2008). The effect of pH on transfection efficiencies of TM_{57}-Bz_{42}-CS/DNA and chitosan/DNA complexes were shown in Fig. 5. TM_{57}-Bz_{42}-CS mediated gene delivery (weight ratio of 16) about 2.5 times and 12 times enhanced gene transfection was observed than that of chitosan (weight ratio of 2) at the medium pH values of 6.5 and 7.4, respectively. pH dramatically affected only chitosan with five times decreasing the transfection efficiency by increasing pH values from 6.5 to 7.4, while TM_{57}-Bz_{42}-CS was not significantly influenced by pH.

Trimethylated chitosan (Mao et al., 2007) and trimethylated chitosan oligomers are promising agents for DNA condensation and promote the transfection efficiency on COS-1, Caco-2 cells

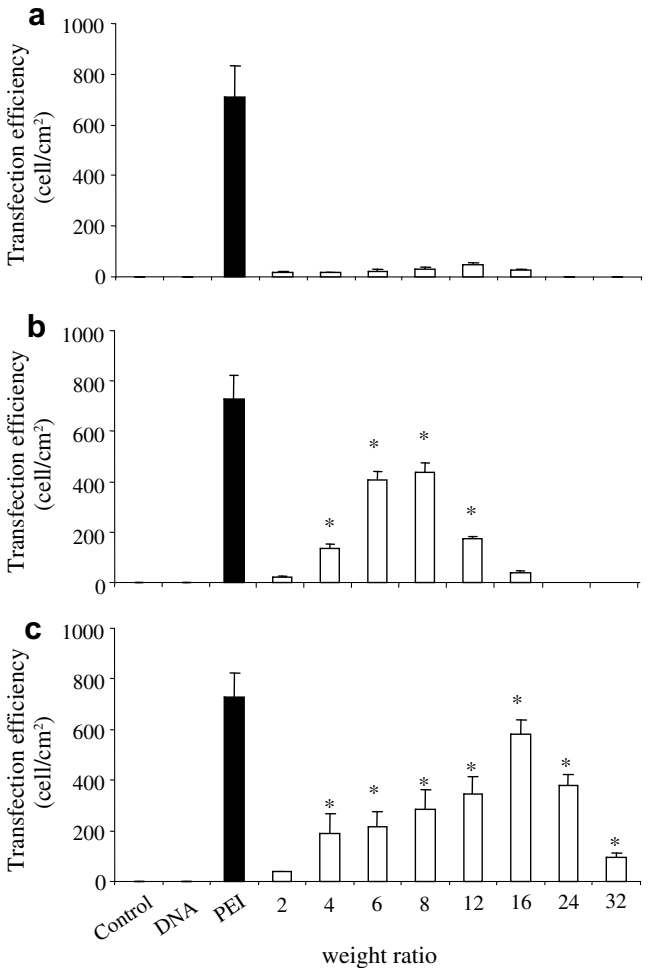


Fig. 4. Transfection efficiencies of TM-Bz-CS/DNA complexes formulated with (a) TM_{57}-Bz_{18}-CS, (b) TM_{47}-Bz_{42}-CS and (c) TM_{57}-Bz_{42}-CS in Huh7 cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < .05$).

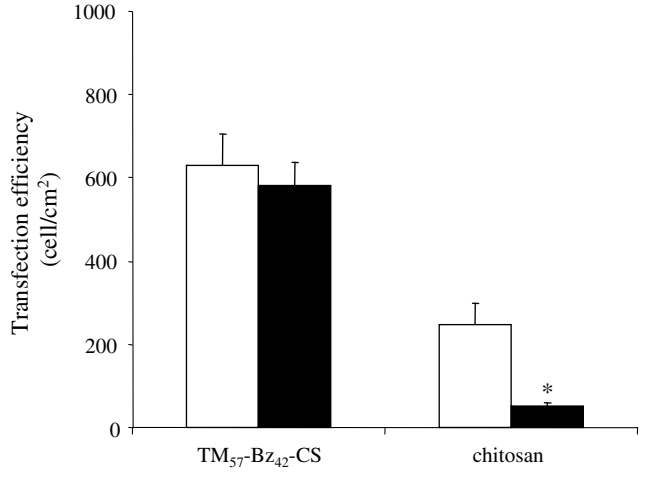


Fig. 5. Effect of pH medium at: (white bar) pH 6.5 and (black bar) pH 7.4; on transfection efficiencies of TM_{57}-Bz_{42}-CS/DNA complexes and chitosan/DNA complexes at the weight ration of 16 and 2, respectively, in Huh7 cells. Each value represents the mean ± SD of three wells. Difference values * were statistically significant ($p < .05$).

(Thanou et al., 2002), COS-7 and MCF-7 cells (Kean, Roth, & Thanou, 2005). This permanent positive charge of the trimethylated

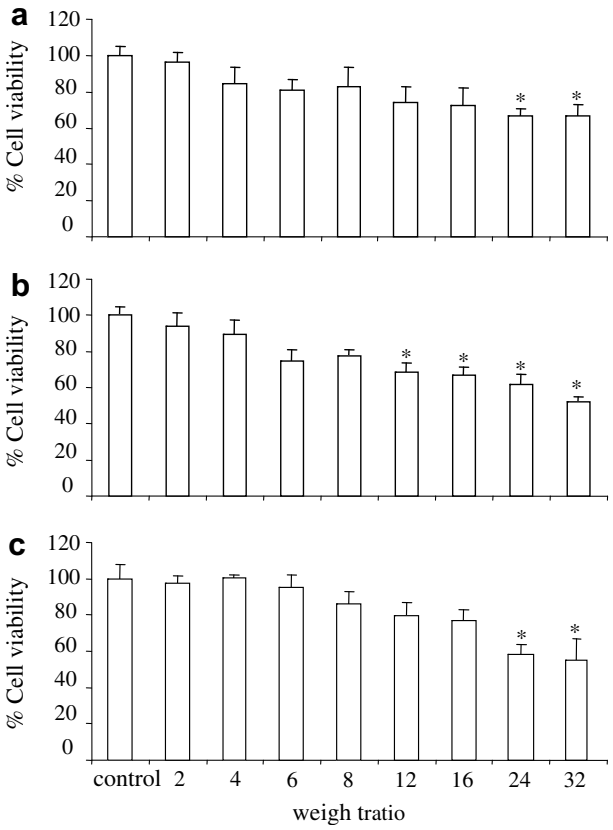


Fig. 6. Cell viability of TM-Bz-CS/DNA complexes formulated with (a) TM_{57}-Bz_{18}-CS, (b) TM_{47}-Bz_{42}-CS and (c) TM_{57}-Bz_{42}-CS in Huh7 cells. Each value represents the mean ± SD of six wells. Difference values * were statistically significant ($p < .05$).

chitosan is a key factor for the condensation and protection of DNA. The introduction of trimethylaminobenzyl group into the chitosan polymer backbone enhances the hydrophobicity which improves the hydrophobic interaction between polymer and DNA and DNA condensation. In addition, it improves hydrophobic interaction with cell membrane (Chae et al., 2005; Doody, Korley, Dang, Zawaneh, & Putnam, 2006). These help the water-soluble chitosan to be an efficient vector. As reported previously, hydrophobically modified cationic polymers or cationic lipids have shown high gene transfection capability as an optimal substitution by increasing cell membrane/carrier interactions or destabilization of the cell membranes (Tian et al., 2007). Although the exact mechanism of TM-Bz-CS mediated efficient gene delivery remained to be further studied, our results showed that could be suitable for non-viral gene carriers.

3.4. Effect of chitosan derivatives/DNA complexes on cell viability

One of the major requirements for cationic polymer vectors for gene delivery is low cytotoxicity. It has been reported that chitosan and chitosan derivatives were less toxic than other cationic polymers such as poly-lysine and polyethyleneimine in vitro and in vivo (Thanou et al., 2002). Various chitosans and its derivatives have been reported for gene delivery. However, the toxicity of those chitosans was different depending on the type of cells and derivatives studied. Therefore, the cytotoxicity study of the chitosan derivatives/DNA complex was performed in Huh7 cells. Fig. 6 shows the effect of TM_{57}-Bz_{18}-CS /DNA (Fig. 6a), TM_{47}-Bz_{42}-CS/DNA (Fig. 6b) and TM_{57}-Bz_{42}-CS/DNA complexes (Fig. 6c) on cell viability. When Huh7 cells were incubated with 1 μg of naked DNA, cell viability remained almost the same as that seen in control non-transfected cells (data not shown). There was significant

decrease in cell viability when Huh7 cells were incubated with various weight ratios of TM-Bz-CS/DNA complexes. The average cell viability decreased when the weight ratios increased. However, the viability was over 80% at the weight ratio from which the highest transfection efficiency was obtained. It was clear that TM-Bz-CS/DNA complexes were safe at the concentration used.

4. Conclusions

In this study, the novel water soluble and high transfection efficiency chitosan derivatives (TM-Bz-CS) were successfully synthesized by chemically modified with hydrophobic moiety of dimethylaminobenzyl and by *N*-quaternization. This study indicated that the degree of dimethylaminobenzyl substitution (DS) was crucial for high transfection efficiency.

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References

Chae, S. Y., Son, S., Lee, M., Jang, M. K., & Nah, J. W. (2005). Deoxycholic acid-conjugated chitosan oligosaccharide nanoparticles for efficient gene carrier. *Journal of Controlled Release, 109*, 330–344.

Curti, E., de Britto, D., & Campana-Filho, S. P. (2003). Methylation of chitosan with iodomethane: Effect of reaction conditions on chemoselectivity and degree of substitution. *Macromolecular Bioscience, 3*, 571–576.

Doody, A. M., Korley, J. N., Dang, K. P., Zawaneh, P. N., & Putnam, D. (2006). Characterizing the structure/function parameter space of hydrocarbon-conjugated branched polyethylenimine for DNA delivery in vitro. *Journal of Controlled Release, 116*, 227–237.

Gao, S., Chen, J., Dong, L., Ding, Z., Yang, Y. H., & Zhang, J. (2005). Targeting delivery of oligonucleotide and plasmid DNA to hepatocyte via galactosylated chitosan vector. *European Journal of Pharmaceutics and Biopharmaceutics, 60*, 327–334.

Germershaus, O., Mao, S., Sitterberg, J., Bakowsky, U., & Kissel, T. (2008). Gene delivery using chitosan, trimethyl chitosan or polyethylenglycol-graft-trimethyl chitosan block copolymers: Establishment of structure–activity relationships in vitro. *Journal of Controlled Release, 125*, 145–154.

Huang, M., Fong, C. W., Khorc, E., & Lim, L. Y. (2005). Transfection efficiency of chitosan vectors: Effect of polymer molecular weight and degree of deacetylation. *Journal of Controlled Release, 106*, 391–406.

Ishii, T., Okahata, Y., & Sato, T. (2001). Mechanism of cell transfection with plasmid/chitosan complexes. *Biochimica et Biophysica Acta, 1514*, 51–64.

Kean, T., Roth, S., & Thanou, M. (2005). Trimethylated chitosans as non-viral gene delivery vectors: Cytotoxicity and transfection efficiency. *Journal of Controlled Release, 103*, 643–653.

Kiang, T., Wen, J., Lim, H. W., & Leong, K. W. (2004). The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials, 25*, 5293–5301.

Kim, T. H., Ihm, J. E., Choi, Y. J., Nah, J. W., & Cho, C. S. (2003). Efficient gene delivery by urocanic acid-modified chitosan. *Journal of Controlled Release, 93*, 389–402.

Kumar, M., Kong, X., Behera, A. K., Hellermann, G. R., Lockey, R. F., & Mohapatra, S. S. (2003). Chitosan IFN-gamma-pDNA nanoparticle (CIN) therapy for allergic asthma. *Genetic Vaccines and Therapy, 1*, 3.

Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews, 104*, 6017–6084.

Lavertu, M., Méthot, S., Tran-Khanh, N., & Buschmann, M. D. (2006). High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. *Biomaterials, 27*, 4815–4824.

Lee, M., Nah, J. W., Kwon, Y., Koh, J. J., Ko, K. S., Kim, S. W., et al. (2001). Water-soluble and low molecular weight chitosan-based plasmid DNA delivery. *Pharmaceutical Research, 18*, 427–431.

Lee, D., Zhang, W., Shirley, S. A., Kong, X., Hellermann, G. R., Lockey, R. F., et al. (2007). Thiolated chitosan/DNA nanocomplexes exhibit enhanced and sustained gene delivery. *Pharmaceutical Research, 24*, 157–167.

Maclaughlin, F. C., Mumper, R. J., Wang, J., Tagliaferri, J. M., Gill, I., & Hinchyiffe, M. (1998). Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. *Journal of Controlled Release, 56*, 259–272.

Mao, Z., Ma, L., Jiang, Y., Yan, M., Gao, C., & Shen, J. (2007). *N*,*N*,*N*-Trimethylchitosan chloride as a gene vector: Synthesis and application. *Macromolecular Bioscience, 7*, 855–863.

Muzzarelli, R. A. A., Tanfani, F., Emanuelli, M., & Mariotti, S. (1982). *N*-Carboxybenzyl chitosans, novel chelating polyampholytes. *Carbohydrate Polymers, 2*, 145–157.

Richardson, S. C., Kolbe, H. V., & Duncan, R. (1999). Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA. *International Journal of Pharmaceutics, 178*, 231–243.

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science, 31*, 603–632.

Rojanarata, T., Petchsangsai, M., Opanasopit, P., Ngawhirunpat, T., Apirakaramwong, A., Ruktanonchai, U., et al. (in press). Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan for a novel effective gene carrier. *European Journal of Pharmaceutics and Biopharmaceutics*.

Romóren, K., Pedersen, S., Smistad, G., Evensen, O., & Thu, B. J. (2003). The influence of formulation variables on in vitro transfection efficiency and physicochemical properties of chitosan-based polyplexes. *International Journal of Pharmaceutics, 261*, 115–127.

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

Sato, T., Ishii, T., & Okahata, Y. (2001). In vitro gene delivery mediated by chitosan: Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. *Biomaterials, 22*, 2075–2080.

Simon, R. H., Engelhardt, J. F., Yang, Y., Zepeda, M., Weber-Pendelton, S., Grossman, M., & Wilson, J. M. (1993). Adenovirus-mediated gene transfer of the CFTR gene to the lung of non-human primates: A toxicity study. *Human Gene Therapy, 4*, 771–780.

Thanou, M., Florea, B. I., Geldof, M., Junginger, H. E., & Borchard, G. (2002). Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. *Biomaterials, 23*, 153–159.

Tian, H., Xiong, W., Wei, J., Wang, Y., Chen, X., Jing, X., & Zhu, Q. (2007). Gene transfection of hyperbranched PEI grafted by hydrophobic amino acid segment PBLG. *Biomaterials, 28*, 2899–2907.

Weecharangsan, W., Opanasopit, P., Ngawhirunpat, T., Apirakaramwong, A., Rojanarata, T., & Ruktanonchai, U. (2008). Evaluation of chitosan salts as nonviral gene vectors in CHO-K1 cells. *International Journal of Pharmaceutics, 348*, 161–168.

Weecharangsan, W., Opanasopit, P., Ngawhirunpat, T., Rojanarata, T., & Apirakaramwong, A. (2006). Chitosan lactate as a nonviral gene delivery vector in COS-1 cells. *AAPS PharmSciTech, 7*, E1–E6.

Yoo, H. S., Lee, J. E., Chung, H., Kwon, I. C., & Jeong, S. Y. (2005). Self-assembled nanoparticles containing hydrophobically modified glycol chitosan for gene delivery. *Journal of Controlled Release, 103*, 235–243.

Zhang, Y., Chen, J., Zhang, Y., Pan, Y., Zhao, J., & Ren, L. (2007). A novel PEGylation of chitosan nanoparticles for gene delivery. *Biotechnology and Applied Biochemistry, 46*, 197–204.