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DC-Chol/DOPE cationic liposomes: A comparative study of the influence factors on plasmid pDNA and siRNA gene delivery

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ABSTRACT

Cationic liposomes (CLs) composed of 3β-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) (DC-Chol/DOPE liposomes) have been classified as one of the most efficient gene delivery systems. Our study aims to examine the effect of the molar ratio of DC-Chol/DOPE, PEGylation and serum on the pDNA (plasmid pDNA) and siRNA (small interfering RNA) transfection of DC-Chol/DOPE liposomes. The results showed that the most efficient DC-Chol/DOPE liposomes for pDNA or siRNA delivery were at a 1:2 or 1:1 molar ratio of DC-Chol/DOPE, respectively. The transfection efficiency of DC-Chol/DOPE liposomes increased along with increased weight ratio of DC-Chol/siRNA. However, the pDNA transfection efficiency decreased along with increased weight ratio of DC-Chol/pDNA from 3/1. As expected, PEGylation decreased siRNA and pDNA transfection efficiency of DC-Chol/DOPE liposomes. In PEGylated DC-Chol/DOPE liposomes, increased weight ratio of DC-Chol/pDNA from 3/1 did not lead to higher pDNA transfection efficiency, whereas increased weight ratio of DC-Chol/siRNA resulted in increased siRNA transfection efficiency. Furthermore, the serum did not significantly inhibit the pDNA and siRNA transfection efficiency of DC-Chol/DOPE liposomes. In conclusion, our results elucidated the influence factors of DC-Chol/DOPE liposome transfection and would reveal that siRNA and pDNA transfection mechanisms were different in DC-Chol/DOPE liposomes.

1. Introduction

Currently, gene delivery systems are generally classified into two categories: viral vectors and nonviral vectors. The nonviral vectors, which are composed of cationic liposomes (CLs) and polymers, offer advantages over the viral delivery systems of being completely biodegradable, less toxic and less immunogenic than the viral vectors (Akhtar and Benter, 2007; Maitani et al., 2007). Notably, CLs composed of 3β-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) (DC-Chol/DOPE liposomes) have been classified as one of the most efficient vectors for the transfection of pDNA into cells and in clinical trials (Igarashi et al., 2006; Wasungu and Hoekstra, 2006).

It is worth noting that the ratio and combination of cationic/helper lipids contributed greatly to transfection efficiency of DC-Chol/DOPE liposomes (Gao and Huang, 1995; Plank et al., 1996). Previous studies demonstrated that DC-Chol/DOPE liposomes of a 3:2 or 1:1 molar ratio of DC-Chol/DOPE had high transfection efficiency (Farhood et al., 1995). Currently, it was reported that DOPE is an important lipid component and liposomes of a 1:2 molar ratio of DC-Chol/DOPE had the most efficient transfection efficiency (Maitani et al., 2007). As a result, it is necessary to examine the optimal DC-Chol/DOPE ratio of DC-Chol/DOPE liposomes.

The short circulation lifetime and potential aggregation of CLs might be overcome by attaching poly(ethylene glycol) (PEG) at the surface of CLs (Dass and Choong, 2006). Although PEGylation could enhance gene transfection efficiency of CLs in few instances (Pannier et al., 2008), it represents a major barrier for CLs internalization and nucleic acid (pDNA and siRNA) endosomal escape, resulting in much reduced transfection efficiency (Oupicky et al.,

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2002; Knorr et al., 2008). However, the effect of PEGylation on DC-Chol/DOPE liposomes transfection has not been investigated up till now. In addition to PEGylation, the CLs-mediated transfection efficiency was greatly lowered in the presence of serum in most cases (Zhang and Anchordoquy, 2004; Han et al., 2008). As a result, most CLs-mediated transfections were performed in absence of serum (Aljaberi et al., 2004; Salvati et al., 2006; Tagami et al., 2006). To simplify the transfection procedure and accelerate the clinical application of CLs, there is an intense effort to develop CLs that efficiently deliver gene in the presence of serum (Zhang and Anchordoquy, 2004). However, little insight was available in exploring the effect of serum on DC-Chol/DOPE liposomes-mediated transfection.

Gene transfection is a multi-step process, which may face several hurdles, including cellular internalization and intracellular barriers such as endosomal escape, cytoplasm trafficking and nucleus entry (Morille et al., 2008; Wasungu and Hoekstra, 2006). In the case of siRNA, nucleus entry can be overcome since the interference mechanism arises in the cytoplasm (de Fougerolles et al., 2007; Morille et al., 2008). However, the large molecules, such as pDNA (plasmid pDNA), still need cytoplasm trafficking and nucleus entry (Wasungu and Hoekstra, 2006; Ikeda and Taira, 2006). Evidently, the mechanisms of siRNA and pDNA gene delivery were different. We could hypothesize that the influence factors of transfection efficiency, such as molar ratio of DC-Chol and DOPE, PEGylation and serum, may have different influence on siRNA and pDNA gene delivery.

In the present study, we evaluated the effects of various influence factors on the pDNA and siRNA transfection efficiency of DC-Chol/DOPE liposomes and examined the differences in the pDNA and siRNA transfection mechanisms. The comprehension of such mechanisms, will lead to the design of better adapted DC-Chol/DOPE liposomes for gene therapy applications.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylethanolamine (DOPE), 3β-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine [methoxy (polyethyleneglycol)-2000] (mPEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other organic reagents were of analytical grade and purchased from Sinopharm (Shanghai, China). The pGPH1/GFP/Neo plasmid, non-labeled and FAM-labeled negative control (NC) siRNA (sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′-ACGUGACACGUUCGGAGAATT-3′), anti-HER2 siRNA (sense 5′-CGUUUGAGUCCAUGCCCAATT-3′, antisense 5′-UUGGGCAUGGACUCAAACGTG-3′) were provided by GenePharma (Shanghai, China). Silencer® Cy™3-labeled negative control siRNA (Cy3-siRNA) was obtained from Ambion (Austin, USA). FITC labeled rhuMAbHER2 (recombinant humanized anti-HER2 monoclonal antibody, 1 mg/ml) was prepared as we described before (Gao et al., 2009). Lipofectamine™ 2000 (lipo2000), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbag, CA, USA).

2.2. Cell lines

The human breast cancer cell line SK-BR3 was purchased from ATCC (American Type Culture Collection, VA, USA). SK-BR3 cells were maintained in DMEM supplemented with 10%FBS, 25 mM HEPES buffer, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Preparation of DC-Chol/DOPE liposomes

DC-Chol/DOPE liposomes were prepared by the dry-film method as described previously (Igarashi et al., 2006). Briefly, all lipids (total lipids were 8 μmol), including DC-Chol, DOPE and mPEG-DSPE, were dissolved in 2 ml chloroform and the solution was evaporated for 30–35 min at 37 °C. After the dried film was formed, N_2 gas was used to remove the chloroform solvent. The dried film was hydrated with 10 mM phosphate buffer saline (PBS, pH 7.4). After sufficient hydration, the film was suspended by vortexing. The liposomes were then sonicated, and extruded 10 times each through two stacks each of progressively decreasing pore-sized polycarbonate membranes (from 200, 100, to 80 nm) (Nucleopore, Whatman), using a hand held extruder (Avestin, Ottawa). Resulting DC-Chol/DOPE liposomes were sterilized by passage through a 0.22 μm sterile filter, and stored at 4 °C. The phospholipids concentration was determined with ammonium ferrothiocyanate method (Stewart, 1980). The DC-Chol concentration was calculated from the phospholipids concentration correspondingly.

2.4. Formation of liposomes/pDNA or liposomes/siRNA complex

Liposome/pDNA or liposome/siRNA complex (lipoplex) at different weight ratio of DC-Chol and pDNA (DC-Chol/pDNA) was formed by dilution method. Briefly, DC-Chol/DOPE liposomes were diluted with PBS to a final DC-Chol concentration of 1 μg/μl. Then the diluted liposomes and appropriate amount of pDNA or siRNA were diluted separately to equivalent volume of PBS, allowed to stand for 5 min, mixed, and incubated at room temperature for a further 20 min.

2.5. Particle size and zeta potential

After the liposomes and lipoplex were dispersed in deionized water, their particle size and zeta potential were analyzed using Zeta sizer Nano-S (Malvern instruments, UK).

2.6. Gel retardation assay

The pDNA or siRNA binding ability of DC-Chol/DOPE liposomes was evaluated by gel retardation assay. The lipoplex containing 1 μg pDNA or 0.3 μg siRNA at various weight ratios of DC-Chol and pDNA or siRNA was loaded into individual wells of 1% (for pDNA) or 2% (for siRNA) agarose gel, electrophoresed and stained with ethidium bromide. The resulting pDNA or siRNA migration pattern was revealed under UV irradiation.

2.7. siRNA loading efficiency

Ultrafiltration centrifugation method is commonly used to evaluate siRNA loading efficiency in liposomes (Sato et al., 2008; Chen et al., 2004). Briefly, the liposomes/siRNA complex was added to Amicon® Ultra-15 centrifugal filter devices (100,000 NMWL, MA, USA) and centrifuged at 3000 × g × 10 min at 4 °C. The siRNA in the flow-through liquid was collected and quantitated by UV (260 nm). This procedure was repeated for five times. siRNA encapsulation efficiency (%) was calculated as the percentage of entrapped siRNA to the total amount of siRNA added. The entrapped siRNA = the total amount of added siRNA − the amount of nonentrapped siRNA in the flow-through liquid.

2.8. In vitro pDNA transfection

For transfection efficiency analysis, SK-BR3 cells were seeded in 48-well plates with a density of 7.5×10^4 cells per well overnight.

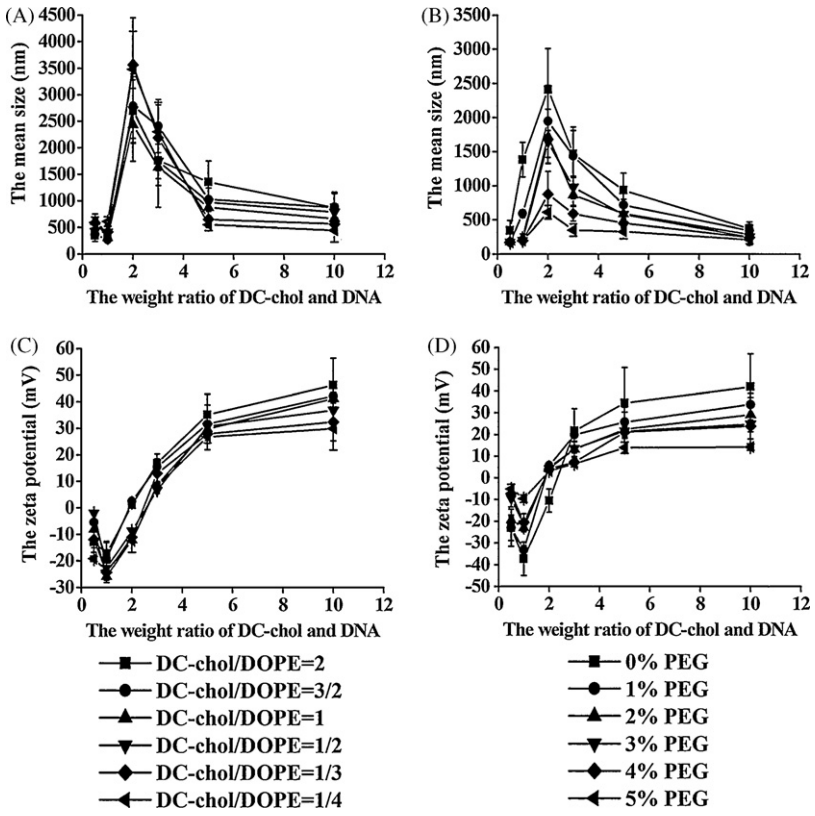

Fig. 1. The size and zeta potential of DC-Chol/DOPE liposomes/pDNA complex (lipoplex). (A) The size of DC-Chol/DOPE lipoplex; (B) the size of PEGylated DC-Chol/DOPE lipoplex; (C) the zeta potential of DC-Chol/DOPE lipoplex; (D) the zeta potential of PEGylated DC-Chol/DOPE lipoplex. Data are presented as mean ± SD ($n = 4$).

The lipoplex containing 0.5 μg pDNA was incubated with the cells for 8 h until fresh culture medium was changed. 48 h later, the cells were trypsinized, washed and analyzed by FCM (flow cytometry). For serum-free transfection, the medium was replaced with serum-free culture medium before transfection and 8 h after transfection, the medium was changed with culture medium containing serum. Transfection using Lipo2000 was performed according to the manufacture's standard protocols. pDNA transfection efficiency was determined using FCM (Becton-Dickinson, San Jose, CA), using 488 nm excitation to detect the green light of GFP (green fluorescence protein) of transfected cells. The transfection efficiency was determined as the percentage of the transfected cells against all cells counted. The GFP fluorescence was also detected by a fluorescence microscope (Olympus, Tokyo, Japan).

2.9. In vitro siRNA transfection

For transfection efficiency analysis, SK-BR3 cells were seeded in 48-well plates with a density of 7.5×10^4 cells per well overnight. The lipoplex containing 0.5 μg FAM-siRNA was incubated with the cells. 8 h later, the cells were trypsinized, washed and analyzed by FCM (flow cytometry). For serum-free transfection, the medium was replaced with serum-free culture medium. Transfection using Lipo2000 was performed according to the manufacture's standard protocols. siRNA transfection efficiency was determined using FCM (Becton-Dickinson, San Jose, CA), using 488 nm excitation to detect the green light of FAM of transfected cells. The transfection efficiency was determined as the percentage of the transfected cells against all cells counted. Although the green fluorescence of FAM-siRNA was easily detected by flow cytometry, it is hard to observe using a fluorescence microscope, owing to the quick quenching of FAM under excitation light. To get bright image of siRNA transfection, Cy3-labeled siRNA was used instead of FAM-siRNA in siRNA transfection. The Cy3 fluorescence was detected by a fluorescence microscope (Olympus, Tokyo, Japan).

For gene silencing analysis, SK-BR3 cells were seeded in 48-well plates with a density of 7.5×10^4 cells per well overnight. The lipoplex containing 0.5 μg anti-HER2 siRNA or NC siRNA was incubated with the cells for 8 h until fresh culture medium was changed. For serum-free transfection, the medium was replaced with serum-free culture medium. 48 h later, the cells were trypsinized, washed and incubated with of FITC labeled rhuMAbHER2 at a final concentration of 1 μg/ml for 45 min at 4 °C. After washing, the HER2 mean fluorescence intensity (MFI) of the cells was analyzed by FCM. HER2 was chosen as an ideal model target in gene silencing analysis, since HER2 was overexpressed in SK-BR3 cells and can be easily detected for protein expression using FCM. The siRNA silencing efficiency (%) was calculated using the following formula: (the HER2 MFI of cells treated with NC siRNA − the HER2 MFI of cells treated with anti-HER2 siRNA)/the HER2 MFI of cells treated with NC siRNA × 100%.

3. Results

3.1. Size and zeta potential of DC-Chol/DOPE liposomes

Before complex formation with pDNA or siRNA, all prepared DC-Chol/DOPE liposomes had a particle size between 130 and 150 nm. However, the size of lipoplex became much larger than that of the liposomes from which they were produced (Figs. 1A and 2A). Whereas the liposomes/pDNA complex prepared at a 2:1 weight ratio had the largest size (Fig. 1A), the weight ratio of the liposomes/siRNA complex which had the largest size was 3 or 5 (Fig. 2A). These results suggest that large lipoplex might form when the charge of lipoplex was neutralized, and the size of lipoplex

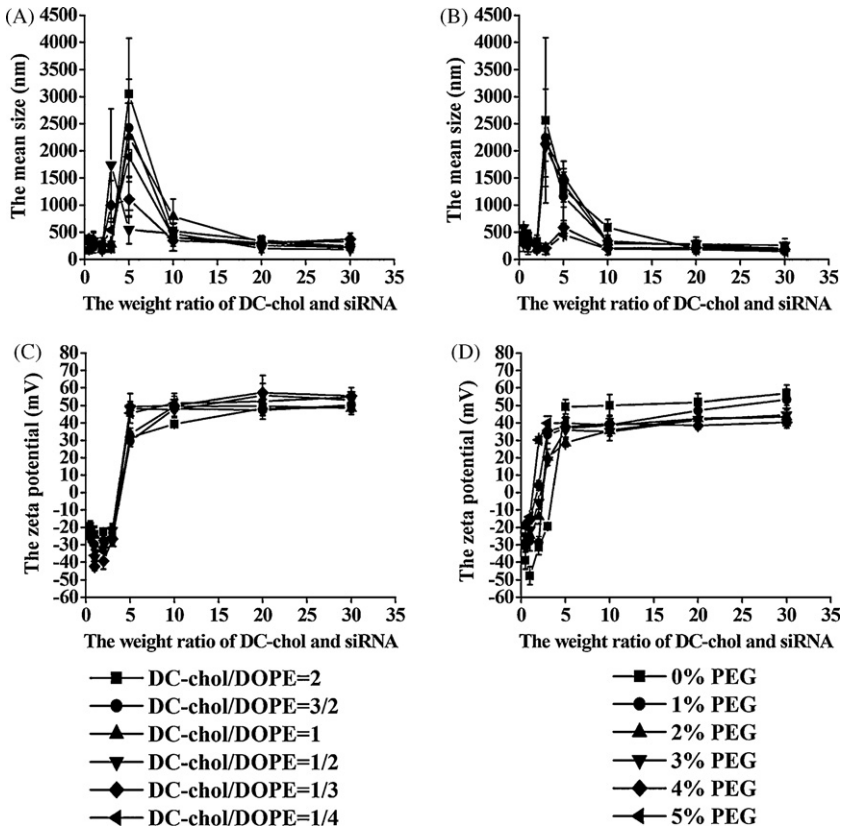

Fig. 2. The size and zeta potential of DC-Chol/DOPE liposomes/siRNA complex. (A) The size of DC-Chol/DOPE liposomes/siRNA complex; (B) the size of PEGylated DC-Chol/DOPE liposomes/siRNA complex; (C) the zeta potential of DC-Chol/DOPE liposomes/siRNA complex; (D) the zeta potential of PEGylated DC-Chol/DOPE liposomes/siRNA complex. Data are presented as mean ± SD ($n = 4$).

could decrease when the charge of lipoplex increase from the neutralization point. The effect of PEGylation on the size of lipoplex was also investigated. At every weight ratio, the size of PEGylated lipoplex gradually decreased when the PEGylation degree increased, indicating that PEGylation could decrease the size of lipoplex (Figs. 1B and 2B).

Zeta potential is another important parameter of CLs. Accompanying with increased weight ratio of DC-Chol to pDNA or siRNA, the zeta potential of liposomes gradually increased, and reaching plateau when the weight ratio was over 5 (Figs. 1C and 2C). In liposomes/pDNA complex, incorporation of PEG to CLs resulted in a reduction in the absolute value of the zeta potential, and the reduction was greater when the PEGylation degree was higher, suggesting that PEGylation can reduce the surface charge density (Fig. 1D).

3.2. pDNA and siRNA binding affinity of DC-Chol/DOPE liposomes

Gel retardation assay was a commonly used method to examine the pDNA binding affinity of CLs (Bajaj et al., 2007; Hattori et al., 2007; Yingyongnarongkul et al., 2009). The migration pattern of pDNA in the lipoplex changed when pDNA was mixed with liposomes at the weight ratios of DC-Chol and pDNA from 1 to 10 (Fig. 3). No pDNA migration was observed at or beyond a weight ratio of 2. These results indicated that complete liposomes/pDNA complex was formed at and above a weight ratio of DC-Chol and pDNA of 2,

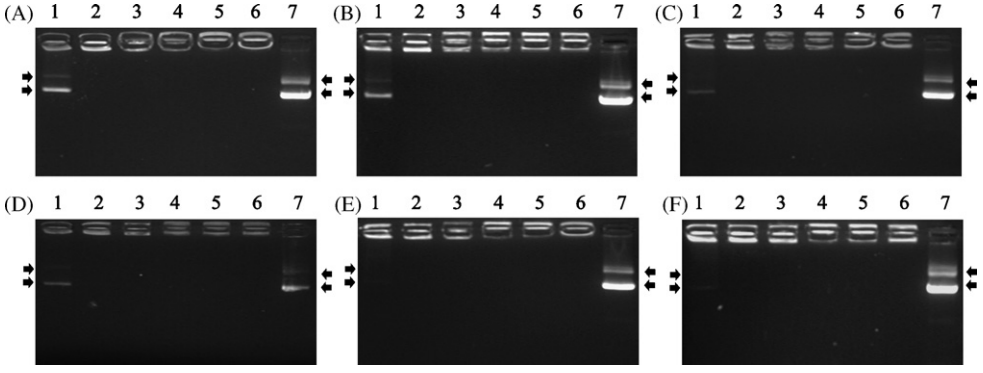

Fig. 3. pDNA gel retardation assays. DC-Chol/DOPE liposomes were complexed with pDNA at various weight ratios, and then run through a 1% agarose gel. The mobility of pDNA was visualized by ethidium bromide staining. The weight ratio of DC-Chol/pDNA was 1, 2, 3, 5, 8 and 10 (lanes 1, 2, 3, 4, 5 and 6, respectively). Lane 7, 1 μg plasmid. (A) DC-Chol/DOPE = 2/1; (B) DC-Chol/DOPE = 3/2; (C) DC-Chol/DOPE = 1; (D) DC-Chol/DOPE = 1/2; (E) DC-Chol/DOPE = 1/3; (F) DC-Chol/DOPE = 1/4. The bars were used to indicate the pDNA bands in the gels.

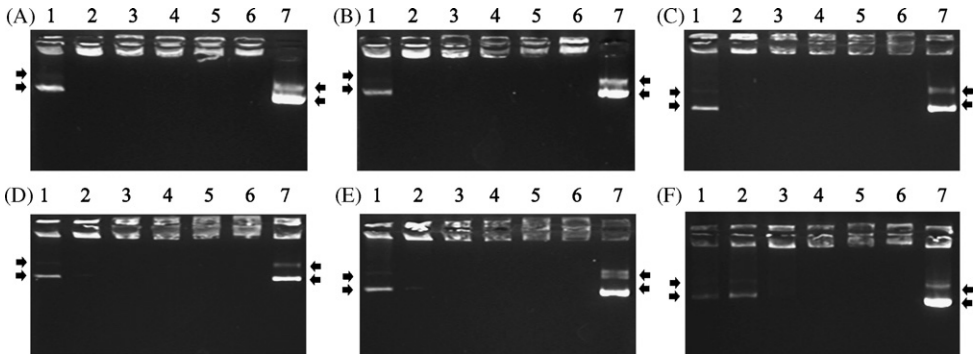

Fig. 4. pDNA gel retardation assays. PEGylated DC-Chol/DOPE liposomes were complexed with pDNA at various weight ratios, and then run through a 1% agarose gel. The mobility of pDNA was visualized by ethidium bromide staining. The weight ratio of DC-Chol/pDNA was 1, 2, 3, 5, 8 and 10 (lanes 1, 2, 3, 4, 5 and 6, respectively). Lane 7, 1 μg plasmid. (A) 0% PEG; (B) 1% PEG; (C) 2% PEG; (D) 3% PEG; (E) 4% PEG; (F) 5% PEG. The bars were used to indicate the pDNA bands in the gels.

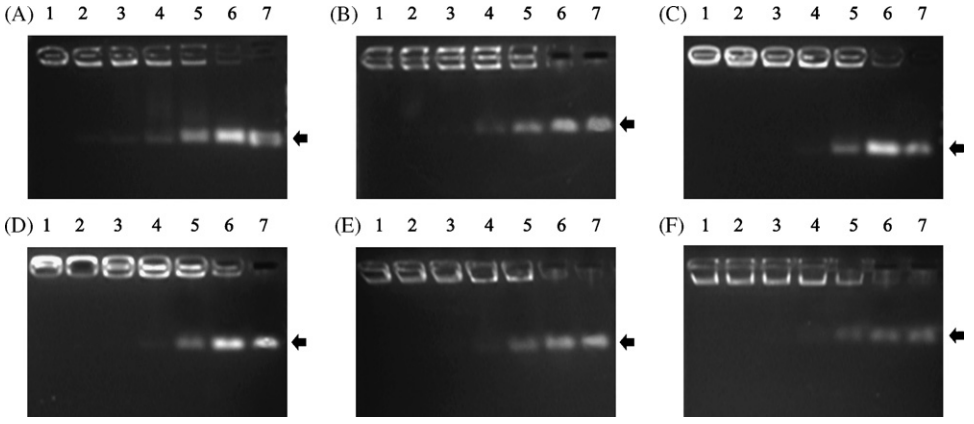

Fig. 5. siRNA gel retardation assays. DC-Chol/DOPE liposomes were complexed with siRNA at various weight ratios, and then run through a 2% agarose gel. The mobility of siRNA was visualized by ethidium bromide staining. The weight ratio of DC-Chol/siRNA was 15, 10, 7.5, 5, 3 and 1 (lanes 1, 2, 3, 4, 5 and 6, respectively). Lane 7, 0.3 μg siRNA. (A) DC-Chol/DOPE = 2/1; (B) DC-Chol/DOPE = 3/2; (C) DC-Chol/DOPE = 1; (D) DC-Chol/DOPE = 1/2; (E) DC-Chol/DOPE = 1/3; (F) DC-Chol/DOPE = 1/4. The bars were used to indicate the siRNA bands in the gels.

which was consistent with the results reported previously (Hattori et al., 2007). The effect of PEGylation on pDNA binding affinity of DC-Chol/DOPE liposomes was also investigated (Fig. 4). When the PEGylation degree was 0–2%, complete lipoplex was formed at or above a weight ratio of 2, indicating pDNA binding affinity was not affected by low PEGylation degree (~2%). However, the pDNA binding affinity decreased accompanied with subsequently increased PEGylation degree (Fig. 4D–F). At the highest PEGylation degree of 5% (Fig. 4F), a slight amount of pDNA was still detected at a weight ratio of 3:1, suggesting that PEGylation can reduce the surface charge density of CLs and have a negative effect on pDNA binding affinity (Zhong et al., 2005; Agarwal et al., 2005).

Similar results were obtained in siRNA gel retardation assay. When the weight ratio of DC-Chol to siRNA was higher than 5 (7.5,

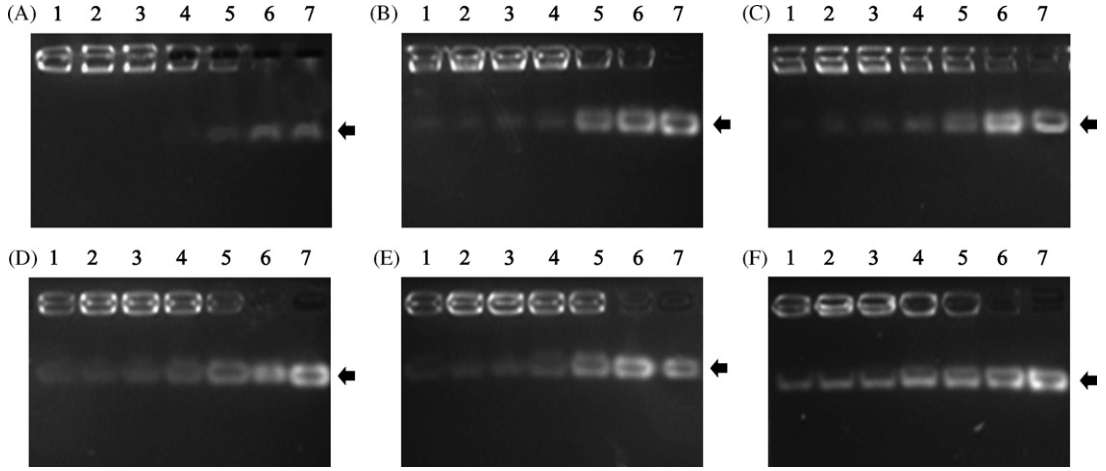

Fig. 6. siRNA gel retardation assays. PEGylated DC-Chol/DOPE liposomes were complexed with siRNA at various weight ratios, and then run through a 2% agarose gel. The mobility of siRNA was visualized by ethidium bromide staining. The weight ratio of DC-Chol/siRNA was 15, 10, 7.5, 5, 3 and 1 (lanes 1, 2, 3, 4, 5 and 6, respectively). Lane 7, 0.3 μg siRNA. (A) 0% PEG; (B) 1% PEG; (C) 2% PEG; (D) 3% PEG; (E) 4% PEG; (F) 5% PEG. The bars were used to indicate the siRNA bands in the gels.

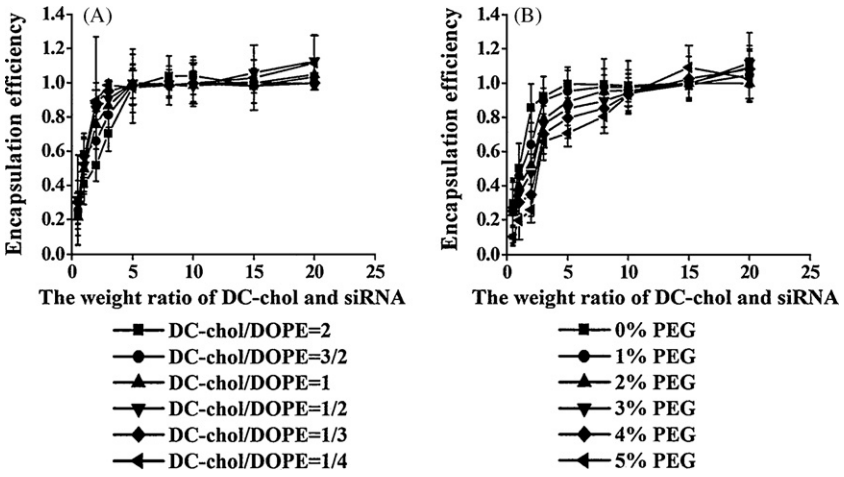

Fig. 7. siRNA encapsulation efficiency. DC-Chol/DOPE liposomes (A) or PEGylated DC-Chol/DOPE liposomes (B) were complexed with siRNA at various weight ratios. Then the liposomes/siRNA complex was added to the centrifugal filter devices and centrifuged at 3000 × *g* for 10 min five times at 4 °C. The siRNA in the flow-through liquid was collected and quantitated. siRNA encapsulation efficiency (%) was the percentage of entrapped siRNA to the total amount of siRNA added. The entrapped siRNA = the total amount of added siRNA − the amount of nonentrapped siRNA in the flow-through liquid. Data are presented as mean ± SD ($n = 5$).

10 and 15), no migration of siRNA was observed (Fig. 5). As shown in Fig. 6, PEGylation significantly weakened the siRNA binding affinity of DC-Chol/DOPE liposomes. When the PEGylation degree was 1%, there was not complete siRNA retardation in liposomes, even at the weight ratio of 15. The siRNA binding affinity of DC-Chol/DOPE liposomes became weaker along with increased PEGylation degree (1–5%) (Fig. 6B–F).

In fact, gel retardation assay was seldom used to prove the siRNA binding affinity of CLs (Han et al., 2008), as the DC-Chol/siRNA electronic interaction was weak (Chen et al., 2004). Ultrafiltration centrifugation method is a common method to evaluate the siRNA binding affinity of CLs (Sato et al., 2008; Chen et al., 2004). As shown in Fig. 7A, more than 90% of siRNA was entrapped at and above the weight ratio of 5 in DC-Chol/DOPE liposomes. PEGylation also reduced the siRNA binding affinity of CLs and PEGylated DC-Chol/DOPE liposomes could entrap >90% of siRNA at and above the weight ratio of 10 (Fig. 7B).

3.3. pDNA and siRNA transfection

The molar ratio of DC-Chol/DOPE played an important role in DC-Chol/DOPE liposomes transfection. Previous studies demon-

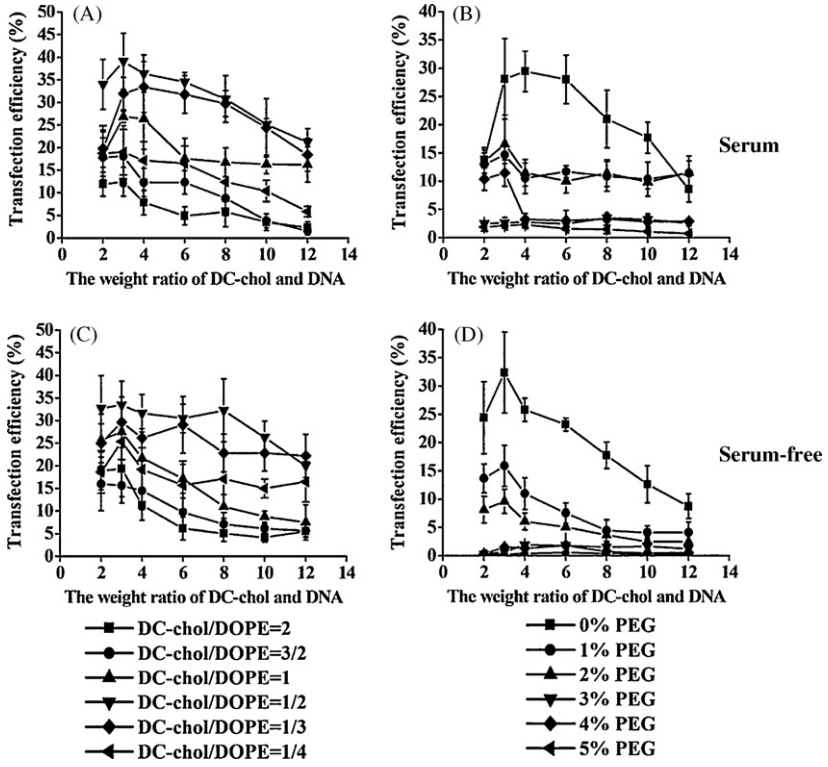

Fig. 8. pDNA transfection assays. SK-BR3 cells were plated at a density of 7.5×10^4 per well overnight. DC-Chol/DOPE liposomes (A and C) or PEGylated DC-Chol/DOPE liposomes (B and D) were complexed with pDNA (a fixed amount of 0.5 μg) at various weight ratios. Then the lipoplex was incubated with SK-BR3 cells for 8 h until fresh culture medium was changed. 48 h later, the cells were trypsinized, washed and analyzed by FCM. For serum-free transfection, the medium was replaced with serum-free culture medium and 8 h after transfection, the medium was changed with culture medium containing serum. Data are presented as mean ± SD ($n = 3$).

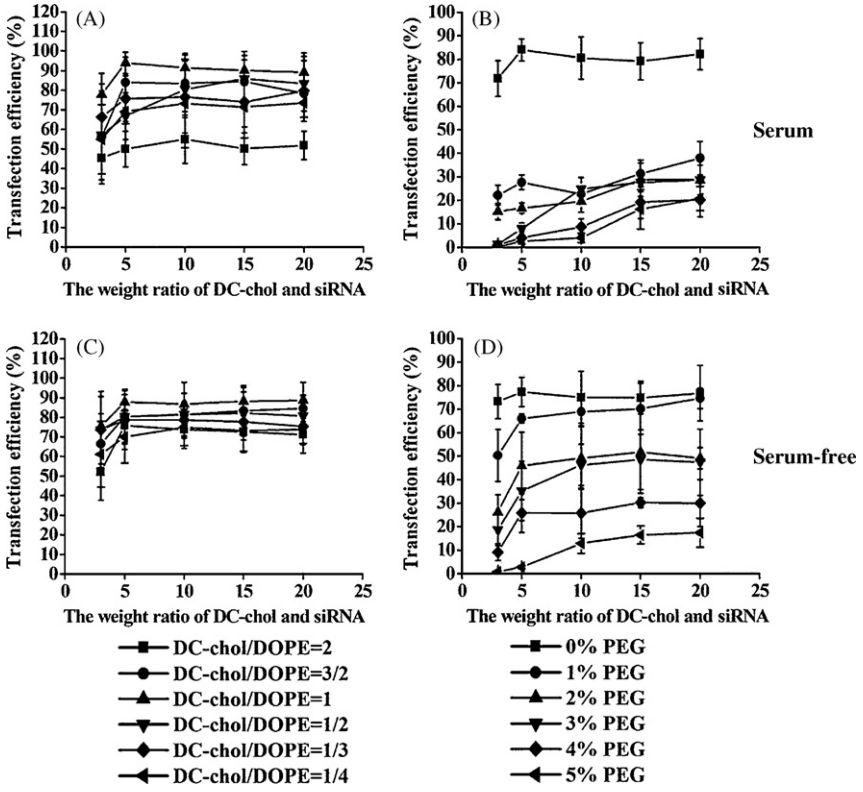

Fig. 9. siRNA transfection assays. SK-BR3 cells were plated at a density of 7.5×10^4 per well overnight. DC-Chol/DOPE liposomes (A and C) or PEGylated DC-Chol/DOPE liposomes (B and D) were complexed with FAM-siRNA (a fixed amount of 0.5 μg) at various weight ratios. Then the lipoplexes were incubated with SK-BR3 cells for 8 h at 37 °C. 8 h later, the cells were trypsinized, washed and analyzed by FCM (flow cytometry). For serum-free transfection, the medium was replaced with serum-free culture medium. Data are presented as mean ± SD ($n = 3$).

strated that a 3:2 or 1:1 molar ratio of DC-Chol/DOPE liposomes resulted in high transfection efficiency (Farhood et al., 1995; Maitani et al., 2007). A set of experiments was performed over a DC-Chol/DOPE molar ratio from 2 to 1/4 (2, 1, 1/2, 1/3, 1/4). The results showed that the most efficient DC-Chol/DOPE liposomes for pDNA transfection were at a 1:2 molar ratio (Fig. 8). When the DC-Chol/DOPE molar ratio was decreased from 1/2 to 1/4 or increased from 1/2 to 2, the pDNA transfection efficiency gradually decreased without exception. siRNA transfection showed the highest transfection efficiency at the DC-Chol/DOPE molar ratio of 1. Similarly, the siRNA transfection efficiency gradually decreased when the DC-Chol/DOPE molar ratio was decreased from 1 to 1/4 or increased from 1 to 2 (Fig. 9).

It is urgent to investigate the effect of PEGylation on DC-Chol/DOPE liposomes transfection. In pDNA transfection, when the PEGylation degree was or over 3%, transfection efficiency was below 5% in most cases (Fig. 8B and D). Also, PEGylation decreased siRNA transfection efficiency in a PEG dose-dependent manner (Fig. 9B and D). The results showed that PEGylation severely decreased pDNA or siRNA transfection efficiency.

The effect of the weight ratio of DC-Chol/pDNA or siRNA on transfection was also investigated. As shown in Fig. 8, increased weight ratio did not lead to higher pDNA transfection efficiency. For most transfection, the most pDNA efficient transfection occurred at the weight ratio of 3. In contrast, increased DC-Chol/siRNA weight ratio resulted in increased siRNA transfection

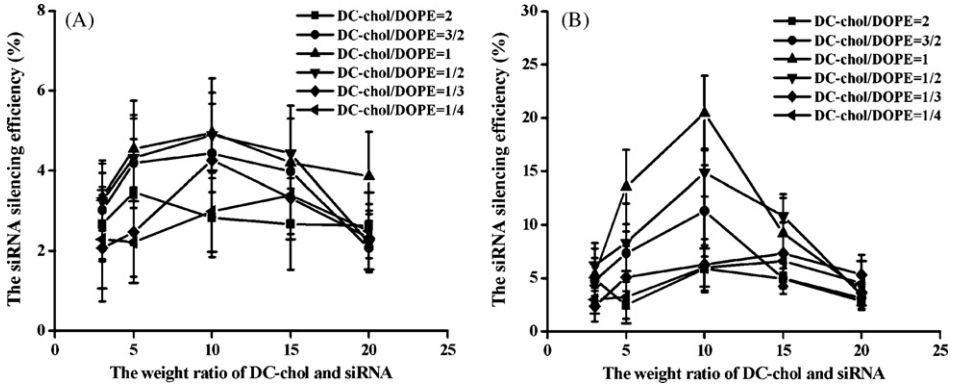

Fig. 10. siRNA silencing efficiency in the presence (A) or absence (B) of serum. The siRNA silencing efficiency (%) was calculated using the following formula: (the HER2 MFI of cells treated with NC siRNA − the HER2 MFI of cells treated with anti-HER2 siRNA)/the HER2 MFI of cells treated with NC siRNA × 100%. The detailed procedure was described in Section 2.9. Data are expressed as mean ± SD ($n = 3$).

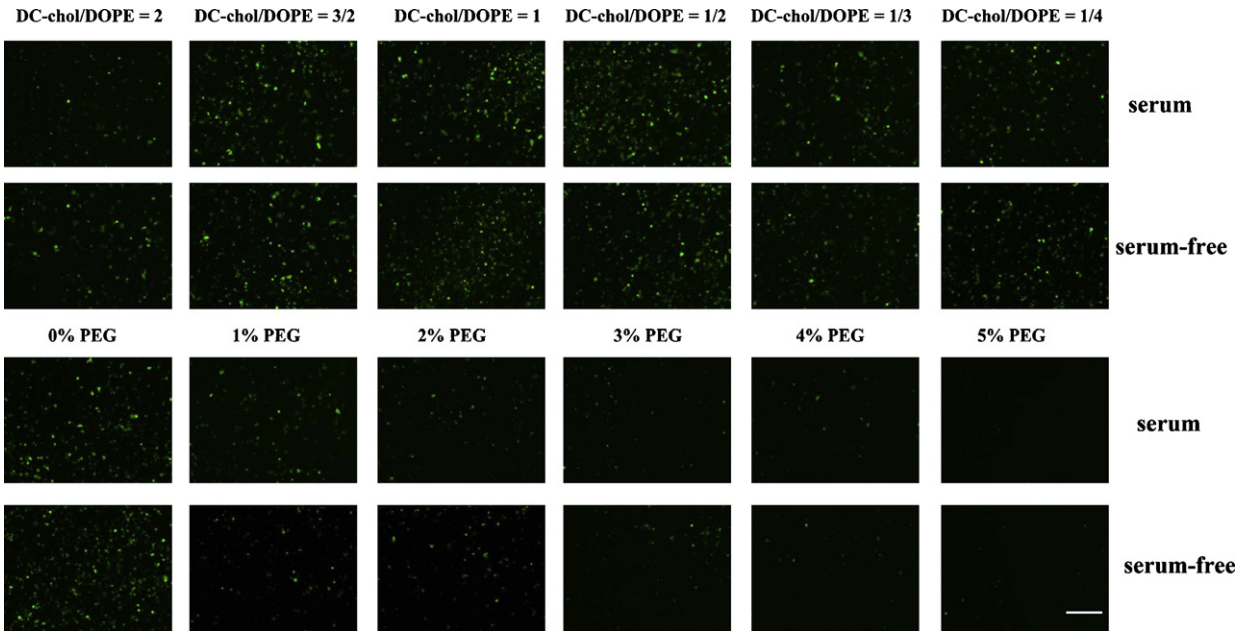

Fig. 11. The representive GFP fluorescence images of Fig. 8. The GFP green fluorescence was detected by a fluorescence microscope (Olympus, Tokyo, Japan). Bar represents 100 μm.

efficiency, reaching plateau when the weight ratio was over 5 or 10 (Figs. 9 and 10).

Serum has been shown to dimish or even completely eliminates CLs-mediated gene delivery (Zuhorn et al., 2002). It is necessary to evaluate the effect of serum on gene transfection. No significant difference was found in serum-containing or serum-free pDNA transfection, suggesting serum have little effect on DC-Chol/DOPE liposomes-mediated pDNA transfection (Fig. 8). As shown in Fig. 9, most DC-Chol/DOPE liposomes-mediated siRNA transfection was not effected by serum, except the liposomes at the DC-Chol/DOPE molar ratio of 2/1, whose transfection efficiency was slightly impaired (Fig. 9A and C). However, the serum-containing transfection efficiency of 1–5% PEG DC-Chol/DOPE liposomes abruptly decreased to below 40% (Fig. 9B), whereas the serum-free transfection efficiency of PEGylated DC-Chol/DOPE liposomes slowly decreased in a PEG dose-dependent manner (Fig. 9D). The representive GFP green fluorescence images of pDNA transfection and Cy3 red fluorescence images of siRNA transfection were shown in Figs. 11 and 12.

The siRNA silencing efficiency was also demonstrated by HER2 expression analysis by FCM. As shown in Fig. 10, in the serum-free transfection, the siRNA silencing efficiency of DC-Chol/DOPE liposomes was <25%. However, the siRNA silencing efficiency of DC-Chol/DOPE liposomes was <6% in the serum-containing transfection. All PEGylated (1–5%) DC-Chol/DOPE liposomes did not show any siRNA silencing efficiency (data not shown, because the siRNA silencing efficiency did not exist).

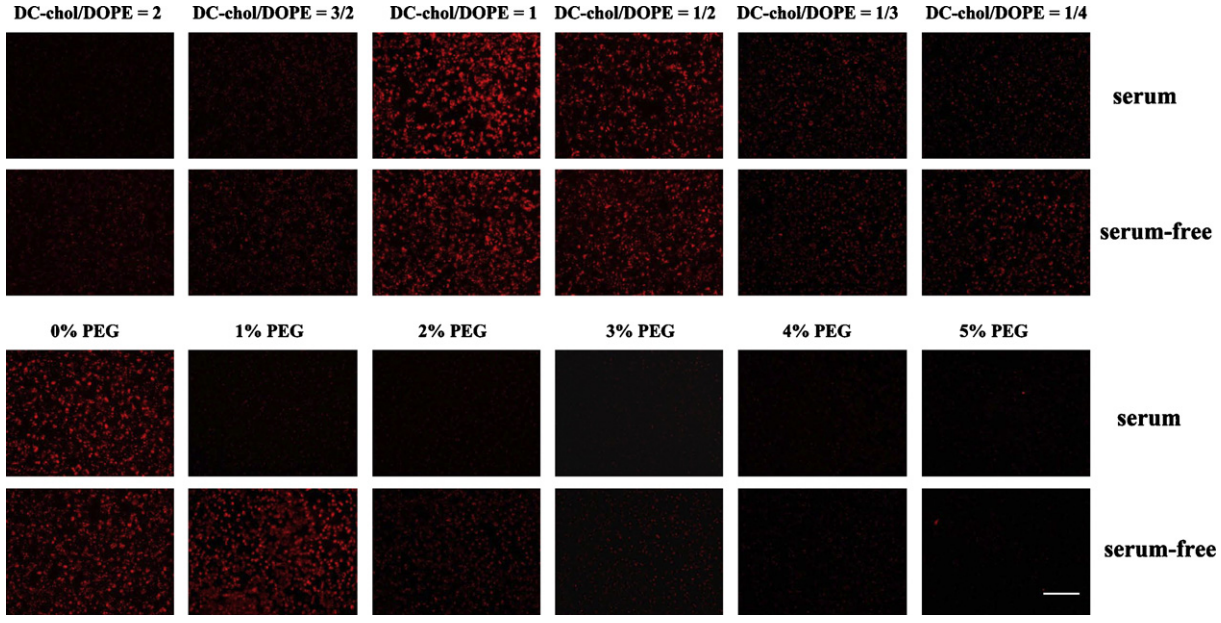

Fig. 12. The representive Cy3 fluorescence images of Fig. 9. The Cy3 red fluorescence was detected by a fluorescence microscope (Olympus, Tokyo, Japan). Bar represents 100 μm. Cy3 fluorescence images. Bar represents 100 μm.

4. Discussion and conclusion

DC-Chol/DOPE liposomes have been classified as one of the most efficient vectors for gene transfection in preclinical experiments and clinical trials (Maitani et al., 2007; Igarashi et al., 2006; Wasungu and Hoekstra, 2006). There is an urgent need to examine the effect of the molar ratio of DC-Chol and DOPE, PEGylation and serum on the pDNA and siRNA transfection efficiency of DC-Chol/DOPE liposomes. Furthermore, the transfection mechanism of pDNA and siRNA was quite different, so the listed above influence factors (DC-Chol and DOPE, PEGylation and serum) may have different effect on pDNA and siRNA transfection.

Gel retardation assay demonstrated that the gel could completely retard pDNA at the DC-Chol/pDNA weight ratio of 2, at which ratio the DC-Chol/DOPE liposomes/pDNA complex possessed the largest size. In case of DC-Chol/DOPE liposomes/siRNA complex, gel retardation assay could only completely retard siRNA at a high weight ratio of 7.5, owing to the weak DC-Chol/siRNA electronic interaction. PEGylated DC-Chol/DOPE liposomes could not even retard siRNA at the highest weight ratio of 15, indicating that gel retardation assay may be not a suitable way to confirm the siRNA binding affinity of PEGylated DC-Chol/DOPE liposomes. Furthermore, DC-Chol/DOPE liposomes of the lower DC-Chol/DOPE molar ratio (the DC-Chol quantity was equal) have the stronger pDNA or siRNA binding affinity (Figs. 3 and 4 and Fig. 6A). We hypothesize that DOPE also has weak nucleic acid binding affinity, which is consistent with the previous results (Zuidam and Barenholz, 1998).

As shown in Fig. 9, siRNA transfection efficiency increased and reached a plateau, when the weight ratio of DC-Chol/siRNA increased. We could hypothesize that in the case of siRNA transfection, the siRNA transfection efficiency was positively associated with the weight ratio of DC-Chol/siRNA and siRNA internalization. In contrast, pDNA transfection efficiency decreased along with the increase of the weight ratio of DC-Chol/pDNA. The difference between pDNA and siRNA transfection lead to the conclusion that pDNA and siRNA possess different transfection mechanisms. When the weight ratio of DC-Chol/siRNA increased, the quantity of liposomes/siRNA complex would increase, resulting in more internalization and more efficient transfection. However, in pDNA transfection, although more liposomes/pDNA complex was internalized when the weight ratio of DC-Chol/pDNA increased, endosomal escape, which represents a major barrier to efficient transfection, would not increase. Even, when the weight ratio of DC-Chol/pDNA increased, the more compact internalized liposomes/pDNA complex would release fewer pDNA loading, resulting in lower transfection efficiency. Another explanation might be that increased weight ratio of DC-Chol/pDNA may have increased cytotoxicity, which could negatively influence the transfection efficiency.

The molar ratio of DC-Chol/DOPE also plays an important role in gene transfection of DC-Chol/DOPE lipsomes. The siRNA transfection results in DC-Chol/DOPE liposomes showed the maximized lipoplex internalization occurred at the molar ratio of 1/1. However, the DC-Chol/DOPE liposomes at a DC-Chol/DOPE molar ratio of 1/2 showed the highest pDNA transfection efficiency. The difference in siRNA and pDNA transfection was attributed to that the endosomal escape and the passage through the membrane of the nucleus is essential for plasmid pDNA but not for siRNA. It is reported that DOPE profoundly affects the polymorphic features of lipoplex in that it may promote the transition from a lamellar to a hexagonal phase, which could facilitate liposomes endosomal escape and enhance transfection efficiency (Morille et al., 2008; Wasungu and Hoekstra, 2006). Our results further confirmed DOPE plays an important role in pDNA transfection.

Our results showed that PEGylation could reduce the size and the surface charge density of DC-Chol/DOPE lipsomes (Figs. 1 and 2). Furthermore, compared with non-PEGylated liposomes, PEGylated DC-Chol/DOPE liposomes showed remarkable reduction in siRNA and pDNA transfection efficiency, which is consistent with the results reported previously (Knorr et al., 2008; Oupicky et al., 2002).

Serum has been found to inhibit gene delivery. Serum proteins may block liposomes/pDNA complex association with cell membranes and reduce their ability to aggregate at the membrane (Ross and Hui, 1999). However, the presence of serum proteins is inescapable in vivo, and is desirable in vitro because it allows for increased cell survival and lowered lipoplex-associated toxicity. The pDNA or siRNA transfection efficiency was not significantly affected by the serum indicating that our prepared DC-Chol/DOPE liposome was stable and exhibited efficient transfection efficiency in the presence of serum. However, the siRNA silencing efficiency of DC-Chol/DOPE liposomes was significantly impaired by the serum (Fig. 10). Furthermore, all PEGylated (1–5%) DC-Chol/DOPE liposomes did not show any siRNA silencing efficiency, indicating PEGylation significantly impaired the siRNA silencing efficiency of DC-Chol/DOPE liposomes.

In conclusion, our results elucidated the influence factors of pDNA and siRNA transfection efficiency in DC-Chol/DOPE liposomes and examined the different transfection mechanisms of pDNA and siRNA. The comprehension of such mechanisms, will lead to the design of better adapted DC-Chol/DOPE liposomes for gene therapy applications.

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