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Enhanced siRNA delivery using cationic liposomes with new polyarginine-conjugated PEG-lipid

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ABSTRACT

Gene therapy based on small interfering RNA (siRNA) has emerged as an exciting new therapeutic approach. However, insufficient cellular uptake and poor stability have limited its usefulness. Here, we report efficient delivery of siRNA via the use of cationic liposomes that contain a new PEG-lipid. The new lipid, poly-L-arginine-conjugated polyethylene glycol (PLR–PEG), was synthesized. To confirm the synthesis of the amino acid-conjugated PEG-lipid, ^{1}H NMR and gel permeation chromatography (GPC) were performed. Cationic liposomes as non-viral vectors were formulated using the cationic lipids 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine propane (DOPE), cholesterol (Chol) and PLR–PEG. Physicochemical properties of cationic liposomes were investigated. A GFP siRNA was used as a model siRNA to test the efficiency of cationic liposome-mediated siRNA delivery. The liposomes could enhance delivery efficiency and decrease cytotoxicity at an optimized lipid composition. The new cationic liposome formulation using a new PEG-lipid (PLR–PEG) showed not only enhanced intracellular delivery of siRNA but also decreased cytotoxicity in H4II-E and HepG2 cell lines. The GFP siRNA delivered by new cationic liposomes using PLR–PEG was effective in reducing the GFP protein expression levels of the gene. These results suggest that the new cationic liposomes could be used for efficient delivery of siRNA therapeutics.

1. Introduction

A small interfering RNA (siRNA) is a short double-stranded RNA that contains 21–23 nucleic acids with a 19-nucleotide duplex region. It associates with a nucleic acid–protein complex called RNA-induced silencing complex (RISC) in the RNAi process. siRNA shows a specific and effective gene silencing activity by the selected specific sequence's down-regulation of a complementary messenger RNA (Milhavet et al., 2003). There have been many reports discussing siRNA as a potential therapeutic agent for the treatment of numerous diseases including cancer, genetic disorders and viral infections (Akhtar and Benter, 2007; de Fougerolles et al., 2007). The challenge, however, is to overcome extracellular and intracellular barriers to achieve efficient target cell delivery of siRNA (Pouton and Seymour, 2001). Accordingly, various non-viral delivery systems have been investigated for siRNA delivery.

Several groups have attempted to deliver siRNA using cationic carriers such as cationic liposomes (Kim et al., 2008), chitosan nanoparticles (Liu et al., 2007) and polyethylenimine nanoparticles (Werth et al., 2006). Among non-viral delivery systems, cationic liposomes are the most widely investigated vectors for the delivery of negatively charged gene medicines by formation of charge complexes (Simões et al., 2005). However, the use of a cationic liposome/siRNA complex (siRNA-lipoplex), such as systemic delivery in a clinical situation, is questionable due to poor blood circulation characteristics. Therefore, the surface of the siRNA-lipoplex is frequently modified by a polyethylene glycol (PEG)-conjugated lipid. PEGylation extends circulation time for liposomes, the mechanism of which is hypothesized to be as follows: the PEG on the liposomal surface attracts a water shell, resulting in the reduced adsorption of opsonins and the recognition of liposomes by the cells of a mononuclear phagocyte system (Papahadjopoulos et al., 1991; Torchilin et al., 1994). Therefore, the PEGylation of siRNA-lipoplexes may also enhance the circulation time of the complex and can increase the amount of siRNA in the targeted tissue. Moreover, arginine has similar characteristics to cell-penetrating peptides (CPPs) (Mitchell et al., 2000; Wender et al., 2000). The CPPs contain a number of basic amino acid residues, and can deliver their associated molecules into cells (Derossi et al., 1994; Vivès et al., 1997; Oehlke et al.,

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1998). Thus, arginine-modified vectors with the ability to cross the plasma membrane are anticipated to result in the efficient delivery of siRNA.

Here, we report the synthesis of a new amino acid-conjugated PEG-lipid, poly-L-arginine-conjugated polyethylene glycol (PLR–PEG). Formulation of PLR–PEG-based cationic liposomes incorporated 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE) and cholesterol (Chol). Cationic liposomes containing PLR–PEG showed high siRNA transfection efficiency and low cytotoxicity compared to conventional cationic liposomes, which are not PEGylated.

2. Materials and methods

2.1. Materials

DOTAP, DOPE, Chol, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Poly-L-arginine hydrochloride (PLR) (molecular weight = 13,300 Da) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco® Invitrogen (NY, USA). All other chemicals used in this study were of reagent grade.

2.2. Synthesis of PLR–PEG

The synthesis procedure of PLR–PEG was based on the previously described method (Huang et al., 2002). Briefly, PLR was dissolved in 25 ml of 50 mM sodium tetraborate buffer (STBB, pH 8.5) per gram of PLR. The resulting solution was stirred vigorously for approximately 30 min and subsequently filtered through a 0.22 μm Durapore® membrane (Sterile Millex GV, Sigma–Aldrich, Buchs, Switzerland) into a sterile culture tube. The appropriate stoichiometric amount of DSPE-PEG powder was then slowly added to the solution while it was continuously stirred. After another 6 h of vigorous stirring at room temperature, the solution was transferred to a dialysis tube (Spectr/Por dialysis tubing, M.W.C.O. of 6–8 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, CA). The synthesized product was dialyzed out for 24 h in 10 mM phosphate-buffered saline (PBS, pH 7.0), followed by an additional 24 h of dialysis in deionized water. The product was then freeze-dried for 48 h at −70 °C with a pressure of 0.2 mbar.

The synthesized PLR–PEG was confirmed by ^{1}H NMR in D_2O and gel permeation chromatography (GPC). Aliquots of 0.0300 g polystyrene standards and samples were dissolved in 10 ml tetrahydrofuran (THF) or dimethylformamide (DMF) at room temperature. A 200 ml injection sample loop was filled with polystyrene standards and samples. THF served as an eluent. The flow rate was set at 1.0 ml/min. The two analytical columns were connected in series at 35 °C. The GPC column effluent was monitored at a wavelength of 254 nm using a UV scanning detector.

2.3. Cell lines

The rat hepatoma cells (H4II-E), stabilized to express green fluorescent protein (GFP), were kindly provided from Dr. Kim SK (Chungnam National University, Daejeon, Republic of Korea). The cells were cultured in DMEM in 100 mm Petri dishes in a humidified incubator supplied with 5% CO_2 and maintained at 37 °C for 24 h. All media were supplemented with 10% heat-inactivated fetal bovine serum, 10,000 units/ml of penicillin (Gibco BRL) and 10 mg/ml of streptomycin (Gibco BRL).

2.4. Preparation of cationic liposomes and transfection complexes

Cationic liposomes were prepared by lipid film method. Stock solutions of each lipid in an organic solvent mixture ($CHCl_3$:MeOH = 2:1, v/v) were mixed in the ratio of lipid as presented in Table 1. The total amount of lipid was 20 μmole per 2 ml PBS buffer (pH 7.4). The organic mixture was removed on a rotary evaporator under reduced pressure with the temperature of water bath adjusted to 40 °C. To this lipid film, 2 ml PBS buffer (pH 7.4) was added and hydrated by vigorous vortexing. The resulting suspension of liposomes was passed through an extruder (Northern Lipids Inc., Canada) equipped with double-layered 0.2 μm polycarbonate membrane filters 10 times.

The cationic liposomes were complexed with siRNA at various molar ratios and formation of these complexes was confirmed by a gel retardation assay. The siRNA for the targeted silencing of GFP was purchased from Bioneer Co. (Daejeon, Republic of Korea). The sequence of siRNA was 5′-GCAUCAAGGUGAACUUCAAA-3′ (sense), and 5′-UUGAAGUUCACCUUGAUGC-3′ (antisense).

2.5. Particle size and zeta potential measurements

Particle size of cationic liposomes was determined by light scattering spectrophotometer (ELS-8000, Photal, Japan). The samples were diluted with deionized water, and then transferred into a quartz cuvette in an ELS-8000 dynamic light scattering instrument. Zeta potential of cationic liposomes was measured with an electrophoretic light scattering spectrophotometer. Data were analyzed using a software package (ELS-8000 software) supplied by the manufacturer.

2.6. RNase protection assay of siRNA in complexes

Twenty picomoles of siRNAs were complexed with cationic liposomes and incubated in the presence of 0.1 mg/ml RNase A (Solgent, Daejeon, Republic of Korea) for 30 min at 37 °C. The solutions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The siRNAs were precipitated with ethanol, electrophoresed on agarose gel (2%) and visualized by ethidium bromide staining (Minakuchi et al., 2004).

2.7. In vitro transfection

H4II-E cells were seeded into 6-well plates at a density of 1×10^5 cells/well in 2 ml complete medium. After overnight incubation, the media was removed and replaced with 500 μl serum-free media and the liposome/siRNA complexes equivalent to 20 pmol siRNA at desired N/P ratios were added to each well. After 4 h, the media was replaced with 2 ml fresh medium containing 10% FBS, and incubated for another 24 h or 48 h at 37 °C (Zhang et al., 2008). Commercially available Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was used as a control. All transfection experiments were performed in triplicate.

2.8. Flow cytometry

For comparison of transfection efficiency, mean GFP intensity was measured by flow cytometry. The cells were transfected with liposome/siRNA complexes prepared in basal DMEM and incubated for 24 h. After medium was removed and the cells were washed twice with pre-cold PBS, cells were transferred into a 5 ml polystyrene round-bottom tube (BD Falcon, Bedford, MA, USA) using 200 μl pre-cold fresh medium and maintained at 4 °C for later flow cytometry analysis. GFP mean intensity of H4II-E cells was

recorded in the FL1 channel using a FACScalibur (Becton Dickinson, USA) flow cytometer (Zou et al., 2005).

2.9. Confocal laser scanning microscopy

To visualize the GFP expression in cells, confocal laser scanning microscopy was used. H4II-E cells were grown on 22 × 22 mm coverslips. The cells were transfected with liposome/siRNA complexes prepared in basal DMEM. Control cells were untreated and maintained in basal DMEM. After the transfection for 24 h, the cells were rinsed with PBS and observed with confocal microscopy (Leica TCS NT, Leica Microsystems, Wetzlar, Germany) equipped with a Diode laser and associated filters for simultaneous 488 nm excitation.

2.10. Cytotoxicity

The cytotoxicity of siRNA alone, vehicles, and liposome/siRNA complexes was determined to indicate the viability and proliferation of cells against transfection complexes. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a yellow salt that is reduced in the mitochondria of viable cells to a blue formazan product, which absorbs maximally at 570 nm. H4II-E cells and HepG2 cells were seeded at 1×10^5/ml in 96-well plates. After overnight incubation, the cells were exposed to siRNA or liposome/siRNA complexes containing media for 24 h. The medium was then removed and 100 μl MTT-containing medium (5 mg/ml) was added to the wells. Following 4 h incubation at 37 °C, the MTT-containing medium was carefully aspirated to avoid disturbing any formazan crystals formed and 100 μl MTT solubilization solution was added to each well. Plates were incubated at room temperature for 30 min and optical densities were recorded at 570 nm using a Microplate reader (Sunrise, Tecan Trading, Switzerland). Cell viability was expressed as a percentage of the untreated control cells.

3. Results

3.1. ^{1}H NMR identification and GPC of PLR–PEG

The ^{1}H NMR chemical shifts (in D_2O) were assigned as follows (Fig. 1a); 1.2–1.5 and 1.5–1.8 ppm (m, $-CH_2-$, β–γ carbons of the arginine side chains), 3.15 ppm (t, 2H, $-CH_2-N-$, methoxy PEG linked to arginine), 3.55 ppm (77H, PEG), 4.20 (1H, –N–CHR–COO–). To confirm conjugation ratio and mean molecular weight of DSPE-PEG, poly-L-arginine hydrochloride, PLR–PEG, physical mixture of DSPE-PEG and poly-L-arginine hydrochloride, we used gel permeation chromatography (Fig. 1b). When comparing PLR–PEG with the physical mixture, we confirmed that the conjugation was performed well and mean molecular weight of PLR–PEG was 216,000 Da.

3.2. Particle size and zeta potential of liposomes

The particle sizes of conventional cationic liposomes, PEGylated cationic liposomes and PLR–PEGylated cationic liposomes were determined by light scattering spectrophotometer. The mean diameters of conventional cationic liposomes, PEGylated cationic liposomes and PLR–PEGylated cationic liposomes were 188.7 nm, 143.9 nm and 148.6 nm, respectively. The zeta potential distributions of all of the cationic liposomes were 20–40 mV. Therefore, PEGylation reduced the particle size of liposomes but increased the zeta potential a little.

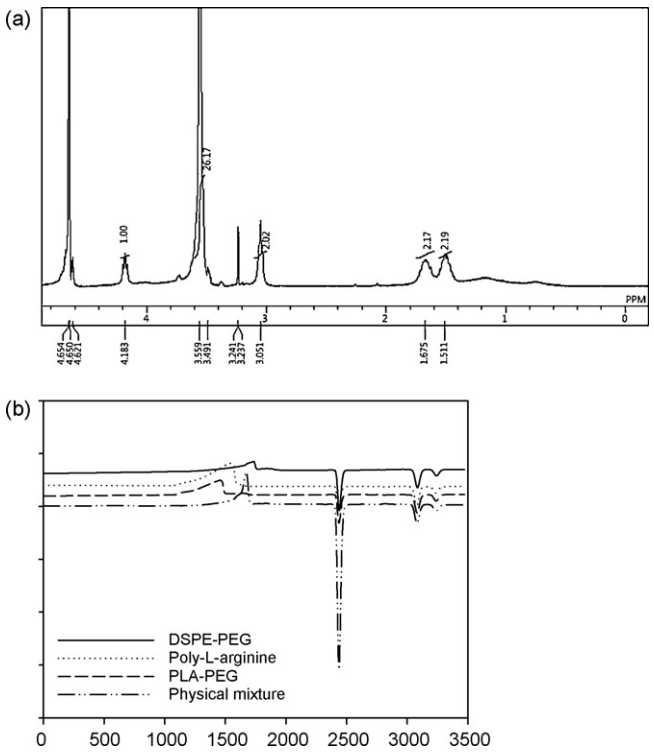

Fig. 1. (a) ^{1}H NMR and (b) gel permeation chromatography of PLR–PEG.

3.3. Complexation of cationic liposomes and siRNA

To confirm the complex formation between cationic liposomes and negatively charged siRNA, we employed an agarose gel retardation assay. PEGylated cationic liposomes showed gel retardation upon complex formation with siRNA. When the complexation molar ratios of total lipids:siRNA were less than 30:1, no retardation of siRNA was observed. However, gel retardation was clearly indicated when the complexation molar ratio of total lipids:siRNA was greater than 30:1 (Fig. 2).

3.4. RNase protection of siRNA/liposome complex

To examine if cationic liposomes formulated with DSPE-PEG blocked degradation of siRNA from nuclease, siRNA alone and liposome/siRNA complex were incubated in the presence of RNase (0.1 mg/ml) for 30 at 37 °C followed by agarose gel electrophoresis. The results indicated that the liposome/siRNA complex showed partial resistance to degradation of siRNA in the presence of nuclease (Fig. 3). Both PEG and liposomes efficiently protected siRNA from RNase.

Table 1
Composition and physicochemical properties of cationic liposomes.

Cationic liposomes	Composition DOTAP:DOPE:Chol:PEG	Particle size (nm)	Zeta potential (mV)
Conventional	75:20:5:0	188.7 ± 19.3	15.4 ± 7.5
PEGylated	70:20:5:5	143.9 ± 7.5	25.3 ± 5.7
PLR–PEGylated	70:20:5:5	148.6 ± 8.5	32.2 ± 3.7

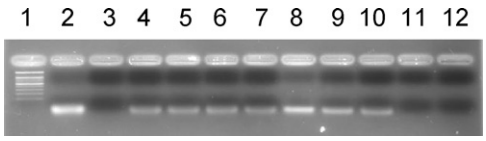

Fig. 2. Gel retardation patterns of cationic liposomes and siRNA complexes. PEGylated liposomes were complexed with siRNA at various molar ratios, and then run through a 2% agarose gel. The mobility of siRNA complexed with cationic liposomes was visualized by ethidium bromide staining. Lane 1, 100 bp size marker; lane 2, siRNA alone; lane 3, Lipofectamine®/siRNA molar ratio 6:1; lane 4, liposome/siRNA molar ratio 0.05:1; lane 5, 0.5:1; lane 6, 1:1; lane 7, 2.5:1; lane 8, 5:1; lane 9, 10:1; lane 10, 20:1; lane 11, 30:1; and lane 12, 40:1.

3.5. In vitro transfection of liposome/siRNA complexes

As in the previous study, the optimum ratio of siRNA to cationic liposomes was considered as 1:30. Altering this ratio, the effect of cationic liposomes on transfection efficiency was evaluated by varying the composition of the cationic liposome. As shown in Fig. 4, GFP expression in the cells was decreased with the increasing N/P ratios up to 1:30 except PEGylated cationic liposomes. The lowest percentages of GFP intensity by conventional, PEGylated, and PLR–PEGylated cationic liposomes were 52.78%, 60.17%, and 42.52%, respectively. At higher N/P ratios than 30:1, the transfection efficiency of liposome/siRNA complexes was reduced, at which GFP intensity was increased. This is able to be explained by limitation of cationic lipid with dose-dependent toxicity (Spagnou et al., 2004; Lv et al., 2006). The PLR–PEGylated cationic liposomes increased approximately 30% the transfection efficiency of siRNA at ratio 30:1.

The intracellular inhibition of siRNA following the in vitro transfection experiment was observed using a confocal microscope. As shown in Fig. 5, the silencing of GFP expression was observed at 24 h after the cationic liposomes-mediated transfection of H4II-E cells. In preliminary study, incubation time was varied from 24 h to 48 h after transfection with ratio 30:1. Since clear fluorescence images of GFP expression in H4II-E cells at 24 h could be obtained compared to those at 48 h due to the reduced silencing effect of siRNA, incubation time was fixed as 24 h for further study. The GFP expression was distinctly observed in intact H4II-E cells as a control (Fig. 5a), whereas the GFP intensity in the cells treated with complexed siRNA was reduced with slight difference depending on formulation in micrographs (Figs. 5c–f). However, naked siRNA showed higher GFP intensity than any other treated groups, showing low transfection efficiency of siRNA alone (Fig. 5b).

3.6. Cell viability

The cytotoxicities of siRNA alone and cationic liposome/siRNA complexes were tested in H4II-E cells and HepG2 cells using MTT assay. The results obtained revealed that the loss of the cell viability depended on the composition of cationic liposomes (Fig. 6). When comparing cationic liposome/siRNA complex with naked siRNA, cell viability of cationic liposome/siRNA complex was high in both cell lines. When treated with the PLR–PEGylated liposome/siRNA complex, cell viability was the highest in liposome/siRNA complexes.

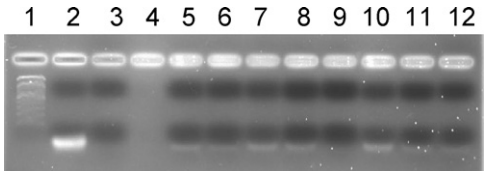

Fig. 3. Cationic liposomes block degradation of siRNA by RNase A. siRNA alone and PEGylated liposome/siRNA complexes were incubated with RNase A for 30 min at 37 °C and then electrophoresed on an agarose gel. The presence of siRNA was revealed by ethidium bromide staining. Lane 1, 100 bp size marker; lane 2, siRNA alone; lane 3, siRNA + RNase; lanes 4–6, liposome/siRNA (25:1) + RNase; lanes 7–9, liposome/siRNA (30:1) + RNase; and lanes 10–12, liposome/siRNA (40:1) + RNase.

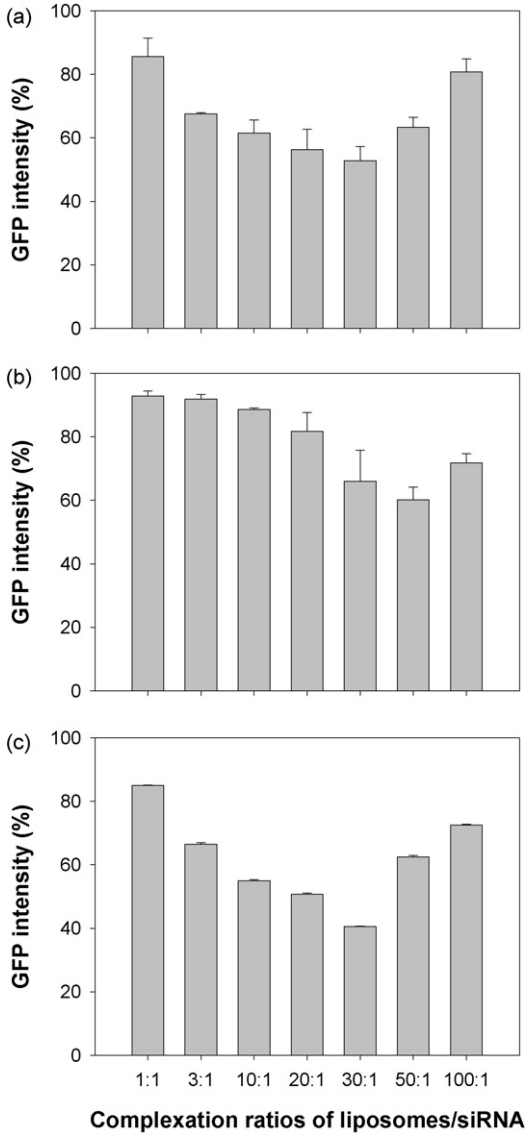

Fig. 4. Comparison of transfection efficiency in H4II-E cell lines with (a) conventional liposome/siRNA complexes, (b) PEGylated liposome/siRNA complexes and (c) PLR–PEGylated liposome/siRNA complexes. The molar ratios of siRNA to cationic liposomes were 1:1, 1:3, 1:10, 1:20, 1:30, 1:50, and 1:100. Results represent the mean ± s.d. ($n = 3$).

4. Discussion

Cationic liposomes have been developed that are capable of delivering DNA or RNA, including both plasmids encoding siRNA sequences and siRNAs, through the cellular membrane, and achieving high activity of RNAi (Zhang et al., 2006; Han et al., 2008). In particular, Lipofectamine® is frequently used for the delivery of siRNA (Dalby et al., 2004; Tseng et al., 2009). Despite the certain success using lipoplexes, these agents allow for little control over the process of their interaction with siRNA leading to final product, siRNA-lipoplex particles, of excessive size, low stability, and with incomplete encapsulation of siRNA molecules, which thereby exposes siRNA to potential enzymatic or physical degradation prior to delivery to cells. In addition, such complexes do not work effi-

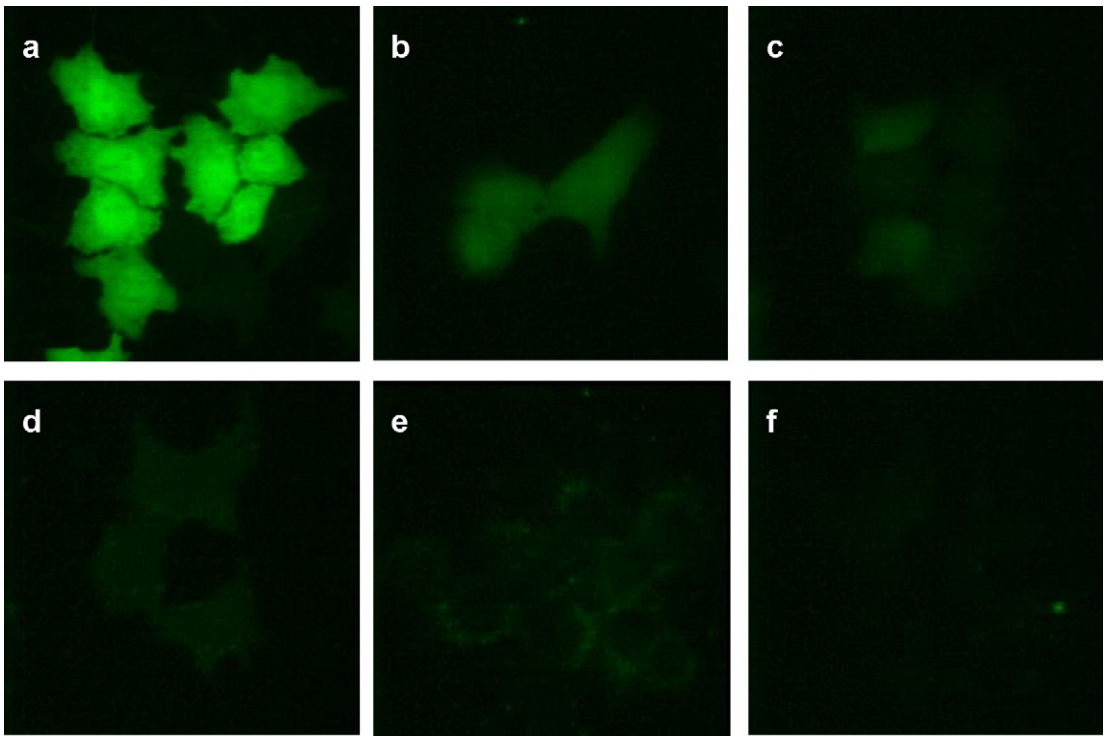

Fig. 5. GFP expression following delivery of GFP-specific siRNA with cationic liposomes. H4II-E cells were treated with GFP-specific siRNA alone or cationic liposome/siRNA complexes. (a) Untreated H4II-E cells, (b) treated with siRNA alone, (c) treated with Lipofectamine®/siRNA complex, (d) treated with conventional liposome/siRNA complex, (e) treated with PEGylated liposome/siRNA complex, and (f) treated with PLR–PEGylated liposome/siRNA complex.

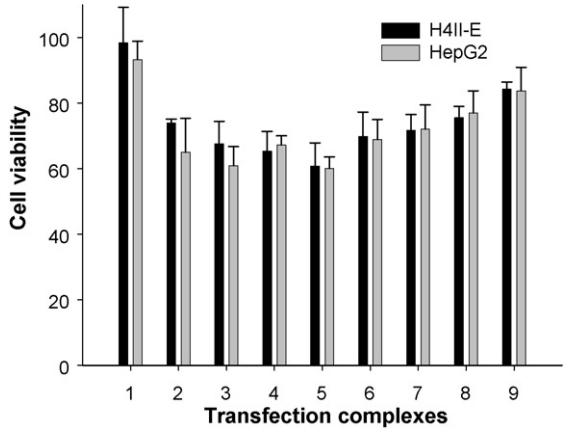

Fig. 6. Cytotoxicity of cationic liposomes in H4II-E cells and HepG2 cells. After treatment of the cells under various conditions for 24 h, cell viability was measured by MTT assay. 1, siRNA alone; 2, Lipofectamine®; 3, Lipofectamine®/siRNA complexes; 4, conventional cationic liposomes; 5, conventional cationic liposome/siRNA complexes; 6, PEGylated cationic liposomes; 7, PEGylated cationic liposome/siRNA complexes; 8, PLR–PEGylated cationic liposomes; 9, PLR–PEGylated cationic liposome/siRNA complexes.

ciently with many cell types and are toxic to cells and experimental animals (Tseng et al., 2009). Therefore, an alternative approach such as synthesis of polyarginine-based derivatives of DSPE-PEG has been adopted to improve stability and cellular uptake of siRNA (Mitchell et al., 2000; Deshayes et al., 2005).

Here, we synthesized the new lipid PLR–PEG and formulated cationic liposomes with DOTAP, DOPE, Chol and PLR–PEG for increased cellular siRNA delivery. DOPE has been widely used as a fusogenic lipid component of cationic liposomes used to deliver plasmid DNA and siRNA. The addition of DOPE to cationic liposomes has been reported to enhance cellular delivery of siRNA and reduce expression of the target genes of the siRNA when compared to the effect of liposomes composed of only cationic lipids (Hassani et al., 2005). Moreover, the addition of DOPE to cationic liposomes has been shown to provide higher transfection efficiency in vivo (Hattori et al., 2005). Cholesterol has previously been used as the major lipid component of liposomes for the delivery of various genes (Li and Huang, 2006; Tagami et al., 2007) and chemical drugs (Maestrelli et al., 2006; Al-Jamal and Kostarelos, 2007), and recently, cholesterol derivatives have been studied for their ability to deliver siRNA. Additionally, cholesterol has been used to modify the structure of siRNA and cholesterol-linked siRNA has been studied to evaluate how useful it is for in vivo systemic application of siRNA (Soutschek et al., 2004). DSPE-PEG was used to synthesize the new polyarginine–PEG-lipid conjugate since PEGylation has an enormous advantage in prolonging the blood circulation time of delivery vehicles for siRNA.

PEGylation of liposomes reduced the particle size and increased the zeta potential (Table 1). Given the comparable particle sizes of the liposomes formulated with DSPE-PEG or PLR–PEG, it is unlikely that the size of the complex contributed to the different delivery efficiencies between the two liposomes. This observation indicates that structures of lipids and the composition of cationic liposomes may be crucial for delivery of siRNA. It has been suggested that biophysical properties, such as size, charge density, and morphology of the resulting DNA complexes, determine the transfection efficiencies of non-viral vectors in serum (Esposito et al., 2006; Zhang and Anchordoquy, 2004). Non-viral vectors producing larger particle sizes with nucleic acids were reportedly effective at protecting DNA from attack by DNase I (García et al., 2007) and increasing serum resistance (Almofti et al., 2003). In addition, the PEGylated liposomes improved the stability of siRNA against RNase (Fig. 3). The PEGylation of siRNA-lipoplexes may also enhance the circulation time of the complex and can increase the amount of siRNA in the targeted tissue, which offers significant potential for developing siRNA-based therapy, particularly for the application of systemic

administration. Thus, PEGylated cationic liposomes could be useful to administer siRNA via parenteral routes because of their particle size and attribution to stability against enzyme.

Non-PEGylated conventional cationic liposomes also demonstrated a high gene silencing at a ratio of 30:1 (lipid to siRNA) (Fig. 4). PEGylated liposomes increased the stability of siRNA against RNase (Fig. 3), but showed lower gene silencing effect than conventional liposomes. PEGylation prolongs the residence time of liposomes in the blood by providing a steric barrier at the liposomal surface that inhibits opsonization. Moreover, PEGylation is known to introduce lipoplexes in a more effective way through the extracellular compartments of the body, as well as to improve pharmacokinetic characteristics of plasmid DNA, antisense ODN, and ribozymes. PEG-lipids play a role in the formulation of nucleic acid materials by inhibiting excessive aggregation and fusion during the critical self-assembly phase when cationic lipids associate with anionic DNA or RNA.

On the contrary, PEGylation seems to decrease the transfection efficiency of lipoplexes once they go on to the intracellular level (Remut et al., 2007). Deshpande et al. reported that PEGylation lowers the cellular interaction and uptake of lipoplexes (Deshpande et al., 2004). However, it was found that the cellular uptake of the PEGylated lipoplexes was unaltered and suggested that the inhibitory effect of PEGylation is situated at the step of endosomal escape (Song et al., 2002). In our study, PLR–PEGylated liposomes showed the highest efficiency of GFP gene silencing at a ratio of 30:1 (lipid to siRNA). These results clearly showed that the transfection efficiency was significantly increased by introducing arginine onto the surface of cationic liposomes. It was attributed to increased specificity by PLR and to increased stability by PEGylation. When several cationic polypeptides showing cell-penetrating property were investigated with polyarginine, polyhistidine and polylysine, a higher efficiency was shown in polyarginine peptides (Mitchell et al., 2000). In particular, polyarginines have also been used for transfer of genes through formation of complexes involving the gene and several polyarginine molecules (Deshayes et al., 2005). Moreover, the liposomes bearing arginine octamer (R8) molecules attached to their outer surface (R8-liposomes) effectively delivered siRNA that inhibited the target gene and significantly reduced the proliferation of cancer cells (Zhang et al., 2006). In addition, poly-L-arginine has been successfully applied for targeted delivery of siRNA with complexed hyaluronic acid (Kim et al., 2009). Furthermore, the toxicity of siRNA alone and PLR has been reported (Kim et al., 2009), but our PLR–PEG conjugate reduced the toxicity of PLR as well as siRNA.

5. Conclusions

PLR–PEGylated liposomes have the required physicochemical characteristics, in terms of particle size and zeta potential, to be used as siRNA carriers. The PLR–PEGylated cationic liposomes showed high siRNA transfection efficiency and low cytotoxicity compared to conventional cationic liposomes. From transfection results, it could be concluded that using PLR–PEGylated liposomes for siRNA delivery overcame the transfection-reducing effect of PEG. Therefore, PLR–PEG may be a useful addition to cationic liposomes for improving the properties of siRNA delivery vehicles. Further studies to determine if PLR–PEG could affect the pharmacokinetics and biodistribution of siRNAs are needed.

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