Water-Soluble Lipopolymer for Gene Delivery

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The use of biocompatible polymeric gene carriers may overcome the current problems associated with viral vectors in safety, immunogenicity, and mutagenesis. Nontoxic water-soluble lipopolymer (WSLP), poly{(ethylenimine)-*co*-[*N*-(2-aminoethyl) ethyleneimin]-*co*-*N*-(*N*-cholesteryloxycarbonyl-(2-aminoethyl)-ethylenimine)} was synthesized using branched poly(ethylenimine) (PEI, mw 1800) and cholesteryl chloroformate. Following synthesis and purification, the structure and molecular weight of WSLP were confirmed by ^{1}H NMR and MADI-TOF mass spectrometry, respectively. The percentage of cholesterol conjugated to PEI was about 47%, and the average molecular weight of WSLP was approximately 2000 Da. WSLP/pDNA complexes were prepared at different N/P (nitrogen atoms of WSLP/phosphate of plasmid DNA) ratios and characterized in terms of particle size, ζ potential, osmolarity, surface morphology, and cytotoxicity. WSLP condensed plasmid DNA when N/P ratio reached 2.5/1 and no free DNA was detected at N/P ratio of 5/1 and above, as determined by agarose gel electrophoresis. The mean particle size was in the range of 25.9 to 148.5 nm and was dependent on N/P ratios. Atomic force microscopy (AFM) showed complete condensation of plasmid DNA with spherical particles of ~50 nm in diameter. WSLP/pDNA complexes or WSLP itself were nontoxic to CT-26 colon adenocarcinoma and 293 T human embryonic kidney transformed cells when formulated at the N/P ratio of 10/1 and below as determined by MTT assay. In contrast, PEI25000/pDNA complexes were toxic to these cells. Erythrocytes aggregated when incubated with PEI25000/pCMV−Luc complexes at high DNA concentrations, but there was little aggregation with WSLP/pCMV−Luc complexes. WSLP/pCMV−Luc complexes demonstrated higher transfection efficiency in both CT-26 and 293 T cells compared to PEI25000- or PEI1800-based formulations. WSLP/pCMV−Luc complexes are nontoxic and showed enhanced in vitro transfection. Thus, WSLP will be a suitable carrier for in vivo gene delivery.

INTRODUCTION

Development of improved gene transfer methods is prerequisite for gene therapy to realize its clinical potential. Although currently less effective than viral vectors, cationic lipids and polymers are increasingly being considered for in vivo gene delivery into the animal models and patients due to their relative stability, greater carrier capacity, and ease of large scale production (*1*). Current cationic liposomes and polymers from complexes with plasmid DNA via electrostatic interaction and tend to form large aggregates on storage. These complexes are efficient at transfecting cells in culture, yet are poor at diffusing within a tissue or escaping from blood vessels (*2*−*5*). Polyethylenimine (PEI) has been reported to be effective for gene delivery, because plasmid DNA can be delivered to the cytoplasm via endosomes due to the proton-sponge effect of PEI (*6*, *7*). However, high molecular weight PEI is toxic to the cells, and PEI/DNA complexes are prone to aggregation (*8*).

The general structure of a cationic lipid has three parts: (i) a hydrophobic lipid anchor group, which helps in forming micellar structure and can interact with cell membranes; (ii) linker group, such as an ester, amide or carbamate; and (iii) a positively charged headgroup, which interacts with plasmid DNA, leading to its condensation. The hydrophobic group lipid anchors can be either a cholesterol group or fatty acid chains (*9*). The linker group is an important component and determines the chemical stability and biodegradability of cationic lipids. These linker groups should be biodegradable yet strong enough to survive in a biological environment. Ester linkage between hydrophobic lipid anchors and cationic headgroups are likely to provide biodegradability to cationic lipids. Cationic lipids with T-shaped headgroups tend to be more effective than linear counterparts (*10*). However, current cationic lipids are water insoluble, and most of them require the formation of liposomes. Complex formation between plasmid DNA and cationic liposomes usually produces large size complexes, which are poorly extravasated through the capillary endothelia.

In this study, we designed an effective water-soluble lipopolymer (WSLP) by combining the advantages both of cationic liposomes and PEI, which condenses DNA and enhances endosomal release due to its tertiary amines, while the lipid coating on the DNA increases its permeability through cell membranes (*11*). Monomolecular pDNA collapse has been suggested to occur only for cationic lipid or lipopolymer concentrations below the critical micellar concenturations (CMC) and are used for complex formation with DNA (*12*). We synthesized WSLP using PEI and cholesteryl chloroformate, and characterized it for its physicochemical properties, cytotoxicity, and in vitro transfection efficiency.

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MATERIALS AND METHODS

Materials. Poly(ethylenimine) (PEI, mw 1800) was purchased from Polysciences, Inc. (Warrington, PA). Cholesteryl chloroformate, PEI (mw 25000), triethyleneamine, glucose, deuterium oxide (D_2O), and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), terrific broth, ampicillin, Rosewell Park Memorial Institute (RPMI-1640) medium, Dulbecco's modified essential medium (DMEM), and 1,4-dioxane-d_8 were purchased from Sigma Chemical Co. (St. Louis, MO). LipofectAMINE, phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and proteinase K were purchased from Gibco-BRL (Gaithersburg, MD). QIAGEN maxi plasmid purification kit were purchased from QIAGEN (Valencia, CA). Methylene chloride and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fair Lawn, NJ). RQ1 RNase-free DNase, λ HindIII, ScaI, buffer B, 100bp DNA stepladder, 1kb DNA stepladder, reporter lysis buffer, and bacterial strain DH5α were purchased from Promega (Madison, WI). Albumin standard and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce Chemical Co. (Rocford, IL). PCMV−Luc was obtained as a kind gift from Dr. Mitsuru Hashida of Kyoto University, Japan.

Synthesis of Water Soluble Lipopolymer. Three grams of PEI (mw 1800) was stirred on ice in a mixture of 10 mL of anhydrous methylene chloride and 100 μL of triethylamine for 30 min. One gram of cholesteryl chloroformate was dissolved in 5 mL of anhydrous ice-cold methylene chloride and then slowly added to the PEI solution for 30 min. The mixture was stirred for 12 h on ice, and the resulting product was dried on a rotary evaporator. The powder was dissolved in 50 mL of 0.1 N HCl and then filtered through the glass microfiber filter. Cholesteryl chloroformate is well soluble in methylene chloride, diethyl ether, and acetone. Therefore, nonreacted cholesteryl chloroformate was removed from the product by washing three times in these different organic solvents as follows: the aqueous solution was extracted three times with 100 mL of methylene chloride to remove nonreacted cholesterol chloroformate, and then extracted aqueous solution was filtered through a glass microfiber filter. The product was concentrated by solvent evaporation, precipitated with large excess acetone, and dried under vacuum. The powder was dissolved in water and precipitated three times with acetone. After washing with methanol, the product was washed three times with diethyl ether. The white powder product was analyzed using matrix-assisted laser desorption-time-of-flight (MALDI-TOF) mass spectrophotometry (Perspective Voyager-DE STR, PE Applied Biosystem Co., Framingham, MA) with *trans*-4-hydroxy-3-methoxycinnamic acid as matrix and 400-MHz ^{1}H nuclear magnetic resonance (^{1}H NMR, Varian, Inc., Palo Alto, CA) and then stored at −20 °C until used.

Critical Micellar Concentration. The critical micellar concentration (CMC) of WSLP was determined using the dye solubilization methods as described previously by Jeong et al. (*13*). Various amounts of WSLP was dissolved in 1 mL of deionized water to adjust the concentrations of WSLP ranging from 9.28 to 76021.76 μg/mL. To prepare 0.4 mM stock solution of 1,6-diphenyl-1,3,5-hexatriene (DPH), 2.2 mg of DPH was dissolved in 23.54 mL of methanol followed by sonication for 15 min. Ten microliters of 0.4 mM DPH was added to 1 mL of WSLP and stored in dark for 6 h. The reference solution was also prepared by adding 10 μL of 0.4 mM DPH to 1 mL of water. The absorption spectra of these samples were measured at 200−600 nm using a UV/vis/NIR spectrometer (Perkin-Elmer Lamda 19). The absorbance at 356 nm was plotted against the concentration of WSLP, and the crossing point of the extrapolated two straight lines was defined as the CMC of WSLP.

Amplification and Purification of Plasmid. Plasmid DNA encoding Luciferase driven by CMV promoter (pCMV−Luc) was constructed by inserting the HindIII/XbaI firefly Luciferase cDNA fragment from pGL3-control vector into the HindIII/XbaI site of pcDNA3 (*14*). This plasmid DNA was transformed in *Escherichia coli* DH5α and amplified in terrific broth media at 37 °C overnight with 225 rpm. The amplified plasmid DNA was purified by QIAGEN Maxi plasmid purification kit. Purified plasmid DNA was dissolved in Tris−EDTA (TE) buffer, and its purity and concentration were determined by ultraviolet (UV) absorbance at 260 nm. The optical density ratios at 260−280 nm of these plasmid preparations were in the range of 1.7−1.8. To confirm that there is no rearrangement of the gene during cloning and propagation of the plasmid DNA, restriction enzyme assay was done using ScaI and HindIII followed by 1% agarose gel electrophoresis.

Gel Retardation and DNase Protection Assays. WSLP/pDNA complexes were prepared at various N/P (nitrogen atoms of WSLP/phosphate of plasmid DNA) ratios ranging from 1.25 to 20.0. In phosphate buffered saline, the positively charged polymer/plasmid complexes tend to aggregate on storage. Therefore, WSLP/pLuc complexes were prepared in the presence of 5% glucose to adjust the osmolality at 290−310 mOsm and incubated for 15−20 min at room temperature to allow complex formation between lipopolymer and pDNA. The samples were electrophoresed on agarose gel in 1× Tris−boric acid−EDTA (TBE) buffer at 80 V until the 1× orange blue loading dye ran through 80% of the gel. A 1kb marker was used as a DNA size marker. The gel was stained with 0.5 μg/mL ethidium bromide for 45 min and analyzed on UV illuminator to show the location of DNA.

For DNase protection assay, WSLP/pDNA (5/1, N/P) complexes were incubated at 37 °C in the presence of DNase I (45 μL). At 0, 5, 10, 15, 30, 60, and 120 min postincubation, 50 μL of the samples was taken into Eppendorf tubes and was mixed with 100 μL of stop solution (400 mM NaCl, 100 mM EDTA) under mild vortexing. The samples were then mixed with 12 μL of 10% sodium dodecyl sulfate (SDS) and incubated at 65 °C overnight. The DNA was then extracted with the mixture of Tris-EDTA saturated phenol: chloroform: isoamyl alcohol (25:24:1, v/v). The extracted DNA was precipitated with 700 μL of absolute ethanol at 12000 rpm for 30 min and washed with 70% ethanol. The DNA precipitate was dried by air followed by dissolving in 10 μL Tris−EDTA buffer, and the plasmid DNA integrity was assessed using 1% agarose gel electrophoresis.

Atomic Force Microscopy. Morphology of WSLP/pDNA complexes was analyzed using atomic force microscopy (AFM) as described by Maheshwari et al. (15). Briefly, red mica was freshly cleaved as a thin wafer and then soaked in 33 mM $MgAc_2$ overnight to favor the replacement of potassium ions by divalent magnesium ions for stronger DNA binding. Mica was then sonicated for 30 min in distilled water, and its surface was subjected to glow discharge for 15 s in a vacuum between 100 and 200 mTorr. As soon as the mica surface was exposed to air, 20 μL of 0.1 mg/mL of WSLP/pDNA complexes was placed on mica surface and allowed to

stick for 2 min. The mica surface was then rinsed gently with deionized water and blow−dried with nitrogen gas. Nanoscope II SFM (Digital Instruments, Santa Barbara, CA) was used for imaging at room temperature in the attractive force regime and under 30−60% relative humidity. The microscope was operated using cantilever oscillation frequencies between 12 and 24 kHz. Force minimization was maintained by reducing the set point voltage to minimize sample damage. To remove the high frequency noise in the slow scan direction, only minimal filtering was applied to the image.

Particle Size and ζ Potential Measurement. ζ potential and particle size of WSLP/pDNA complexes were measured as described by Mahato et al. (*16*). Briefly, polymer/pDNA complexes were prepared as discussed above and were diluted four times in the cuvette, and the electrophoretic mobility was determined with Zeta-PALS (Brookhaven Instruments Corp, Holtsville, NY). All experiments were performed at 25 °C, pH 7.0 and 677 nm wavelength at a constant angle of 15°. The ζ potential was automatically calculated from the electrophoretic mobility based on Smoluchowski's formula. Following the determination of electrophoretic mobility, the samples were subjected to mean particle size measurement by the same equipment using the same light source and wavelength. The particle size was reported as effective mean diameter.

Cell Lines. 293T human embryonic kidney transformed cells were obtained from ATCC (Rockville, MD) and were grown and maintained in DMEM medium containing 10% FBS, 100 units/mL penicillin and 2.2 mg/mL sodium bicarbonate at 37 °C humidified 5% CO_2.

CT-26 colon adenocarcinoma cell lines was a kind gift from Dr. Charles Tannenbaum of Cleveland Clinic Foundation, Cleveland, OH (*17*). Tumor cells were grown and maintained in RPMI 1640 medium which was supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 2.2 mg/mL sodium bicarbonate at 37 °C and humidified 5% CO_2.

Cytotoxicity Assay. Cytotoxicity of WSLP/pDNA complexes prepared at various charge ratios were assessed as described by Fisher et al. (*18*). The fraction of nitrogen atoms that are protonated at neutral pH is unknown. Therefore, the PEI/DNA ratios are usually calculated on the basis of PEI nitrogen per DNA phosphate (i.e., N/P ratio) (*19*). The optimal N/P ratio of PEI/pLuc complexes was determined, according to several publications, to be around 5/1 (*6*, *20*−*23*). However, Boussif et al. mentioned that the transfection efficiency of PEI (800 and 50 kDa) was highest at 13/1 N/P ratio (*19*). In their studies, the transfection efficiency was reported in terms of relative light units (RLU). Transfection efficiency/cytotoxicity balance is known to increase with increase in the polymer molecular weight and charge (±) or N/P ratios. Although PEI (mw 800 or 50 kDa)/pDNA complexes showed high transfection at 13/1 N/P ratio, many cells might have died due to high toxicity. To normalize the cytotoxic effect of the polymer, we reported our transfection results in terms of RLU/mg of total proteins. CT-26 cells and 293T cells were seeded in 96 well plates at 4000 cells/well and incubated at 37 °C for 24 h. After checking the cell confluency, which was over 70%, gene carriers were added to the cells. Following 48 h of incubation at 37 °C, 25 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) stock solution in phosphate-buffered saline (5 mg/mL) was poured into each well until it reached a final concentration of 0.5 mg/mL MTT. The plate was then incubated at 37 °C in 5% CO_2 for 5 h. The medium was removed, and 150 μL of DMSO was added to dissolve the formazan crystals. The plate was read spectrophotometrically at 570 nm using an ELISA plate reader. The relative cell viability was calculated as $[Abs]_{sample}/[Abs]_{control} \times 100$.

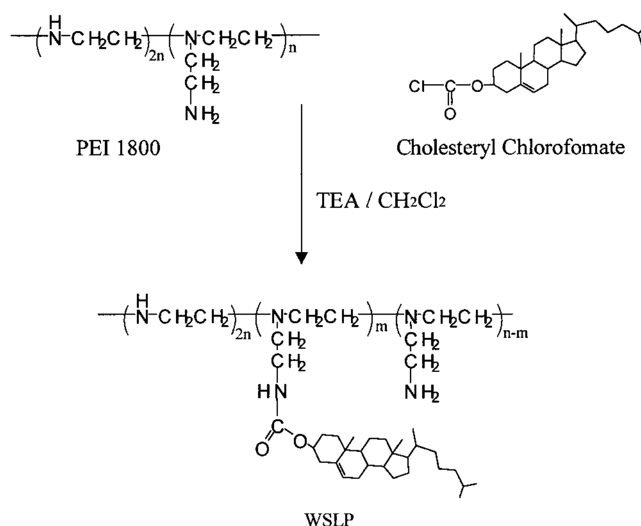

Figure 1. Sythesis scheme of poly{(ethylenimine)-*co*-[*N*-2-(aminoethyl)ethylenimine]-*co*-[*N*-(*N*-cholesteryloxycarbonyl-(2-aminoethyl))ethylenimine]}.

Erythrocyte Aggregation Assay. Erythrocyte aggregation assay was done as described by Ogris et al. (*24*). Briefly, fresh blood was collected from a BALB/c female mouse and immediately mixed with sodium citrate to a final concentration of 25 mM. Erythrocytes were washed on ice three times in Ringer's solution. Five and ten microliters of erythrocytes/well of six well plate were mixed with WSLP/pCMV−Luc (10/1, N/P) complexes at a dose of 20 μg DNA/well. PEI 25000/pCMV−Luc (5/1, N/P) complexes were used as a control. The cells containing erythrocytes were incubated for 1 h at 37 °C.

In Vitro Transfection. CT-26 cells and 293 T cells were seeded separately in six well tissue culture plates at 3×10^5 cells per well in 10% FBS containing RPMI 1640 or DMEM media. Cells achieved 70% confluency within 24 h after which they were transfected with WSLP/pDNA complexes prepared at different charge (N/P) ratios ranging from 5/1 to 20/1. The total amount of plasmid DNA loaded was maintained constant at 2.5 μg/well and transfection was carried out in absence of serum. The cells were allowed to incubate at 37 °C in the presence of complexes for 6 h in CO_2 incubator followed by replacement of 2 mL of RPMI 1640 or DMEM containing 10% FBS. Thereafter the cells were incubated 37 °C for additional 36 h. Cells were lysed using 1X lysis buffer after washing with cold PBS. Total protein assays were carried out using BCA protein assay kit. Luciferase activity was measured in terms of relative light units (RLU) using 96 well plate Luminometer (Dynex Technologies Inc, Chantilly, VA). The Luciferase activity was monitored and integrated over a period of 30 s. The final values of Luciferase were reported in terms of RLU/mg total protein. In all the above experiments, both naked DNA as well as untreated cultures were used as positive and negative controls, respectively.

RESULTS

Synthesis of Water-Soluble Lipopolymer. Water-soluble lipopolymer (WSLP) was synthesized using branched PEI of 1800 Da and cholesteryl chloroformate

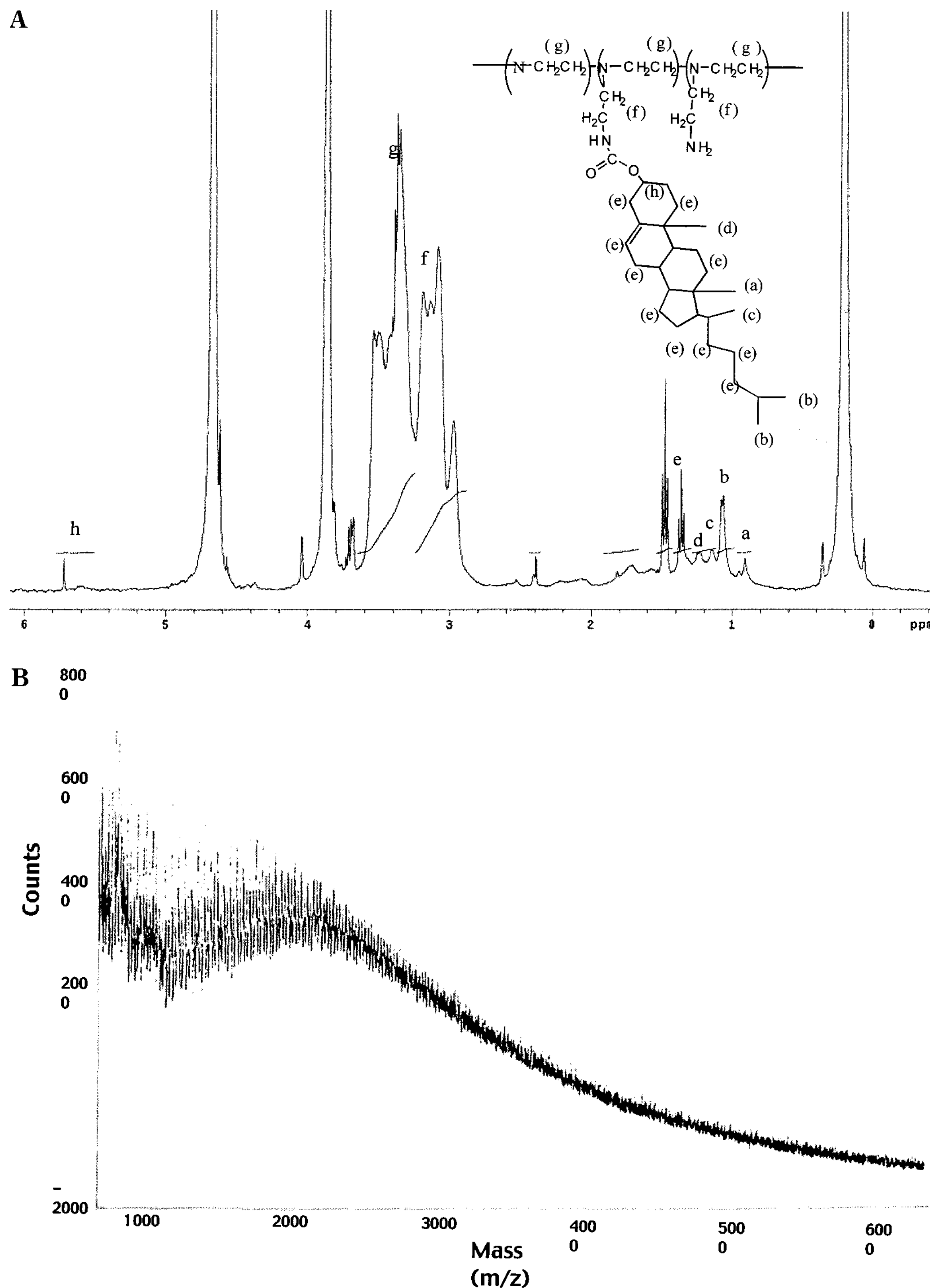

Figure 2. Determination of chemical structure and molecular weight of water-soluble lipopolymer (WSLP) by ^{1}H NMR spectra (A) and MALDI-TOF mass spectra (B).

(Figure 1). Following synthesis and purification, the structure and molecular weight of WSLP were determined using 400-MHz ^{1}H NMR and MALDI-TOF mass spectrometry. The NMR results are as follows (Figure 2 A): ^{1}H NMR (400 MHz, D_2O + 1,4-dioxane-d_6) δ 0.93 (s, 4.28 H of CH_3 from cholesterol (a)); δ 1.08 (d, 17.89 H of $(CH_3)_2$ from cholesterol (b)); δ 1.14 (d, 8.9 H of CH_3 from cholesterol (c)); δ 1.24 (d, 9.0 H of CH_3 from cholesterol (d)); δ1.35 and δ 1.48 (t, 16.68 and 17.53 H from CH_2-CH_2 and $CHCH_2$ from cholesterol (e)) δ 3.1 (59.6 H of $NHCH_2CH_2$ from the side chain of PEI (f)); δ 3.4 (80.8 H of $=NCH_2CH_2NH_2$ from backbone of PEI (g)); δ 5.65 (1 H of $=C=CHC$ from cholesterol (h)). The peak appearing at δ 0.8–1.9 was from cholesterol. The amount of cholesterol conjugated to PEI was determined to be about 47%. MALDI-TOF mass spectrophotometric analysis of WSLP showed its molecular weight to be approximately 2000 (Figure 2B). Many intensity peaks appeared from 1000 to 3500 (*m*/*z*); however, the majority peaks were at around 2000 (*m*/*z*). Expected position is 2414: 1800 (mw of PEI) + 449−35 (mw of cholesteryl chloroformate after removal of one chloride). The data suggest that the majority of WSLP synthesized were of 1/1 molar ratio of cholesterol and PEI of 1800 Da, although some were either not conjugated or conjugated at the molar ratio of 2/1 (cholesterol/PEI). The molecular weight of WSLP could not be measured using MALDI-TOF mass spectrophotometry at various concentrations and matrices.

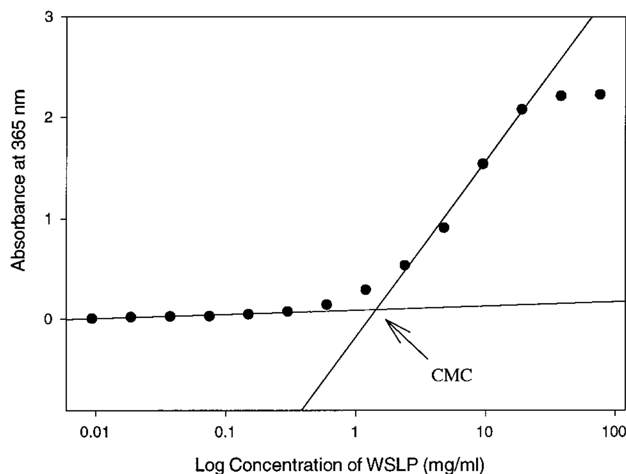

Figure 3. Determination of critical micellar concentration of water-soluble lipopolymer (WSLP) by dye solubilization methods.

Critical Micellar Concentration. WSLP is amphiphilic in nature because water is a poor solvent for hydrophobic cholesterol while it is a good solvent for hydrophilic PEI. With increasing its concentration, WSLP may form mutimolecular micelles or micellar aggregates. The solubility of WSLP in water greatly depends on the molar ratio of PEI and cholesterol, and thus it is important to maintain the hydrophobic−hydrophilic balance. DPH is a hydrophobic dye, which has a significantly lower absorptivity at 356 nm in an aqueous environment compared with that in the hydrophobic environment. With the formation of micelles, DPH is preferentially partitioned into the hydrophobic core of micelles, resulting in the increase in the absorbance of DPH (*13*). The abrupt increase in absorbance reflects the micelle formation. As show in Figure 3, CMC of WSLP was determined to be 1.43 mg/mL which was the cross-point of expolating the absorbance at low and high concentration regions.

Band Retardation and DNase Protection Assays. DNA condensing ability of WSLP was determined by gel retardation assay. The positively charged headgroup of WSLP makes strong complexes with the negatively charged phosphate ions on the base backbone on plasmid DNA. When the value of N/P reached 5/1, free DNA could not be detected on agarose gel electrophoresis (data not shown). WSLP could protect pDNA from digestion by DNase at least for 2 h at 37 °C, whereas naked DNA was completely digested by DNase within 5−10 min of incubation at 37 °C (data not shown).

Surface Morphology. DNA condensation, its reduction in overall size and the resulting surface morphology were captured by AFM (Figure 4). Plasmid DNA when complexed with WSLP at the N/P ratio of 10 was completely condensed and formed spherical particles of ∼50 nm. The AFM image revealed that the naked pDNA was condensed from its natural extended state in water to about one-tenth of its size in the complex. The small-sized complexes might be of special significance, as it could facilitate enhanced mobility of the complex through the plasma membrane as well as trafficking across the cytoplasm.

Particle Size and ζ Potential. The compaction of plasmids into particulates of defined colloidal and surface

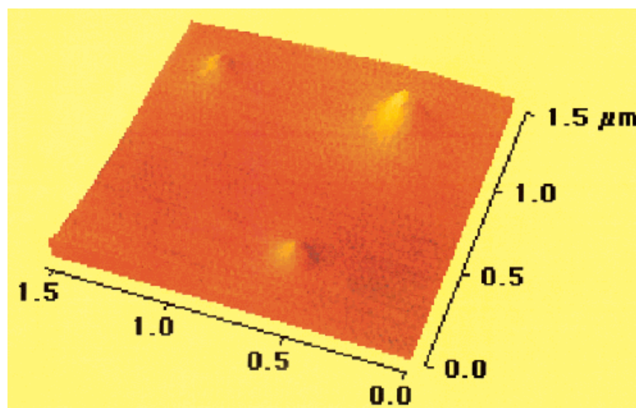

Figure 4. Atomic force microscopy (AFM) image of WSLP/pCMV−Luc complexes (10/1, N/P). Condensed plasmid DNA forms an ellipsoidal structure as seen in *z*-direction.

properties plays a major role in controlling stability, biodistribution, and intracellular fate of formulated plasmids. Interaction with the tissue components and transport in the interstitial space and uptake characteristics by the cells. Low aggregate formulation and a small-sized complex would enhance the diffusivity and cellular uptake of plasmid DNA in the tissues. The particle size distribution of WSLP/pDNA complexes was determined by dynamic lighter scattering. In contrast to the large size of naked DNA (*25*), complex formation between WSLP and plasmid DNA produced small size particles. The mean particle size of the complexes was in the range of 25.9 to 148.5 nm and was dependent on N/P ratios (Table 1). At the N/P ratio of 1.25, plasmid DNA was not completely neutralized, resulting in the formation of heterogeneous particles with the mean diameter of 148.5 nm. In contrast, the mean particle size of WSLP/pDNA complexes 40 nm or below, when the complexes were formed at the N/P ratios of 2.5 and 10. However, at 20/1 (N/P) ratio these complexes had narrow particle size distribution with a mean diameter of 61.8 nm.

ζ potential of WSLP/pDNA complexes prepared at different charge ratios was greatly dependent on the charge ratio and linearly increased with the increase in the charge ratios. At the N/P ratio of 2.5/1, it was −41.35 mV, but increased to 8.23 mV when formulated at N/P ratio of 5/1. The gel retardation data confirm the above finding, as there was no DNA movement seen at the N/P ratio of 2.5/1 or above.

Cytotoxicity Assay. The cytotoxicity of WSLP and WSLP/pCMV−Luc complexes after 24 h of incubation at 37 °C was determined using MTT assay on CT-26 colon adenocarcinoma (Figure 5 A) and 293 T human embryonic kidney transformed cells (Figure 5 B). The cytotoxicity of WSLP and WSLP/pCMV−Luc complexes was compared with commercially available cationic liposomes (LipofectAMINE/pCMV−Luc, 5/1 w/w) and branched PEI of 25000 Da (PEI25000/pCMV−Luc, 5/1 N/P) complexes. LipofectAMINE reagent is 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-*N*-[2(sperminecarboxyamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA, mw 867) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE, mw 744) in membrane-filtered water. Based on its chemical structure, LipofectAMINE has two primary amines, two

Table 1. Mean Particle Size and ζ Potential of WSLP/pDNA Complexes

N/P ratios of WSLP/pDNA	1.25/1	2.5/1	5.0/1	10.0/1	20.0/1
mean particle size (nm)	148.5 ± 0.18	25.9 ± 0.02	41.6 ± 0.05	42.1 ± 0.53	61.8 ± 0.07
mean ζ potential (mV)	−59.12 ± 2.79	−41.35 ± 2.18	8.23 ± 0.25	37.40 ± 0.46	61.67 ± 0.06

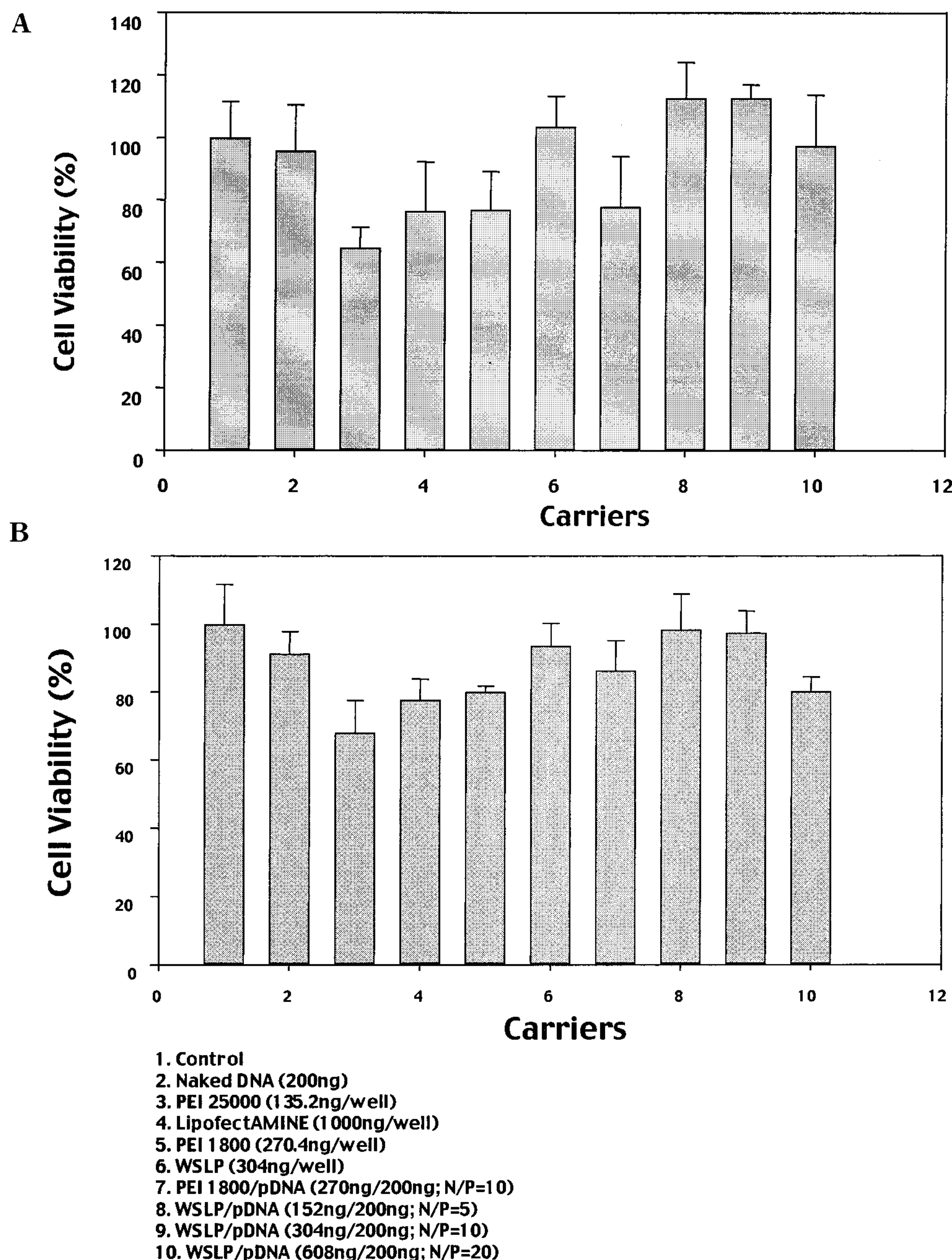

Figure 5. Cell viability assay of CT-26 colon adenocarcinoma (A) and 293 T human embryonic kidney transformed (B) cell lines after transfection with WSLP/pCMV−Luc complexes prepared at different N/P ratios in 5% (w/v) glucose. Naked pCMV−Luc, PEI 25000 (25 Kda) and LipofectAMINE were used for comparison. Relative cell viability was at least 85% for all WSLP/pCMV−Luc complexes. In contrast, PEI 25000/pCMV−Luc (5/1, N/P) complexe-based transfection resulted in less than 60% cell viability.

secondary amines, and one quaternary amine, total five positive charges per molecule (*26*). Subsequent calculations show that 5/1 (w/w) corresponds to 6.82/1 (±) for LipofectAMINE/pDNA complexes. Following normalization by (±) charge ratios, we thus confirmed that WSLP/pDNA complexes or WSLP itself were indeed nontoxic to the cell when formulated at N/P ratio of 10 and below (Figure 5). In contrast, LipofectAMINE/pDNA and PEI 25000/pDNA complexes were toxic to the cells. Compared to 293T cells, these complexes were more toxic to CT-26 cells, which granulated and the cell population decreased under the same or lower concentration as WSLP. Similar to WSLP, PEI of 1800 Da was less toxic to both CT-26 and 293T cells compared to PEI 25000 or LipofectAMINE (Figure 5).

Interaction with Erythrocytes. To investigate the interaction of WSLP/pDNA complexes with erythrocytes, an erythrocyte aggregation assay was performed (Figure 6). Mouse erythrocytes were incubated with either WSLP/pCMV−Luc (10/1, N/P) or PEI25000/pCMV−Luc (5/1, N/P) complexes at a dose of 20 μg DNA/well. After 2 h of incubation at 37 °C, several erythrocytes were aggregated, when the cells were incubated with PEI25000/pCMV−Luc complexes. In case of WSLP/pCMV−Luc complexes, the extent of erythrocyte aggregation was minimal (Figure 6). Although we did not check the effect of N/P ratios on erythrocyte aggregation, we expect significant increase in erythrocyte aggregation with the increase in N/P ratios of WSLP/pCMV−Luc complexes.

In Vitro Transfection. WSLP/pCMV−Luc complexes formulated at different N/P ratios in 5% (w/v) glucose were evaluated for their transfection efficiency in CT-26 colon carcinoma and 293 T human embryonic kidney transformed cells. Following transfection, cells were

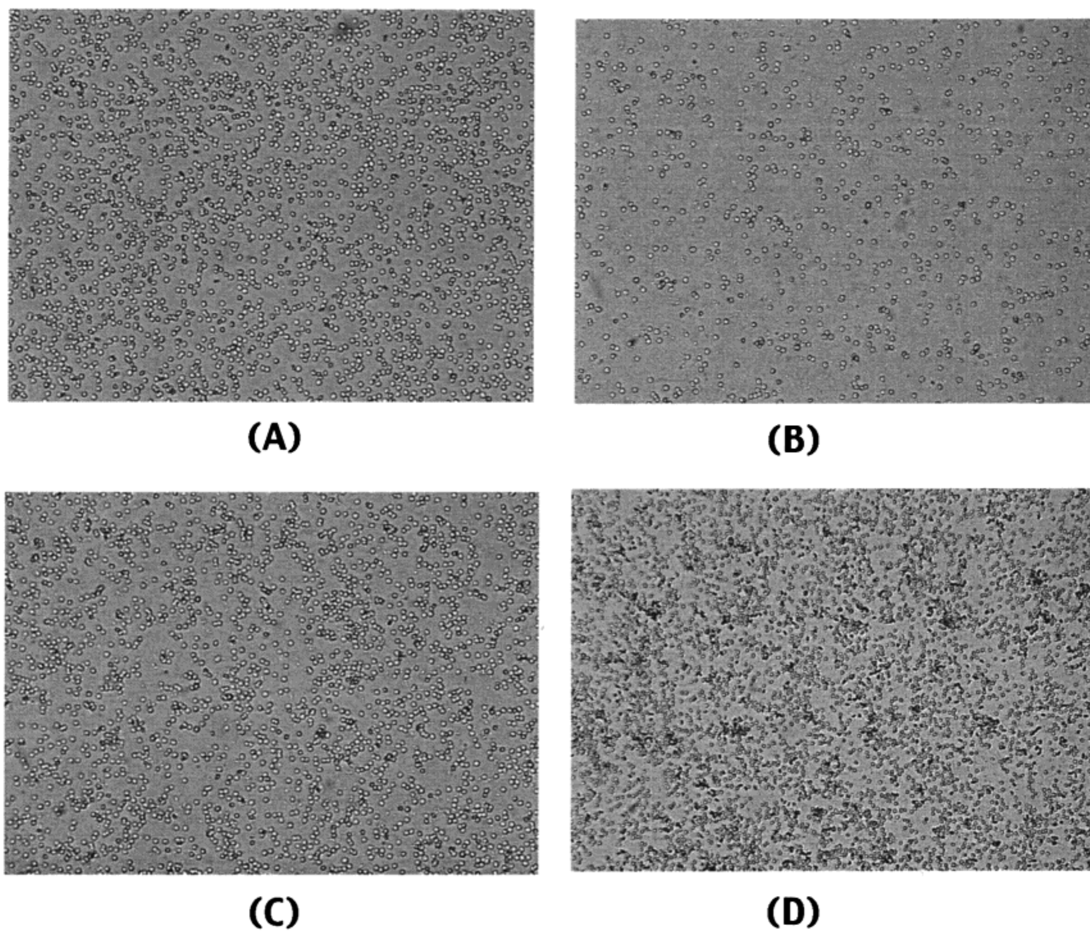

Figure 6. Interaction of WSLP/pCMV−Luc complexes with ertyrocytes. Freshly collected erythrocytes were washed thrice with Ringer's solution and incubated with WSLP/pCMV−Luc (10/1, N/P) containing 20 μg DNA/well of six well plates. Untreated erythrocytes were used as control. PEI25000/pCMV−Luc (5/1, N/P) complexes treated erythrocytes were used for comparison. (A) Nontreated erythrocytes, (B) erythrocytes treated with 5 μL of WSLP/pCMV−Luc (10/1, N/P), (C) erythrocytes treated with 10 μL of WSLP/pCMV−Luc (10/1, N/P), and (D) erythrocytes treated with 10 μL of PEI25000/pCMV−Luc (5/1, N/P) complexes.

lysed, and RLU and total protein concentration were measured. Transfection efficiency increased with the increase in N/P ratios. Among the N/P ratios tested, highest Luciferase activity (RLU/μg total protein) was observed at N/P ratio of 20/1 for both CT-26 and 293T cells (Figure 7). The RLU values for WSLP/pCMV−Luc complexes gave several orders of magnitude higher Luciferase levels compared to naked pCMV−Luc. The Luciferase activity of WSLP/pCMV−Luc complexes was also higher than PEI25000/pCMV−Luc (5/1, N/P) complexes when formulated at the N/P ratio of 20/1, but similar when formulated the N/P ratio of 10/1.

DISCUSSION

Current cationic lipid and polymer-based gene carriers are not satisfactory due to the low transfection efficiency, high toxicity, and poor storage stability. In an attempt to combining these two modalities, namely cationic liposomes and polycations, Huang and associates (*27*) synthesized lipopolysine for gene transfer. However, treatment for endosomal disruption, such as chloroquine treatment or liposome preparation with colipid DOPE was essential for enhanced gene transfer by lipopolysine (*28*). Behr (*29*) synthesized lipopolyamine, which mediates transfection by itself and does not require any phospholipid to form liposomes. In other words, a micellar complex of lipopolyamine and DNA was sufficient for transfection. Pitard et al. (*30*) attempted to determine the conditions under which DNA molecules can collapse into oligomolecular complexes rather than forming large multimolecular aggregates when mixed with lipopolyamine micelles. As lipopoly(L-lysine) requires the addition of chloroquine or the formation of liposomes with colipid DOPE for efficient transfection, Yamazaki et al. (*11*) grafted cetyl groups as hydrophobic lipid anchors on to PEI with molecular weights of 1800 and 25000 Da and prepared polycation liposomes for gene transfer. However, there is the growing need to avoid the preparation of cationic liposomes and the variability associated with it, especially because the large DNA particles are not good for many in vivo applications, due to their poor extravasation through the capillary endothelia and dispersibility inside the tumor tissues.

Several cholesterol-based cationic lipids have been proven been used for gene transfer. Gao and Huang (*2*) were the first to synthesize a cholesterol-based cationic lipid 3β[N, (*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) using cholesteryl chloroformate and *N*,*N*-dimethylethylenediamine and mixed with colipid DOPE to form cationic liposomes that efficiently transfect mammalian cells. DC-Chol liposomes have already been used in gene therapy applications in the clinical settings (*31*, *32*). These early successes spurred recent interest in the development of novel cholesterol-based cationic lipids. Spermine cholesteryl carbamate and spermidine cholesteryl carbamate have been shown to be more active than DC-Chol, which has only a single protonatable amine. Furthermore, spermine cholesteryl carbamate,

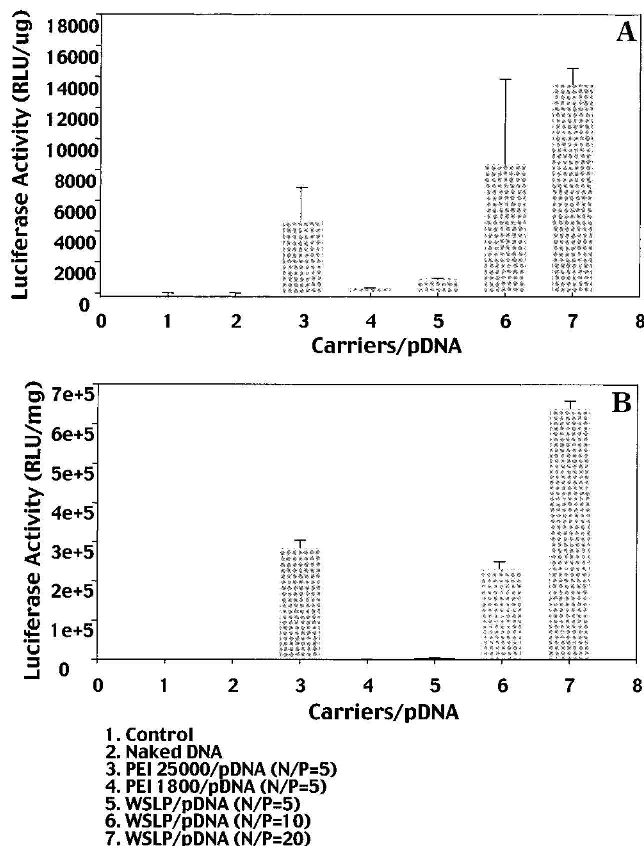

Figure 7. Luciferase activity in cultured CT-26 colon carcinoma (A) and 293 T human embryonic kidney transformed (B) cells after transfection with WSLP/pCMV−Luc complexes prepared at different (N/P) charge ratios in 5% (w/v) glucose. Naked pCMV−Luc, PEI 1800/pCMV−Luc and PEI 25000/pCMV−Luc complexes were used for comparison. Nontransfected cells (in vitro) were used as negative controls. Luciferase activity is expressed as RLU/mg of total protein.

which contains three protonatable amines, was more effective compared to two protonatable spermidine cholesteryl carbamate (*10*).

In this study, we conjugated cholesterol as a hydrophobic lipid anchor to PEI, a cationic polymer known to have high transfection activity. PEI is a cationic polymer composed 25% primary amines, 50% secondary amines, and 25% tertiary amines. Massive vesicular ATPase-driven proton accumulation followed by passive chloride influx into endosomes buffered with PEI should cause osmotic swelling and subsequent endosome disruption (*19*). For gene transfer, PEI of high molecular weight, namely 25000 Da or above, is commonly used, which is highly toxic to the cells even at low N/P ratios, and PEI25000/pDNA complexes are prone to aggregation (*33*). Since micellization may concentrate positive charge on the lipid surface, polycations with high molecular weight might not be required. Furthermore, anchoring of polymers with lipids may strongly be observed with polymers of low molecular weight (*11*). Thus, in the present study, we used PEI of 1800 Da, which has about 41 monomer units of aziridine and around 13 units of primary amines in one molecule. WSLP can destabilize the endosomal membrane by protonation of PEI itself without the requirement of nonbilayer lipids. PEI has many protonation sites, which may cause influx of chloride ions into the endosomes, and as a result of this proton-sponge effect, endosomes are broken to release their internal contents (*1*). Thus, this water-soluble lipopolymer (WSLP) should deliver plasmid DNA without the requirement of any colipid as a liposomal compartment. The percentage of cholesterol conjugated to PEI was about 47%, and the average molecular weight of WSLP was about 2000 Da (Figure 2). WSLP forms micelles in water with a critical micellar concentration value of 1.43 mg/mL (Figure 3). Since WSLP was found to form micellar solutions, we investigated its potential usefulness as transfection reagent by direct mixing its solution with plasmid containing solution. The particle size of WSLP/pDNA complexes was in the range of 25.9 to 148.5 nm and was dependent on N/P ratios. Atomic force micrographs suggest that these complexes are spherical particles of ~50 nm (Figure 4).

WSLP and WSLP/pDNA complexes were not toxic to CT-26 colon carcinoma and 293T human embryonic kidney transformed cells even at N/P charge ratios, whereas both PEI25000 and LipofectAMINE-based formulations were fairly toxic to these cells (Figure 5). WSLP/pDNA complexes did not cause any aggregation of freshly prepared murine erythrocytes, whereas PEI25000/pDNA complexes aggregated the erythrocytes (Figure 6). This result suggests that cholesterol of WSLP may provide some shielding effect to WSLP/pDNA complexes and thus preventing erythrocytes aggregation.

WSLP/pDNA complexes efficiently transfected CT-26 colon carcinoma and 293T human embryonic kidney transformed cells (Figure 7). The transfection efficiency of WSLP was dependent on the N/P ratios, and the highest Luciferase activity was observed at the N/P ratio of 20/1 (Figure 7). Our findings on the usefulness of water soluble lipopolymers are in good agreement with Vigneron et al. (*34*), who reported the synthesis of guanidinium cholesterol lipids and their use for the efficient transfection of various mammalian cell lines as water-soluble lipopolymer as well as in the form of cationic liposomes.

In summary, we designed a water soluble nontoxic lipopolymer, which efficiently condenses and protects plasmid DNA from degradation by nucleases, and promotes enhanced transfection into mammalian cells.

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