

Research paper

## Methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan for novel effective gene carriers

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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<sub>47</sub>-Bz<sub>42</sub>-CS, using the plasmid DNA encoding green fluorescent protein (pEGFP-C2) on human hepatoma cell lines (Huh7 cells), in comparison to quaternized chitosan (TM<sub>43</sub>-CS) and chitosan (CS). Factors affecting the transfection efficiency, such as the carrier/DNA weight ratio, the pH of the culture medium, and the presence of serum, have been investigated. The results revealed that TM<sub>47</sub>-Bz<sub>42</sub>-CS was able to condense with pDNA. As illustrated by the agarose gel electrophoresis, the complete complexes of TM<sub>47</sub>-Bz<sub>42</sub>-CS/DNA were formed at a weight ratio of above 0.5, whereas those of TM<sub>43</sub>-CS/DNA and CS/DNA were formed at a ratio of above 1. TM<sub>47</sub>-Bz<sub>42</sub>-CS showed superior transfection efficiency to TM<sub>43</sub>-CS and CS at all weight ratios tested. Higher transfection efficiency and gene expression were observed when the carrier/DNA weight ratios increased. The highest transfection efficiency was found at a weight ratio of 8. The results indicated that the improved gene transfection was due to the hydrophobic group (*N,N*-dimethylaminobenzyl) substitution on CS, which promoted the interaction and condensation with DNA, as well as *N*-quaternization, which increased the CS water solubility. During cytotoxicity studies, it was found that high concentrations of TM<sub>47</sub>-Bz<sub>42</sub>-CS and TM<sub>43</sub>-CS could decrease the Huh7 cell viability. In conclusion, this novel CS derivative, TM<sub>47</sub>-Bz<sub>42</sub>-CS, shows promising potential as a gene carrier by efficient DNA condensation and a mediated higher level of gene transfection in Huh7 cells.

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**Keywords:** Trimethylaminobenzyl chitosan; Gene delivery; Huh7 cells; Transfection efficiency

### 1. Introduction

Gene delivery has been regarded as a powerful tool for curing disease by replacing defective genes, substituting missing genes, or silencing unwanted gene expression. The two main types of vectors used in gene therapy are viral and non-viral. Viral vectors are the most effective

because of their evolutionary optimization for this purpose, but recently reported safety issues, such as random recombination, oncogenic potential, and immunogenicity, have set back the rapid development of viral vectors [1–2]. In light of these safety concerns, non-viral delivery systems have been developed. Among these, cationic liposomes are widely used for almost all animal cells because they have non-specific ionic interactions and low toxicity [3–5]. There are some limitations, however, because these liposomes are unstable when used for *in vivo* transfection. Therefore, many polymeric cationic systems, such as gelatin, polyethyleneimine (PEI), poly(L-lysines), tetraminofullerene, poly(L-histidine)-graft-poly(L-lysines), DEAE-

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dextrans, cationic dendrimers, and chitosan, have been studied for in vitro as well as in vivo applications [6–7].

Chitosan (CS) [(1 → 4) 2-amino-2-deoxy-β-D-glucan] is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) produced by the alkaline deacetylation of chitin. CS is a weak base with a pKa value of the D-glucosamine residue of about 6.2–7.0; therefore, it is insoluble at neutral and alkaline pH values but soluble in acidic mediums such as acetic acid, citric acid, glutamic acid, aspartic acid, hydrochloric acid, and lactic acid. CS has been used in drug delivery as an absorption enhancer [8] and as a vector for gene delivery. In addition, CS 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 [9–13]. At acidic pH, below their pKa, the primary amines in the chitosan backbone become positively charged. These protonated amines enable CS to bind to negatively charged DNA and condense DNA into particles. Moreover, CS has shown a capability to protect DNA from DNase I and II degradation [14]. Formulation parameters such as molecular weight ( $M_w$ ), degree of deacetylation (DD), *N/P* ratio (ratio of positively charged chitosan to negatively charged DNA), and pH of the transfection medium were found to affect the transfection efficiency of CS/DNA complexes [15–18].

The main drawback of CS is its poor water solubility at physiological pH, and its low transfection efficiency. Several CS derivatives have been synthesized in the last few years to obtain a modified carrier with altered physico-chemical characteristics. Using modified CS, such as glycol CS or PEGylated CS [19–20], low molecular weight soluble CS [9], and quaternized CS [21], could be possible ways to circumvent the known solubility issues. To improve gene transfection, chemically modified CS, such as quaternized CS [21], urocanic acid-modified CS [22], galactosylated CS [23], deoxycholic acid CS oligosaccharide nanoparticle [24], and thiolate CS [25], was reported.

Although many researches have synthesized CS derivatives as alternatives for gene carriers, few were successful in increasing the transfection efficiency. In this study, the water-soluble CS derivatives, which favor the interaction with pDNA, have been synthesized and evaluated for their in vitro transfection efficiency and cytotoxicity. CS was substituted with an *N,N*-dimethylbenzyl group to provide the hydrophobic moiety for the improved hydrophobic interaction with pDNA, and it was quaternized to render CS soluble. A number of variables that influence the transfection efficiency, such as carrier/DNA weight ratio, particle size, ζ-potential, morphology, and the pH of the culture medium and serum, were investigated.

## 2. Materials and methods

### 2.1. Materials

CS was purchased from Seafresh Chitosan Lab (Bangkok, Thailand) with a  $M_w$  of 267 kDa and a 94% degree

of deacetylation. Sodium cyanoborohydride and polyethylenimine (PEI),  $M_w$  25 kDa, were purchased from Aldrich (Milwaukee, USA). Iodomethane, 4-*N,N*-dimethylamino-benzaldehyde, sodium iodide, and 1-methyl-2-pyrrolidone were purchased from Fluka (Deisenhofen, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was 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 pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP), was obtained from Clontech, Palo Alto, USA. The λHindIII were obtained from Promega (Madison, WI USA). Huh7 (Human hepatocellular carcinoma) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

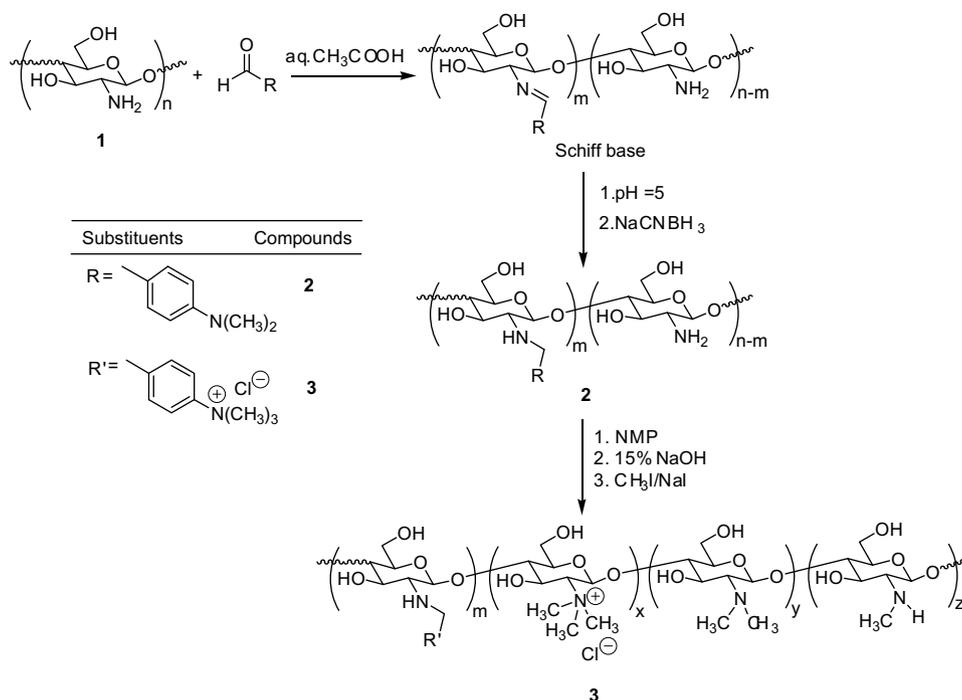
### 2.2. Synthesis of methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (TM-Bz-CS)

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

DM-Bz-CS 2 was prepared as previously reported [26]. Briefly, 1.00 g of chitosan 1 (6.11 meq/GlcN) was dissolved in 0.2 M acetic acid (pH 4, 70 mL). The solution was diluted with ethanol (70 mL), and an aromatic aldehyde (4-*N,N*-dimethylamino benzaldehyde (3.0 meq/GlcN) was added and stirred at room temperature for 1 h. The pH of the solution was adjusted to 5 with 1 N NaOH. Subsequently, 1.54 g of NaCNBH<sub>3</sub> was added and stirred at room temperature for 24 h, followed by a 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 trimethylated CS and CS derivatives

Compound 1 or 2 (0.50 g) was dispersed in 25 mL of *N*-methyl pyrrolidone (NMP) for 12 h at room temperature. Then, 1.5 g of 15% sodium iodide 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 a 15% (w/v) NaCl solution to replace the iodide ion with 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 compounds (TM-CS and TM-Bz-CS 3) were collected and dried overnight at room temperature under a stream of nitrogen (Scheme 1). TM-CS, after being obtained from the first methylation, was subjected to repeated methylation to yield a higher tri-methyl substitution.

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

### 2.3. Characterization

FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared (FT-IR) spectrometer, and all samples were prepared as potassium bromide pellets. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were measured on a Mercury Varian 300 MHz spectrometer. All measurements were performed at 300 K, using a pulse accumulation of 64 scans and the LB parameter of 0.30 Hz.  $\text{D}_2\text{O}/\text{CF}_3\text{COOD}$  (1% (v/v)) and  $\text{D}_2\text{O}$  were used as solvents for 10 mg of chitosan and its derivatives, respectively.

### 2.4. Plasmid preparation

The pEGFP-C2 was propagated in *Escherichia coli* DH5- $\alpha$  and purified by using the Qiagen endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA, USA). The 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 at pH 8.0, using  $\lambda\text{DNA}/\text{HindIII}$  as a DNA marker.

### 2.5. Preparation and characterization of CS derivatives / DNA complexes

The CS derivatives/DNA complexes were prepared at various carrier/DNA weight ratios by adding the DNA solution to the CS derivative solution. The mixture was gently mixed using a 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  $\mu\text{g}/\text{mL}$ ). The electrophoresis was carried out for 60 min at 100 V. A CS derivatives/DNA complex sample (15  $\mu\text{L}$ ) containing 1  $\mu\text{g}$  of DNA was loaded in the well.

### 2.6. Size and $\zeta$ potential measurements

The particle size and surface charge of CS 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 a 0.22  $\mu\text{m}$  membrane filter prior to use. All samples were measured in triplicate.

### 2.7. Morphology

The morphology of the polyplexes was analyzed by atomic force microscope (AFM). Complexes of plasmid DNA and  $\text{TM}_{47}\text{-BZ}_{42}\text{-CS}$  were prepared at the weight ratio of 8. The complexes were diluted with distilled water and applied to freshly cleaved mica. After allowed to bind for 20–120 s, the mica was then rinsed twice with 100 mL distilled water and the excess liquid wicked away at the mica edge with a tissue. The mica was then blown until completely dry with a stream of nitrogen gas and put into a vacuum desiccator until it could be imaged by AFM.

### 2.8. *In vitro* transfection of CS/DNA complexes in Huh 7 cells

Huh7 cells were seeded into 24-well plates at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> 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 a humidified atmosphere (5% CO<sub>2</sub>, 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 CS derivatives/DNA complexes at various *N/P* ratios containing 1 µg of pDNA for 24 h at 37 °C under 5% CO<sub>2</sub> 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 a culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

### 2.9. Evaluation of cell viability

The evaluation of cytotoxicity was performed by the MTT assay. Huh7 cells were seeded in a 96-well plate at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> in 200 µL of growth medium and incubated for 24 h at 37 °C under 5% CO<sub>2</sub> atmosphere. Prior to transfection, the medium was removed, and the cells were rinsed with PBS and then supplied with the CS derivatives/DNA complexes in the same concentrations as in the *in vitro* transfection experiment. After treatment, solutions of the CS derivatives/DNA complexes were removed. Finally, the cells were incubated with 100 µL MTT containing medium (1 mg/mL) for 4 h. The medium was subsequently 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. The 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). The viability of non-treated control cells was arbitrarily defined as 100%.

### 2.10. Statistical analysis

The statistical significance of differences in the transfection efficiency and cell viability was examined using one-way analysis of variance (ANOVA) followed by an LSD *post hoc* test. The significance level was set at  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Synthesis and characterization of CS derivatives

As previously reported, DM-Bz-CS 2 was synthesized by reductive amination of the corresponding Schiff base intermediates (Scheme 1) [26]. The extent of *N*-substitution

(ES), determined by <sup>1</sup>H NMR, varied due to the different ratio of aldehydes to GlcN in CS. The FT-IR spectra of DM-Bz-CS were similar to that of CS, except that additional absorption bands at wavenumbers 1605, 1526, and 811 cm<sup>-1</sup> were observed. These bands were assigned to the C=C stretching and C-H deformation (out of plane) of the aromatic group. The <sup>1</sup>H NMR spectrum of DM-Bz-CS exhibited a broad singlet,  $\delta$  7.5 ppm, in the aromatic region and another singlet at  $\delta$  3.1 ppm assigned to *N,N*-dimethyl protons. The <sup>1</sup>H NMR spectra of TM-Bz-CS were similar to that of the corresponding TM-CS, except for additional signals at  $\delta$  3.5 ppm, which corresponded to the *N,N,N*-trimethyl protons on the 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 CS using procedures that were slightly modified from the method previously described [27]. The quaternization of DM-Bz-CS 2 with methyl iodide yielded TM-Bz-CS 3, which could occur at both the aromatic substituent and primary amino group of the GlcN of chitosan. The results clearly demonstrated that the *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<sub>Ar</sub> values equal to the corresponding ES's (Table 1). In addition to *N,N,N*-trimethylation, *N,N*-dimethylation, and *N*-monomethylation at the primary amino group of GlcN of chitosan were also observed. In this case, *O*-methylation at the GlcN of CS is also observed by the appearance of small signal at  $\delta$  3.3 and 3.4 ppm.

**TM-CS.** FT-IR (KBr):  $\nu$  3444, 1475, 1107, 1071, and 1057 cm<sup>-1</sup>. <sup>1</sup>H NMR (D<sub>2</sub>O):  $\delta$  (ppm) 5.42 (br s; 1H H1, H1'), 4.40–3.01 (br o; 23H -NH-CH<sub>2</sub>-, H2, H3, H4, H5, H6 and H6', s; OCH<sub>3</sub>, br s; N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub>), 2.71 (br m; 6H N(CH<sub>3</sub>)<sub>2</sub>), 2.31 (s; 3H NHCH<sub>3</sub>), 1.97 (s; 3H NHC(O)CH<sub>3</sub>). <sup>13</sup>C NMR (D<sub>2</sub>O):  $\delta$  (ppm) 96.55 (C1), 77.62 (C4), 74.74 (C5), 68.85 (C5), 60.0–55.5 (C2 and C6), 54.43 (N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub>), 42.71 (N(CH<sub>3</sub>)<sub>2</sub>).

**TM-Bz-CS.** FT-IR (KBr):  $\nu$  3442, 1559, 1475, 1147, 1104, 1059, and 850 cm<sup>-1</sup>. <sup>1</sup>H NMR (D<sub>2</sub>O):  $\delta$  (ppm) 7.75–7.50 (dd; 4H Ph), 5.40, 4.96 (s; 2H H1, H1'), 4.42–3.13 (br m; 32H -NH-CH<sub>2</sub>-, H2, H3, H4, H5, H6 and H6', br s; N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub> Ph, s; OCH<sub>3</sub>, br s; N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub>), 2.71 (br m; 6H N(CH<sub>3</sub>)<sub>2</sub>), 2.31 (s; 3H NHCH<sub>3</sub>), 1.97 (s; 3H NHC(O)CH<sub>3</sub>). <sup>13</sup>C NMR (D<sub>2</sub>O):  $\delta$  (ppm) 145.52, 141.53, 130.98, 119.56 (C-Ph), 96.55 (C1), 77.13–58.67 (C2, C3, C4, C5, and C6), 56.98 (N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub> Ph), 53.88 (N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub>), 41.77 (N(CH<sub>3</sub>)<sub>2</sub>), 36.30 (NCH<sub>3</sub>).

### 3.2. Characterization of CS derivatives/DNA complexes

To determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between either CS or CS derivatives and DNA at different CS concentrations. The formation of complexes between CS derivatives and the pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. By varying the concentration

Table 1  
Quaternization of chitosan and *N*-benzyl chitosan derivatives

Samples	ES (%)	DQ <sub>T</sub> (%)		DM-CS (%)	M-CS (%)	Recovery (%)
		DQ <sub>Ar</sub> (%)	DQ <sub>CS</sub> (%)			
TM <sub>43</sub> -CS	–	–	43	48	–	80
TM <sub>47</sub> -Bz <sub>42</sub> -CS	42	42	5	Trace	7	78

ES is the extent of *N*-substitution; DQ<sub>Ar</sub> is degree of quaternization at aromatic substituents; DQ<sub>CS</sub> is degree of quaternization; DM-CS is *N,N*-dimethylation of GlcN of CS; M-CS is *N*-monomethylation GlcN of CS; Recovery (%) is [weight of product (g)/weight of starting reactant (g)] × 100.

of CS and fixing the DNA concentration, the weight ratios of positively charged CS (due to amine groups) to negatively charged DNA (due to the phosphate groups), referred to as weight ratios of the particle formulations, were varied (Fig. 1; lanes 3–8, CS/DNA complexes with weight ratio of 0.5, 1, 2, 4, 6, and 8). As shown in Fig. 1, TM<sub>47</sub>-Bz<sub>42</sub>-CS showed different gene condensation patterns. In the case of TM<sub>47</sub>-Bz<sub>42</sub>-CS (Fig. 1a), complete complexes were formed at carrier/DNA weight ratios above 0.5. In contrast, DNA complexes with TM<sub>43</sub>-CS or CS had carrier/DNA weight ratios above 1. These results revealed that the hydrophobic interactions between trimethylaminobenzyl moieties of TM<sub>47</sub>-Bz<sub>42</sub>-CS and charge-neutralized DNA segments were possibly responsible for the enhanced gene condensation. The hydrophobic moiety-dependent gene condensation capacity phenomenon is in close agreement with the previously reported results [24].

The particle size and  $\zeta$  potential were plotted against weight ratios of the formulated CS derivatives/DNA complexes (Fig. 2). The particle size of the TM<sub>47</sub>-Bz<sub>42</sub>-CS/DNA complexes increased with the increasing weight ratio from 0.5 to 1 and decreased to a constant value in the range of 200 to 300 nm after a weight ratio of 2 (Fig. 2a). At an *N/P* ratio of 1, TM<sub>47</sub>-Bz<sub>42</sub>-CS/DNA complexes had the largest particle size. An initial negative value of the  $\zeta$  potential was observed at a low charge ratio of 0.5. At weight ratios between 0.5 and 1, the  $\zeta$  potential was approximately neutral, but the particle size of the TM<sub>43</sub>-CS/DNA complexes slightly increased with an increasing weight ratio

from 1 to 2 and decreased to a constant value in the range of 176–300 nm after a weight ratio of 2 (Fig. 2b). The  $\zeta$  potential of the complexes was found to increase with an increase in the weight ratios of CS derivatives due to the higher density of protonated amines in the CS backbone. A similar result was observed in CS (Fig. 2c).

The morphological examination of the TM<sub>47</sub>-Bz<sub>42</sub>-CS/DNA complexes at a weight ratio of 8 was performed by AFM. The AFM images revealed that the complexes were spherical with nano size.

### 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 the CS derivative-mediated gene transfection efficiencies, an *in vitro* gene transfection assay was performed with human hepatoma cell lines (Huh7 cells) using the pEGFP-C2 plasmid encoding green fluorescent protein (GFP). CS derivatives/DNA complexes were formulated at various weight ratios [2,4,6,8,12,16,24,32] 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, no transfection was seen in the control (cells without complexes) and naked DNA. As shown in Fig. 3 (at pH 7.4), the gene transfection efficiencies were significantly influenced by the carrier/DNA ratios. By increasing the ratios, the transfection efficiencies reached the highest values, with a decrease by further increment of the ratios. Among the carriers, TM<sub>47</sub>-Bz<sub>42</sub>-CS showed the highest transfection efficiency (Fig. 3a). Its transfection efficiency (carrier/DNA weight ratio of 8) was 6 and 140 times higher in gene transfection than that of TM<sub>43</sub>-CS (Fig. 3b) and CS (Fig. 3c), respectively. These results revealed that not only the trimethyl groups but also the hydrophobic groups (trimethylaminobenzyl moieties) affected the gene transfection efficiency. Previous studies reported that the transfection efficiency of CS was dependent on pH and serum. Chitosan-mediated high gene transfection was observed at pH values below 6.5 [28]. Cationic liposome-associated gene

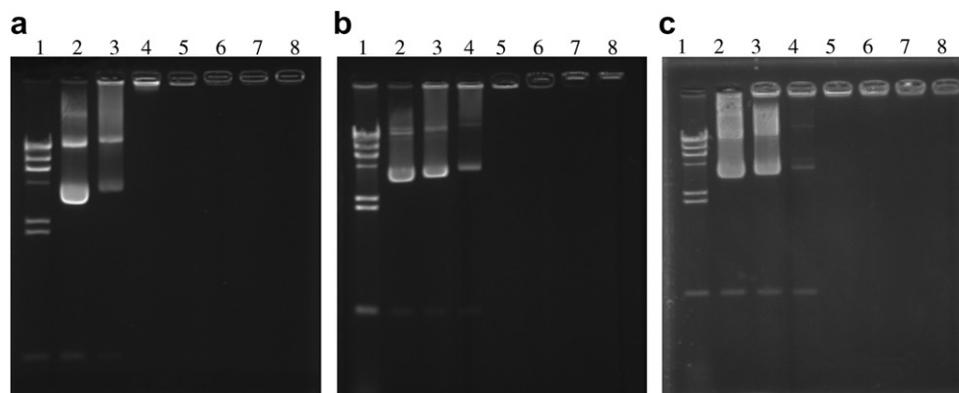


Fig. 1. Gel retardation analysis of CS derivatives/DNA complexes formulated with (a) TM<sub>47</sub>-Bz<sub>42</sub>-CS, (b) TM<sub>43</sub>-CS, and (c) CS. Lane 1, DNA marker; lane 2 pEGFP-C2 plasmid; lanes 3–8, CS derivatives/DNA complexes at weight ratios of 0.5, 1, 2, 4, 6, and 8, respectively.

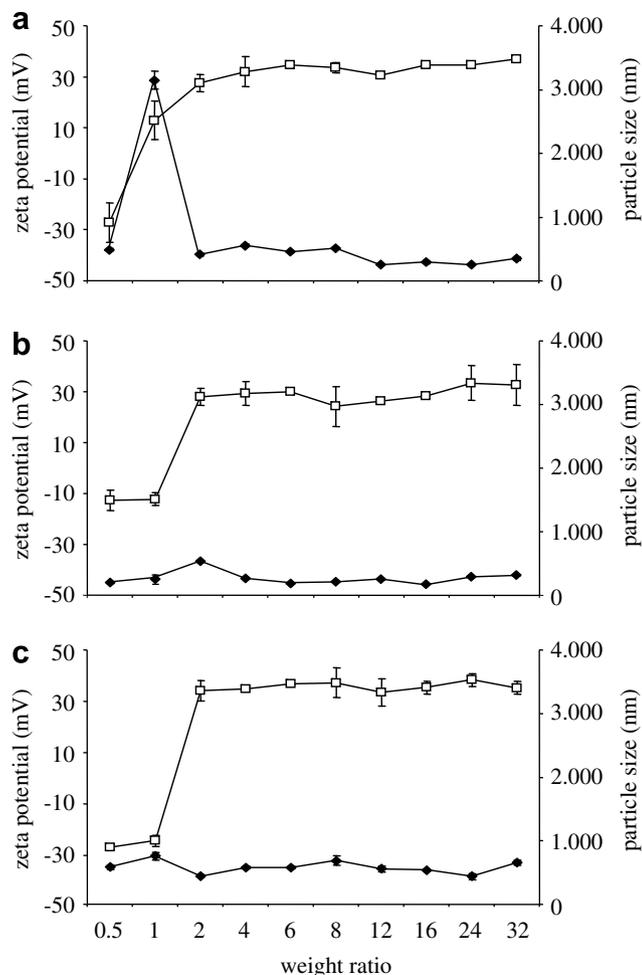


Fig. 2.  $\zeta$  Potential ( $\square$ ) and particle size ( $\blacklozenge$ ) at varying weight ratios of CS derivatives/DNA complexes formulated with (a) TM<sub>47</sub>-Bz<sub>42</sub>-CS, (b) TM<sub>43</sub>-CS, and (c) CS. Each value represents means  $\pm$  SD of three measurements.

expression was inhibited by serum, while CS showed resistance to serum [15]. As shown in Fig. 4, TM<sub>47</sub>-Bz<sub>42</sub>-CS and CS associated gene expression was inhibited by the presence of 10% serum. Changes in pH dramatically affected the transfection efficiency of CS by causing a decrease in efficiency with an increase in pH from 6.5 to 7.4. In contrast, TM<sub>47</sub>-Bz<sub>42</sub>-CS was not influenced by pH.

Partially quaternized polymers have been previously referred to as promising transfection agents. Cationic polymers containing quaternary charged trimethylamino ethyl-methacrylate (TMAEM) copolymerized with hydrophilic-(2-hydroxypropyl) methacrylates (HPMA) were found to condense DNA and transfect 293 cells [29]. Trimethylated chitosan oligomers are promising agents for DNA condensation and promote the transfection efficiency on COS-1 and Caco-2 cells [21] and COS-7 and MCF-7 cells [30]. This permanent positive charge of the trimethylated chitosan is a key factor for the condensation and protection of DNA. The introduction of an *N*-benzyl group into the CS polymer backbone enhances the hydrophobicity, which improves the hydrophobic interactions between the poly-

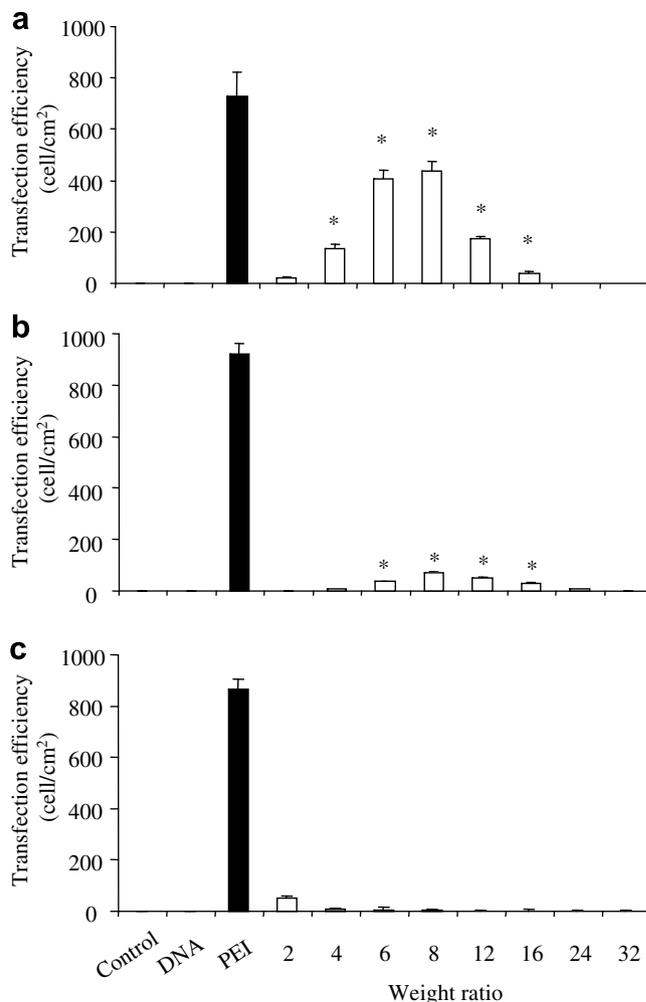


Fig. 3. Transfection efficiencies of CS derivatives/DNA complexes formulated with (a) TM<sub>47</sub>-Bz<sub>42</sub>-CS, (b) TM<sub>43</sub>-CS, and (c) CS in Huh7 cells. Each value represents means  $\pm$  SD of three wells. Difference values \* were statistically significant ( $p < 0.05$ ).

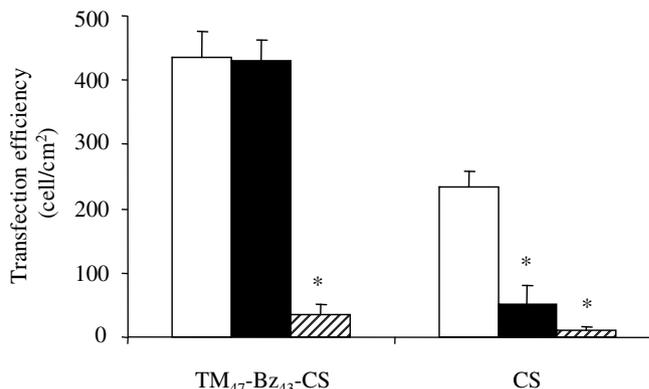


Fig. 4. Effect of pH medium and serum at ( $\square$ ) pH 7.4 without serum ( $\blacksquare$ ) pH 6.2 without serum and ( $\text{hatched}$ ) pH 7.4 with 10% serum, on transfection efficiencies of TM<sub>47</sub>-Bz<sub>42</sub>-CS/DNA complexes and CS/DNA complexes at a weight ratio of 8 in Huh7 cells. Each value represents means  $\pm$  SD of three wells. Difference values \* were statistically significant ( $p < 0.05$ ).

mer and DNA, and DNA condensation. In addition, it improves hydrophobic interactions with the cell membrane [24,31]. These help the water-soluble CS 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 the destabilization of the cell membranes [32]. Although the exact mechanism of TM<sub>47</sub>-Bz<sub>42</sub>-CS mediated efficient gene delivery and the optimal degree of trimethylated and hydrophobic groups (*N*-benzyl group) must be studied further, our first results showed that TM<sub>47</sub>-Bz<sub>42</sub>-CS could be a potential candidate for non-viral gene carriers.

### 3.4. Effect of CS 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 CS and CS derivatives were less toxic than other cationic polymers, such as poly-lysine and polyethyleneimine, *in vitro* and *in vivo* [21]. Various chitosans and chitosan derivatives have been reported for gene delivery,

but the toxicity of those chitosans varied depending on the type of cells and derivatives studied. Therefore, the cytotoxicity study of the CS derivatives/DNA complexes was performed in Huh7 cells. Fig. 5 shows the effect of TM<sub>47</sub>-Bz<sub>42</sub>-CS/DNA (Fig. 5a), TM<sub>43</sub>-CS/DNA (Fig. 5b), and CS/DNA complexes (Fig. 5c) on cell viability. When Huh7 cells were incubated with 1 µg of naked DNA, the cell viability remained almost the same as that seen in non-transfected cells used as a control (data not shown). There was a significant decrease in cell viability when Huh7 cells were incubated with various weight ratios of both TM<sub>47</sub>-Bz<sub>42</sub>-CS/DNA and TM<sub>43</sub>-CS/DNA complexes compared to that seen for CS/DNA complexes. Though their average cell viability decreased when the carrier/DNA weight ratios increased, the viability was over 80% at a weight ratio of 8, from which the highest transfection efficiency was obtained. Therefore, from this study, TM<sub>47</sub>-Bz<sub>42</sub>-CS has clearly been proven to be safe.

## 4. Conclusion

In this study, a novel water-soluble chitosan derivative (TM<sub>47</sub>-Bz<sub>42</sub>-CS) was successfully synthesized with the goal of improving the transfection efficiency. This was done by chemically modifying chitosan with a hydrophobic moiety of *N,N*-dimethylaminobenzyl and increasing the solubility by quaternization. The results of this study suggest that TM<sub>47</sub>-Bz<sub>42</sub>-CS is safe and exhibits significantly improved gene delivery potential *in vitro*.

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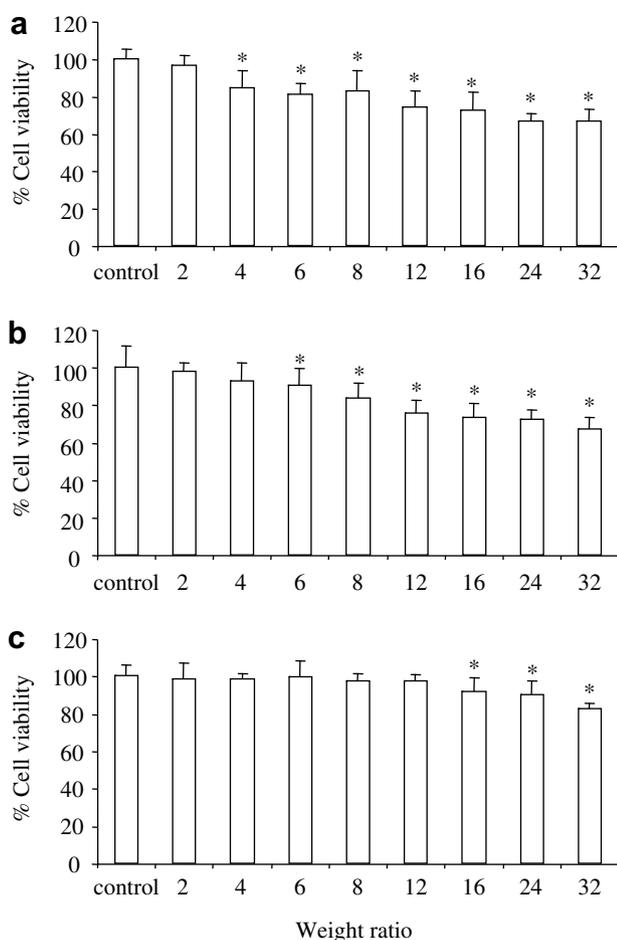


Fig. 5. Cell viability of CS derivatives/DNA complexes formulated with (a) TM<sub>47</sub>-Bz<sub>42</sub>-CS, (b) TM<sub>43</sub>-CS and (c) CS in Huh7 cells. Each value represents means  $\pm$  SD of six wells. Difference values \* were statistically significant ( $p < 0.05$ ).

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