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Evaluation of chitosan salts as non-viral gene vectors in CHO-K1 cells

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Abstract

The aim of this study was to investigate chitosan/DNA complexes formulated with various chitosan salts (CS) including chitosan hydrochloride (CHy), chitosan lactate (CLa), chitosan acetate (CAc), chitosan aspartate (CAs) and chitosan glutamate (CGl). They were assesed for their DNA complexing ability, transfection efficiency in CHO-K1 (Chinese hamster ovary) cells and their effect on cell viability. CHy, CLa, CAc, CAs and CGl, MW 45 kDa formed a complex with pcDNA3-CMV-Luc at various N/P ratios. CGl/DNA complexes were formulated with various chitosan molecular weights (20, 45, 200 and 460 kDa). The CS/DNA complexes were characterized by agarose gel electrophoresis and investigated for their transfection efficiency in CHO-K1 cells. The cytotoxicity of the complexes was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in CHO-K1 cells. Gel electrophoresis illustrated that complete complexes formed at N/P ratios above 2 in all CS of MW 45 kDa. The transfection efficiency of CS/DNA complexes was dependent on the salt form and MW of chitosan, and the N/P ratio of CS/DNA complexes. Of different CS, the maximum transfection efficiency was found in different N/P ratios. CHy/DNA, CLa/DNA, CAc/DNA, CAs/DNA and CGl/DNA complexes showed maximum transfection efficiencies at N/P ratios of 12, 12, 8, 6 and 6, respectively. Cytotoxicity results showed that all CS/DNA complexes had low cytotoxicity. This study suggests CS have the potential to be used as safe gene delivery vectors.

Keywords: Chitosan salt; Transfection efficiency; Gene delivery; CHO-K1 cells

1. Introduction

Chitosan [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. It makes salts with inorganic and organic acids such as hydrochloric acid and acetic acid, which allows it to be soluble in water. Due to its specific properties, chitosan has been used in drug delivery as an absorption enhancer (Tengamnuay et al., 2000; Maestrelli et al., 2004; Florea et al., 2006) and as a vector for gene delivery (Kumar et al., 2003; Zhao et al., 2006).

As a non-viral vector for gene delivery, chitosan has several advantages over viral vectors such as virally induced inflammatory responses, immunological reactions and oncogenic effects (Simon et al., 1993; Ferber, 2001; Somia and Verma, 2000). Chitosan can be used for the efficient transfection of cells with DNA. 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 (Lee et al., 2001; Thanou et al., 2002; Corsi et al., 2003; Weecharangsan et al., 2006; Özgel and Akbuğa, 2006).

At acidic pH, below pK_a, the primary amines in the chitosan backbone become positively charged. These protonated amines enable chitosan to bind to negatively charged DNA and

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condense it into particles. Chitosan has shown promise to protect DNA from DNase I&II degradation (Richardson et al., 1999; Köping-Höggård et al., 2001; Huang et al., 2005) and transfect into different cell types (MacLaughlin et al., 1998; Ishii et al., 2001; Sato et al., 2001; Corsi et al., 2003). 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 chitosan/DNA complexes (Ishii et al., 2001; Sato et al., 2001; Romóren et al., 2003; Kiang et al., 2004; Lavertu et al., 2006).

Although chitosan salts (CS) were reported to be used for drug delivery, protein delivery and transfection of DNA, these methods employed the dissolution of the chitosan base with acidic solutions (Tengamnuay et al., 2000; Hino et al., 2000; Lavertu et al., 2006; Zhao et al., 2006) or spray-dried chitosan salts (HCl, glutamate, aspartate, lactate, etc.) (Luangtana-anan et al., 2005; Weecharangsan et al., 2006). We were interested in investigating the different CS obtained from respective acid solutions on the transfection of DNA. Different acids possess different physicochemical properties such as pK_a, solubility, charge and size, and these properties might affect the physicochemical properties of respective chitosan, resulting in different transfection efficiencies. Although chitosan and chitosan derivatives have been extensively reported to be effective methods for transfection, each experiment was separately performed by different investigators. No previous study compared the effect of different salt forms on the chitosan/DNA complexes and transfection efficiency in the same experiment. Our previous study compared the transfection efficiency of the chitosan/DNA complex formulated with CLa and CAc, and pSVβ-Gal in COS-1 cells (Weecharangsan et al., 2006). We found that CLa and CAc had efficient transfection abilities. In this study, chitosan/DNA complexes formulated with different CS including CAc, CAs, CGl, CHy and CLa were investigated for their ability to form complexes with the pcDNA3-CMV-Luc plasmid, and for their transfection efficiencies in a Chinese hamster ovary cell line (CHO-K1). Different chitosan molecular weights (20, 45, 200 and 460 kDa) on transfection ability was investigated. Particle size and zeta potential of CS/DNA complexes was evaluated. In addition, the cytotoxicity of CS/DNA complexes was investigated in CHO-K1 cells.

2. Materials and methods

2.1. Materials

Chitosan was purchased from the Seafresh Chitosan Lab (Bangkok, Thailand) with MWs of 20, 45, 200 and 460 kDa and a DD of 87%. Acetic, hydrochloric and lactic acid and DMSO were purchased from Fisher Scientific (Fairlawn, NJ, USA). Aspartic and glutamic acid were purchased from ACROS Organics (Geel, Belgium). Polyethylenimine (PEI, branched, MW 25 kDa) was purchased from Aldrich (Milwaukee, WI, USA). Agarose was purchased from Sigma (St. Louis, MO, USA). The Endotoxin-free Plasmid Maxiprep Kit was purchased from Qiagen (Santa Clarita, CA, USA). RPMI-1640, fetal bovine serum (FBS), trypsin–EDTA and penicillin–streptomycin were purchased from Gibco BRL (Rockville, MD, USA). Twenty-four-well and 96-well plates were purchased from TPP (Trasadingen, Switzerland). Luciferase assay reagent was purchased from Promega (Madison, WI, USA). Bicinchoninic acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma. CHO-K1 cells were provided by Dr. Robert J. Lee, Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, Ohio, USA. All other chemicals were of cell culture and molecular biology quality.

2.2. Plasmid preparation

pcDNA3-CMV-Luc plasmid DNA encoding the firefly luciferase reporter gene under control of the cytomegalovirus enhancer/promoter was used. Plasmid DNA was purified from DH5-α *E. coli* using the Qiagen endotoxin-free plasmid purification kit (Qiagen). DNA concentrations were quantified by measurement of UV absorbance at 260 and 280 nm (OD_{260}/OD_{280} ratio ~1.9) using a spectrophotometer (UV-160U; Shimadzu, Tokyo, Japan). The purity of the plasmid was checked by gel electrophoresis (0.8% agarose gel) in Tris acetate–EDTA buffer, pH 8.0.

2.3. Complex formation between CS and plasmid DNA

CHy, CLa, CAc, CAs and CGl were prepared by dissolving chitosan base in 0.6% solution of hydrochloric acid, lactic acid, acetic acid, aspartic acid and glutamic acid, respectively, with gentle stirring for 12 h. The final concentration of CS solution was 1 mg/ml. CS/DNA complexes were formulated at N/P ratios of 0.5, 1, 2, 4, 6, 8 and 12 by adding the DNA solution to the CS solution. The mixture was gently pipetted and vortexed for 3–5 s to initiate complex formation and left for 15 min at room temperature for the complexes to completely form. The pH of complex solutions was adjusted to pH 6.5 by adding 0.1N NaOH. Complex formation was confirmed by electrophoresis. Agarose gels were prepared with 0.8% agarose solution in TAE buffer with ethidium bromide (0.4 μg/ml). The electrophoresis was carried out for 45 min at 100 V. The volume of the sample loaded in the well was 15 μl of CS/DNA complex containing 0.9 μg of DNA.

2.4. Size and zeta potential measurements

The particle size and surface charge of CS/DNA complexes were determined by laser Doppler anemometry using a Zetasizer 3000 (Malvern Instruments, Southborough, MA, USA). The CS/DNA complexes were prepared in sterile water (500 μl) at N/P of 1, 2, 6, 8 and 12. The pH of the complex solutions was adjusted to pH 6.5 by adding 0.1N NaOH. The measurements were performed using the aqueous flow cell in the automatic mode at 25 °C.

2.5. In vitro transfection of CS/DNA complexes in CHO-K1 cells

CHO-K1 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 (RPMI-1640 containing 10% FBS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). Prior to transfection, the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4), and then supplied with 150 μl of fresh culture medium without FBS. The cells were incubated with 100 μl of the CS/DNA complexes (N/P ratios of 2, 4, 6, 8 and 12) containing 5 μg of pcDNA3-CMV-Luc for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells transfected with naked plasmid and PEI/DNA complexes at an N/P ratio of 4 were used as controls. After transfection, the medium was replaced with 1 ml of fresh growth medium, and the cells were incubated for 24 h at 37 °C under 5% CO_2 atmosphere. All transfection experiments were performed in triplicate.

2.6. Luciferase activity assay

Cells were harvested by removing the medium and then adding 100 μl of 1× Glo lysis buffer (Promega), and incubating for 5 min prior to gentle scraping of the plate. The cell lysate was centrifuged at 10,000 rpm for 3 min and the supernatant was collected. Ten microliters of the supernatant was placed into a 1.5 ml eppendorf tube (Eppendorf, Hamburg, Germany) in which 50 μl of luciferase substrate (Promega) was added. The luminescence was measured using a Mini-Lum luminometer (Bioscan Inc., Washington, DC, USA) immediately after mixing the cell lysate with the luciferase substrate. The transfection efficiency was defined as relative light unit (RLU) standardized with a protein concentration determined by the bicinchoninic acid (BCA) protein assay (Pierce) using bovine serum albumin as a standard.

2.7. Evaluation of cytotoxicity

Evaluation of cytotoxicity was performed by the MTT assay. CHO-K1 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 phosphate-buffered saline (PBS, pH 7.4), and then supplied with 15 μl of fresh culture medium without FBS. The cells were then treated with 10 μl of CS/DNA complexes (N/P ratios of 2, 4, 6, 8 and 12) containing 0.5 μg of pcDNA3-CMV-Luc for 24 h at 37 °C under 5% CO_2 atmosphere. Non-treated cells and cells treated with naked plasmid and PEI/DNA complexes at an N/P ratio of 4 were used as controls incubated for the same duration of time. After treatment, CS/DNA complexes solutions were removed. Finally, the cells were incubated with 20 μl MTT containing medium (0.5 mg/ml MTT in medium) 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 (340 ATTC; SLT Lab Instruments, Salzburg, Austria). Viability of non-treated control cells was arbitrarily defined as 100%.

2.8. 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

3.1. Characterization of CS/DNA complexes

The formation of complexes between CS and the pcDNA3-CMV-Luc plasmid formulated with CHy, CLa, CAc, CAs and CGl MW 45 kDa was observed by agarose gel electrophoresis. Fig. 1a shows the naked DNA and CS/DNA complexes at N/P ratios of 0.5, 1, 2, 4, 6, 8 and 12. The naked DNA lane showed the DNA band, whereas complexed DNA was completely retained within the gel loading well for all chitosan salts at N/P ratios above 2, illustrating that complete CS/DNA complexes were

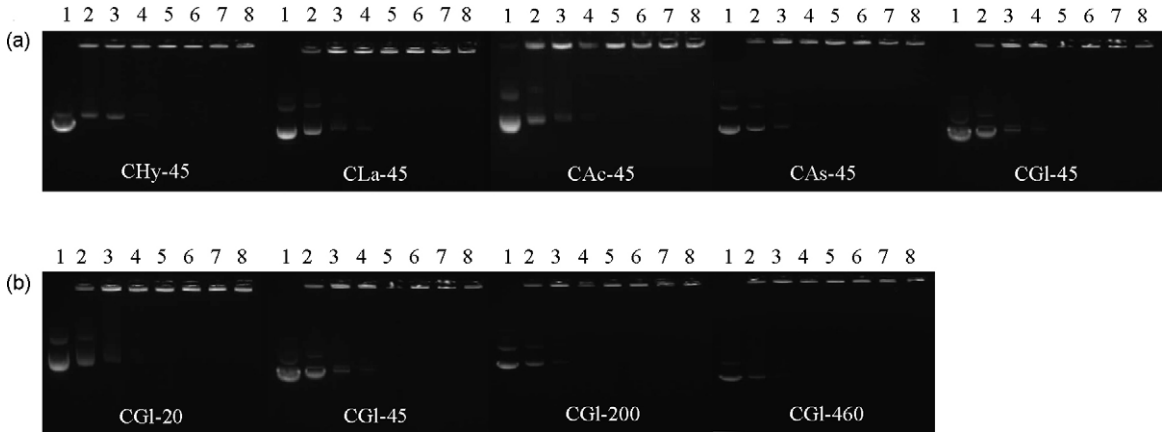

Fig. 1. Gel retarding analysis of chitosan/DNA complexes (a) formulated with chitosan MW 45 kDa of different salts and (b) chitosan/DNA complexes with CGl of different MWs (20, 45, 200, 460 kDa). Lane (1) pcDNA3-CMV-Luc plasmid (0.9 μg); lanes (2–8) chitosan/DNA complexes at N/P ratios of 0.5, 1, 2, 4, 6, 8 and 12, respectively. CHy, Chitosan hydrochloride; CLa, chitosan lactate; CAc, chitosan acetate; CAs, chitosan aspartate; CGl, chitosan glutamate.

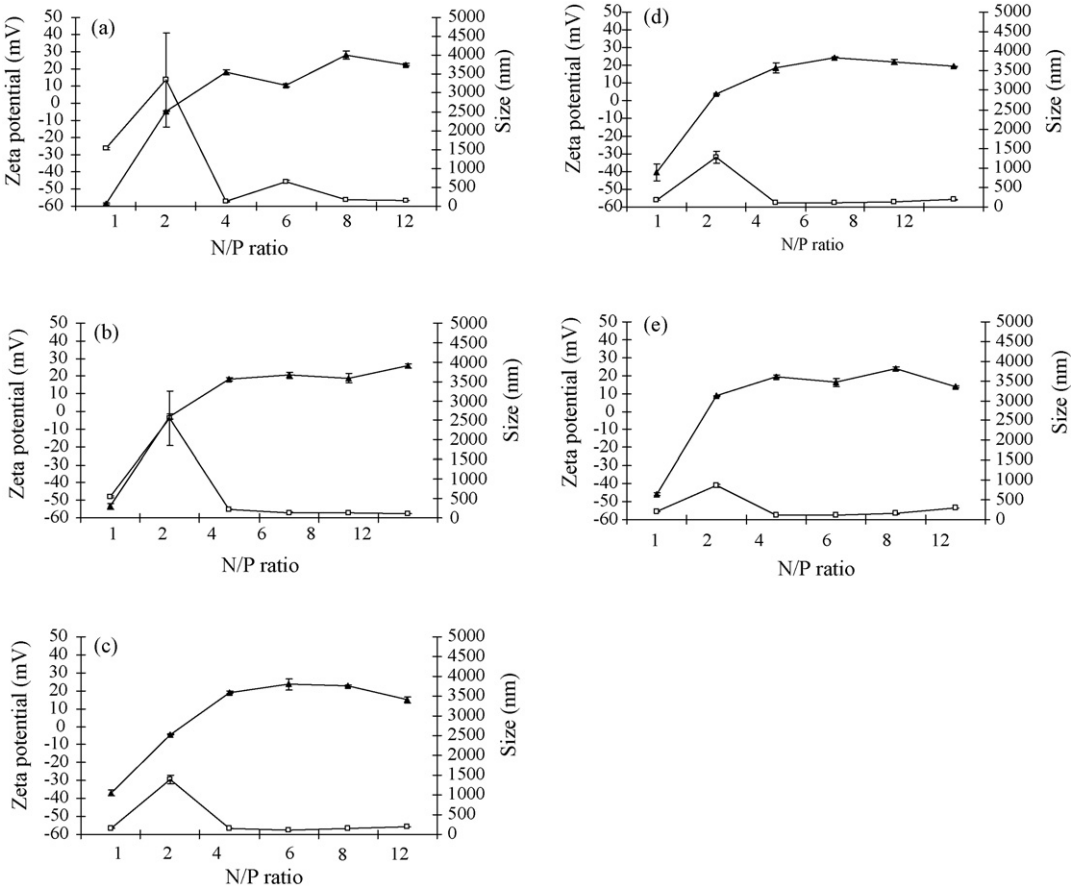

Fig. 2. Zeta potential (▲) and particle size (□) at varying N/P ratios of chitosan-45 kDa/DNA complexes formulated with (a) chitosan hydrochloride, (b) chitosan lactate, (c) chitosan acetate, (d) chitosan aspartate and (e) chitosan glutamate. Each value represents the mean ± S.D. of three measurements.

formed. In order to investigate the effect of MW on complex formation, CGl/DNA complexes with chitosan of different MWs (20, 45, 200 and 460 kDa) were formulated. Fig. 1b shows that complexes were completely formed at N/P ratios above 1 for chitosan MW 200 and 460 kDa, whereas complete complexes for chitosan MW 20 and 45 kDa were formed at N/P ratios above 2.

Particle size and the zeta potential were plotted against N/P ratios of CS/DNA complexes formulated with chitosan, MW 45 kDa (Fig. 2). The particle size of the complexes increased with increasing charge ratio from 1 to 2 and decreased to constant value in the range of 101–299 nm after a charge ratio of 2. At the N/P ratio of 2, all CS showed the largest particle size and the size ranks of the complexes were CHy (3346 nm) > CLa (2553 nm) > CAc (1390 nm) > CAs (1276 nm) > CGl (856 nm). An initial negative value of the zeta potential was observed at a low charge ratio of 1. At the N/P ratio of 2, the zeta potential was approximately neutral. At N/P ratios greater than 2, the zeta potential reached a plateau of about +15 to +28 mV. Higher chitosan MW (200 and 460 kDa) had higher particle size and zeta potential than lower chitosan MW (20 and 45 kDa) (Fig. 3).

3.2. Effect of CS on transfection efficiency in CHO-K1 cells

CS/DNA complexes were formulated with various CS in order to investigate the effect of salt form of chitosan on transfection efficiency. Polyethylenimine (PEI, 25 kDa) was used as a positive control. The transfection efficiency of PEI/DNA complexes at an N/P ratio of 4 was 2.59×10^5 RLU/mg protein. Fig. 4a shows the transfection efficiency of CS/DNA complexes formulated with chitosan MW 45 kDa at N/P ratios of 2, 4, 6, 8 and 12. The transfection efficiency had a tendency to increase as the N/P ratio increased. At N/P ratios of 2 and 4, the transfection efficiencies of all CS/DNA complexes were not significantly different from naked DNA ($P > 0.05$). At an N/P ratio higher than 4, the transfection efficiencies of all CS/DNA complexes were higher than naked DNA. However, in different CS, the maximum transfection efficiency was found at different N/P ratios studied. CHy/DNA, CLa/DNA, CAc/DNA, CAs/DNA and CGl/DNA complexes showed maximum transfection efficiency at N/P ratios of 12, 12, 8, 6 and 6, respectively.

3.3. Effect of MW of chitosan on transfection efficiency in CHO-K1 cells

CGl/DNA complexes were formulated with chitosan of various MWs (20, 45, 200 and 460 kDa) in order to investigate the effect of MW on transfection efficiency. The transfection efficiencies of CGl/DNA complexes are shown in Fig. 4b. The transfection efficiency had a tendency to increase as the N/P ratio increased. At N/P ratios of 2 and 4, the transfection efficiencies

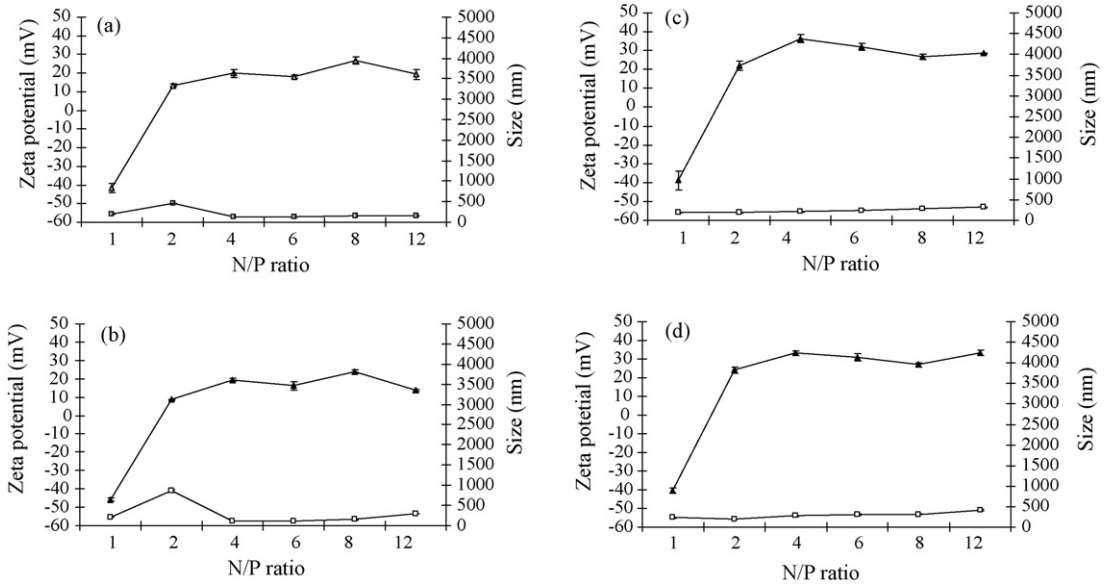

Fig. 3. Zeta potential (▲) and particle size (□) at varying N/P ratios of chitosan glutamate/DNA complexes formulated with chitosan MW (a) 20 kDa, (b) 45 kDa, (c) 200 kDa and (d) 460 kDa. Each value represents the mean ± S.D. of three measurements.

of all MW of CGl/DNA complexes were not significant different from the naked DNA ($P > 0.05$). At N/P ratios higher than 4, the transfection efficiency of all MW of CGl/DNA complexes was higher than that of naked DNA. However, in different MW of CGl, the maximum transfection efficiency was found in different N/P ratios studied. CGl/DNA complexes of MW 20, 45, 200 and 460 kDa showed maximum transfection efficiency at N/P ratios of 12, 6, 6 and 6, respectively.

3.4. Cytotoxicity of CS/DNA complexes on CHO-K1 cells

A cytotoxicity assay was performed in order to evaluate the potential of CS as a vector for safe gene delivery. Therefore, the cytotoxicity of the CS/DNA complexes at various N/P ratios was examined in CHO-K1 cells. Cells without treatment of the CS/DNA complexes were considered as a control with a cell viability of 100%. Fig. 5 shows the effect of CS (Fig. 5a) and

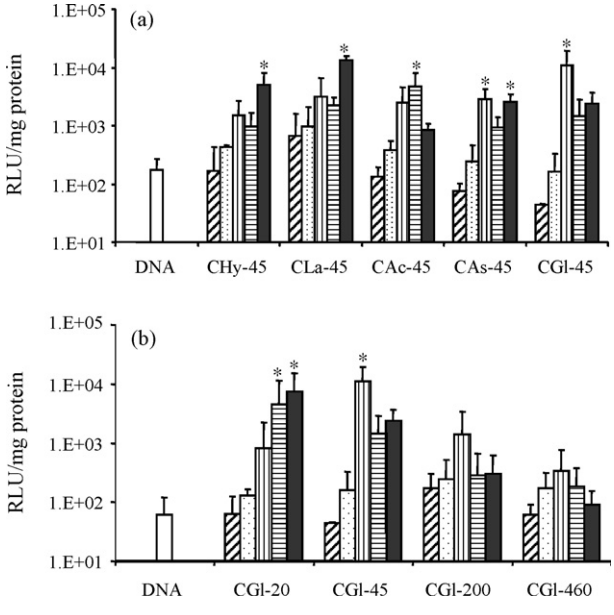

Fig. 4. Transfection efficiency of chitosan/DNA complexes (a) formulated with chitosan MW 45 kDa of different salts and (b) formulated with chitosan glutamate of different MWs (20, 45, 200, 460 kDa) in CHO-K1 cells (▨, N/P ratio of 2; ⁙, N/P ratio of 4; ▥, N/P ratio of 6; ▤, N/P ratio of 8; ■, N/P ratio of 12). Each value represents the mean ± S.D. of three wells. Differences values were statistically significant ($^{*}P < 0.05$). CHy, Chitosan hydrochloride; CLa, chitosan lactate; CAc, chitosan acetate; CAs, chitosan aspartate; CGl, chitosan glutamate; RLU, relative light unit.

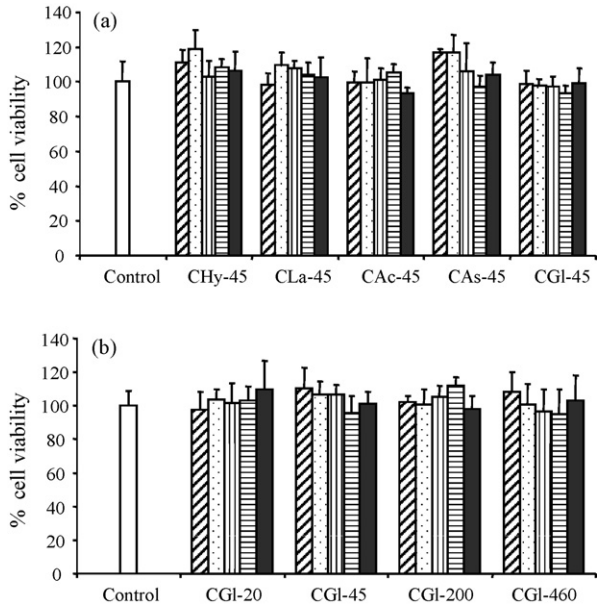

Fig. 5. Effect of chitosan/DNA complexes (a) formulated with chitosan MW 45 kDa of different salts and (b) formulated with chitosan glutamate of different MWs (20, 45, 200, 460 kDa) on CHO-K1 cell viability (▨, N/P ratio of 2; ⁙, N/P ratio of 4; ▥, N/P ratio of 6; ▤, N/P ratio of 8; ■, N/P ratio of 12). Each value represents the mean ± S.D. of six wells. CHy, Chitosan hydrochloride; CLa, chitosan lactate; CAc, chitosan acetate; CAs, chitosan aspartate; CGl, chitosan glutamate.

MW of CS (Fig. 5b) on cell viability. The results showed that CS/DNA complexes formulated with various CS and MW of CS at various N/P ratios were not significantly different from the untreated cells ($P > 0.05$). Average cell viability was over 90%.

4. Discussion

CS including CHy, CLa, CAc, CAs and CGl has been used to formulate CS/DNA complexes and chitosan/DNA nanoparticles for gene delivery (Ishii et al., 2001; Sato et al., 2001; Corsi et al., 2003; Kiang et al., 2004; Huang et al., 2005; Lavertu et al., 2006; Weecharangsan et al., 2006; Zhao et al., 2006). However, the effect of different salt forms of chitosan on transfection efficiency has not been elucidated in the same experiment. In this study, CS/DNA complexes formulated with various CS including CHy, CLa, CAc, CAs and CGl were investigated for their transfection abilities. The pcDNA3-CMV-Luc plasmid was used as the reporter gene to monitor the transfection efficiency in CHO-K1 cells. It was found that CS/DNA complexes could sufficiently transfect CHO-K1 cells with low cytotoxicity.

Analysis by gel electrophoresis indicated that all CS formulated with chitosan MW 45 kDa had similar abilities to form complexes with DNA (N/P ratio of 4, at pH 6.5). At this pH, all CS were equally ionized, despite their different pK_a values. As a result, the number of protonated amine groups available to interact with the phosphate groups of DNA was similar. In our preliminary study, the N/P ratio to form the complete complex between CS and DNA at pH 3 and 5 was 1 and 2, respectively. These indicated that the degree of binding between CS and DNA was largely dependent on the pH of the complex solution, which possibly affected the degree of protonation of amine groups in CS.

The MW of chitosan affected complex formation. Lower MW chitosan (20 and 45 kDa) required a higher N/P ratio (N/P ratio of 4) to completely form complexes compared to higher MW chitosan (200 and 460 kDa, N/P ratio of 2). The influence of MW in complex formation can be attributed to a chain entanglement effect. Chain entanglement contributes less to complex formation as the MW of CS decreases since an increase in the number of CS chains is required to achieve the same N/P ratio compared to higher MW CS. Longer CS chains in high MW CS more easily entangle and trap free DNA once the initial electrostatic interaction has occurred. The need for more CS chains with low MW CS/DNA binding may not be energetically favorable for complex formation (Kiang et al., 2004).

At the same pH (pH 6.5), the particle size of CS/DNA complexes formulated with different salts depended on the type of salt (counter ions), the N/P ratio and MW of chitosan. At neutral zeta potential (N/P ratio 2), the size of CS/DNA complexes varied. This variation could be the effect from different counter ions formed from acids with different pK_a values. At the same pH, acids with lower pK_a dissociate to yield a greater amount of anions than those with higher pK_a. These ions could neutralize the positive charge of chitosan, thereby decreasing the interaction between chitosan and DNA. As a result, the binding of chitosan and DNA is loose and the size of the complex likely becomes larger. The acids used to form CS in this study can be classified into two groups; amino acids (aspartic acid, pK_a 3.65 and glutamic acid, pK_a 4.25) and non-amino acids (hydrochloric acid, 100% dissociation, lactic acid, pK_a 3.86 and acetic acid, pK_a 4.75). The size of the chitosan complexes decreased in the order of CHy > CLa > CAc for non-amimo acids and CAs > CGl for amino acids, which corresponds to the increasing order of pK_a. At N/P ratios above 4, the particle size of all CS/DNA complexes increased with increasing N/P ratio. This was due to the intermolecular cross-linking between DNA strands by self-aggregates with an excess amount of CS. The increase in particle size of CS/DNA complexes with increasing chitosan molecular weight was also observed by MacLaughlin et al. (1998).

The zeta potential of the complexes was found to increase with an increase in N/P ratios and MW of CS due to their higher density of protonated amines in the chitosan backbone. A similar result was observed in previous studies (Kiang et al., 2004; Lavertu et al., 2006). At N/P ratios lower than 2, the CS/DNA complex had a negative zeta potential. The negative charges from phosphates are exposed at the complex surface, confirming that not all amines available participate in complex formation. At an N/P ratio of 2, where the amines are balanced with the phosphates, CS/DNA complexes had zero zeta potential. However, at N/P ratios higher than 2, an excess of amine groups is made available for complex formation, and the final zeta potentials plateau to almost equivalent levels (+15 to +28 mV) for all CS compounds studied.

At the same pH (pH 6.5), the transfection efficiency was affected by salt form (counter ion), MW of CS and N/P ratio. Of the different CS molecules, the maximum transfection efficiency was dependent on different N/P ratios. CHy/DNA, CLa/DNA, CAc/DNA, CAs/DNA and CGl/DNA complexes showed the maximum transfection efficiency at N/P ratios of 12, 12, 8, 6 and 6, respectively. This discrepancy could be due to the effect of counter ions that hinder the interaction between CS and DNA. In the case of hydrochloric acid which highly dissociates into anionic chloride ion, more chitosan was required to maintain a positive charge for a complete interaction with DNA. The maximum transfection efficiency of the CHy/DNA complex was at an N/P ratio of 12. The N/P ratio at which the transfection efficiency was maximum for CLa/DNA was also higher than for CAc/DNA because lactic acid can ionize more than acetic acid. However, those differences could not be observed between CAs/DNA and CGl/DNA. These might be due to other factors such as solubility, molecular size and so on.

The transfection efficiencies of CS/DNA complexes were also dependent on N/P ratio. At a low N/P ratio (N/P ratio of 2 and 4), the transfection efficiency of all CS/DNA complexes was not different from that of naked DNA. This might be because the amount of positively charged amines in chitosan in the CS/DNA complexes at low N/P ratios was not sufficient to transfect cells. The CS/DNA complexes achieved sufficient transfection efficiencies at higher N/P ratios (N/P ratios of above 4). This might be because an increase in the concentration of chitosan at higher N/P ratios could yield a higher amount of positively charged complexes to successfully transfect cells. The increase in transfection ability with an increase in

chitosan concentration in chitosan/DNA complexes was also demonstrated by Lavertu et al. (2006).

The MW of chitosan and N/P ratio of chitosan/DNA complexes is one of the important formulation parameters that affect transfection efficiency. In CGl/DNA complexes, there was an interaction between both the MW and the N/P ratio, where a high MW chitosan gave a higher transfecton efficiency at a low N/P ratio, and a low MW chitosan required a higher N/P ratio. This could be due to optimal association and dissociation between chitosan and DNA in the CGl/DNA complexes with an optimal MW of chitosan and N/P ratio of CGl/DNA complexes that resulted in a high transfection efficiency. This event was in agreement with previous studies (Romóren et al., 2003; Lavertu et al., 2006).

The MW of chitosan in the range studied (20, 45, 200 and 460 kDa) affected transfection efficiency. Chitosan MW 20, 45 and 200 kDa had effective transfection efficiencies, whereas MW 460 kDa had a slightly higher transfection efficiency than naked DNA. This could be due to the high association between chitosan and DNA that prevented dissociation once inside the cells. This observation is in agreement with previous studies (MacLaughlin et al., 1998; Ishii et al., 2001; Sato et al., 2001). On the other hand, high transfection efficiency of chitosan/DNA complexes formulated with high MW chitosan was observed by Zhao et al. (2006).

A required characteristic of a gene delivery system is that it is not cytotoxic. The cytotoxicity of chitosan/DNA complexes was found to be low compared to other cationic complexes (Lee et al., 2001; Thanou et al., 2002; Corsi et al., 2003; Weecharangsan et al., 2006). The cytotoxicity of CS has been reported in B16F10 cells (Carreño-Gómez and Duncan, 1997). However, there was no report of cytotoxicity available on CHO-K1 cells. Therefore, the CS/DNA complexes were investigated for a possible cytotoxic effect. Cell viability was monitored using the MTT assay after 24 h incubation with CS/DNA complexes. The CS/DNA complexes formulated with chitosan MW 45 kDa and CGl/DNA complexes with various chitosan MW (20, 45, 200 and 460 kDa) did not affect the viability of CHO-K1 cells. It was clear that CS/DNA complexes formulated with CAc, CAs, CGl, CHy and CLa were safe.

5. Conclusion

The CS/DNA complexes formulated with various CS including CAc, CAs, CGl, CHy and CLa had the ability to transfect CHO-K1 cells. CS effectively condensed plasmid DNA and yielded nanosized particles. The transfection efficiency of the CS/DNA complexes was dependent on the salt form and MW of chitosan, and the N/P ratios of chitosan/DNA complexes. All CS/DNA complexes tested had low cytotoxicity in CHO-K1 cells. This study suggests CS have the potential to be used as safe gene delivery vectors.

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