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Cationic Solid Lipid Nanoparticles Reconstituted from Low Density Lipoprotein Components for Delivery of siRNA

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Abstract: Cationic solid lipid nanoparticles (SLN), reconstituted from natural components of protein-free low-density lipoprotein, were used to deliver small interfering RNA (siRNA). The cationic SLN was prepared using a modified solvent-emulsification method. The composition was 45% (w/w) cholesteryl ester, 3% (w/w) triglyceride, 10% (w/w) cholesterol, 14% (w/w) dioleoylphosphatidylethanolamine (DOPE), and 28% (w/w) 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol (DC-chol). The SLN had a mean diameter of 117 $\pm$ 12 nm and a surface zeta potential value of +41.76 $\pm$ 2.63 mV. A reducible conjugate of siRNA and polyethylene glycol (PEG) (siRNA−PEG) was anchored onto the surface of SLN via electrostatic interactions, resulting in stable complexes in buffer solution and in even 10% serum. Under an optimal weight ratio of DC-chol of SLN and siRNA−PEG conjugate, the complexes exhibited higher gene silencing efficiency of GFP and VEGF than that of polyethylenimine (PEI) 25K with showing much reduced cell cytotoxicity. Flow cytometry results also showed that siRNA−PEG/SLN complexes were efficiently taken up by cells. Surface-modified and reconstituted protein-free LDL mimicking SLN could be utilized as noncytotoxic, serum-stable, and highly effective carriers for delivery of siRNA.

Keywords: Gene delivery; low density lipoprotein; siRNA−PEG conjugate; solid lipid nanoparticles; vascular endothelial growth factor

Introduction

Small interfering RNA (siRNA) with 21−25 bp in length, a mediator of RNA interference phenomenon, silences a specific gene expression by triggering the cleavage of a target mRNA (mRNA) in the cytoplasm of mammalian cells.[1,2] Recently siRNA has generated a great deal of interest in treatment of inherited and acquired diseases due to the selective knockdown ability of a gene of interest with much reduced doses. Although siRNA is a promising nucleic acid drug in gene therapy, various intra- and extracellular barriers seriously hamper therapeutic applications.[3–5] Negatively charged siRNA shows extremely low cellular uptake and transfection efficiency, and undergoes rapid chemical degradation when administered intravenously. To overcome the

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delivery obstacles, cationic polymers and lipids were used to form polyelectrolyte complex nanoparticles.[5,6]

Among the cationic polymers, polyethylenimine (PEI) has been widely utilized to form polyplexes with plasmid DNA, antisense oligonucleotides, and siRNA because of its superior buffering capacity and high gene transfection efficiency.[7,8] There have been a number of studies of using chemically modified PEI derivatives, such as poly(ethylene glycol) (PEG) conjugated PEI and targeting ligand tethered PEI, for prolonged circulation in the blood stream and target-specific cellular uptake.[9,10] We previously reported that siRNA was conjugated with PEG via a cleavable disulfide linkage to form polyelectrolyte complex (PEC) micelles with branched PEI (MW 25K). The resultant siRNA−PEG/PEI PEC micelles having a core/shell type structure with an inner core of charge neutralized siRNA/PEI complex and a PEG shell layer showed much better gene silencing effect in vitro and in vivo, as compared to siRNA/PEI complexes.[11] Nevertheless, PEI-based delivery systems often induced severe cytotoxicities in various cells through necrosis or apoptosis.[12–15] Although low MW linear PEI or polyethylene glycol (PEG)-grafted PEI copolymer exhibited less cytotoxicity, their intracellular transfection efficiencies were still lower than those of high MW branched PEI ones.[16] Thus, it is essential to develop alternative cationic and noncytotoxic core condensing agents for clinical applications of siRNA−PEG conjugate based PEC micelles.

There have been several studies using nanoscale cationic lipid microemulsions for delivery of plasmid DNA.[17–19] Various microemulsion droplets composed of linseed oil, soybean oil, and squalene were prepared using 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) as a cationic emulsifier. They exhibited less cytotoxicity than PEI. When complexed with plasmid DNA, the resultant complexes demonstrated enhanced stability, and showed comparable transfection efficiency in the presence of serum, to that of PEI. However, it appears that the colloidal stability of cationic microemulsion carriers was problematic for practical uses. Low density lipoprotein (LDL) complexes are naturally occurring nanoparticles abundant in the blood, transporting lipids, cholesterol, proteins, and hydrophobic drugs to extrahepatic tissues throughout systemic circulation.[20–23] Natural LDL nanoparticles were previously employed for delivery of plasmid DNA by incorporating with stearyl-poly(L-lysine) (stearyl-PLL) as

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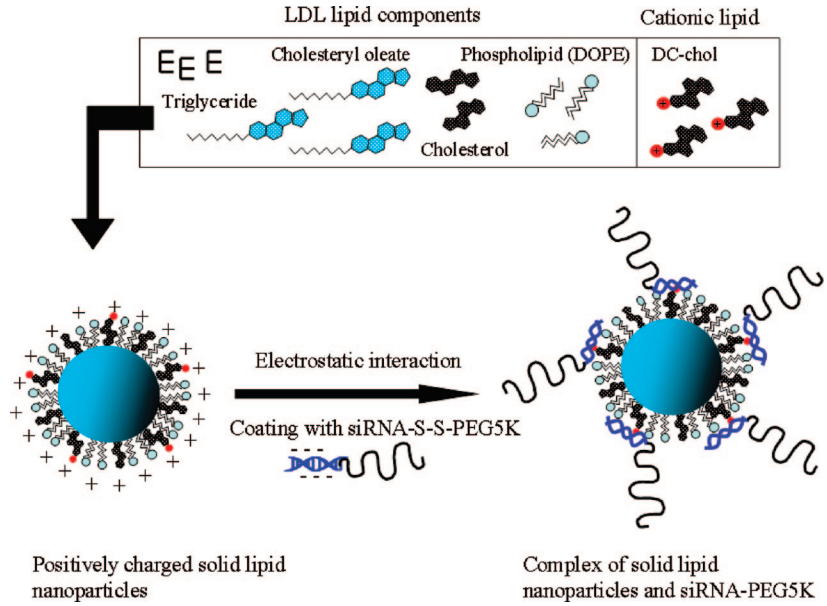

Figure 1. Schematic diagram of the assembly of lipid portions of low density lipoprotein (LDL), DOPE, and DC-chol for preparation of solid lipid nanoparticles (SLN). The formulation of siRNA−PEG/SLN complexes via electrostatic interactions between the positively charged SLN surface and negatively charged siRNA is also illustrated.

a cationic surface modifier (Terplex system).[24–26] It was shown that the Terplex system carrying vascular endothelial growth factor (VEGF) plasmid DNA improved a left ventricular function in a rabbit model of myocardial infarction.[26] However, the isolation procedure of natural LDL from the blood is too sophisticated and time-consuming a process; reconstituted LDL-like solid lipid nanoparticles (SLN) were developed as an alternative LDL mimicking model, which were prepared from phospholipids and cholesterol esters without incorporating apolipoprotein.[27–29] SLN has been shown to be very stable due to the solidlike nature of main lipid components in contrast to the microemulsion oil droplets. In addition, reconstituted LDL-like SLN behaves like native LDL when injected into the bloodstream in animal studies and clinical treatments.[29]

In the present study, surface-modified SLN, reconstituted from apolipoprotein-free low density lipoprotein components, were prepared for siRNA delivery by a modified solvent-emulsification method. siRNA was conjugated with PEG via a disulfide linkage, and the resultant siRNA−PEG conjugate was anchored on the cationic surface via charge interactions to produce siRNA−PEG/SLN complexes that had protective PEG chains outward. Figure 1 illustrates the scheme for preparation of SLN and SLN-based siRNA−PEG conjugate complexes. Various siRNA−PEG/SLN complexes were prepared and characterized by measuring size and surface charge values as a function of weight ratio between cationic lipid and siRNA−PEG conjugate used in the formulation. The cellular uptake and gene silencing effect of siRNA−PEG/SLN complexes, formulated with employing green fluorescent protein (GFP) and vascular endothelial growth factor (VEGF) siRNA, were evaluated in the presence of 10% serum media.

Materials

Cholesteryl oleate, glyceryl trioleate (triglyceride), and cholesterol were purchased from Sigma (St. Louis, MO). L-α-Dioleoyl phosphatidylethanolamine (DOPE) and 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride were provided by Avanti Polar Lipids (Alabaster, AL). VEGF and GFP siRNA modified with a hexylamine group at the 3′ end of its sense strand were obtained from Bioneer Co. (Daejeon, South Korea). For FACS analysis, VEGF siRNA modified with a hexylamine group at the 3′ end and an indocarbocyanine dye (Cy3) at the 5′ end of its sense strand was purchased from Dharmacon Research

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Methods

Preparation of Solid Lipid Nanoparticles (SLN). Solid lipid nanoparticles (SLN) were prepared by a modified solvent-emulsification method. Typically, cholesteryl oleate (22.5 mg, 45% w/w), glyceryl trioleate (1.5 mg, 3% w/w), DOPE (7 mg, 14% w/w), cholesterol (5 mg, 10% w/w), and DC-cholesterol (14 mg, 28% w/w) were dissolved in a chloroform/methanol (2 mL) mixture (2:1, v/v) in a vial. Deionized water (10 mL) was added to the vial and vortexed thoroughly. The suspension was sonicated using Branson Sonifier 450 (20 kHz, duty cycle = 40, output control = 3.5) for 3 min. The microemulsion solution was transferred to a rotary evaporator and the solvent was removed above 52 °C, the melting point of cholesteryl oleate. The prepared SLN was stocked at 4 °C.

Transmission Electron Microscopy (TEM). The morphology of SLN was visualized using transmission electron microscopy (TEM). The SLN (20 μL) dispersed in aqueous solution (5 mg/mL) was deposited three times successively on a Formvar/carbon support grid with 300 mesh. The grid was dried for 2 min and observed using a Zeiss Omega 912 TEM (Carl Zeiss, Oberkochen, Germany) operating at 80 kV.

Synthesis of siRNA−PEG Conjugate. siRNA was conjugated with PEG through a disulfide bond as described previously.[11] The sequences of GFP siRNA were 5′-AACUUCAGGGUCAGCUUGCdTdT-3′ (sense) and 5′-GCAAGCUGACCCUGAAGUUdTdT-3′ (antisense). The sequences of VEGF siRNA were 5′-GGAGUACCCUGAUGAGAUCdTdT-3′ (sense) and 5′-GAUCUCAUCAGGGUACUCCdTdT-3′ (antisense). Briefly, siRNA modified by a hexylamine group at the 3′ end of a sense strand (300 μg, 20 nmol of VEGF or GFP siRNA), was dissolved in PBS (pH 7.5) solution. Then 20 μL (400 nmol) of SPDP stock solution (20 mM) in DMSO was added to the siRNA solution. After reacting for 3 h at room temperature, excess SPDP was removed by using gel permeation chromatography (D-SaltTM dextran desalting column, Pierce, Rockford, IL). Four micromoles of mPEG-SH dissolved in PBS (pH 7.5) solution was added to the purified siRNA−SPDP conjugate. The reaction was carried out for 3 days at room temperature. Unreacted mPEG was separated by dialysis (MWCO 10,000) against deionized water. The siRNA−PEG conjugate was concentrated by using a speed-vacuum evaporator. The purity and concentration were determined by measurement of UV absorbance at 260 and 280 nm. The purified siRNA−PEG conjugate was stored at −80 °C.

Formation of siRNA−PEG/SLN Complexes. SLN was incubated with siRNA−PEG conjugate at 0, 1.4, 2.8, 4.2, 5.6 and 8.4 weight ratios of DC-chol (of SLN)/siRNA at room temperature for 15 min in PBS solution (pH 7.4 and 150 mM NaCl) or deionized water. The resultant complexes were characterized by gel retardation assay for binding of siRNA−PEG conjugate to SLN.

Size and Zeta Potential Measurements. The diameter and surface zeta-potential value of the SLN or their complexes with siRNA−PEG conjugate were measured by a dynamic light scattering (DLS) instrument (Zeta-Plus, Brookhaven Instrument Co., NY) equipped with a He−Ne laser at a wavelength of 632 nm at a 90° detection angle. For size measurement, each sample was properly diluted in deionized water in order to maintain the number of counts per second between 104 and 105. For stability study of the complexes in serum, FBS (10%) containing RPM-1 media 1640 was added.

Gel Retardation Analysis of siRNA−PEG/SLN Complexes. siRNA−PEG/SLN complexes formulated with different weight ratios of DC-chol (of SLN)/siRNA were run on agarose (2%) gel electrophoresis. The visualization of siRNA−PEG conjugate was illuminated with ethidium bromide staining, and the gel image was taken under UV.

Cell Culture. PC3 cells (human prostate cancer cell line) provided from the Korean Cell Line Bank (Seoul, Korea) were grown on RPM-1 medium 1640 with heat-inactivated fetal bovine serum (10% v/v), penicillin (100 UI/mL), and streptomycin (100 μg/mL). GFP overexpressing MDAMB435 cells (human breast cancer cells) provided from Samyang Corporation (Daejeon, Korea) were grown on DMEM supplemented with serum (10%) and the antibiotics above. Cells were cultivated at 37 °C in a humidified atmosphere of CO_2 (5%).

Gene Silencing Efficiency. GFP overexpressing MDAMB435 cells were seeded in a 12 well plate at a density of 2×10^5 cells per well in DMEM media supplemented with FBS (10%) and antibiotics. The cells were incubated 24 h before the transfection experiment. The media was removed, and the cells were washed with PBS (pH 7.4) solution three times. One microgram of siRNA−PEG conjugate alone or siRNA−PEG/SLN complexes formulated at different weight ratios of DC-chol (of SLN)/siRNA (0, 1.4, 2.8, 5.6, 8.4, and 16.8) were transfected to the cells in FBS (10%) containing culture media for 4 h at 37 °C. For a dose-dependent experiment, different concentrations (0, 15, 37.5, 75, 150, and 300 pmol/mL) of siRNA−PEG conjugate were employed for the formation of complexes with SLN prepared at the DC-chol (of SLN)/siRNA−PEG weight ratio of 8.4. After the supernatant was replaced with fresh serum-supplemented media, the transfected cells were further cultivated for 48 h and treated by Triton X-100 (0.1%)/PBS solution. The fluorescence intensity of cell lysates was measured at a wavelength of 525 nm (excitation at 488 nm) using a spectrofluorophotometer (SLM-AMINCO 8100, SLM Instruments Inc., Rochester, NY). The relative GFP expression percent was expressed as the GFP expression level of

transfected cells divided by that of nontransfected cells. For VEGF gene inhibition experiment, PC3 cells were transfected with various formulations as described above. After 4 h post-transfection, cell media was replaced with fresh serum-supplemented media and incubated for 6 h. The media were discarded in order to eliminate endogenously secreted VEGF. Then, the media were replaced with new RPM-1 media 1640 supplemented with FBS (10%) and heparin (20 μg/mL). After 16 h further incubation, VEGF containing cell supernatant was collected. The concentration of released VEGF from the cells was determined by using Quantikine human VEGF immunoassay kit (R&D System, Minneapolis, MN) according to the manufacturer's protocols.

Cytotoxicity Assay. MDAMB435 cells were seeded in a 96 well plate (10^4 cells per well) 24 h before the assay. Different concentrations (3, 6, 12, 18, 24, 36, 48, and 72 μg/mL) of SLN and PEI were prepared within RPM-1 media 1640 containing FBS (10%). Culture media were aspirated, and the prepared samples (100 μL) were added to the well. After 24 h incubation at 37 °C, Cell Counting Kit-8 (CCK-8, 10 μL) solution (Dojindo, MD) was added in each well. Cells were further incubated at 37 °C for 4 h, and then absorbance at 450 nm was measured using a microplate reader (BioRad Model 550).

Flow Cytometry. For cellular uptake experiment, PC3 cells were seeded in a 6 well plate at a density of 5×10^5 cells per well in RPM-1 media 1640 supplemented with FBS (10%) and antibiotics, and cultivated for 24 h at 37 °C. The media was withdrawn, and cells were washed three times with PBS solution (pH 7.4). One microgram of Cy3-siRNA−PEG conjugate was complexed with PEI25K or SLN (5.6 and 8.4 weight ratio of DC-chol (of SLN)/siRNA) and incubated for 2 h at 37 °C. The cellular uptake was stopped by removing the culture media. The transfected cells were gently washed three times with cold PBS solution and fixed with paraformaldehyde solution (1% w/v). The cellular uptake was monitored by flow cytometry (FACScan, Becton & Dickinson).

Statistical Analysis. All experiments were done in triplicate and were repeated independently at least three times. The mean and standard deviation were calculated from the data obtained and represented in all the figures. Standard Student's *t* test was used for significant statistical difference with a minimum confidence level of 0.05.

Results and Discussion

Characterization of SLN. The aim of this study is to produce positively charged and reconstituted apolipoprotein-free LDL mimicking nanoparticles for delivery of siRNA. Natural LDL consists of polar phospholipids, apolipoproteins, cholesterol, and nonpolar neutral lipids predominantly composed of cholesterol esters and triglycerides. Nonpolar lipid cores are stabilized by polar phospholipids, cholesterol, and apolipoproteins that act as emulsifying agents on the surface.[27] The compositions for natural LDL and LDL mimicking SLN prepared by the modified solvent emulsification are listed in Table 1. In the SLN formulation, 22% (w/w) phospholipids and 20% (w/w) apolipoprotein present in the natural LDL were replaced with 14% (w/w) DOPE and 28% (w/w) DC-chol while maintaining the same composition of 10% (w/w) cholesterol and nonpolar lipids in the core. Typically, LDL-like SLN consisted of 45% (w/w) cholesteryl ester, 3% (w/w) triglyceride, 14% (w/w) dioleoylphosphatidylethanolamine (DOPE), 10% (w/w) cholesterol, and 28% (w/w) 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]-cholesterol (DC-chol). Both DOPE and cholesterol are commonly used as helper lipids in the liposomal formulations for gene therapy, which are known to improve the stability, enhance the gene transfection efficiency, and reduce the cytotoxicity of cationic lipids.[31–33] Particularly, DOPE was added to destabilize the membrane of endosome vesicles by facilitating fusion of cationic lipids of SLN with endosomal membrane phospholipids.[34–37] Cholesterol was incorporated to provide morphological rigidity to the surface packing of

Table 1. Composition of Natural LDL and LDL-Mimicking SLN

	natural LDL[30]		SLN	
	components	portion (%) (w/w)	components	portion (%) (w/w)
core	cholesteryl ester	45	cholesteryl oleate	45
	triglyceride	3	triglyceride	3
surface	cholesterol	10	cholesterol	10
	phospholipid	22	DOPE	14
	apolipoprotein B-100	20	DC-chol	28

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DC-chol, and to promote the stability of SLN.[38] DC-chol was introduced as major cationic lipids to generate positively charged SLN, since it is considered to be less toxic than other cationic lipids. DC-chol based gene carriers were recently approved in many clinical treatments such as melanoma, cystic fibrosis, cervical carcinoma, breast and ovarian cancer.[39] In the SLN formulation, the molar ratio of DOPE:cholesterol:DC-chol was 9.4:13:26 and that of cationic lipid:helper lipid was 1:16, which were reported to be near optimal molar ratios for efficient gene transfection for liposome formulations.[40,41] The mean diameter of SLN as measured by laser light scattering was 117 ± 12 nm (Figure 2A), and the TEM observation confirmed the size with revealing that SLN had a spherical shape with a core−shell structure (Figure 2B). The surface zeta potential value of SLN was + 41.76 ± 2.63 mV. Natural LDL nanoparticles had an average size of 18−25 nm with a surface charge value of −11.4 ± 2.63 mV.[42] It was likely that the incorporation of DC-chol and DOPE increased the size and changed the surface charge value from negative to positive. The reconstituted SLN in aqueous solution maintained excellent stability without exhibiting any aggregation, precipitation, and phase separation at room temperature for several weeks after preparation (data not shown). This was because the SLN was immediately solidified at physiological temperature due to the presence of cholesteryl oleate in the core, which has a melting temperature at 52 °C. Thus, the far enhanced stability of SLN can be attributed to the rocklike solid nature in the core, providing a superior stability advantage to the conventional DC-chol/DOPE liposome and microemulsion formulations that often elicited severe stability problems for a long-term storage period. From the standpoint of stability and biocompatibility, cationic LDL-mimicking SLN carriers would provide highly desirable physicochemical characteristics as delivery vehicles of siRNA therapeutics.

Characterization of siRNA−PEG/SLN Complexes. In order to characterize siRNA−PEG/SLN complexes, siRNA−PEG conjugate was complexed with SLN as a function of weight ratio of DC-chol (of SLN)/siRNA at room temperature in deionized water. The size and zeta potential value were monitored by a dynamic light scattering (DLS) method.

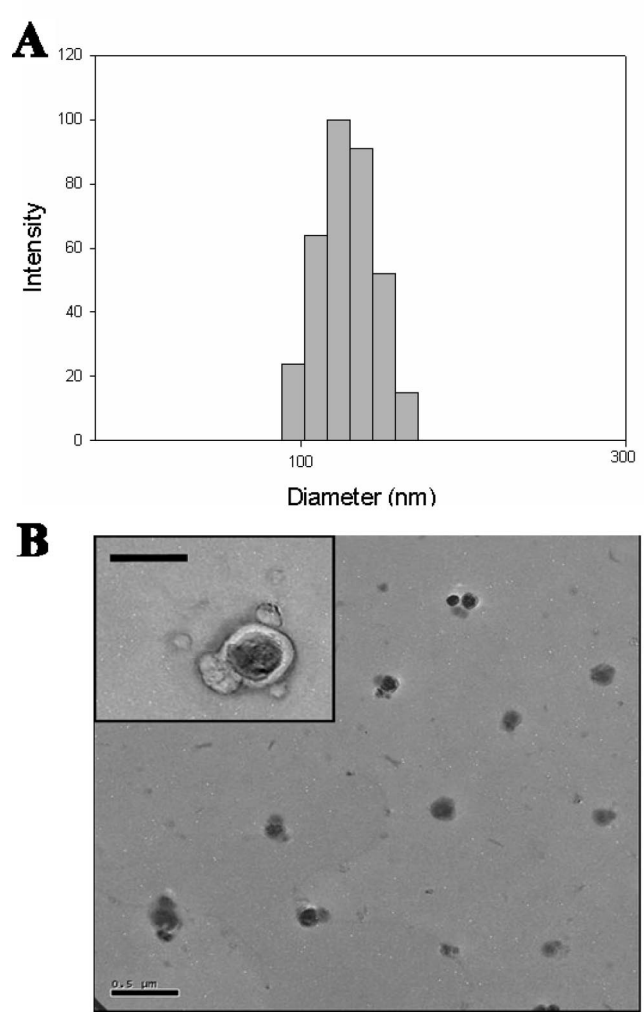

Figure 2. (A) Size distribution of SLN measured by dynamic light scattering (DLS) and (B) TEM images of SLN. Scale bar is 500 nm in the large panel and 200 nm in the upper small panel.

As shown in Figure 3A, the zeta potential value of SLN increases from −13.8 ± 3.9 to +35.67 ± 1.2 mV with increasing weight ratio of DC-chol (of SLN)/siRNA from 1 to 4.67, but was unchanged above a weight ratio of 4.7. The data suggest that a negatively charged siRNA part in the conjugate was gradually interacted onto the cationic surface of SLN up to the weight ratio of 4.7, above which the charge interactions were likely to be saturated. The sizes of SLN coated with the siRNA−PEG conjugate were almost constant near 100 nm over an entire weight ratio range of DC-chol (of SLN)/siRNA−PEG, showing that the formulated siRNA−PEG/SLN complexes did not aggregate (Figure 3A). This indicates that positively charged siRNA−PEG/SLN complexes were very stable in aqueous solution. Gel retardation assay was also carried out in the weight ratio range of DC-chol (of SLN)/siRNA from 1.4 to 8.4. The agarose gel electrophoresis results displayed that siRNA−PEG/SLN was fully retarded at weight ratio of 5.6 and 8.4 (Figure 3B). Both zeta potential data and gel retardation assay results revealed that complete complexation could be attained above the weight ratio of ∼5.

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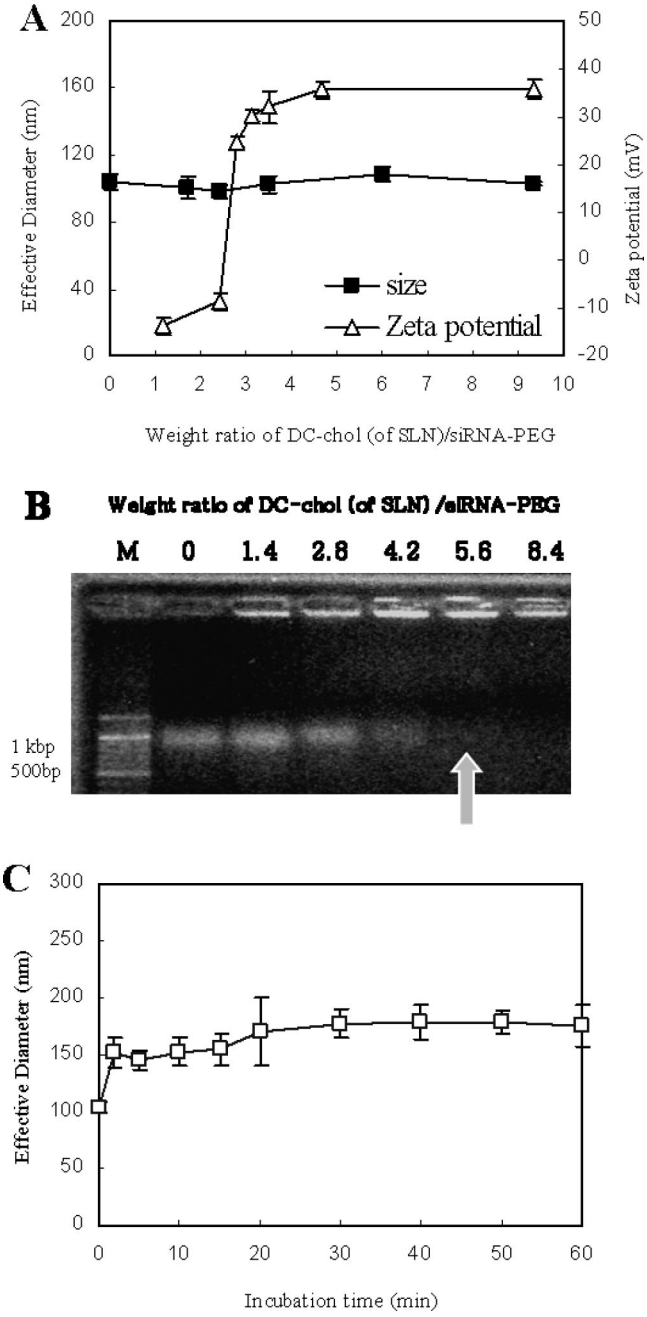

Figure 3. Characterization of the siRNA−PEG/SLN complexes. (A) Measurements of size and zeta potential and (B) gel retardation assay of siRNA−PEG/SLN complex as a function of weight ratio (1.4−8.4) of DC-chol (of SLN)/siRNA−PEG. (C) Sizes of siRNA−PEG/SLN complex at the ratio of 8.4 of DC-chol (of SLN)/siRNA−PEG in 10% serum containing RPM-1 media 1640 measured by DLS. In the B panel, M and 0 weight ratio corresponds to the marker and siRNA−PEG only as control, respectively, and completed siRNA−PEG coating ratio is indicated by an arrow.

Stability of siRNA−PEG/SLN Complexes in 10% Serum Media. Injectable nanoparticulate drug carriers are often surface modified with hydrophilic, flexible, and nonionic PEG chains to avoid recognition by macrophages of mononuclear phagocyte system (MPS), and to achieve prolonged circulation in the blood stream.[43] In this study, PEG 5K was used to conjugate siRNA via a disulfide linkage to produce a siRNA−PEG conjugate that can be cleavable under reductive conditions, such as intracellular cytoplasmic region, after endocytic cellular uptake.[11] The anionic siRNA part in the conjugate was expected to anchor on the cationic surface of SLN through charge interactions, while the PEG part was exposed outward to generate PEGylated complexes. After forming siRNA−PEG/SLN complexes in PBS solution, they were transferred into 10% serum containing culture media, and their size was measured as a function of time using DLS. As shown in Figure 3C, the size of siRNA−PEG/SLN complexes (~100 nm prior to the addition of serum) was sharply increased to 151.2 ± 13.2 nm at 2 min, and gradually changed to 170.3 ± 29.9 nm at 20 min. The complex sizes, however, were stably maintained at ~170 nm up to 60 min. It was possible that adsorption and rearrangement of plasma proteins on the cationic surface of siRNA−PEG/SLN complexes initially occurred, mediating the partial aggregation of the protein adsorbed complexes for the next 20 min incubation time, as reported previously.[44] However, from 20 to 60 min of incubation time, the size of the complexes was almost constant, implying that surface exposed PEG chains on the surface of SLN functioned as a protective shell layer to prevent any further nonspecific protein adsorption, and consequently to suppress the interparticulate aggregation in a steric repulsive manner. The results suggest that the PEG decoration on the surface of siRNA−PEG/SLN complexes played a key role in contributing to the high stability in the serum containing media. When unPEGylated siRNA alone was complexed with SLN, siRNA/SLN complexes were severely clustered to form larger aggregates (data not shown).

Gene Silencing Efficiency of siRNA−PEG/SLN Complexes. To optimize the weight ratio DC-chol (of SLN)/siRNA−PEG for efficient gene silencing, two separate gene inhibition experiments employing GFP and VEGF siRNA were carried out at various weight ratios using two different cell lines. Gene silencing efficiencies of GFP siRNA−PEG/SLN complexes were determined with GFP overexpressing MDAMB435 cells in the serum containing media (Figure 4A). As increasing the weight ratio of DC-chol (of SLN)/siRNA up to 8.4, the GFP gene expression level decreased continuously. At the weight ratio of 8.4, the GFP expression level was 41.1 ± 4.9%. Gene silencing efficiency of VEGF siRNA−PEG/SLN was also measured in VEGF releasing

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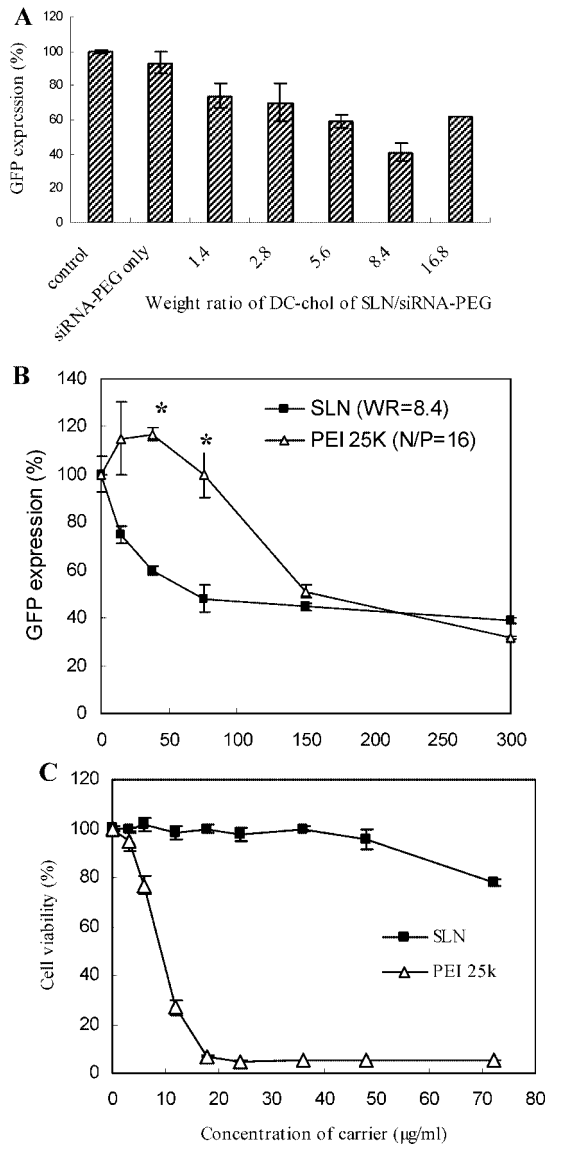

Figure 4. (A) Suppression of GFP expression as a function of weight ratio (DC-chol (of SLN)/siRNA−PEG) by transfection of siRNA−PEG/SLN complex in GFP overexpressing MDAMB435 cells. (B) Dose-dependent transfection curve of GFP siRNA−PEG/SLN at weight ratio of 8.4 and GFP siRNA−PEG/PEI 25k at N/P ratio of 16. The GFP expression extents were statistically different (denoted by ∗) between siRNA−PEG/PEI and siRNA−PEG/SLN samples at the same concentration of siRNA−PEG. (C) Cytotoxicity assay of gene carriers in MDAMB435 cells. Solid square and open triangle symbols represent SLN and PEI 25K, respectively, in C. All experiments were performed in the condition of 10% serum containing DMEM media.

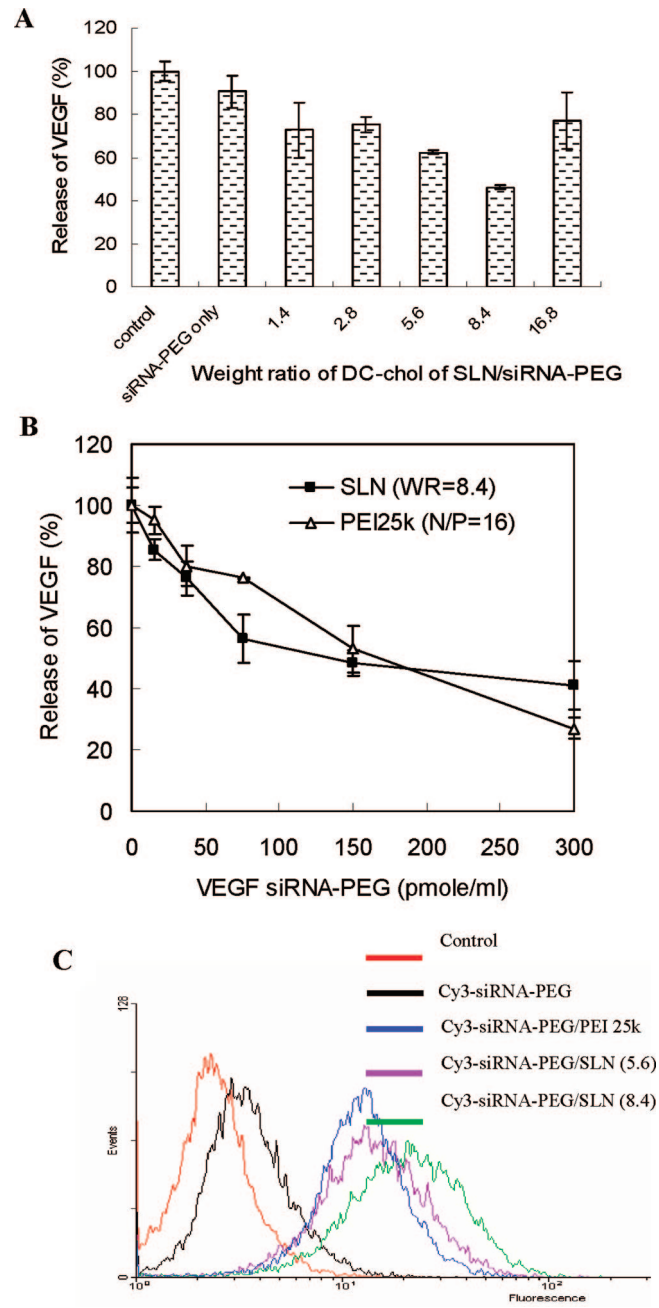

Figure 5. (A) Suppression of VEGF release as a function of weight ratio (DC-chol (of SLN)/siRNA−PEG) by transfection of siRNA−PEG/SLN complex in VEGF releasing PC3 cells. (B) Dose-dependent transfection curve of VEGF siRNA−PEG/SLN at weight ratio of 8.4 and GFP siRNA−PEG/PEI 25k at N/P ratio of 16. (C) Flow cytometry of Cy3 dye-labeled siRNA−PEG/SLN complex in PC-3 cells after 2 h incubation. All experiments were performed in the condition of 10% serum containing media.

PC3 cells as shown in Figure 5A. The silencing profile of VEGF in PC3 cells was very similar to that of GFP in MDAMB435 cells. At the weight ratio of 8.4, the VEGF expression level was 46.2 ± 0.9%. At the weight ratio of 16.8, both GFP siRNA−PEG/SLN and VEGF siRNA−PEG/SLN complexes significantly reduced the gene silencing extents: 62.0 ± 0.3% for GFP expression and 77.2 ± 13.0% for VEGF expression. This can be attributed to the fact that, at that weight ratio, uncomplexed cationic SLN species might be present in the media and they were likely to compete with siRNA−PEG/SLN complexes for cellular uptake. It should be noted that, at a fixed amount of siRNA−PEG, the increasing weight ratio of DC-chol (of SLN)/siRNA−PEG

played a critical role in influencing both gene silencing efficiency and complex stability in an opposite direction. Since the number of SLN in the transfection media increased proportionally with increasing the charge weight ratio between DC-chol and siRNA, the loading amount of siRNA−PEG per SLN decreased with concomitantly reducing the extent of PEG shielding effect. The excessive PEG decoration on the surface of polyplexes and lipoplexes is known to lower the extent of their cellular uptake, but to enhance the complex stability. Thus it appears that at the weight ratio of 8.4, siRNA−PEG/SLN complexes demonstrated the most pronounced gene silencing efficiency with optimal complex stability in the presence of serum proteins.

Dose-Dependent Gene Silencing Efficiencies of siRNA−PEG/SLN and siRNA−PEG/PEI 25K Complexes. Previously we used PEI 25K as a core forming agent for the formation of siRNA−PEG/PEI polyelectrolyte complex micelles.[11] For siRNA−PEG/PEI formulations, it was shown that a maximum VEGF gene silencing efficiency was attained at the N/P ratio (PEI/siRNA) of 16. In order to compare the silencing efficiency between siRNA−PEG/SLN and siRNA−PEG/PEI 25K, siRNA dose-dependent transfection experiments were performed to MDAMB435 cells for GFP silencing and PC3 cells for VEGF silencing. The weight ratio of DC-chol (of SLN)/siRNA−PEG was adjusted at 8.4 for the SLN formulation, and the N/P ratio of PEI/siRNA was set at 16 for the PEI formulation. In the case of GFP siRNA−PEG/SLN, the extent of GFP gene expression decreased in a dose-dependent manner up to 75 pmol/mL of siRNA−PEG, where the GFP expression percent was 48.1 ± 5.6% (Figure 4B). At 300 pmol/mL of siRNA−PEG, the GFP expression percent reached 38.9 ± 1.3%. On the other hand, GFP siRNA−PEG/PEI complexes did not exhibit any significant gene silencing effect up to 75 pmol/mL of siRNA−PEG, but thereafter demonstrated sharp silencing effects, comparable to that of siRNA−PEG/SLN at the siRNA−PEG concentration of 300 pmol/mL. The MDAMB435 cells that overexpressed GFP in a highly stable manner inherently limited further gene silencing below ~40%. Figure 5B shows dose-dependent VEGF expression levels for both siRNA−PEG/SLN and siRNA−PEG/PEI complexes. Both of the formulations exhibited gradual decrease in gene silencing efficiency over the entire concentration range, in contrast to those for GFP gene silencing behaviors of MDAMB435 cells. At 300 pmol/mL of siRNA−PEG, the VEGF expression percent reached 41.0 ± 7.8%. Since the VEGF siRNA used in this study downregulated VEGF expression in a highly sequence-specific manner,[11,45] the observed gene silencing effects were due to the inherent RNAi effect, not by "off-target" effects.

Overall, both GFP and VEGF siRNA−PEG/SLN complexes demonstrated similar dose-dependent gene silencing efficiencies for MDAMB435 and PC3 cells, respectively, but siRNA−PEG/PEI complexes showed different profiles for the two different cells with different targeting genes. It is possible that the extent of gene silencing was largely affected by various factors including cell types, target genes, kinds of cationic carriers, and surface charge and size values of the complexes. For example, MDAMB435 cells overexpressing exogenous GFP and PC cells expressing inherent VEGF might have different extents of gene silencing for the siRNA−PEG/SLN and siRNA−PEG/PEI complexes. Probably, VEGF secreting PC3 cells are more susceptible to the cellular uptake and gene silencing effect of the PEI based siRNA formulations. It should be noted that siRNA−PEG/SLN complexes were formed mainly by electrostatic adsorption of siRNA−PEG conjugate onto the surface of SLN, while siRNA−PEG/PEI complexes were preferentially produced via the formation of siRNA/PEI polyelectrolyte complex inner cores. siRNA−PEG/PEI complexes were likely to be more stable than siRNA−PEG/SLN complexes. The enhanced complex stability, however, would be beneficial for cellular uptake, but unfavorable for decomplexation of siRNA−PEG from the complexes transported within cells. From the comparative dose-dependent studies for SLN and PEI carriers, it can be concluded that both of the formulations resulted in similar gene silencing efficiencies at higher siRNA−PEG doses above an siRNA concentration of 2 μg/mL.

Cytotoxicity and Cellular Uptake of siRNA−PEG/SLN and siRNA−PEG/PEI 25K Complexes. In order to investigate whether the gene silencing results observed in the dose-dependent gene silencing studies was due to the cytotoxic effect, in vitro cytotoxicity assay was performed for SLN and PEI 25K using MDAMB435 cells. The concentration of SLN and PEI 25K was varied from 3 μg/mL to 72 μg/mL, the same range for the transfection experiments. While PEI 25K demonstrated only 6.9 ± 0.4% cell viability at 18 μg/mL after 24 h incubation (IC_{50} value: about 9 μg/mL), SLN did not induce any damage to cells up to 48 μg/mL (Figure 4C). At the concentration of 72 μg/mL, SLN exhibited 78 ± 1% cell viability. The comparison of cytotoxicity between PEI 25K and SLN clearly revealed that SLN was far less toxic than PEI 25K, suggesting that SLN could be used as noncytotoxic core condensing materials for siRNA−PEG conjugate. Figure 5C shows cellular uptake extents of VEGF siRNA−PEG/SLN and siRNA−PEG/PEI 25K as measured by flow cytometry. Cy3-labeled siRNA−PEG only, Cy3-siRNA−PEG/PEI 25K (N/P ratio: 16) and Cy3-siRNA−PEG/SLN complexes (weight ratio: 5.6 and 8.4) were transfected to PC3 cells for 2 h in 10% serum containing media. It can be seen that Cy3-siRNA−PEG/SLN complexes formulated at the weight ratios of 5.6 and 8.4 are internalized within cells to a greater extent than Cy3-siRNA−PEG/PEI 25K, supporting that the enhanced cellular uptake extent was directly related to the increased gene silencing efficiency (Figure 5C).

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Conclusions

We have developed surface-modified and reconstituted protein-free LDL mimicking solid lipid nanoparticles (SLN) as noncytotoxic and cationic carriers for delivery of siRNA. Surface modified SLN by incorporating DOPE and DC-chol showed the formation of stable complexes with siRNA−PEG conjugate via electrostatic interactions in serum containing media. siRNA−PEG/SLN complexes exhibited comparable gene silencing efficiency to siRNA−PEG/PEI complexes while showing very low cytotoxicity. It is reasonable to say that when the siRNA−PEG/SLN complexes were injected into the blood stream, plasma apolipoproteins would be adsorbed on the surface, thereby mimicking natural apolipoprotein-containing LDL nanoparticles. Since elevated levels of low density lipoprotein receptor (LDLR) are overexpressed in various cancer cells such as myeloid leukemic cells, colon, kidney, and brain tumor cells.[46,47] SLN might be useful nanocarriers for LDLR-mediated siRNA tumor targeting therapy. We are currently working on in vivo systemic delivery of siRNA−PEG/SLN for treatment of tumor in an animal model.

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