International Journal of Pharmaceutics 410 (2011) 161–168

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Chitosan enhances transfection efficiency of cationic polypeptides/DNA complexes

Praneet Opanasopit, Jintana Tragulpakseerojn, Auayporn Apirakaramwong, Tanasait Ngawhirunpat, Theerasak Rojanarata*

Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand

ARTICLE INFO

Article history:
Received 27 January 2011
Received in revised form 1 March 2011
Accepted 6 March 2011
Available online 11 March 2011

Key words:
Cationic polypeptides
Poly-L-arginine
Chitosan
Gene delivery

ABSTRACT

The aim of this research was to investigate the effect of cationic polypeptides mixed with chitosan (CS) on in vitro transfection efficiency and cytotoxicity in human cervical carcinoma cells (HeLa cells). The polypeptides/DNA complexes and ternary complexes (CS, polypeptides and DNA) at varying weight ratios were formulated and characterized by using gel electrophoresis. Their particle sizes and charge were evaluated. The effect of the type and molecular weight (MW) of polypeptides, the weight ratio, order of mixing, the pH and serum on transfection efficiency and cytotoxicity were evaluated in HeLa cells. Three types of polypeptides (poly-L-lysine; PLL, poly-L-arginine; PLA and poly-L-ornithine; PLO) were able to form complete complex with DNA at weight ratio above 0.1. The PLA MW >70 kDa showed the highest transfection efficiency. The order of mixing between CS, PLA and DNA affected the transfection efficiency. The highest transfection efficiency was observed in ternary complexes of PLA/DNA/CS (2:1:4) equal to PEI/DNA complex. For cytotoxicity studies, over 80% the average cell viabilities of the complexes were observed by MTT assay. This study suggests that the addition of CS to PLA/DNA is easy to prepare, safe and exhibits significantly improved DNA delivery potential in vitro.

1. Introduction

Gene therapy requires safe and efficient vehicles to transfer and deliver expressible genetic material to target tissues. The most extensively used delivery tools are viral-based vectors, which are highly effective. In this type of approach, the viruses can act so as to sneak foreign genes into cells. Unfortunately, viruses, even disabled ones, can cause serious side effects (Lundstrom, 2003). The second approach involves using a nonviral vector such as cationic lipids and cationic polymers (Wong et al., 2007; Midoux et al., 2008, 2009; Ruozi et al., 2003). Many polymeric cationic systems such as gelatin, polyethyleneimine (PEI), poly(L-lysines), tetraminofullerene, poly(L-histidine)-graft-poly(L-lysines), diethylamino ethyl-dextrans, cationic dendrimers, cationic polyrotaxanes and chitosan and its derivatives have been studied for in vitro as well as in vivo application (Dang and Leong, 2006; Weecharangsan et al., 2006; Opanasopit et al., 2008, 2009; Kim et al., 2003a; Chae et al., 2005). Chitosan [(1 → 4) 2-amino-2-deoxy-β-D-glucan] is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) produced by alkaline deacetylation of chitin. Chitosan 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 medium such as acetic acid, citric acid, glutamic acid, aspartic acid, hydrochloric acid and lactic acid.

Cationic polypeptides have been used as tools for gene carriers. The main reason for the using cationic polypeptides is that cationic polymers vectors provide the flexibility of DNA carrying capacity and simplicity of use. The nature of the basic peptide in efficient protein transduction and in nucleic acid binding capability made them possible to be the carrier for DNA transfection (Saccardo et al., 2009; Ferrer-Miralles et al., 2008). The most used DNA-condensing cationic peptides in gene delivery systems are poly-L-lysines (PLL) and related peptides. The peptides can bind the negatively charged backbone of DNA chain, not only promoting its condensation but also favoring the interaction of the nanoparticle with the cell membrane and the consequent internalizations. However, the degree of polymerization has shown to be directly related to toxic effects, the longer the lysine chain, the more the cytotoxic PLL (Martin and Rice, 2007; Plank et al., 1999). The cationic poly-L-arginine (PLA) and poly-L-ornithine (PLO) has been used in the recent years as an alternative to lysine in non-viral gene delivery systems (Tecle et al., 2003). From the study of polypeptide/DNA complexes, the physicochemical properties and the transfection efficiency of the complexes could be resulted from the effect of type and MW of polypeptide. In addition, it has been reported that the biological activities of the transfection reagents are highly associated with their physicochemical properties (Thomas and Klibanov, 2003). The variable type and MW of polypeptide, weight ratio and pH

* Corresponding author. Tel.: +66 34 255800; fax: +66 34 255801.
E-mail address: teerasak@su.ac.th (T. Rojanarata).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.03.008

of transfection medium influenced the physicochemical properties and transfection efficiency of polypeptide/DNA complexes (Pouton and Seymour, 1998; Ramsay et al., 2000; Choi et al., 2006; Goparaju et al., 2009).

Recently, cationic polypeptide was reported to be able to translocate through cell membrane very easily, and these cell penetrating peptides (CPPs) containing arginines and lysines, have been used as DNA and oligo DNA carriers (Takeuchi et al., 2006). Arginine residues were finally applied to polymeric gene delivery carriers. These arginine residues have been conjugated to poly (amido amine) (PAMAM) dendrimer (Nam et al., 2008), poly (N-isopropylacrylamide) (Cheng et al., 2006) and chitosan (Gao et al., 2008), and they showed significantly higher transfection efficiency than unmodified polymers. Therefore, combining the unique properties of biodegradable polymers such as chitosan with the advantages of cationic polypeptides (PLL, PLO and PLA), we expect that this self-assembly vector was easy to prepare without any chemical synthesis and would show high transfection efficiency with its low cytotoxicity. In this study, cationic polypeptides/DNA complexes and ternary complexes (CS, PLA and DNA) were prepared and their physicochemical properties intended for gene delivery such as the complex formation, particle size and zeta potential of complexes were investigated with the complex solution of different type and MW of polypeptides, weight ratio and pH of transfection medium. A number of variables influencing transfection efficiency and cell cytotoxicity such as carrier/DNA weight ratio, type and molecular weight of polypeptides, order of mixing, pH of culture medium and serum were also investigated.

2. Materials and methods

2.1. Materials

Poly-L-arginine hydrochloride (PLA; MW 5–15, 15–70 and >70 kDa), poly-L-ornithine hydrobromide (PLO; MW 30–70 kDa), poly-L-lysine hydrobromide (PLL; MW 30–70 kDa) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Chemical Co. (St. Louis, MO, USA). Polyethylenimine (PEI; MW 25 kDa) was purchased from Aldrich (Milwaukee, USA). Chitosan with MW of 45 kDa and 87% degree of deacetylation was purchased from Seafresh Chitosan Lab., Thailand. Chitosan salt (CS; chitosan HCl) was prepared as previously described (Weecharangsan et al., 2008). Briefly, chitosan was dissolved in distilled water containing hydrochloric acid at 1:1 molar ratio. The solution was stirred for 12 h and spray-dried. MEM medium, 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, USA. The λ*Hin*dIII were obtained from Promega (Madison, WI, USA). HeLa (human cervical carcinoma) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology quality.

2.2. Methods

2.2.1. Plasmid preparation

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

2.2.2. Complex formation of polypeptides/DNA

The polypeptides/DNA complexes were prepared at various weight ratios by adding the DNA solution to the cationic polypeptide solution, then mixed by pipetting up and down and tapping the tubes gently. They were incubated for 30 min at room temperature to ensure complex formation.

2.2.3. Ternary complexes formation of CS and PLA/DNA

In this study, CS and DNA were fixed at 4 μg and 1 μg, respectively, with various weight ratios of PLA MW >70 kDa (0–50) and the order of mixing. Three type of order of mixing use in this study were: (1) adding the DNA solution to PLA solution and then added CS solution (PLA/DNA/CS); (2) adding the DNA solution to CS solution and then added PLA solution (CS/DNA/PLA); and (3) adding the PLA solution to CS solution and then added DNA solution (PLA/CS/DNA). These mixtures were gently mixed using pipette for 3–5 s to initiate complex formation and then left for 30 min at room temperature.

2.2.4. Characterization of complexes

The complex formation was confirmed by electrophoresis (MyRun intelligent, Cosmo Bio, Japan). Agarose gels were prepared with 1% agarose solution in TAE buffer with ethidium bromide (0.5 μg/ml). The electrophoresis was carried out for 60 min at 100 V. The volume of the sample loaded in the well was 15 μl of complex containing 1 μg of DNA.

2.2.5. Size and zeta potential measurements

The particle size and surface charge of CS/DNA complexes with and without polypeptide were determined by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at room temperature. The complexes were diluted with distilled water which was passed through 0.22 μm membrane filter prior to use. All samples were measured in triplicate.

2.2.6. Binding affinity

Ethidium bromide (EtBr) displacement assay was performed in order to study the ability of CS and polypeptide to bind with DNA. EtBr (0.1 mg/ml) was dissolved in 0.01 M phosphate buffered saline (PBS, pH 7.4). 20 μl of EtBr solution was added to a 20 μl of 500 μg/ml DNA solution. The steady state fluorescent measurement was performed on a spectrofluorometer (RF-1501, Shimadzu, Tokyo, Japan) at an excitation wavelength of 560 and an emission wavelength of 605 nm. An aliquot CS solution (1 mg/ml) or PLA (1 mg/ml) was then titrated into the DNA/EtBr solution to the varied weight ratios of DNA/CS/PLA complexes (1:4:0 to 50) with varying order of mixing. The fluorescent intensity calculated based on the fluorescent intensity of the DNA/EtBr solution is shown in Eq. (1). The recorded fluorescent intensity (FI) was expressed relative to the fluorescent intensity of the DNA/EtBr solution in the absence of CS or polypeptide (FI_0), after subtracting the fluorescence of EtBr in the absence of DNA under the same buffer conditions (FI_{buff}). Data are presented as mean ± SD. The assay was performed in triplicate.

$$\text{FI}\ (\%) = \left[\frac{\text{FI} - \text{FI}_{\text{buff}}}{\text{FI}_0 - \text{FI}_{\text{buff}}}\right] \times 100 \quad (1)$$

2.2.7. In vitro transfection

HeLa cells were seeded into 24-well plates at a density of 4×10^4 cells/well in 1 ml of growth medium (MEM containing 10% FBS, supplemented with 2 mM L-glutamine, 1% non-essential amino acid solution, 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown under humidified atmosphere (5% CO_2, 95% air, 37 °C) for 24 h. Prior to transfection, the medium was removed and the cells were rinsed with PBS, pH 7.4. The cells were incubated with

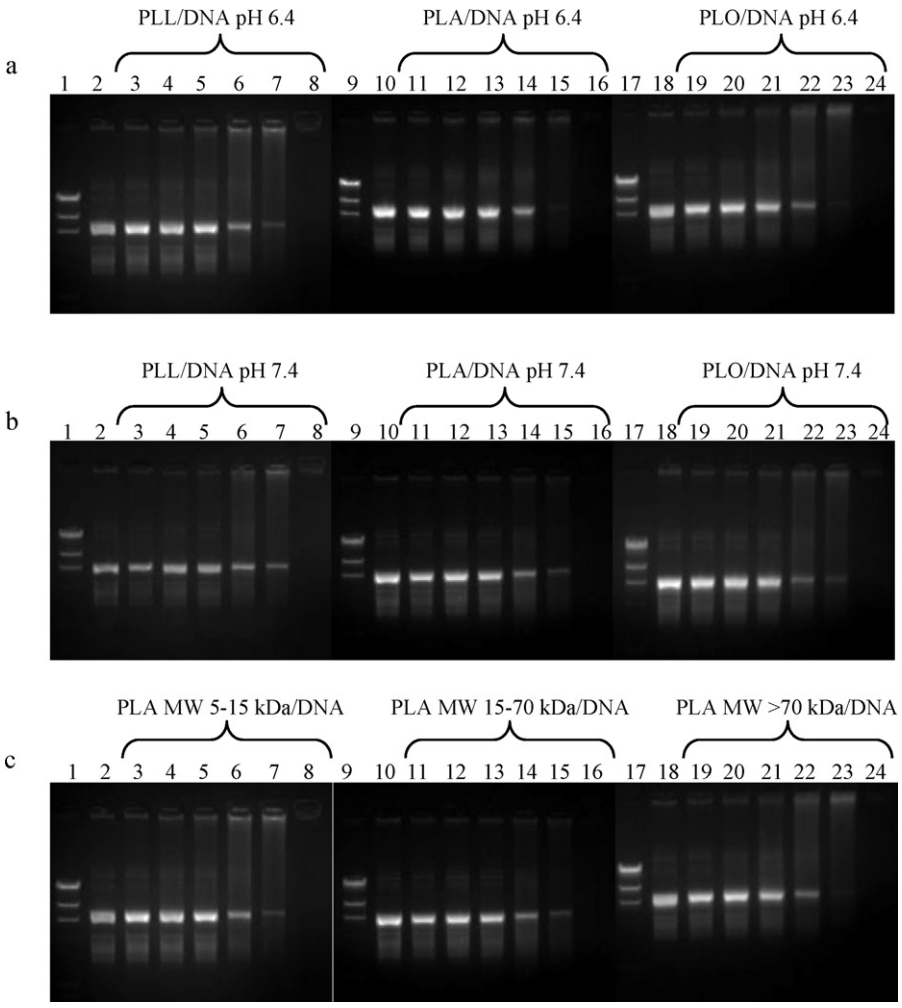

Fig. 1. Gel retarding analysis of polypeptides/DNA complexes (a) at pH 6.4, (b) at pH 7.4 and (c) PLA/DNA complexes with different molecular weight at pH 7.4. Lanes 1, 9, 17 DNA marker; lanes 2, 10, 18 pEGFP-C2 plasmid; lanes 3–8, 11–16, 19–24 of polypeptides/DNA complexes at weight ratios of 0.001, 0.005, 0.01, 0.05, 0.1 and 0.5, respectively.

0.5 ml of the polypeptides/DNA complexes or ternary complexes of CS and PLA/DNA at various weight ratios and order of mixing 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 at the weight ratio of 1 (yielding the maximum transfection efficiency) were used as controls. After transfection, the cells were washed twice with PBS and grown in culture medium for 48 h to allow for GFP expression. All transfection experiments were performed in triplicate.

2.2.8. Evaluation of cell viability

Evaluation of cytotoxicity was performed by the MTT assay. HeLa cells were seeded in a 96-well plate at a density of 8×10^3 cells/well in 100 μl of growth medium and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Prior to evaluation, the medium was removed and the cells were rinsed with PBS, and then supplied with the polypeptides/DNA complexes or ternary complexes of CS and PLA/DNA at various weight ratios and order of mixing in the same concentrations as in vitro transfection experiment. After treatment, the complexes solutions were removed. Finally, the cells were incubated with 100 μl MTT containing medium (1 mg/ml) for 4 h. Then the medium was removed, the cells were rinsed with PBS, pH 7.4, and formazan crystals formed in living cells were dissolved in 100 μl DMSO per well. Relative viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100%.

2.2.9. Statistical analysis

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

3. Results and discussion

3.1. Characterization of polypeptide/DNA complexes

In order to determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between polypeptide and DNA at different polypeptide type and molecular weight. The formation of complexes between polypeptide and the pEGFP-C2 plasmid DNA was visualized by agarose gel electrophoresis. The results showed that three types of cationic polypeptides (PLL, PLA and PLO), MW of PLA (5–15, 15–70 and >70 kDa), and pH (6.4 and 7.4) did not affect the complex formation (Fig. 1). The complete complexes were formed at weight ratio above 0.1. These results indicated that DNA binding to polypeptides was independent from the type, molecular weight of polypeptides and pH of complexes solution.

Accordingly, the complete complexes were able to form at the weight ratio above 0.1, regardless to the type of polypeptide. To deliver DNA into cells, the particle size and zeta potential of a complex are the important factors that influence the access and passage of the complex through the targeting site. The effect of pH on the particle size and zeta potential of polypeptide/DNA complexes were investigated in the complex solution at pH 6.4 and 7.4, and the results are shown in Table 1. It was found that their particle size and zeta potential were dependent on the weight ratio. The particle size of three cationic polypeptides (PLL, PLA and PLO)/DNA complexes at pH 7.4 were in nanometer (130–560 nm) and slightly decreased with an increasing weight ratio from 1 to 4. Whereas the zeta-potential of these complexes slightly increased with an increasing weight ratio from 1 to 4 and had the positive charge in the range of +15 to +30 mV. In addition, as the MW of PLA increased, the particle size of the complexes had a trend showing a slightly increase (data not shown). The particle size of PLL/DNA and PLA/DNA complexes at pH 6.4 was a slightly larger than those at pH 7.4. However at pH 6.4, the all complexes were nanosize (200–800 nm). The particle sizes of PLO/DNA complexes were slightly larger than PLL/DNA and PLA/DNA complexes. The charge densities of polypeptides increase when the pH of the polypeptide solution decreases from high to low pH. Due to the increase charge density of polypeptide at low pH, the slight increase of particle size could be resulted from the exclusion of redundant positive charge provided by polypeptide inside nanoparticles.

3.2. Characterization of ternary complexes of CS and PLA/DNA

Physicochemical properties of ternary complexes of CS and PLA/DNA including complex formation, binding affinity, particle size and zeta potential were characterized because these properties affect the transfection efficiency. PLA (MW >70 kDa) was selected to form complexes with CS/DNA because PLA showed the highest transfection efficiency. The CS/DNA complexes with PLA were prepared by charge interaction induced self-assembly at various weight of PLA ranging from 0 to 50 μg, whereas DNA and CS were fixed at 1 μg and 0.01 μg, respectively. Three types of order of mixing were (1) PLA/DNA/CS; (2) CS/DNA/PLA and (3) PLA/CS/DNA. Fig. 2 illustrates gel electrophoresis of these CS/DNA complexes without polypeptide (Fig. 2a) and three types of mixing (Fig. 2b). For CS/DNA complexes, at pH 6.4 the migration of DNA was completely retarded when the weight ratio was above 0.1. The migration of PLA/DNA/CS, CS/DNA/PLA and PLA/CS/DNA complexes was completely retarded when the weight ratio was above 0.5, 0.5 and 0.1, respectively. These results indicated that the order of mixing affected the formation of complete complexes. This might due to the different in binding affinity.

The particle size and zeta potential of those complexes were elucidated. The CS/DNA complexes with PLA were prepared by three types of order of mixing at various weight of PLA ranging from 0 to 4, whereas, DNA and CS were fixed at 1 μg and 4 μg, respectively. As shown in Table 2, the particle size was dependent on both weight ratio and order of mixing between CS, PLA and DNA. The particle size of all complexes was nanosize (130–400 nm), showing that complete complexes were formed. The zeta-potential was positive and rather constant in the range of 23–40 mV.

The binding affinity of CS or PLA to DNA was studied by ethidium bromide (EtBr) displacement assay. EtBr intercalates between the base pairs of the DNA double helix, yielding a highly fluorescent EtBr/DNA complexes. As polycation was introduced to the DNA, the fluorescence emitted because EtBr/DNA complexes decreased until a minimum level was reached close to baseline fluorescence. The degree of displacement of EtBr by CS or PLA illustrates the binding affinity, indicating the relative strength of the interaction between CS or PLA and DNA. Fig. 3 shows the change in fluores-

Table 1
The particle sizes and zeta-potential of the polypeptides/DNA complexes at pH 6.4 and 7.4.

Weight ratio	PLL/DNA				PLA/DNA				PLO/DNA			
	pH 6.4		pH 7.4		pH 6.4		pH 7.4		pH 6.4		pH 7.4	
	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)
1	362 ± 22	45 ± 1.6	257 ± 18	15 ± 0.6	282 ± 34	27 ± 0.5	267 ± 15	16 ± 0.6	474 ± 38	50 ± 13	557 ± 78	19 ± 1.3
2	541 ± 71	48 ± 5.3	337 ± 39	20 ± 1.3	252 ± 12	35 ± 3.4	317 ± 6	20 ± 0.3	500 ± 20	53 ± 1.1	340 ± 10	22 ± 1.4
3	478 ± 44	57 ± 1.7	377 ± 22	27 ± 0.9	340 ± 23	39 ± 2.4	331 ± 13	26 ± 3.9	283 ± 24	23 ± 2.1	554 ± 24	23 ± 0.7
4	402 ± 36	59 ± 7.1	364 ± 25	27 ± 0.4	342 ± 18	39 ± 0.8	309 ± 46	27 ± 0.6	752 ± 37	52 ± 6.4	134 ± 27	29 ± 3.4

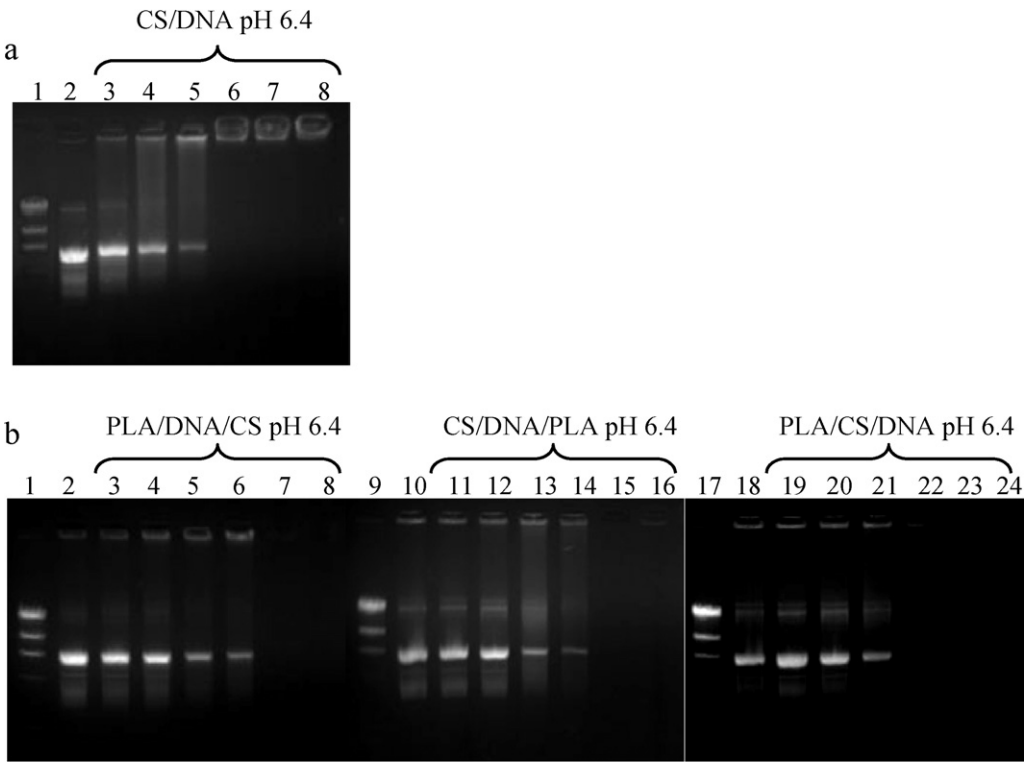

Fig. 2. Gel retarding analysis of (a) CS/DNA complexes at pH 6.4. Lane 1, DNA marker; lane 2, pEGFP-C2 plasmid; lanes 3–8, CS/DNA complexes at weight ratios of 0.01, 0.05, 0.1, 0.05, 1 and 4. (b) CS/DNA at pH 6.4 with increasing weight ratio of PLA and different order of mixing, DNA and CS were fixed at 1 μg and 0.01 μg, respectively. Lanes 1, 9, 17 DNA marker; lanes 2, 10, 18 pEGFP-C2 plasmid; lanes 3–8, 11–16, 19–24 of PLA/DNA complexes at weight ratios of 0.01, 0.05, 0.1, 0.5, 1 and 2, respectively, with three different orders of mixing (PLA/DNA/CS, CS/DNA/PLA and PLA/CS/DNA).

Table 2
The particle sizes and zeta-potential of the ternary complexes at pH 6.4.

Amount of PLA (μg)	PLA/DNA/CS		CS/DNA/PLA		PLA/CS/DNA	
	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)
1	229 ± 8	33 ± 2.3	377 ± 18	39 ± 1.2	398 ± 5.9	39 ± 1.3
2	248 ± 6.2	33 ± 2.8	237 ± 3	39 ± 1.2	353 ± 31	34 ± 1.6
3	175 ± 3.4	31 ± 1.3	269 ± 9	39 ± 0.9	261 ± 7.6	30 ± 1.5
4	231 ± 18	33 ± 0.4	409 ± 30	35 ± 3.4	134 ± 27	29 ± 3.4

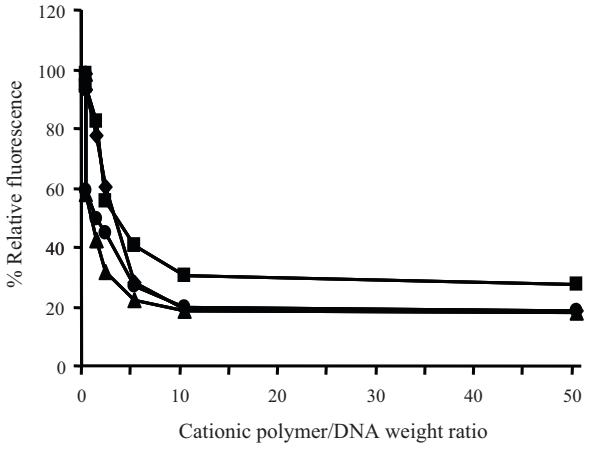

Fig. 3. Percentage relative fluorescent intensity of DNA/EtBr complex containing 10 μg DNA in the complex formulation with increasing amounts of CS and PLA for CS/DNA (■) and PLA/DNA (♦) complexes, respectively. For CS/DNA/PLA (▲) and PLA/DNA/CS (●) complexes CS was fixed at 4 μg with increasing amounts of PLA. Each value represents the mean ± SD of three measurements.

cence intensity of samples relative to a control containing 10 μg DNA in the complex formulation with increasing amounts of CS and PLA for CS/DNA and PLA/DNA complexes, respectively. For CS/DNA/PLA and PLA/DNA/CS complexes, CS was fixed at 4 μg with increasing amount of PLA. The DNA/EtBr complex without titration of CS or PLA was considered as a control with a relative fluorescent intensity of 100%. With the increase in the amount of CS or PLA, the fluorescent intensity showed a decreasing trend, indicating EtBr was replaced by the added CS or PLA, that is, CS or PLA bound selectively to DNA. This could be the increase in the interaction of CS or PLA and DNA when the amount of CS or PLA increased. In addition, the binding affinity reached a plateau at high weight ratio. The rank of binding affinity of the CS or PLA to DNA were PLA/DNA/CS ≈ CS/DNA/PLA > CS/DNA ≈ PLA/DNA. The result is in close agreement with the results previously reported by Liu et al. (2005) and Strand et al. (2005). They found that the binding affinity of chitosan to DNA increased with increasing the N/P ratio. In addition, the binding affinity reached a plateau at high N/P ratio. Our data on ethidium exclusion indicated that the mixture of CS and PLA showed a stronger binding between polycation and DNA than CS or PLA alone.

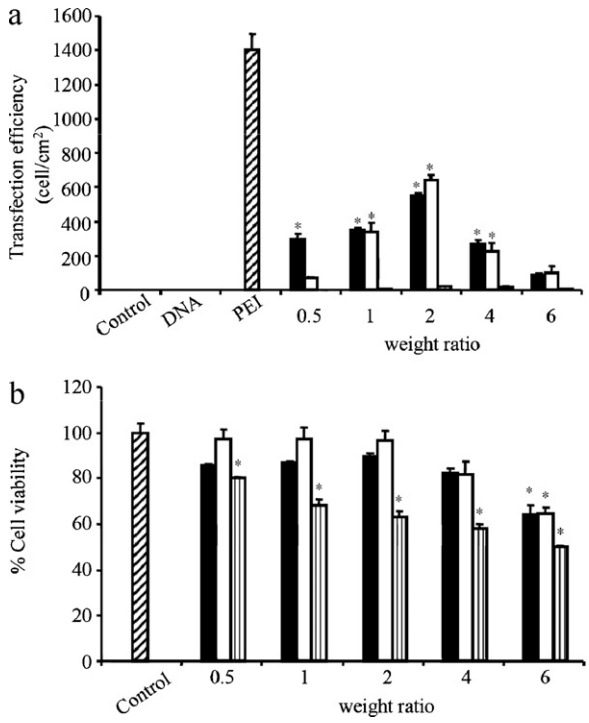

Fig. 4. Effect of weight ratios on (a) transfection efficiency and (b) cell viability of polypeptides/DNA complexes with various weight ratios of 0.5–6 at pH 7.4 in HeLa cells; (■) PLL MW 30–70 kDa, (□) PLA MW 15–70 kDa and (▯) PLO MW 30–70 kDa. Values shown are the means ± SD of triplicate experiments (* indicates $p \leq 0.05$).

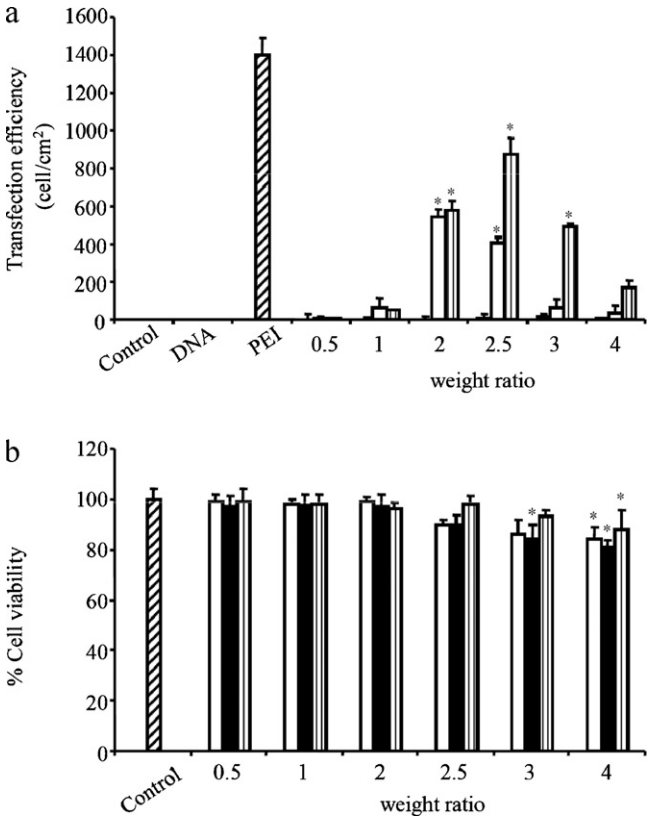

Fig. 5. Effect of molecular weight of PLA on (a) transfection efficiency and (b) cell viability of polypeptides/DNA complexes with various weight ratios of 0.5–6 at pH 6.4 in HeLa cells; (■) PLA MW 5–15 kDa, (□) PLA MW 15–70 kDa and (▯) PLA MW >70 kDa. Values shown are the means ± SD of triplicate experiments (* indicates $p \leq 0.05$).

3.3. *In vitro transfection and cell viability of polypeptide/DNA complexes*

The gene delivery potential of polymer in mammalian cells depends on several factors such as type of polymer, polymer MW, weight ratio and pH of transfection medium/complex solution (Sato et al., 2001). Therefore, transfection conditions such as type and MW of polypeptides, weight ratio and pH of transfection medium were investigated in HeLa cells by transfected-cell counting using images obtained by fluorescence microscope. In the first experiment, we studied the effect of polypeptides type (PLL, PLA and PLO) on transfection efficiency by various weight ratios (0.5, 1, 2, 4, 6) of polypeptides/DNA complexes. 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. 4a (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. The maximum transfection efficiency achieved at the weight ratio of 2. Among the carriers, PLA showed the highest transfection efficiency. The rank of maximum transfection efficiency of the polypeptides were PLA > PLL > PLO. To test whether pH had an effect on transfection, HeLa cells were transfected with PLL/DNA, PLA/DNA and PLO/DNA complexes at pH 6.4. The results revealed that transfection efficiency of cationic polypeptides at pH 6.4 was not significantly different to that of pH 7.4 (data not shown). The highest transfection efficiency was obtained with PLA/DNA complexes. The viability of HeLa cells was also tested in the presence of polypeptides/DNA complexes at various weight ratios as studied in the transfection experiment (Fig. 4b). The naked DNA did not show any cytotoxicity effect on the cells, and the cell viability was maintained around 100%. The results showed that cationic polypeptides showed a significant decrease in cell viability with increasing in weight ratio of polypeptides/DNA complexes. PLL/DNA and PLA/DNA complexes at weight ratio less than 4, approximately 80% of cells were viable. PLO/DNA complexes had the highest cytotoxicity level and the average cell viability was less than 80% at the weight ratio more than 0.5. The cytotoxicity of cationic polymer was probably caused by polymer aggregation on cell surfaces impairing the important membrane function (Jiang et al., 2007). These results illustrated that there is the extreme importance of polypeptide type to gene transfer mediated, which is a unique property of this vector. Therefore, the PLA/DNA complexes were the condition chosen for further exploring experiments.

In order to investigate the effect of MW of polypeptides on the transfection efficiency and on the cell viability, PLA with different MW (5–15, 15–70 and >70 kDa) was used and the results of the transfection efficiencies and the cell viability are shown in Fig. 5a and b, respectively. As the weight ratio increased, the transfection efficiency had a tendency to increase, whereas cell viability significantly decreased. However, there was no significant difference in the cell viability in the different MW of PLA. At different MW of PLA, the maximum transfection efficiency was found at different weight ratio. PLA/DNA complexes of MW 5–15, 15–70 and >70 kDa showed maximum transfection efficiency at the weight ratios of 3 (17 ± 3 cell/cm^2), 2 (545 ± 36 cell/cm^2) and 2.5 (872 ± 40 cell/cm^2), respectively. MW >70 kDa showed the highest transfection efficiency, whereas, the cell viability was not significant different. Thus, the MW of PLA in the range studied affected the transfection efficiency. The high transfection efficiency of high MW cationic polymer might be attributed to the highly positive charge complexes that be able to be uptaken by the cells (Zhao et al., 2006). Kim et al. (2003a,b) reported that the efficiency of arginine 15 residues is almost sevenfold higher than that of commercial transfection agents (Lipofectin)

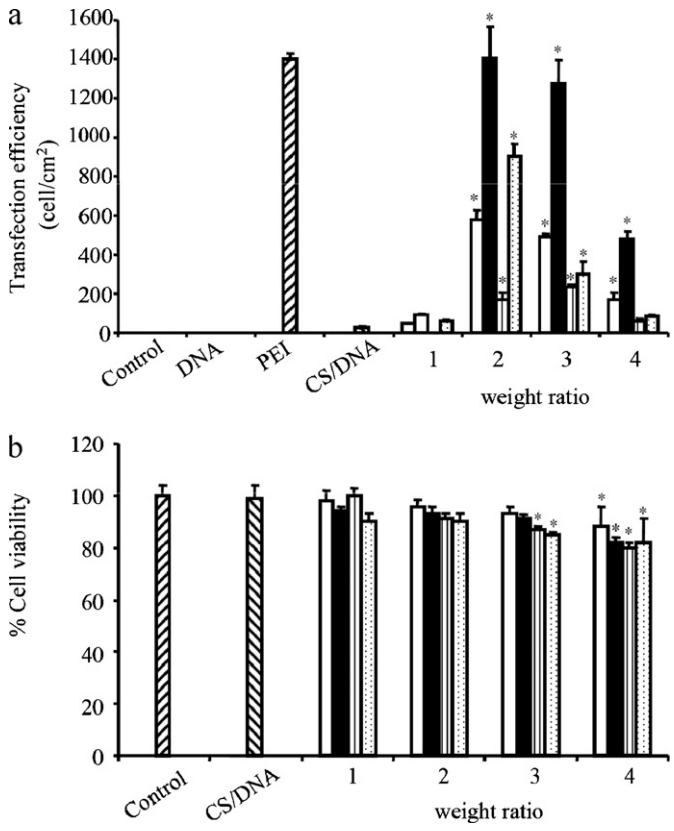

Fig. 6. Effect of order of mixing on (a) transfection efficiencies (b) cell viability of CS/DNA (4:1) complexes at varying weight ratios of PLA with three different order of mixing; at pH 6.4 in HeLa cells; (□) PLA/DNA, (■) PLA/DNA/CS, (◫) CS/DNA/PLA, and (◧) PLA/CS/DNA. Each value represents the mean ± SD of three wells (* indicates $p \leq 0.05$).

and arginine 12 and 9 residues in 293T cells. The main drawback of these low MW peptide–DNA formulations is their instability during in vitro and in vivo gene delivery because the poor peptide–DNA binding leads to easy DNA release.

3.4. In vitro transfection and cell viability of CS and PLA/DNA complexes

Previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH values below 6.5 and the maximum transfection efficiency of the CS/DNA complex achieved at the weight ratio of 4 (Weecharangsan et al., 2008). Therefore, in this study CS and DNA were fixed at 4 μg and 1 μg, respectively, with various weight ratios of PLA MW >70 kDa and order of mixing. Three types of order of mixing used in this study were (1) PLA/DNA/CS, (2) CS/DNA/PLA and (3) PLA/CS/DNA. The results of the transfection efficiencies and the cell viability are shown in Fig. 6a and b, respectively. The mixing of two polymers (CS and PLA) complexed with DNA showed higher transfection efficiency than that of CS/DNA complexes (weight ratio of 4 about 30 ± 4 cell/cm^2) or PLA/DNA complexes (weight ratio of 2 about 578 ± 34 cell/cm^2). The transfection efficiency was dramatically depended on the order of mixing of these CS, PLA and DNA. All types of complexes had a tendency to increase the transfection efficiency as the weight ratio of PLA increased (Fig. 6a). At the low weight ratio of 1, the transfection efficiency of all complexes was not different in comparing with CS/DNA. This might be resulted from the insufficient amount of PLA. Among the order of mixing, PLA/DNA/CS (2:1:4) showed the highest transfection

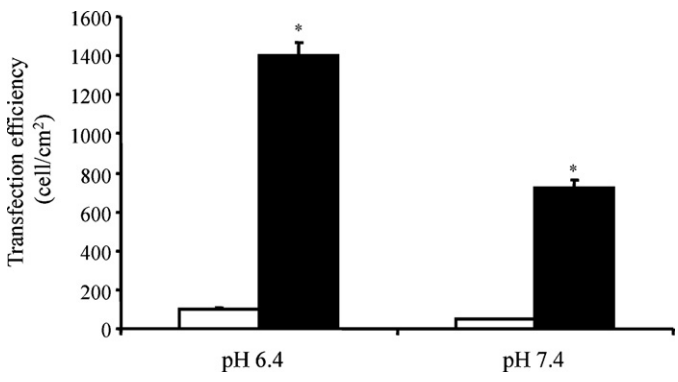

Fig. 7. Effect of pH medium at pH 7.4 and pH 6.4 with 10% serum (□) and without serum (■), on transfection efficiencies of PLA/DNA/CS complexes at the weight ratio of 2:1:4 in HeLa cells. Each value represents the mean ± SD of three wells (* indicates $p \leq 0.05$).

efficiency (1407 ± 155 cell/cm^2). Its transfection efficiency was 6 and 1.6 times higher than that of CS/DNA/PLA (4:1:3; 230 ± 12 cell/cm^2) and PLA/CS/DNA (2:4:1; 900 ± 66 cell/cm^2), respectively. The transfection efficiency of PLA/DNA/CS complexes was not significantly different in comparing with that of PEI/DNA complex at weight ratio of 1 (1400 ± 28 cell/cm^2). On the other hand, it was significantly higher than that of PLA/DNA complexes (2.4 times) and CS/DNA complexes (47 times), respectively. The transfection efficiency of carriers could be ranked as follows: PLA/DNA/CS > PLA/CS/DNA > PLA/DNA > CS/DNA/PLA > CS/DNA.

Our results clearly demonstrate that order of mixing in ternary complexes, resulting from the association of CS to PLA/DNA complexes, are significantly more efficient in mediating transfection than other complexes. Although the exact mechanism of CS mediated efficient gene delivery of PLA/DNA complexes remains to be further studied, our preliminary study in determining the uptake pathways of those complexes using endocytosis inhibitors showed that entry of PLA/DNA/CS complexes may differ from that of PLA/DNA complexes and CS/DNA complexes. Previous studies reported that the transfection efficiency of CS was dependent on pH. Chitosan-mediated high gene transfection was observed at the medium pH values below 6.5 (Weecharangsan et al., 2008). As shown in Fig. 7, pH dramatically affected CS with PLA with decreasing the transfection efficiency by increasing pH values from 6.4 to 7.4. In addition, 10% serum containing media significantly decreased the transfection efficiency both in pH 6.4 and 7.4. Although the exact mechanism of CS to PLA/DNA complexes mediated efficient gene delivery remains to be further studied, our results clearly demonstrate that the addition of CS to PLA/DNA could thereby influence the physical properties of the heteroplex such as particle size, surface charge distribution, DNA condensation, and intracellular stability. These effects could influence the transfection efficiency of the complex and moreover, could be potential candidate for non-viral gene carriers. The ternary complexes displayed low cytotoxicity (Fig. 6b) and had lower cytotoxicity than the complex of PEI 25 kDa. There was a slight decrease in cell viability when HeLa cells were incubated with increasing amount of PLA and the cell viability was over 80% in all order of mixing complexes. This might be the result of biodegradable property of polypeptide and CS that leads to lower cytotoxicity (Wong et al., 2006).

4. Conclusion

Our results clearly demonstrate that ternary complexes, resulting from association of CS to PLA/DNA complexes, are significantly more efficient in mediating transfection than the corresponding

PLA/DNA or CS/DNA complexes and had low cytotoxicity. Our findings suggest that at optimal ratio the high gene expression can be achieved by adding CS to PLA/DNA complexes without a covalent conjugation.

Acknowledgments

The authors would like to acknowledge the financial support from Silpakorn University Research and Development Institute (SURDI 53/01/19), Commission of Higher Education (Thailand) and the Thailand Research Fund (Project No. DBG5180005) and National Nanotechnology Center (NANOTEC), Thailand (grant number: NN-B-22-EN3-17-52-10).

References

Chae, S.Y., Son, S., Lee, M., Jang, M.K., Nah, J.W., 2005. Deoxycholic acid-conjugated chitosan oligosaccharide nanoparticles for efficient gene carrier. J. Control. Release 109, 330–344.

Cheng, N., Liu, W., Cao, Z., Ji, W., Liang, D., Guo, G., Zhang, J., 2006. A study of thermoresponsive poly(N-isopropylacrylamide)/polyarginine bioconjugate non-viral transgene vectors. Biomaterials 27, 4984–4992.

Choi, H.S., Kim, H.H., Yang, J.M., Shin, S., 2006. An insight into the gene delivery mechanism of the arginine peptide system: role of the peptide/DNA complex size. Biochim. Biophys. Acta 1760, 1604–1612.

Dang, J.M., Leong, K.W., 2006. Natural polymers for gene delivery and tissue engineering. Adv. Drug Deliv. Rev. 58, 487–499.

Ferrer-Miralles, N., Vázquez, E., Villaverde, A., 2008. Membrane-active peptides for non-viral gene therapy: making the safest easier. Trends Biotechnol. 26, 267–275.

Gao, Y., Xu, Z., Chen, S., Gu, W., Chen, L., Li, Y., 2008. Arginine-chitosan/DNA self-assemble nanoparticles for gene delivery: in vitro characteristics and transfection efficiency. Int. J. Pharm. 359, 241–246.

Goparaju, G.N., Satishchandran, C., Gupta, P.K., 2009. The effect of the structure of small cationic peptides on the characteristics of peptide–DNA complexes. Int. J. Pharm. 369, 162–169.

Jiang, H.L., Kim, Y.K., Arote, R., Nah, J.W., Cho, M.H., Choi, Y.J., Akaike, T., Cho, C.S., 2007. Chitosan-graft-polyethylenimine as a gene carrier. J. Control. Release 117, 273–280.

Kim, T.H., Ihm, J.E., Choi, Y.J., Nah, J.W., Cho, C.S., 2003a. Efficient gene delivery by urocanic acid-modified chitosan. J. Control. Release 93, 389–402.

Kim, H.H., Lee, W.S., Yang, J.M., Shin, S., 2003b. Basic peptide system for efficient delivery of foreign genes. Biochim. Biophys. Acta 1640, 129–136.

Liu, W., Sun, S., Cao, Z., Zhang, X., Yao, K., Lu, W.W., Luk, K.D., 2005. An investigation on the physicochemical properties of chitosan/DNA complexes. Biomaterials 26, 2705–2711.

Lundstrom, K., 2003. Latest development in viral vectors for gene therapy. Trends Biotechnol. 21, 117–122.

Martin, M.E., Rice, K.G., 2007. Peptide-guided gene delivery. AAPS J. 9, E18–E29.

Midoux, P., Breuzard, G., Gomez, J.P., Pichon, C., 2008. Polymer-based gene delivery: a current review on the uptake and intracellular trafficking of polyplexes. Curr. Gene Ther. 8, 335–352.

Midoux, P., Pichon, C., Yaouanc, J.J., Jaffrès, P., 2009. Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers. Br. J. Pharmacol. 157, 166–178.

Nam, H.Y., Hahn, H.J., Nam, K., Choi, W.H., Jeong, Y., Kim, D.E., Park, J.S., 2008. Evaluation of generations 2, 3 and 4 arginine modified PAMAM dendrimers for gene delivery. Int. J. Pharm. 363, 199–205.

Opanasopit, P., Sajomsang, W., Ruktanonchai, U., Mayen, V., Rojanarata, T., Ngawhirunpat, T., 2008. Methylated N-(4-pyridinylmethyl) chitosan as a novel effective safe gene carrier. Int.J. Pharm. 364, 127–134.

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

Plank, C., Tang, M.X., Wolfe, A.R., Szoka Jr., F.C., 1999. Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and in vitro activity of DNA polyplexes. Hum. Gene Ther. 10, 319–332.

Pouton, C.W., Seymour, L.W., 1998. Key issues in non-viral gene delivery. Adv. Drug Deliv. Rev. 34, 3–19.

Ramsay, E., Hadgraft, J., Birchall, J., Gumbleton, M., 2000. Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine, as a basis for their differential gene transfection in-vitro. Int. J. Pharm. 210, 97–107.

Ruozi, B., Forni, F., Battini, R., Vandelli, M.A., 2003. Cationic liposomes for gene transfection. J. Drug Target. 11, 407–414.

Saccardo, P., Villaverde, A., González-Montalbán, N., 2009. Peptide-mediated DNA condensation for non-viral gene therapy. Biotechnol. Adv. 27, 432–438.

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

Strand, S.P., Danielsen, S., Christensen, B.E., Vårum, K.M., 2005. Influence of chitosan structure on the formation and stability of DNA–chitosan polyelectrolyte complexes. Biomacromolecules 6, 3357–3366.

Takeuchi, T., Kosuge, M., Tadokoro, A., Sugiura, Y., Nishi, M., Kawata, M., Sakai, N., Matile, S., Futaki, S., 2006. Direct and rapid cytosolic delivery using cell-penetrating peptides mediated by pyrenebutyrate. ACS Chem. Biol. 1, 299–303.

Tecle, M., Preuss, M., Miller, A.D., 2003. Kinetic study of DNA condensation by cationic peptides used in nonviral gene therapy: analogy of DNA condensation to protein folding. Biochemistry 42, 10343–10347.

Thomas, M., Klibanov, A.M., 2003. Non-viral gene therapy: polycation-mediated DNA delivery. Appl. Microbiol. Biotechnol. 62, 27–34.

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

Weecharangsan, W., Opanasopit, P., Ngawhirunpat, T., Apirakaramwong, A., Rojanarata, T., Ruktanonchai, U., Lee, R.J., 2008. Evaluation of chitosan salts as nonviral gene vectors in CHO-K1 cells. Int. J. Pharm. 348, 161–168.

Wong, K., Sun, G., Zhang, X., Dai, H., Liu, Y., He, C., Leong, K.W., 2006. PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo. Bioconjug. Chem. 17, 152–158.

Wong, S.Y., Pelet, J.M., Putnam, D., 2007. Polymer systems for gene delivery-past, present, and future. Prog. Polym. Sci. 32, 799–837.

Zhao, X., Yu, S.B., Wu, F.L., Mao, Z.B., Yu, C.L., 2006. Transfection of primary chondrocytes using chitosan-pEGFP nanoparticles. J. Control. Release 112, 223–228.