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Methylated *N*-(4-pyridinylmethyl) chitosan as a novel effective safe gene carrier

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ABSTRACT

The objective of this study was to study the transfection efficiency of quaternized *N*-(4-pyridinylmethyl) chitosan; TM-Py-CS, using the pDNA encoding green fluorescent protein (pEGFP-C2) on human hepatoma cell lines (Huh 7 cells). The factors affecting the transfection efficiency, e.g. degree of quaternization (DQ), the extent of *N*-pyridinylmethyl substitution (ES) and weight ratio, have been investigated. The results revealed that TM-Py-CS was able to condense with pDNA. Illustrated by agarose gel electrophoresis, complete complexes of $TM_{69}Py_{62}CS$/DNA were formed at weight ratio above 1.1, whereas those of $TM_{53}Py_{40}CS$/DNA and $TM_{52}Py_{13}CS$/DNA were above 1.8 and 8, respectively. $TM_{69}Py_{62}CS$ showed superior transfection efficiency to $TM_{53}Py_{40}CS$, $TM_{52}Py_{13}CS$, $TM_{65}CS$ and $TM_{43}CS$ at all weight ratios tested. The highest transfection efficiency of $TM_{69}Py_{62}CS$/DNA complexes was found at weight ratio of 4. The results indicated that the improved gene transfection was possibly due to 4-pyridinylmethyl substitution on CS which promoted the interaction and condensation with DNA as well as *N*-quaternization which increased CS water solubility. In cytotoxicity studies, high concentration of TM-Py-CS and TM-CS could decrease the Huh 7 cell viability. In conclusion, this novel CS derivative, $TM_{69}Py_{62}CS$, showed promising potential as a gene carrier by efficient DNA condensation and mediated higher level of gene transfection.

1. Introduction

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. The main objective in gene therapy is successful in vivo transfer of the genetic materials to the targeted tissues in order to replace defective genes, substitute missing genes or silence unwanted gene expression (Zhang et al., 2004). However, naked therapeutic genes are rapidly degraded by nucleases and show poor cellular uptake, so the development of safe and efficient gene carriers is one of the prerequisites for the success of gene therapy (Rolland, 2005). The development of new delivery systems for the administration of gene therapeutics is a field of great interest. One approach is a non-viral delivery system based on supramolecular assembly. Cationic lipids (Lonez et al., 2008) and cationic polymers (Garnett, 1999) have been employed as non-viral gene transfer agents. These cationic substances form complexes with anionic DNA by electrostatic interaction. The resultant cationic DNA complexes are taken up by cells through electrostatic interaction because the cell surface is negatively charged. Transfection efficiencies of these complexes have been investigated in vivo and in vitro. However, before putting these complexes to practical use, there are several problems to overcome: insufficient transfection efficiency; strong cytotoxicity; and inhibition by serum. Biodegradable and biocompatible polymers are suitable for human use and can be prepared as particle complexes of various sizes. Particles with suitable sizes and charges can enter mammalian cells by several routes, such as pinocytosis, phagocytosis, receptor-mediated uptake, etc. and this may improve the chances of cellular entry.

Chitosan (CS) [a (1 → 4) 2-amino-2-deoxy-β-D-glucan] is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine produced by alkaline deacetylation of chitin. Chitosan is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0; therefore, it is insoluble at neutral and alkaline pH values. However, CS is soluble in acidic medium such as acetic acid, citric acid, glutamic acid, aspartic acid, hydrochloric acid, lactic acid, etc. Chitosan has been used in drug delivery as an absorption enhancer and as a vector for gene delivery. In addition, chitosan is biocompatible, biodegradable and nontoxic; therefore, it has been proposed as a safer alternative to other non-viral vectors such as cationic lipids and cationic polymers (Lee et al., 2001; Ishii et al., 2001; Kiang et al., 2004; Weecharangsan et al., 2006). At acidic pH, below pK_a, the primary amines in the chitosan backbone become positively

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charged. 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&II degradation (Huang et al., 2005). Formulation parameters such as molecular weight (MW), degree of deacetylation (DD), N/P ratio (ratio of positively charged chitosan to negatively charged DNA), and pH of transfection medium were found to affect the transfection efficiency of CS/DNA complexes (Sato et al., 2001; Romóren et al., 2003; Lavertu et al., 2006, Weecharangsan et al., 2008).

The main drawback of CS is insoluble at physiological pH and low transfection efficiency. Several CS derivatives have been synthesized in the last few years in order to obtain modified carrier with altered physicochemical characteristics. To address the solubility issue, modified CS such as glycol CS or PEGylated CS (Yoo et al., 2005), low molecular weight soluble CS (Lee et al., 2001) and quaternized CS (Thanou et al., 2002) could be another possible way to escape the solubility issues. To improve gene transfection, chemically modified CS, such as quaternized CS (Thanou et al., 2002), urocanic acid-modified CS (Kim et al., 2003), galactosylated CS (Gao et al., 2005), deoxycholic acid CS oligosaccharide nanoparticle (Chae et al., 2005), and thiolated CS (Lee et al., 2007) were synthesized in order to obtain modified carrier with altered physicochemical characteristics. Although many researchers synthesized CS derivatives as alternative for gene carrier, a few were successful in increased transfection efficiency. Recently, our research group have successfully synthesized the novel water-soluble CS derivatives namely methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan. This CS derivatives showed elevated potential as gene carrier by efficient DNA condensation and mediated higher level of gene transfection with negligible cytotoxicity in Huh 7 cells (Rojanarata et al., 2008). However, the chemical structure requirements related to the ability of synthetic chitosan derivatives to improve the transfection efficiency remain largely obscure. The novel derivatives with desired properties are of interest and deserve the intensive investigation. The pyridinium surfactant have been developed and showed high transfection efficiency and nontoxic in vitro gene delivery (van der Woude et al., 1997). Therefore, the pyridinium group was introduced into the chitosan in order to enhance the transfection efficiency and lower cytotoxicity. In this study, the novel water-soluble CS derivatives, methylation of chitosan containing pyridinylmethyl substituent have been synthesized, and evaluated for their in vitro transfection efficiency and cytotoxicity. CS was substituted with *N*-pyridinylmethyl group to produce hydrophobicity for improved hydrophobic interaction with pDNA and it was quaternized to produce soluble CS. A number of variables that influenced transfection efficiency such as extent of *N*-pyridinylmethyl substitution (ES), 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 (Huh 7 cells) were evaluated.

2. Materials and methods

2.1. Materials

Chitosan, CS, (MW, of 276 kDa), was purchased from Seafresh Chitosan (lab) Co., Ltd. in Thailand. 4-Pyridinecarboxaldehyde and iodomethane were purchased from Riedal-deHaen, Seelze, Germany. Sodium cyanoborohydride and polyethyleneimine (PEI), MW 25 kDa, were purchased from Aldrich, Germany. 1-Methyl-2-pyrrolidone (NMP) was purchased from Fluka, Germany, and all other reagents were distilled before use. 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 were 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 N-pyridinylmethyl chitosans (Py-CS)

The synthesis protocol for *N*-pyridinylmethyl chitosans (Py-CS) was shown in Scheme 1. In brief, chitosan was deacetylated to obtain 94% degree of deacetylation (DD; determined by ^{1}H NMR) (Lavertu et al., 2003). 1.00 g of chitosan was dissolved in 70 mL of 1% acetic acid solution. The solution was diluted with 70 mL ethanol, and then 0.58–1.16 mL of 4-pyridinecarboxaldehyde was added to the solution. The reaction mixture was stirred at room temperature for 24 h. At this point the pH of the solution was adjusted to 5 by adding 15% NaOH. Then, 1.54 g of $NaCNBH_3$ was added, and the resulting solution was allowed to stir at room temperature for 24 h, followed by adjusting the pH to 7 with 15% NaOH. The aqueous solution was dialyzed against de-ionized (DI) water using dialysis tubing with MW cut-off of 12,000–14,000 g/mol (Aldrich, Germany) for 3 days, followed by freeze drying.

2.3. Methylation of chitosans (TM-CS) and N-(4-pyridinylmethyl) chitosans (TM-Py-CS)

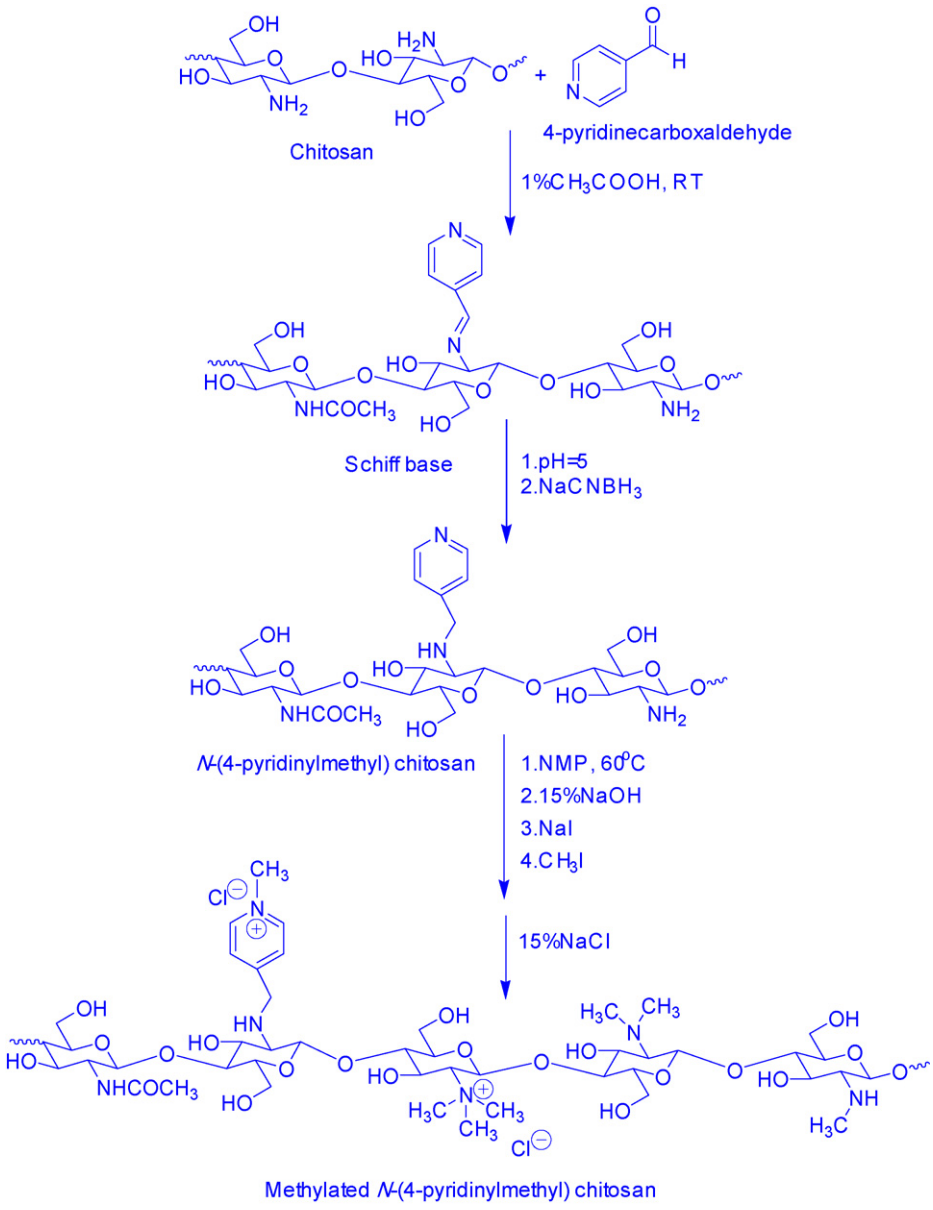
Either chitosans (CS) or *N*-(4-pyridinylmethyl) chitosans (Py-CS) was methylated in accordance to a reported procedure (Scheme 1) in order to enhance the solubility in water (Rodrigues et al., 1998; Sajomsang et al., 2008). In brief, 1.00 g of either CS or Py-CS was dispersed in 50 mL of 1-methyl-2-pyrrolidone (NMP) at room temperature and the mixture was stirred for 12 h. Then 8 mL of 15% aqueous NaOH was dropped slowly in the solution (for high degree of quaternization of CS, 20% aqueous NaOH was used instead of 15% aqueous NaOH). Sodium iodide (3.0 g) was added and the mixture was stirred at 60 °C for 15 min. Subsequently, 3, 3 and 2 mL of iodomethane was added three times, respectively every 4 h and the mixture was stirred at 60 °C for 12 h. After methylation, methylated chitosan and its derivatives were precipitated in 300 mL of acetone. The precipitate was dissolved in 15% NaCl solution in order to replace the iodide counter-ion with a chloride counter-ion. The suspension was dialyzed with de-ionized water for 3 days to remove inorganic materials and then freeze-dried.

2.4. Characterizations of chitosan and its derivatives

The chemical structures of chitosan and its derivatives were confirmed by Fourier Transform Infrared (FT-IR) spectra (Nicolet Impact 410 FT-IR spectrometer) and all samples were prepared as potassium bromide pellets. The degree of deacetylation (DD), the extent of *N*-substitution (ES) and degree of quaternization (DQ) were calculated based on ^{1}H NMR spectra, which were obtained using a Bruker AVANCE 500 MHz Spectrometer. All measurements were performed at 300 K, using pulse accumulating of 64 scans and the LB parameter of 0.30 Hz. 1% of D_2O/CF_3COOD and D_2O were used as the solvents for dissolving 5 mg of CS, Py-CS and TM-Py-CS, respectively.

2.5. Plasmid preparation

pEGFP-C2 was propagated in *Escherichia coli* DH5-α and purified by using the Qiagen endotoxin-free plasmid purification kit



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

(Qiagen, Santa Clarita, CA, USA). DNA concentration was quantified by measurement of UV absorbance at 260 nm and 280 nm using a GeneRay UV Photometer (Biometra® λ260/280 nm). The purity of the plasmid was checked by gel electrophoresis (1% agarose gel) in Tris acetate-EDTA (TAE) buffer, pH 8.0 using λDNA/*Hin*dIII as a DNA marker.

2.6. Preparation and characterization of CS derivatives/DNA complexes

The CS derivatives/DNA complexes were prepared at various charges or weight ratios by adding the DNA solution to the CS derivatives solution. The mixture was gently mixed by pipetting up and down for 3–5 s to initiate complex formation. After left for 15 min at room temperature for the complete reaction, the complex formation was confirmed by agarose gel electrophoresis, prepared from 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 CS derivatives/DNA complex containing 1 μg of DNA.

2.7. 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 and the solution was passed through 0.22 μm membrane filter before used. All samples were measured in triplicate.

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

Huh 7 cells were seeded 24 h 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 and 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 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 with PBS twice and grown in culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

2.9. Evaluation of cell viability

Cytotoxicity of CS derivatives/DNA complexes was evaluated by the MTT assay. Huh 7 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, and then supplied with the CS derivatives/DNA complexes in the same concentrations as in vitro transfection experiment. After treatment, CS 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 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%.

2.10. 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 $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of N-(4-pyridinylmethyl) chitosans and methylated N-(4-pyridinylmethyl) chitosans

N-Pyridinylmethyl chitosans (Py-CS) were synthesized by reductive amination. The formation of Py-CS occurred via the corresponding Schiff base intermediates which were prior to borohydride reduction (Scheme 1). The pyridine carboxaldehydes at different mole ratios with respect to the glucosamine residues of CS were used. It was found that the *N*-substitutions (ES) were in the range of 13–62%, which was depended on molar ratio of aldehydes (Table 1). At the lower molar ratio of aldehyde to GlcN (1:1), ES was 40% while the higher ratio of aldehyde, the ES was 62%. The ^{1}H NMR spectrum of *N*-pyridinylmethyl chitosan was used to determine the ES by comparing the integral area of the H2 + H2′ + 1/3 *N*-acetyl proton signals with those of the aromatic protons of pyridinyl group (Crini et al., 1997). The methylation of CS and Py-CS were carried out using iodomethane under basic condition which yielded methylated chitosan and methylated Py-CS. The methylation occurred at primary amino groups of chitosan and N atom at *N*-pyridinylmethyl substituent. The degree of quaternization (DQ) of chitosan and its methylated derivatives were determined by ^{1}H NMR spectroscopic method which was calculated from the relative peak area of *N*,*N*,*N*-trimethyl protons of GlcN to H1′ proton of the GlcN (Sieval et al., 1998). The DQ was depended on ES and the sodium hydroxide concentration. In our previously study, increasing the sodium hydroxide concentration led to the increase in DQ_{Ch} and *O*-methylation. Nevertheless, increasing ES did not increase DQ_{Ch}, due to lower numbers of unsubstituted GlcN units (Sajomsang et al., 2008). In addition, *O*-methylation at 3-hydroxyl and 6-hydroxyl positions was observed which found in the range of 5–10%.

The characterization of Py-CS and TM-Py-CS were confirmed by FT-IR and ^{1}H NMR spectra. The FT-IR spectra of chitosan exhibited the absorption bands at wave numbers 3430 cm^{-1} due to OH and NH_2 groups, 1648 and 1377 cm^{-1} corresponded to the C=O and C–O stretching of amide group, 1594 cm^{-1} due to N–H deformation of amino groups, 1155, 1081 and 1033 cm^{-1} corresponded to the symmetric stretching of the C–O–C and involved skeletal vibration of the C–O stretching (Brugnerotto et al., 2001). The FT-IR spectrum of Py-CS and TM-Py-CS were shown in Fig. 1. The absorption bands were similar to that of CS except the absorption band of C=C stretching corresponding to the aromatic group at wavenumbers 1607 and 1562 cm^{-1}, C–H stretching of quaternary ammonium group at wavenumber 1470 cm^{-1} and C–H deformation (out of plane) at wavenumber 846 cm^{-1}. The typical ^{1}H NMR spectrum of CS, Py-CS and TM-Py-CS was shown in Fig. 2. The CS exhibited the characteristic ^{1}H NMR pattern such as the singlet at δ 4.9 ppm due to H1, the multiplet at δ 4.4–3.5 ppm due to H3, H4, H5, H6, H6′ and two singlets at δ 3.2 and 2.1 ppm due to the H2 proton of the GlcN and *N*-acetyl protons of GlcNAc, respectively (Lavertu et al., 2003). Both spectra of Py-CS and TM-Py-CS exhibited a doublet of doublets at δ 8.6–8.0 ppm due to the protons of pyridine ring. For the additional signal, H1′ proton of the GlcN of TM-Py-CS appeared at δ 5.40 ppm and other additional signals at δ 4.2 ppm were due to the methyl protons at the N atom of pyridine ring, and the signals at δ 3.2, 2.7 and 2.3 ppm were assigned to *N*,*N*,*N*-trimethyl protons, *N*,*N*-dimethyl protons, and *N*-methyl protons of the GlcN of TM-Py-CS, respectively. In the present study, ES was determined from the relative peak area of pyridine protons to H2 proton of GlcN and *N*-acetyl protons of GlcNAc while DQ was calculated from the relative peak area of *N*,*N*,*N*-trimethyl protons of GlcN to H1′ proton of the GlcN.

Table 1
Methylation of chitosans and *N*-(4-pyridinylmethyl) chitosans

Samples	ES (%)	DQ_{Py} (%)	DQ_{CS} (%)	DQ_{Total} (%)	$N(CH_3)_2$ (%)	$NHCH_3$ (%)	Recovery (%)
$TM_{43}CS$	–	–	43	43	48	ND	80
$TM_{65}CS$	–	–	65	65	23	ND	90
$TM_{52}Py_{13}CS$	13	13	39	52	20	7	76
$TM_{53}Py_{40}CS$	40	40	13	53	2	7	73
$TM_{69}Py_{62}CS$	62	62	7	69	10	13	70

ES is the extent of *N*-substitution; DQ_{Py} is degree of quaternization at N atom of pyridine ring; DQ_{CS} is degree of quaternization at GlcN of chitosan; $N(CH_3)_2$ is *N*,*N*-dimethylation at GlcN of chitosan; $NHCH_3$ is *N*-methylation at GlcN of chitosan; Recovery (%) is dried weight of methylated product (g)/dried weight of chitosan (g) or dried weight of *N*-(4-pyridinylmethyl) chitosan (g) × 100; ND is non-detectable.

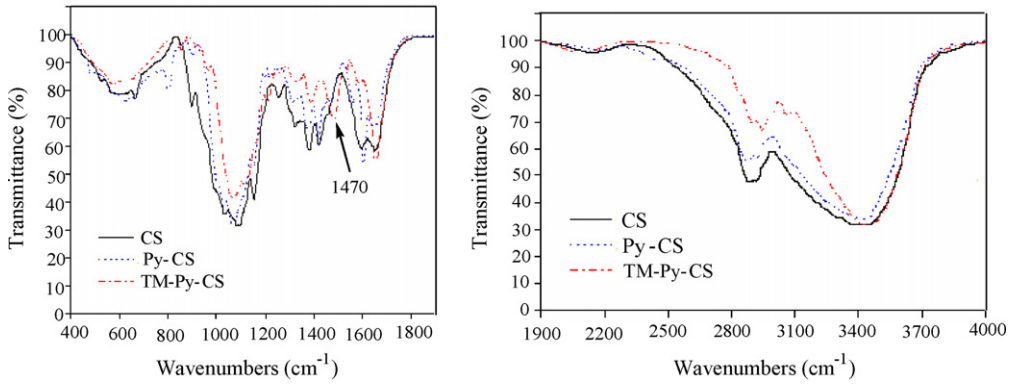


Fig. 1. FT-IR spectra of chitosan (CS), *N*-(4-pyridinylmethyl) chitosan (Py-CS) and methylated *N*-(4-pyridinylmethyl) chitosan (TM-Py-CS).

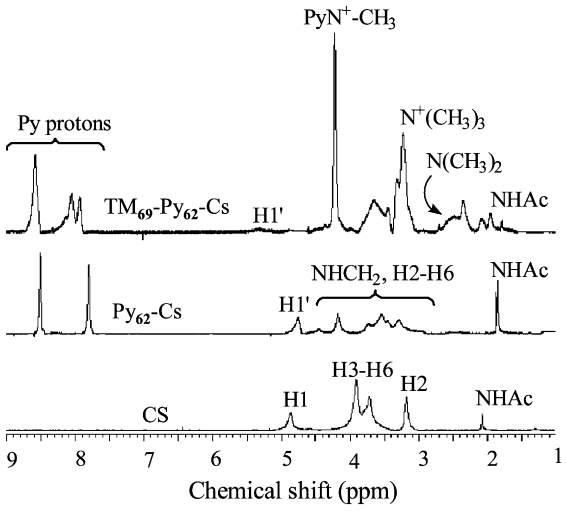


Fig. 2. ^{1}H NMR spectra of chitosan (CS), *N*-(4-pyridinylmethyl) chitosan (Py_{65}-CS) and methylated *N*-(4-pyridinylmethyl) chitosan ($TM_{69}Py_{62}CS$).

3.2. Characterization of CS derivatives/DNA complexes

In order to investigate the optimal conditions for the complex formation, it was necessary to evaluate the degree of binding between CS derivatives and DNA at different CS derivatives and DNA concentrations. The formation of complexes between CS derivatives and pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. Fig. 3a shows the naked DNA (Lane 2) and $TM_{52}Py_{13}CS$/DNA complexes at weight ratios of 2, 4, 6, 8, 12 and 16 (Lane 3–8). Compared with the naked DNA, DNA which formed complexes was retarded in migration. For $TM_{52}Py_{13}CS$, the entire DNA appeared to be retained in the loading well, illustrating that DNA had completely formed the complexes with $TM_{52}Py_{13}CS$ at weight ratio higher than 8. In a case of $TM_{53}Py_{40}CS$, complexes were completely formed at the weight ratio above 1.8 (Fig. 3b), whereas for $TM_{69}Py_{62}CS$ the weight ratio was above 1.1 (Fig. 3c). These results clearly showed that the complete formation of complex occurred at lower weight ratio if the CS with higher degree of *N*-4-pyridinylmethyl substitution were used. Since the structure of DNA comprised of aromatic bases, these charge-neutralized moieties probably interact via hydrophobic force with *N*-4-pyridinylmethyl groups of CS derivatives and accountable for the enhanced DNA condensation. The hydrophobic moiety-dependent gene condensation capacity phenomenon is in close agreement with the previously reported results (Chae et al., 2005).

Particle size and the zeta potential were plotted against weight ratios of CS derivatives/DNA complexes formulated (Fig. 4). The particle size of the complete CS derivatives/DNA complexes were in the range of 200 to 400 nm. Initially, negative values of zeta potentials were observed at low weight ratio. However, when the complete complexes were formed ($TM_{52}Py_{13}CS$ above weight ratio of 8; Fig. 4a, $TM_{53}Py_{40}CS$ above weight ratio of 1.4; Fig. 4b and $TM_{69}Py_{62}CS$ above weight ratio of 1.1; Fig. 4c), the zeta potential values became approximately neutral. The zeta potential of

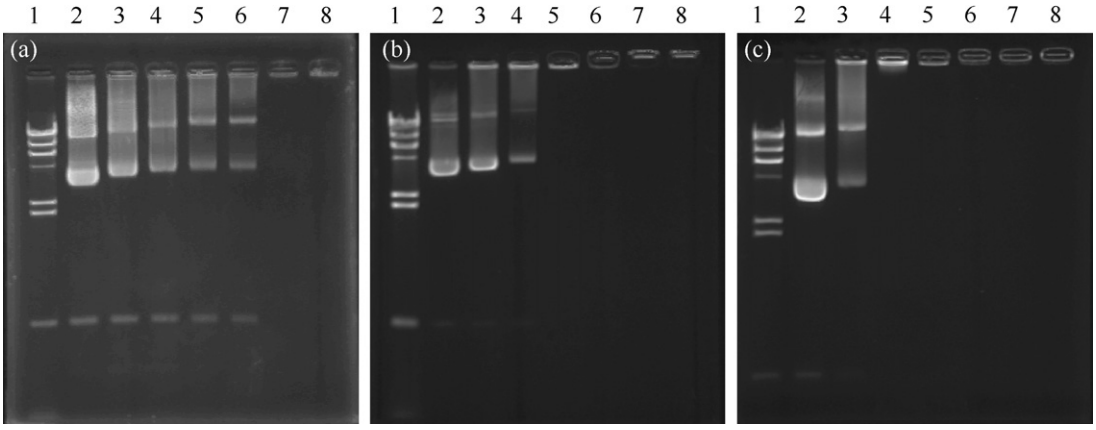


Fig. 3. Gel retarding analysis of CS derivatives/DNA complexes formulated with (a) $TM_{52}Py_{13}CS$, (b) $TM_{53}Py_{40}CS$ and (c) $TM_{69}Py_{62}CS$. Lane 1, DNA marker; Lane 2, pEGFP-C2 plasmid; Lanes 3–8, $TM_{52}Py_{13}CS$/DNA complexes (weight ratios of 2, 4, 6, 8, 12 and 16) $TM_{53}Py_{40}CS$/DNA complexes (weight ratios of 1.4, 1.8, 2.1, 4, 6 and 8) and $TM_{69}Py_{62}CS$/DNA complexes (weight ratios of 1.1, 1.6, 2, 4, 6 and 8).

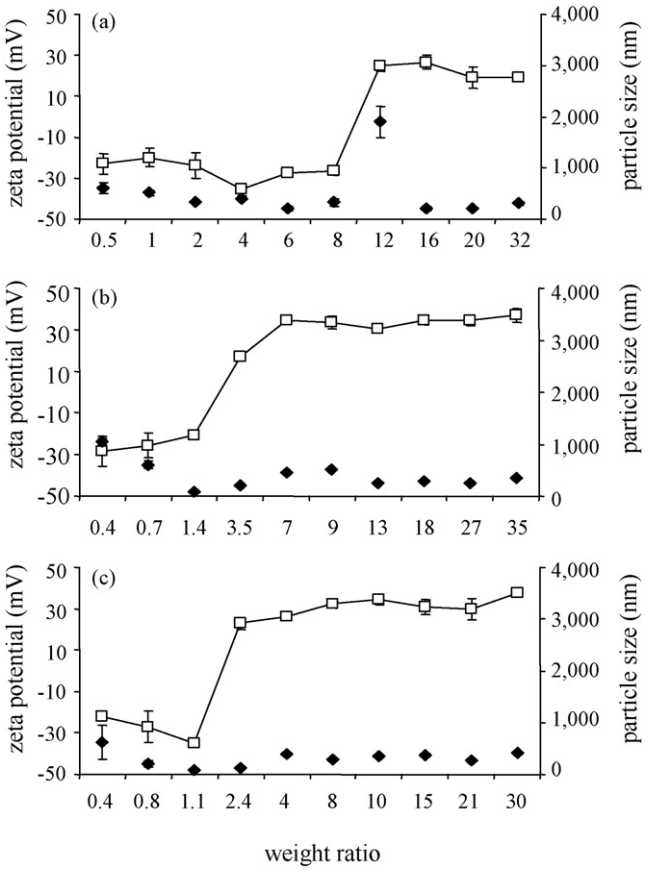


Fig. 4. Zeta potential (□) and particle size (♦) at varying weight ratios of CS derivatives/DNA complexes formulated with (a) $TM_{52}Py_{13}CS$, (b) $TM_{53}Py_{40}CS$ and (c) $TM_{69}Py_{62}CS$. Each value represents the mean ± S.D. of three measurements.

the complexes was found to be more positive with the increase in weight ratios of CS derivatives due to their higher density of quaternization in the CS backbone.

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, in vitro gene transfection assay was performed with human hepatoma cell lines (Huh 7 cells) using pEGFP-C2 plasmid encoding green fluorescent protein (GFP). CS derivatives/DNA complexes were formulated with various weight ratios 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. 5, the gene transfection efficiencies at pH 7.4 were significantly influenced by weight ratios. By increasing the weight ratios, the transfection efficiencies reached the highest values with a decrease by further increment of the ratios. Among the carriers, $TM_{69}Py_{62}CS$ showed the highest transfection efficiency (Fig. 5c). Its highest transfection efficiency at weight ratio of 4 was 33 times higher in gene transfection than that of TM_{65}-CS (Fig. 5e). These results revealed that not only the trimethyl quaternization but also the *N*-4-pyridinylmethyl substitution affected the gene transfection efficiency. Increasing the *N*-4-pyridinylmethyl substitution increased the gene transfection efficiency (Fig. 5a–c). In comparison, the highest transfection efficiency of $TM_{69}Py_{62}CS$ (weight ratio of 4), $TM_{53}Py_{40}CS$ (weight ratio of 4) and $TM_{52}Py_{13}CS$ (weight ratio of 16) were 1137, 248 and 83 cells/cm^2, respectively. Partially quaternized polymers have been previously referred as promising transfection agents. Cationic polymers containing quaternary charged trimethylamino ethylmethacrylate (TMAEM) copolymerized with hydrophilic-(2-hydroxypropyl) methacrylates (HPMA) were found to condense DNA and transfect 293 cells (Curti et al., 2003). Trimethylated chitosan oligomers are promising agents for DNA condensation and promote the transfection efficiency on COS-1, Caco-2 cells (Thanou et al., 2002), COS-7 and MCF-7 cells (Kean et al., 2005). This permanent positive charge of the trimethylated chitosan is a key factor for the condensation and protection of DNA. The introduction of *N*-4-pyridinylmethyl group into the CS 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 et al., 2006). 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 destabilization of the cell membranes (Tian et al., 2007). In our previous study, the introduction of 4-*N*,*N*-dimethylaminobenzyl group into the CS polymer backbone enhanced the hydrophobicity also increased the transfection efficiency (Rojanarata et al., 2008). Moreover, *N*-4-pyridinylmethyl moieties on the CS backbone showed more efficient gene transfection than 4-*N*,*N*-dimethylaminobenzyl moieties. Our results reveal that the chemical structures of hydrophobic moieties play an important role for gene transfection. Although the exact mechanism of $TM_{69}Py_{62}CS$ mediated efficient gene delivery remain to be further studied, our results showed that $TM_{69}Py_{62}CS$ could be 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 (Thanou et al., 2002). Various chitosans and chitosan 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 CS derivatives/DNA complex was performed in Huh 7 cells. Fig. 6 shows the effect of $TM_{52}Py_{13}CS$/DNA (Fig. 6a), $TM_{53}Py_{40}CS$/DNA (Fig. 6b), $TM_{69}Py_{62}CS$/DNA (Fig. 6c), $TM_{43}CS$/DNA (Fig. 6d), and $TM_{65}CS$/DNA complexes (Fig. 6e) on cell viability. When Huh 7 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). The significant decrease in cell viability was observed when Huh 7 cells were incubated with increasing weight ratios of $TM_{43}CS$/DNA and $TM_{65}CS$/DNA complexes, whereas a slight decrease was found in $TM_{52}Py_{13}CS$/DNA, $TM_{53}Py_{40}CS$/DNA, and $TM_{69}Py_{62}CS$/DNA complexes. However, the viability was over 80% at the weight ratio of 4 where the highest transfection efficiency was obtained. Therefore, from this study, the $TM_{69}Py_{62}CS$ is clearly proved to be safe.

The transfection results of $TM_{69}Py_{62}CS$/DNA complexes (Fig. 5c) showed that when the weight ratio was increased above 4, transfection efficiency significantly decreased, whereas cytotoxicity slightly decreased, except for that at weight ratio of 48. These results revealed that cytotoxicity hardly involved in the reduced level of transgene expression by $TM_{69}Py_{62}CS$/DNA complexes prepared at higher weight ratios. Therefore, other factors might be involved in the reduced transgene expression. The decrease in transfection efficiency of the complexes might be explained by large

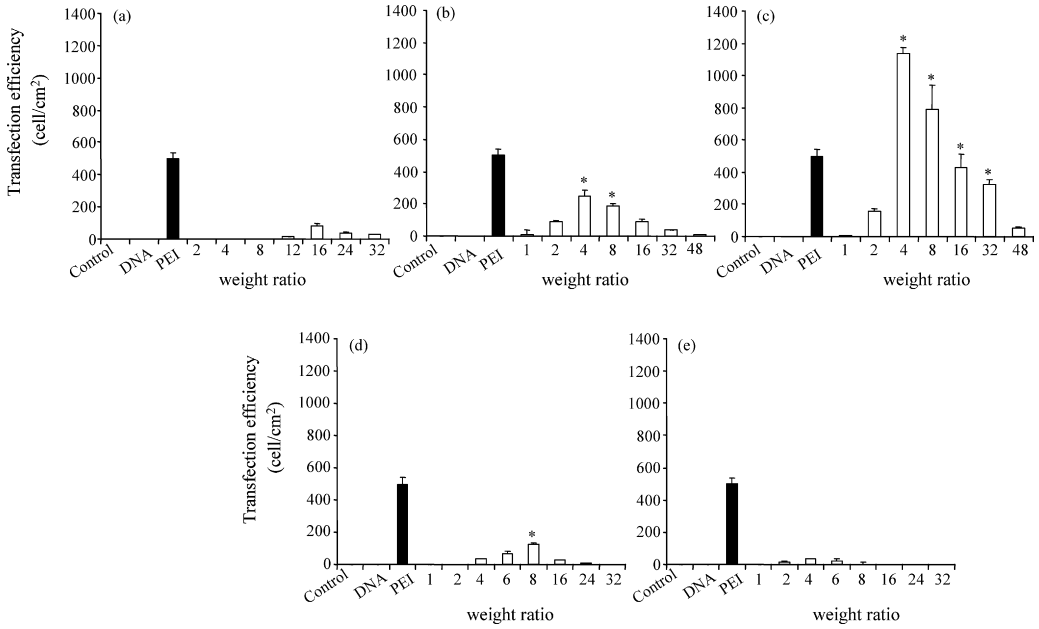


Fig. 5. Transfection efficiencies of CS derivatives/DNA complexes formulated with (a) $TM_{52}Py_{13}CS$, (b) $TM_{53}Py_{40}CS$, (c) $TM_{69}Py_{62}CS$, (d) TM_{43}-CS and (e) TM_{65}-CS in Huh 7 cells. Each value represents the mean ± S.D. of nine wells. Difference values * were statistically significant ($p < 0.05$).

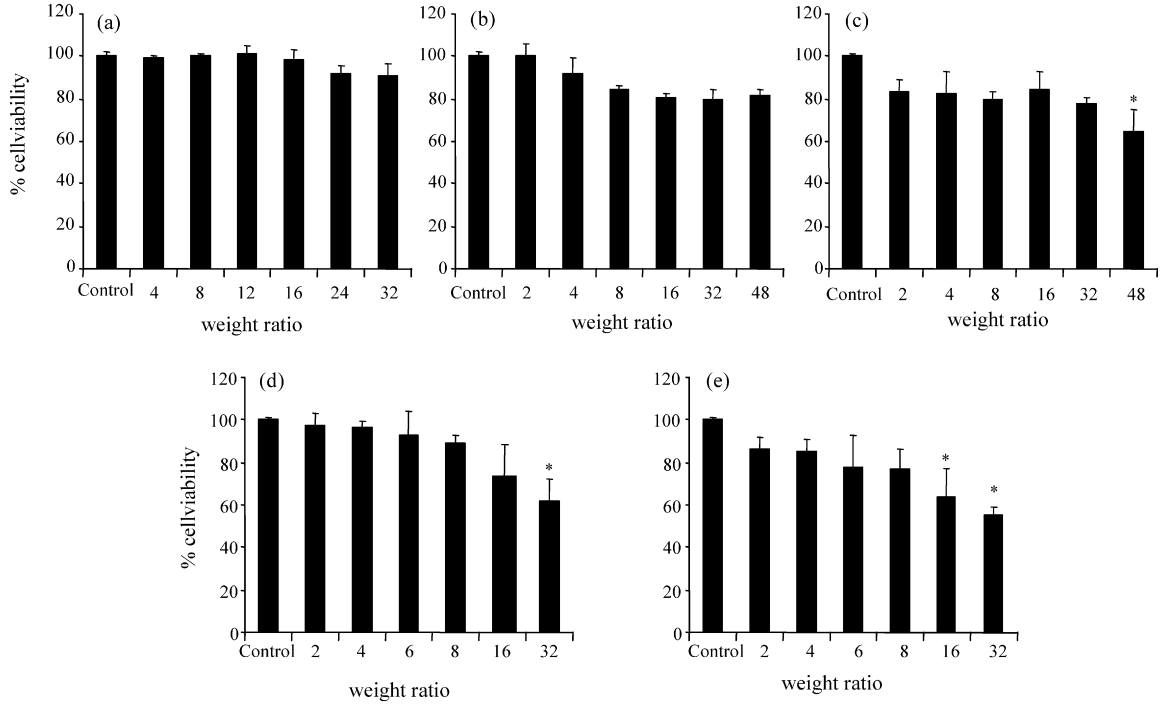


Fig. 6. Cell viability of CS derivatives/DNA complexes formulated with (a) $TM_{52}Py_{13}CS$, (b) $TM_{53}Py_{40}CS$, (c) $TM_{69}Py_{62}CS$, (d) TM_{43}-CS and (e) TM_{65}-CS in Huh 7 cells. Each value represents the mean ± S.D. of nine wells. Difference values * were statistically significant ($p < 0.05$).

amount of polymer which inhibited the cellular internalization of complexes (Furuhata et al., 2008) or the release of DNA from the complexes into cytoplasm (Wang et al., 2002).

4. Conclusion

In this study, the novel water-soluble chitosan derivatives ($TM_{69}Py_{62}CS$) were successfully synthesized for improving transfection efficiency by chemically modified with hydrophobic moiety of *N*-4-pyridinylmethyl and increasing solubility by *N*-quaternization. This study suggests that $TM_{69}Py_{62}CS$ is safe and exhibits significantly improved gene delivery potential in vitro.

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