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Liposomes modified with polycation used for gene delivery: Preparation, characterization and transfection *in vitro*

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Abstract

Gene therapy provides great opportunities for treating diseases from genetic disorders, infections and cancer. The development of efficient and safe gene transfer systems could be one of the most important factors for successful gene therapy. In the present study, an amphiphilic compound, polyethylenimine (PEI, MW 800)-cholesterol (PEI 800-Chol), firstly designed to modify the surface of liposomes, was synthesized. Polycation liposomes (PCLs) composed of soybean phospholipids (SPL), cholesterol (Chol) and PEI 800-Chol were prepared using film hydration method. The mean particle size of the PCLs was 133.0 nm and the zeta potential was 50.1 ± 2.6 mV. Due to the PEI anchored onto the surface of liposomes, higher buffering capacity of PCLs was observed, indicating the potential for buffering in the acidic pH environment of the endosomes. Compared to Lipofectamine™2000, PCLs have equivalent transfection efficiency with significantly low cytotoxicity. Interestingly, the transfection activity of PCLs was not influenced in the presence of serum. Furthermore, we constructed another PCL composed of PEI 800-Chol and DOPE, and transfection efficiency increased notably. In conclusion, the PCLs described in this study have high transfection efficiency with low cytotoxicity, as well as the protection ability from serum, which suggests PCLs would be a potential non-viral gene delivery system.

Keywords: Polycation; Liposomes; Gene delivery vector

1. Introduction

Gene therapy has become the research focus for many laboratories in pharmacy, medicine, biochemistry and chemical engineering. However, the growing potential of gene therapy for both genetically based and infectious diseases will not achieve its goals until the issue of gene delivery has been resolved (El-Aneed, 2004). The success of gene therapy is predicated on the development of gene transfer vectors that are safe and efficacious. Non-viral vectors have been paid more attention due to safety concerns with viral vectors (Kim et al., 1998; Cho et al., 2001; Nakamura et al., 2006). Among the non-viral vectors, cationic polymers (Park et al., 2005; Lee and Kim, 2005; Neu et al., 2005) and cationic lipids (Ciani et al., 2004; Kim et al., 2004) are mostly investigated for gene delivery. Cationic lipid-based liposomes have better biocompatibility and are quite effective for the delivery of DNA into the cytosol through the endosomal pathway (Harashima et al., 2001), while cationic polymers, such as polyethlenimine (PEI), can condense DNA efficiently and are more stabile (Zhang et al., 2004). In addition, it is believed that PEI can escape from endosome through the proton-sponge mechanism (Pack et al., 2005) and facilitate gene entry into the nucleus (Godbey et al., 1999).

In our previous works, we have successfully constructed solid-core liposomes for efficient drug loading and controlled release (Huang et al., 2005) and applied liposomes for anti-cancer agent intracellular delivery (Zhao et al., 2006). We also used non-ionic surfactants to modified the surface of cationic liposomes, which was effective for gene transfer (Huang et al., 2006). Kim and his group (Han et al., 2001; Lee et al., 2003) have reported a water-soluble lipopolymer (PEI-Chol) for gene delivery which has efficient transfection *in vitro* and *in vivo*. Based on our research, we presumed polymers combining with liposomes would increase the DNA loading, the affinity with cells and transfection efficiency. Therefore, we firstly designed the novel polycation liposomes (PCLs) constructing from polyethylenimine (PEI, MW 800)-cholesterol (PEI 800-Chol), which combined the advantage of both cationic

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polymers and cationic lipids. For this report, PCLs were shown lower cytotoxicity and higher transfection efficiency, especially not influenced in the presence of 10% serum. These characteristics are favorable for *in vivo* application of gene delivery systems.

2. Materials and methods

2.1. Materials

Polyethylenimine (PEI, MW 800) and cholesteryl chloroformate were purchased from Aldrich Chemical Co., Ltd. (Milwaukee, WI, USA). Sulforhodamine B (SRB) and cholesterol were purchased from Sigma (St. Louis, MO, USA). Soybean phospholipids (SPL) and dioleoylphosphatidylethanolamine (DOPE) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Lipofectamine^{TM}2000 was purchased from Invitrogen Corporation (CA, USA). Dulbecco's modified eagle's medium (DMEM) and trypsin were purchased from Gibco BRL (Gaithersberg, MD, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Co., Ltd. (Hangzhou, China). All other chemical were analytical grade.

2.2. Cell lines

HeLa (Human cervical adenocarcinoma cell line) cells were obtained from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in DMEM medium containing 10% FBS at 37 °C and humidified 5% CO_2.

2.3. Plasmid DNA

Plasmid DNA (pEGFP-N1) was kindly provided by Institute of Infectious Diseases, Zhejiang University (Hangzhou, China). The plasmid DNA was amplified using Escherichia coli DH5α and purified using V-gene Ultrapure Plasmid DNA Midi-prep Kit (Axygene Biotechnology Limited, Hangzhou, China).

2.4. Synthesis and characterization of PEI 800-Chol

PEI 800-Chol was synthesized as described previously by literature (Han et al., 2001) with some modifications. Briefly, PEI (MW 800) was stirred on ice in a mixture of anhydrous methylene chloride and triethylamine. Cholesteryl chloroformate dissolved in anhydrous methylene chloride was slowly added to the PEI solution. The mixture was stirred for 12 h on ice and the resulting product was dried on a rotary evaporator. The powder was dissolved in 0.1 N HCl, and then the solution was extracted with methylene chloride for several times. The white powder product was obtained by lyophilizing.

The structure of product was analyzed by using 400-MHz ^{1}H nuclear magnetic resonance (Ae-80, Brnker, Germany) and product was stored at −20 °C until used.

2.5. Preparation of PCLs

PCLs were prepared according to the method we previously reported (Huang et al., 2006): SPL, cholesterol, PEI 800-Chol (50:45:5 as a molar ratio) were dissolved in chloroform, dried under vacuum to form a thin film, and then hydrated by double distilled water. The suspension was sonicated and the resulting liposomes were extruded through 0.22 μm filter.

PEI 800-Chol/DOPE liposomes (PEI 800-Chol and DOPE, 50:50, mol/mol) and convention liposomes (SPL and cholesterol, 65:35, mol/mol) were prepared with the same method.

2.6. Preparation and characterization of complexes

PCLs/pEGFP-N1 complexes were prepared by mixing equal volumes of the PCLs and pEGFP-N1 at various N/P ratios (PEI nitrogen per DNA phosphate), and incubated for 20 min at room temperature before use. Convention liposomes/pEGFP-N1 and Lipofectamine^{TM}2000/pEGFP-N1 complexes were prepared as the same method.

The mean particle size and zeta potential of PCLs and PCLs/pEGFP-N1 complexes were determined by laser diffraction spectrometry (Malvern Zetasizer 3000HS, Malvern, UK).

2.7. Determination of buffer capacity

The ability of PCLs to buffer at the drop in pH from 10 to 2 was determined by acid–base titration as described by Wang et al. (Wang et al., 2002). PEI 800 solution, liposomes and PCLs were prepared (the concentration of PEI 800 and PEI 800-Chol was 0.3 mg/ml, 30 ml), the pH raised to 10 using 1 M NaOH, and then titrated with 0.1 N HCl, the volumes of HCl consumed were recorded. The pH was measured at the same time using a pH-meter.

2.8. Cytotoxicity assay

Cytotoxicity of PCLs was studied using a SRB assay (Skehan et al., 1990). HeLa cells were seeded at a density of 5×10^3 cells/well in 96-well plates. After 24 h, fresh DMEM medium without 10% FBS was replaced and PCLs with different amount were added and then incubated for 24 h in CO_2 incubator. After incubation, medium was aspired off and 200 μl PBS was added to each well. Cold (4 °C) 50% trichloroacetic acid (TCA, 50 μl) was added to produce a final TCA concentration of 10%. Plates were left for 30 min at 4 °C and subsequently washed five times with double distilled water. Plates were then dried at room temperature for at least 24 h. SRB (0.4%) in 1% acid solution was added (70 μl/well) and left for 20 min at room temperature. SRB was removed and the plates were washed five times with 1% acetic acid followed by air drying. Bound SRB was dissolved with 200 μl 10 mM unbuffered Tris-base solution and the plates were shaken for 15 min. The absorbance was read at 570 nm in microplate reader (ELx 800, Bio-Tek, USA).

Convention liposomes and Lipofectamine^{TM}2000 were used as control groups.

2.9. Transfection efficiency of PCLs in HeLa cells

HeLa cells (1×10^5 cells/well) were seeded in 24-well plates and culture in DMEM medium with 10% FBS overnight. Before transfection experiment, the medium was replaced with fresh DMEM medium with or without 10% FBS. PCLs/pEGFP-N1 complexes (1 μg pEGFP-N1/well) with different N/P ratios (5, 10, 20, 30) were added to each well followed by 6 h incubation at 37 °C in CO_2 incubator. Thereafter the medium was replaced with DMEM containing 10% FBS, and the incubation was continued for additional 24 h. Inverted fluorescent microscope (HBO 100, Zeiss, Germany) and flow cytometry (Beckman Coulter, USA) were used to evaluate the transfection efficiency of the complexes. Liposomes/pEGFP-N1 complexes and Lipofectamine^{TM}2000/ pEGFP-N1 complexes were negative and positive control, respectively.

2.10. Statistical analysis

Statistical analyses were performed using Student's *t*-test.

3. Results and discussion

3.1. Synthesis of PEI 800-Chol

Different molecular weight of PEI was used for PEI-Chol synthesis in our previous work. However, the viscosity of high molecular weight PEI (25 kDa) caused difficulty for synthesis and purification of product. In addition, liposomalization may concentrate polycations on the surface of liposomes, polycations with high molecular weight might not be required (Yamazaki et al., 2000). Thus, PEI-Chol was synthesized using branched PEI of 800 Da and cholesteryl chloroformate.

The NMR results were as follows (Fig. 1C): ^{1}H NMR (400 MHz, $CDCl_3$) δ ~0.693 ppm (H of CH_3 from cholesterol (a)); δ ~0.904 ppm (H of $(CH_3)_2$ from cholesterol (b)); δ ~0.919 ppm (H of CH_3 from cholesterol (c)); δ ~1.072 ppm (H of CH_3 from cholesterol (d)); δ ~1.216–2.021 ppm (H from CH_2–CH_2 and $CHCH_2$ from cholesterol (e)); δ ~5.357 ppm (H of =CH–C from cholesterol (f)); δ ~2.551–3.475 ppm (H of CH_2 from cholesterol (g)); δ ~4.457 ppm (H of =C=CHC from cholesterol (h)); δ ~2.309 ppm (H of CH_2CH_2 from backbone of PEI (i)). The NMR results indicated that cholesterol has conjugated to the backbone of PEI.

3.2. Characteristics of PCLs and PCLs/pEGFP-N1 complexes

The mean diameter of PCLs was 133.0 nm and zeta potential was 50.1 ± 2.6 mV. When N/P ratio of PCLs/DNA complexes increased from 2.5 to 40, zeta potential notably increased from −17.2 to 42.7 mV. On the other hand, mean particle sizes decreased from 325.9 to 152.4 nm (Fig. 2). The results indicated that PEI modified on the surface of liposomes could efficiently condense DNA to form complexes.

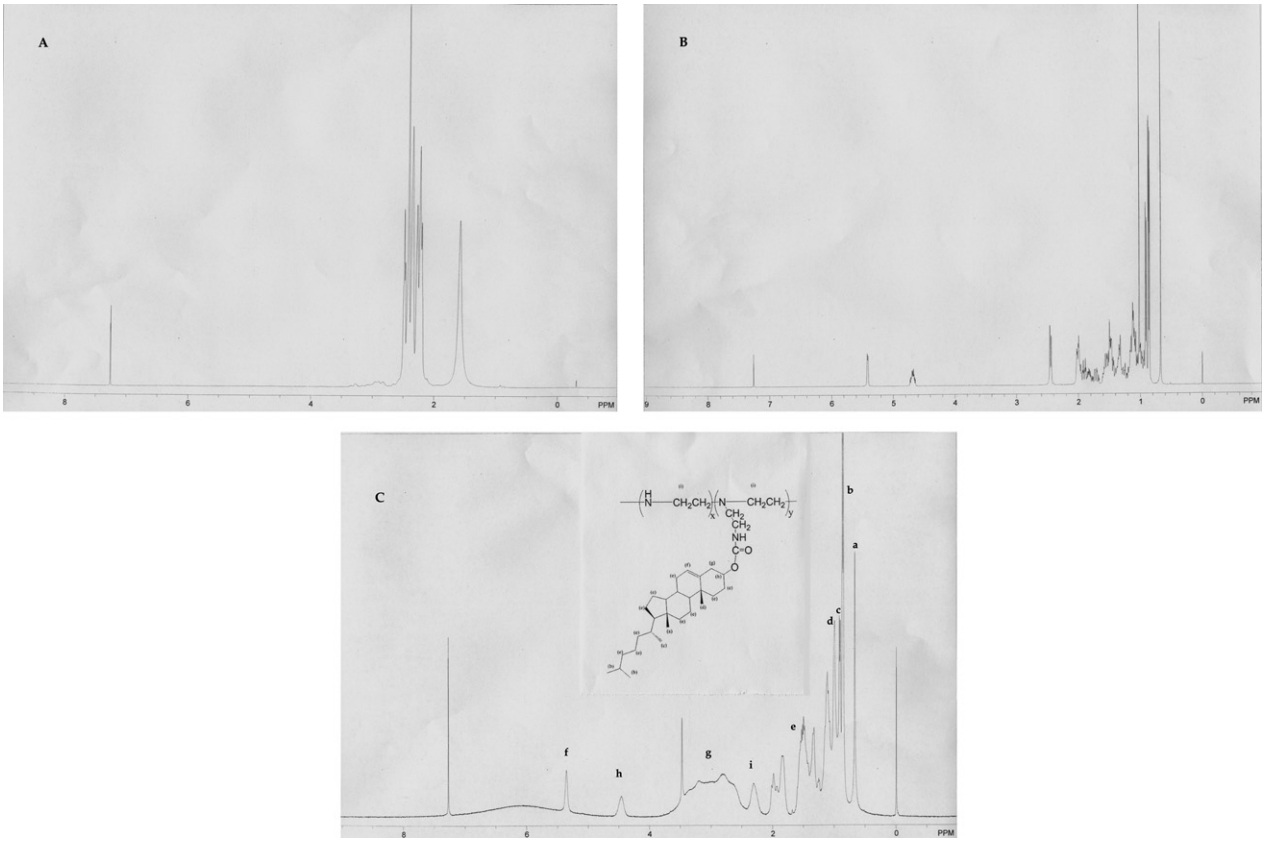

Fig. 1. The ^{1}H NMR characterization for PEI 800 (A), cholesteryl chloroformate (B) and PEI 800-Chol (C).

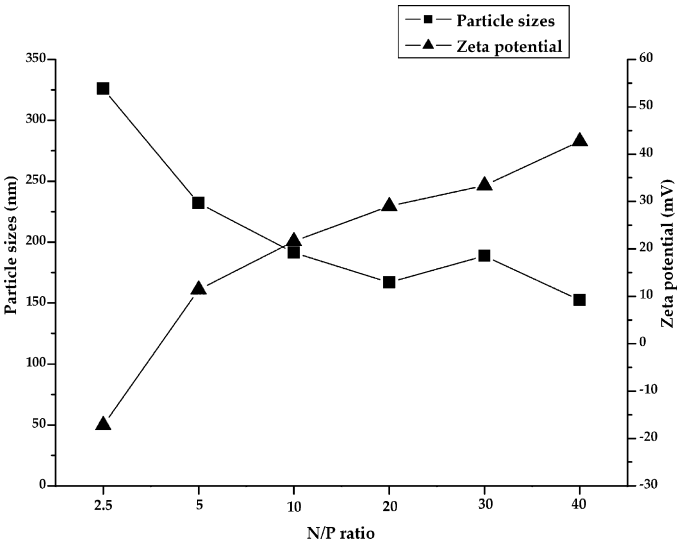

Fig. 2. The mean particle sizes and zeta potential of PCLs/pEGFP-N1 complexes with various N/P ratios.

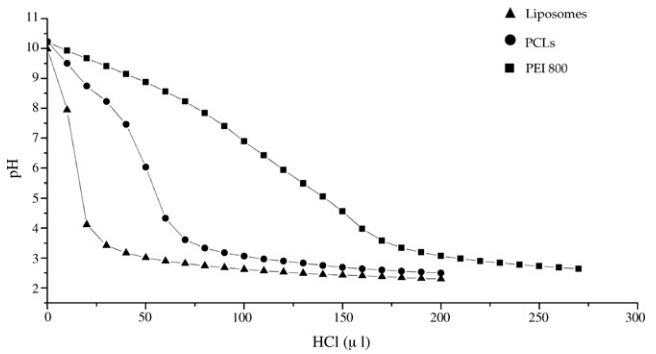

Fig. 3. Determination of the buffer capacity of liposomes, PCLs and PEI 800 by acid–base titration.

3.3. Buffer capacity of PCLs

Molecules entering cells on endocytic pathways will experience a drop in pH from neutral to 5.0 (Maxfield and McGraw, 2004), and lysosomal degradation of therapeutic genes was one of the main cellular barriers to effective gene transfer (Wiethoff and Middaugh, 2003). The "proton sponge" nature of PEI is thought to lead to buffering inside endosomes. The additional pumping of protons into the endosome, along with the concurrent influx of chloride ions to maintain charge neutrality, increases ionic strength inside the endosome. This is then thought to cause osmotic swelling and physical rupture of the endosome, resulting in the escape of the vector from the degradative lysosomal trafficking pathway (Akinc et al., 2005).

The environment of endosomes was simulated to assess the capacity of PCLs to buffer the pH. The acid–base titration profiles obtained for liposomes, PCLs and PEI 800 were shown in Fig. 3. The results showed that PEI modified onto the surface of liposomes still has considerable buffer capacity as compared with convention liposomes. It is indicated that PCLs could swell and disrupt the endosome through the proton-sponge mechanism.

3.4. Cell viability

Sulforhodamine B (SRB) protein staining has been widely used for cell proliferation and chemosensitivity testing, substituting for tetrazolium-based assays (Papazisis et al., 1997). The results of SRB assay were relatively stable and not influenced by some factors, such as detecting time which induce the fluctuation of data by MTT assay. Thus, in this study, SRB assay was used to investigate the cytotoxicity of liposomes. No significant difference in cytotoxicity was found between PCLs and Lipofectamine^{TM}2000 at the low lipid concentration; with the increasing lipid concentration, PCLs and Lipofectamine^{TM}2000 showed cytotoxicity. However, compared with Lipofectamine^{TM}2000, the cytotoxicity of PCLs was notably lower (Fig. 4A), which indicated more extensive dosage range of PCLs for gene transfer could be selected. Fig. 4B also showed that the IC_{50} of PCLs against HeLa cells was ~12.6-fold

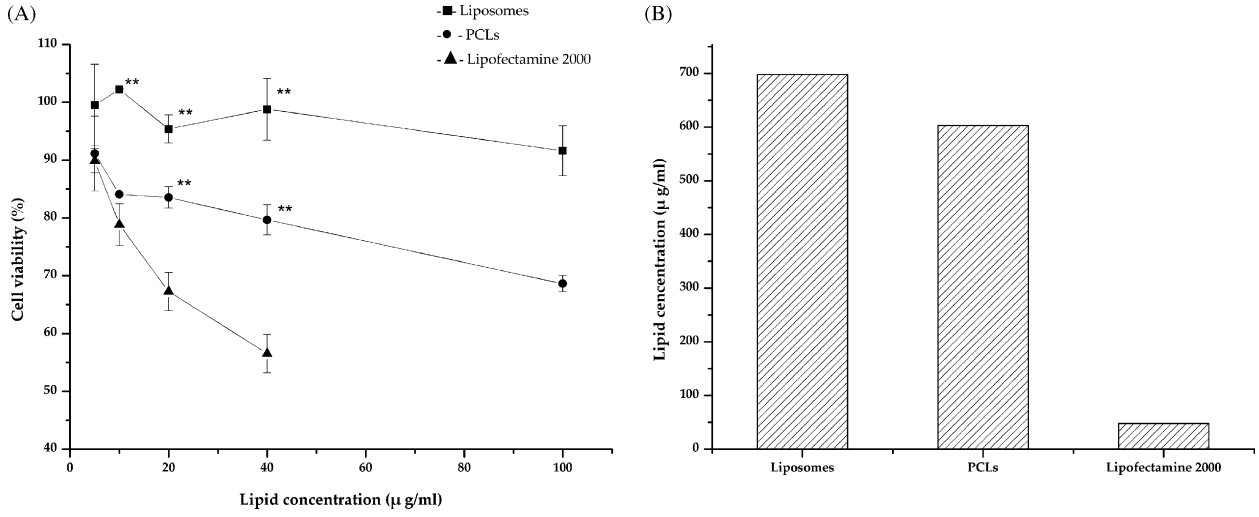

Fig. 4. The cell viability (A) and the IC_{50} (B) of liposomes, PCLs and Lipofectamine^{TM}2000. $^{**}p < 0.01$ indicates significant difference when compared with Lipofectamine^{TM}2000.

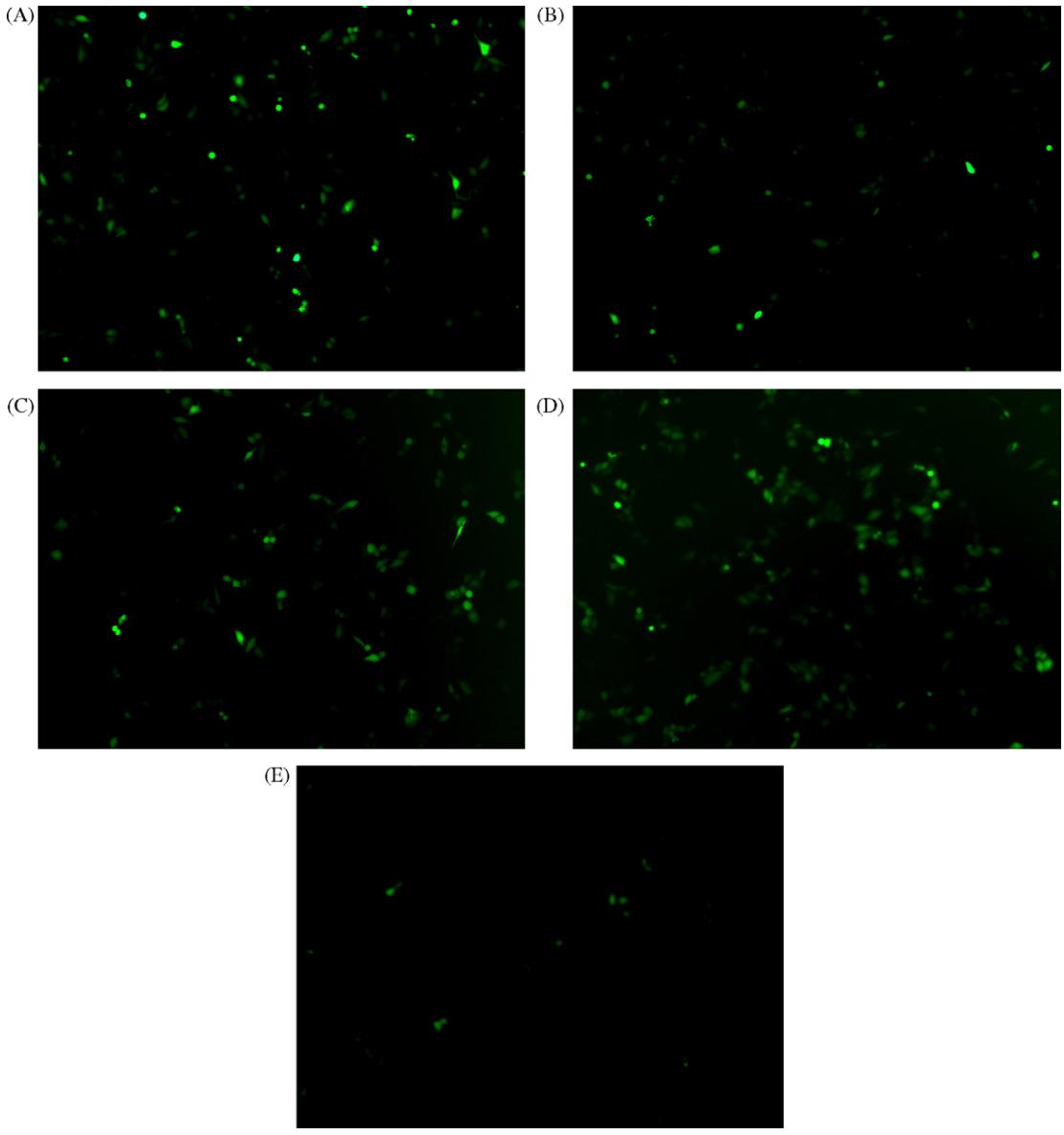

Fig. 5. Fluorescence image observations for GFP expression in HeLa cells with mediated by Lipofectamine^{TM}2000 (4 μg) (A, B); PCLs with N/P ratio of 10 (C, D); liposomes (E). (A, C and E) In the absence of serum; (B and D) in the presence of serum. (pEGFP-N1 1 μg/well).

higher than that of Lipofectamine^{TM}2000, while little difference was found between PCLs and convention liposomes.

3.5. *In vitro transfection*

PCLs/pEGFP-N1 complexes at different N/P ratios (N/P = 5, 10, 20, 30) were evaluated for their transfection efficiency in HeLa cells by using fluorescence microscope. Both liposomes/pEGFP-N1 complexes and Lipofectamine^{TM} 2000/pEGFP-N1 complexes were also used as control. Weak fluorescence was observed when pEGFP-N1 mediated by liposomes (Fig. 5E) and Lipofectamine^{TM}2000 showed high transfection activity (Fig. 5A). The N/P ratios were important to the transfection efficiency of PCLs/pEGFP-N1 complexes. In our study, the highest green fluorescent protein (GFP) expression mediated by PCLs was observed at N/P ratio of 10 (Fig. 5C), and GFP expression was slightly reduced below or upon that N/P ratio (data not shown). Considering the results of mean particle size and zeta potential of complexes (Fig. 2), it is suggested that larger particle size and insufficient surface potential of complexes at low N/P ratios resulted to lower GFP expression. While at high N/P ratios, too strong interaction between PCLs and DNA might prevent the release of the DNA in the cytoplasm (De Smedt et al., 2005).

It is well known that the transfection efficiency of conventional cationic liposomes is suppressed in the presence of serum. In order to evaluate the effect of serum to transfection, the transfection efficiency of PCLs in the absence or presence of serum was investigated. As shown in Fig. 5, Lipofectamine^{TM}2000 showed high GFP expression in the absence of serum (Fig. 5A), though expression was suppressed markedly by serum (Fig. 5B).

In contrast, the transfection efficiency of PCLs was almost the same, even slightly increased in the presence of serum (Fig. 5C and D). PCLs/pEGFP-N1 complexes at other N/P ratios also showed the same results (data not shown).

It is reported that cationic polymer/DNA complexes are more stable than cationic lipids (De Smedt et al., 2000), and cationic polymers are able to condense more DNA than lipids (Gao and Huang, 1996). The transfection results of PCLs in the presence of serum revealed that PEI modifying to the surface of liposomes might increase the stability of PCLs and enhance their transfection efficiency. These results agreed with those reported by Yamazaki (Yamazaki et al., 2000) and Garcia (Garcia et al., 2007), whereas the exact mechanism was still not clear. Though Yamazaki et al. supposed PCLs and DNA form smaller and rather homogeneous aggregates in the presence of serum than that in the absence of serum, no special data was reported (Yamazaki et al., 2000). It is found in our work that the size of PCLs/pDNA complexes is increased and the zeta potential is decreased in the present of serum (data not shown). Therefore, we presumed that the factors which affect the transfection efficiency of PCLs in the serum may be complicated. Further investigations are still under way.

Moreover, we combined PEI and DOPE to form a novel gene carrier for enhancing the gene expression efficiency of PCLs constructed. A key cellular barrier impeding the transfection efficiency of non-viral gene vectors is the inefficient release of endosomally trapped DNA into the cell cytosol (Singh et al., 2004). The relatively high gene-transfer activity of PEI is believed to be due in large part to efficient endocytic pathway through the proton-sponge mechanism (Pack et al., 2005). On the other hand, DOPE is an important co-lipid which is widely used for preparing cationic liposomes. A transition from the lamellar lipoplex phase into a non-lamellar structure under acidic pH, which may facilitate fusion with or destabilization of target membranes, in particular endosomal membranes is observed (Almofti et al., 2003). In the previous work, we constructed cationic liposomes with DOPE and DC-Chol and found them entering the cells through two pathways, endocytosis and cytomembrane fusion (Huang et al., 2006). We presumed that addition of DOPE to the PCLs would improve transfection efficiency.

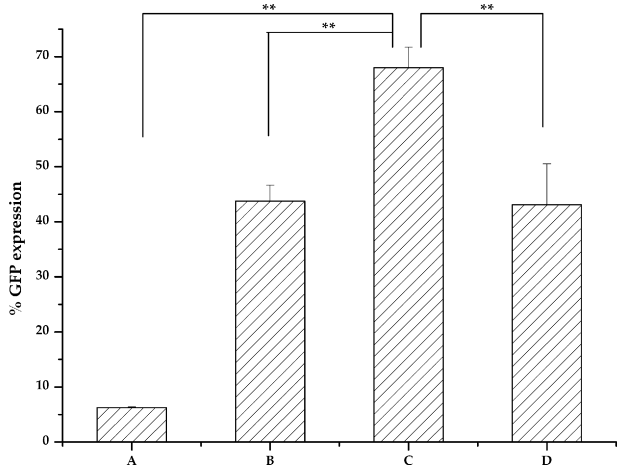

Fig. 6. The percentage of GFP expression determined by flow cytometry after pEGFP-N1 transfection in HeLa cells mediated by (A) liposomes, (B) PCLs (SPL, Chol and PEI 800-Chol; N/P = 10), (C) PCLs (PEI 800-Chol and DOPE; N/P = 40) and (D) Lipofectamine^{TM}2000 (4 μg). $^{**}p < 0.01$ (pEGFP-N1 1 μg/well).

Therefore, we design another PCL composed of PEI 800-Chol and DOPE with equal molars. Flow cytometry was used to quantitate the transfection efficiency of PCLs (PEI 800-Chol and DOPE) (Fig. 6). The GFP expression percentage of PCLs (PEI 800-Chol and DOPE) was significantly increased in comparison with that of PCLs (SPL, Chol and PEI 800-Chol) and Lipofectamine^{TM}2000. Our results suggested that the protonation of PEI in cooperation with the membrane destabilization of DOPE leads to higher transfection efficiency.

4. Conclusion

The present study has demonstrated that PCLs simply prepared by modifying liposomes with PEI 800-Chol, combined the advantage of both cationic polymers and cationic lipids, and showed high transfection efficiency with low cytoxicity in HeLa cells. Interestingly, the presence of 10% serum did not inhibit the transfection efficiency of PCLs. Furthermore, incorporation of DOPE would remarkably increase the transfection activity of PCLs. Taken together; PCLs would be a promising non-viral gene delivery system for further *in vivo* study.

Acknowledgements

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