

Novel Branched Poly(Ethylenimine)–Cholesterol Water-Soluble Lipopolymers for Gene Delivery

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A novel water-soluble lipopolymer was synthesized by linking cholesteryl chloroformate to the secondary amino groups of branched poly(ethylenimine) (PEI) of 1800 and 10000 Da. Conjugation through PEI secondary amines gives this newly synthesized lipopolymer (abbreviated as PEI–Chol) special advantage over our previously synthesized lipopolymers, which utilized the primary amino groups for conjugation, as the primary amino groups have a significant role in DNA condensation. Also, significantly, only one cholesterol molecule was grafted onto each PEI molecule (confirmed by ¹H NMR and MALDI–TOF mass spectrometry), leaving enough space for the steric interactions of the PEI's primary amines with the DNA. The PEI–Chol lipopolymer was characterized for the critical micellar concentration (cmc), buffer capacity, DNA condensation (by band retardation and circular dichroism), in vitro transfection efficiency, and cell viability. The cmcs of PEI–Chol 1800 and PEI–Chol 10000 were 496.6 and 1330.5 μg/mL, respectively. The acid–base titration indicated high buffering capacity of the polymers around the pH range of 5–7, which indicated their potential for buffering in the acidic pH environment of the endosomes. The band retardation studies indicated that efficient condensation of the plasmid DNA could be achieved using these lipopolymers. The circular dichroism spectra indicated a change in DNA conformation and adoption of lower energy state upon condensation with these lipopolymers when an N/P ratio of 2.5/1 or above was formulated. The mean particle size of these complexes was in the range 110–205 nm, except for the complexes prepared using PEI of 1800 Da, which had a mean particle size of 384 ± 300 nm. The ζ potential of DNA complexes prepared using PEI–Chol 1800, PEI–Chol 10000 and PEI of 1800, 10000, and 25000 Da at an N/P ratio of 15/1 was in the range 23–30 mV and was dependent on the N/P ratios. The in vitro transfection of PEI–Chol/pCMS-EGFP complexes in Jurkat cells showed high levels of expressed Green Fluorescent Protein (GFP) with little toxicity as determined by flow cytometry. These novel water-soluble lipopolymers provided good transfection efficiency with other desirable characteristics such as water solubility, free primary amino groups for efficient DNA condensation and high buffer capacity that indicated the possibility of efficient endosomal release.

Introduction

Nonviral gene delivery systems utilize synthetic gene carriers to condense and protect plasmid DNA (pDNA) from premature degradation during storage and transportation from the site of administration to the site of gene expression.^{1–3} Plasmid DNA is condensed into a highly organized structure through a complex self-assembly process.⁴ The commonly utilized synthetic gene carriers are cationic lipids, polymers, and peptides that condense pDNA by virtue of their electrostatic interactions with the anionic phosphate backbone of the nucleic acid chain.^{5–7} Cationic copolymers synthesized by grafting PEI with nonionic polymers, such as poly-

(ethylene oxide) (PEO) or Pluronic 123 have also been used for gene delivery.⁸ However, noncondensing polymers, such as poloxamers and poly(vinylpyrrolidone) (PVP) also being investigated for gene delivery to muscle and tumor tissues.^{9,10}

Most cationic lipids used for gene transfer have three parts: (i) a hydrophobic lipid anchor group; (ii) linker group, such as an ester, amide, or carbamate; and (iii) a positively charged headgroup, which interacts with pDNA, leading to its condensation.¹¹ Cholesterol is a naturally occurring lipid and is metabolized in the body. The early success of the 3-β[*N*-(*N'*,*N'*-dimethylaminoethane)carbonyl]cholesterol (DC–Chol) lipid-based gene delivery system spurred interest in the development of novel cholesterol-based cationic lipids.^{6,12,13} Among all the basic components of the cationic lipid, such as the hydrophobic lipid anchor, linker, and cationic headgroup, the type of the headgroup has been shown to have a dominant role in transfection efficiency and cytotoxicity. The levels of gene expression obtained with

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spermine cholesteryl carbamate and spermidine cholesteryl carbamate was 50–100-fold higher both *in vitro* and *in vivo* than that observed with DC-Chol, which has only a single protonatable amine.¹² Polyethylenimine (PEI) of 25 kDa or above has been reported to be effective for gene delivery, because pDNA can be delivered to the cytoplasm after endosomal disruption due to the proton-sponge effect of PEI, in which unprotonated amino moieties on the polymer buffer at the pH inside the endocytic vehicle.^{14,15} However, high molecular weight PEI is toxic to the cells, and PEI/pDNA complexes are prone to aggregation.

To make gene therapy clinically acceptable, delivery systems, or vectors, must deliver DNA to the cells in a transcriptionally active form and fulfill all regulatory agency mandates to be considered safe for use in humans. We recently designed a water-soluble lipopolymer (WSLP) by combining the advantages of both cationic liposomes and PEI, which condenses DNA and enhances endosomal release due to its secondary and tertiary amines, while the lipid coating on the DNA increases its permeability through cell membranes.^{16,17} In that study, cholesteryl chloroformate was directly linked to branched PEI of 1800 Da through primary amines, which are important for efficient DNA condensation. Therefore, in the present study, we first blocked the primary amines of PEI with benzyloxycarbonyl (CBz) to allow conjugation through the secondary amines, which is expected to result in a “T-shaped” lipopolymer. Following synthesis and purification, the structure and molecular weight of these lipopolymers were by ¹H NMR and MALDI-TOF mass spectrometry. The physicochemical properties of PEI-Chol were determined by measuring critical micellar concentration (cmc), buffer capacity assay, and DNA condensation by circular dichroism (CD) spectroscopy. The mean particle size and ζ potential of PEI-Chol/pDNA and PEI/pDNA complexes were also determined using a dynamic light scattering technique. Further, the *in vitro* transfection efficiency and cytotoxicity of PEI-Chol/pDNA complexes was evaluated in murine Jurkat T-cell lines at different N/P ratios. In addition, we determined the effect of the orientation and molecular weight of the cationic headgroup (PEI 1800 and PEI 10000) as well as the effect of serum on transfection efficiency and cytotoxicity.

Experimental Procedures

Materials. Branched polyethylenimine (PEI; mw 1800 and 10000 Da) was purchased from Polysciences, Inc. (Warrington, PA). Benzylchloroformate, (dimethylamino)pyridine (DMAP), NaHCO₃, agarose, phosphate buffered saline (PBS, pH 7.4), ethidium bromide (EtBr), 2-propanol, terrific broth, glycerol, D-(+)-glucose, dimethyl sulfoxide (DMSO), EDTA, and Luria agar were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesteryl chloroformate, imidazole, 10% palladium on activated carbon (Pd/C), chloroform-*d*₁, *trans*-4-hydroxy-3-methoxycinnamic acid, and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Triethylamine, methylene chloride, acetone, ethyl ether, citric acid, ethyl acetate, acetic acid, NaOH, acetonitrile, trifluoroacetic acid (TFA) and ethyl

alcohol were purchased from Fisher Scientific (Fair Lawn, NJ). The Enhanced Green Fluorescent Protein expression plasmid (pCMS-EGFP) was purchased from BD Biosciences Clontech (Palo Alto, CA). Tris-Boric acid-EDTA Buffer (10X) and S.O.C. medium were respectively purchased from Promega, Inc. (Madison, WI) and Gibco Inc (Gaithersburg, MD). Supercompetent cells for plasmid propagation were purchased from Stratagene Inc. (La Jolla, CA) and QiaFilter Plasmid Maxi Kit for plasmid purification was purchased from Qiagen (Valencia, CA).

Synthesis of PEI-Chol. Stage I. Benzylchloroformate (1.76 g, 1.5 mL, 10.36 mmol) was dissolved in methylene chloride (5 mL) and placed in a three-neck flask under nitrogen atmosphere. Imidazole (1.4 g, 20.6 mmol) was dissolved in methylene chloride (20 mL) and placed in an addition funnel. The three-neck flask was cooled to 0 °C using ice and the imidazole solution was added gradually over 30 min. The mixture was stirred at room temperature for 2 h. Methylene chloride (25 mL) and citric acid (10%; 25 mL) were added. The layers were separated and the organic fraction was washed with citric acid (10%; 25 mL). The organic component was dried over magnesium sulfate and concentrated *in vacuo*. The residue was dried under high vacuum for 2 h at ambient temperature.

Stage II. To the product of Stage I (CBz) were added DMAP (35 mg) and methylene chloride (25 mL), and the mixture was cooled to 0 °C under nitrogen atmosphere. To an addition funnel was added a solution of PEI (MW 1800; 1.7 g, ~10 mmol PEI repeat unit) in methylene chloride (25 mL). The PEI solution was added gradually over 30 min. The reaction mixture was stirred 18 h at ambient temperature. The product was concentrated *in vacuo* and thoroughly washed three times with ethyl ether.

Stage III. Cholesteryl chloroformate (600 mg, 1.34 mmol) was dissolved in methylene chloride (10 mL) and added dropwise quickly with the product of Stage II (PEI-CBz) and triethylamine (1 mL) in methylene chloride (10 mL) cooled to under -10 °C by ice-salt bath, under nitrogen atmosphere, under stirring. The reaction mixture was stirred for 6 h at 0 °C and for another 6 h at ambient temperature. The product was concentrated *in vacuo* at ambient temperature. The residue was thoroughly washed three times with ethyl ether. The unreacted cholesteryl chloroformate was removed by washing with ethyl ether.

Stage IV. The product of stage III (Chol-PEI-CBz) was dissolved in 25 mL of acetic acid and 50 mg of 10% palladium on activated carbon (Pd/C) was added. The solution was purged with nitrogen and stirred under hydrogen at atmospheric pressure. The hydrogenolysis was allowed to proceed for ~8 h. The reaction mixture was filtered and the catalyst was washed with 200 mL of ethyl acetate/acetic acid (9/1 v/v) and the filtrate was concentrated *in vacuo* to give a residue. The crude product was dissolved in 20 mL of 1 N NaOH and precipitated with excessive acetone below 0 °C. Finally, the product was washed three times with ethyl ether.

For the product synthesized through CBz protection, ¹H NMR (Varian Inc., 500 MHz, Palo Alto, CA) analysis was carried out to characterize the presence of cholesterol on the

backbone of PEI and also to determine whether the location of the cholesterol grafting is on the primary amine or secondary amine. Chloroform- d_1 was employed as the solvent. Molecular weight of the synthesized product was determined using matrix-assisted laser desorption–time-of-flight (MALDI–TOF) mass spectrometry (Perspective Voyager, PE Appl. Biosys. Co., Framingham, MA). As the matrix, *trans*-4-hydroxy-3-methoxycinnamic acid was employed. The solvent was acetonitrile, 3% TFA in deionized water. The final concentration of the lipopolymer sample was 10^{-4} M (10^2 pmol/ μ L) and the matrix concentration was 10 mg/mL. The sample and matrix were mixed before measurement.

Determination of Critical Micellar Concentration. The critical micellar concentration (cmc) of PEI–Chol was determined using dye solubilization method as described by Jeong et al.¹⁸ Briefly, the samples were respectively dissolved in 1 mL of deionized water and were adjusted to the concentrations ranging approximately 8–128000 μ g/mL. A stock solution of 1,6-diphenyl-1,3,5-hexatriene (DPH) (0.4 mM) was prepared by dissolving 2.2 mg of DPH into 23.54 mL of methanol followed by sonication for 15 min. Ten microliters of DPH stock solution was added to 1 mL sample solution and stored in dark for 6 h. The blank control was also prepared by adding 10 μ L of DPH stock solution into 1 mL of water. The absorption spectra of these samples were measured at 200–500 nm with a UV/Vis/NIR spectrometer (Perkin-Elmer, Norwalk, CT). The absorbance at 356 nm was plotted against the concentration of the samples.

Determination of Buffer Capacity. The ability of PEI–Chol to *buffer* at the drop in pH and obtain a positive charge over a 10 to 2 pH range was determined by acid–base titration as described by Benn et al.¹⁹ Thirty milliliters of polymer solution was prepared for each polymer, the pH raised to 10 using 1 M NaOH and divided into small aliquots, into each of which was added a different volume of 1 N HCl and the pH of all the solutions was measured at the same time using a pH-meter. The experiment was done at room temperature, concentration of the polymer used was 0.2 mg/mL, each data point was collected at about 10 min after addition of the acid, and this time was the same for all the samples. The solution was then titrated with 1 N HCl given in 10 μ L increments. The pH profile was obtained for each polymer and graphs of the data were generated accordingly.

Amplification and Purification of pDNA. The SURE-2 supercompetent cells were transformed as per the protocol provided by Stratagene (La Jolla, CA). The transformed bacteria were grown on LB agar overnight at 37 °C. Some colonies corresponding to single clones were grown for 8 h in terrific broth media. The plasmid was extracted from the bacterial cultures using the Concert Miniprep protocol, digested with Eco R1 restriction enzyme and electrophoresed on 1% agarose gel to confirm that there was no rearrangement of the genes during cloning and propagation. The clones containing the right plasmid were identified, and one of them was grown into larger quantity overnight in terrific broth media. The pDNA was purified the following day using the Qiafilter plasmid purification kit procured from Qiagen and

the purified plasmid was diluted with purified water and stored at -80 °C until further use. Plasmids were characterized using the following methods: 1) UV spectrophotometry at 260/280 nm and 1% agarose gel electrophoresis to determine the purity, integrity, and concentration of the plasmids.

Band Retardation. PEI–Chol/pDNA complexes were prepared using different ratios of positive charges on the nitrogen atoms of the carrier to the negative charges on the phosphate backbone of DNA at the N/P ratios of 1/0 (naked DNA), 1/1, 2.5/1, 5/1, 10/1, 15/1, 20/1, and 25/1. pDNA and the carriers were mixed together in 5% dextrose solution and incubated for 45 min at room temperature to allow complex formation. Ten microliters of sample containing 1X loading dye was used for electrophoresis on 1% w/v agarose gel in 0.5X TBE buffer at 5 V/cm. The DNA band was stained in the gel using 0.5 μ g/mL ethidium bromide intercalating dye and the migration of the DNA toward anode was compared among the samples.

Circular Dichroism. Circular dichroism (CD) spectra were obtained with Aviv 62A (Lakewood, NJ). A CD quartz cuvette with a path length of 1 cm (0.5 mL) was used. The scans for each sample were repeated four times at 20 °C yielding the average values. The scanning integration time was set to 1s. The scanning slit and step was 1 nm. The aqueous solution containing 5% glucose was used for background normalization. PEI–Chol 1800/pDNA and PEI–Chol 10000/pDNA complexes at the N/P ratios of 2.5/1, 5/1, 10/1, and 20/1 prepared in 5% dextrose were used at 50 μ g/mL DNA (1.5×10^{-4} M, based on phosphate content) concentration. Naked pDNA, PEI–Chol 1800, PEI–Chol 10000, PEI 1800, and PEI 10000 were used as controls.

Measurement of Particle Size and ζ Potential. The particle size and ζ potential of PEI–Chol/pDNA and PEI/pDNA complexes were determined by dynamic light scattering (DLS) at 25 °C with using a Malvern Zetasizer (Southborough, MA 01772) as described by Mahato et al.²⁰ Briefly, 200 μ L of polymer/pDNA complexes were prepared at 0.1 mg/mL DNA and 5–30 N/P ratios in 5% glucose. Following particle size measurement, the complexes were mixed with 3 mL of 0.1 N NaOH and used for measurement of the ζ potential.

In Vitro Transfection. Transfection studies were carried out using the complexes of pCMS-EGFP with PEI–Chol 1800 and 10000 lipopolymers in murine Jurkat T-cell lines. The cells were grown in 2% HEPES media containing 10% FBS, 2% antibiotics (penicillin, streptomycin, and amphotericin B) and 2.5% L-glutamine in 5% CO₂ incubator at 37 °C. The cells were counted using hemocytometer with the aid of trypan blue dye and 2×10^6 cells per well were resuspended in serum-free HEPES media for the experiment. PEI–Chol/pDNA complexes were prepared at the N/P charge ratios of 5/1, 10/1, 15/1, 20/1, 25/1, and 30/1 and 2 μ g of DNA equivalent of the complexes were mixed with serum-free media and added to the cells. Plasmid DNA complexed with PEI of 1800, 10000, and 25000 Da were used as positive controls. We also studied the effect of serum on transfection efficiency by incubating cells with PEI–Chol/pDNA and PEI/pDNA complexes in the presence of 10%

fetal bovine serum (FBS). At 5 h post-transfection, 1.5 mL of serum containing media was added, and the cells were incubated for additional 36 h. The samples were then evaluated for expression of the green fluorescent protein (GFP) using flow cytometry. Flow cytometry was carried out by counting the number of green fluorescent protein expressing cells in the population of 5000 viable cells. First, the instrument was adjusted with the negative control (non transfected) cells to identify the region of the graph occupied by the viable and healthy cells and then the samples were run and the count of positive cells was recorded.

Measurement of Transfection and Cell Viability by Flow Cytometry. Cytotoxicity is an important parameter to assess the clinical applicability of gene medicines. We used advanced Annexin V PE/7-AAD assay (Pharmingen) technique to differentiate apoptotic and dead cells from the healthy cells expressing green fluorescent protein (GFP); which is superior to the conventionally used calorimetric assays like MTT. Annexin V is a 35–36 kDa phospholipids-binding protein that has high affinity for phosphatidylserine present in the plasma membranes. Staining with Annexin V in conjunction with 7-aminoactinomycin D (7-AAD) allows identification of early apoptotic cells. Therefore, 7-AAD was used in conjunction with phycoerythrin conjugated Annexin V (Annexin V-PE) and fluorescein isothiocyanate (FITC) conjugated Annexin V for the exclusion of nonviable cells in flow cytometric assays.

Results

Synthesis of PEI–Chol. The synthesis scheme of PEI–Chol is shown in Figure 1. The cholesterol moiety was conjugated to PEI with CBz protection of the primary amines and following synthesis of PEI–Chol the protecting group was removed. The NMR results are as follows (Figure 2a–c). ^1H NMR (500 MHz, chloroform- d_1): $\delta \sim 0.65$ ppm (H of CH_3 from cholesterol (a)); $\delta \sim 0.85$ ppm (H of $(\text{CH}_3)_2$ from cholesterol (b)); $\delta \sim 0.95$ ppm (H of CH_3 from cholesterol (c)); $\delta \sim 1.10$ ppm (H of CH_3 from cholesterol (d)); $\delta 0.70$ – 2.50 ppm (H from CH_2 – CH_2 and CHCH_2 from cholesterol (e)); $\delta \sim 5.30$ ppm (H from $=\text{CH}$ – from cholesterol (f)); $\delta \sim 4.50$ ppm (H from $-\text{CH}-\text{COO}$ from cholesterol (g)); $\delta 2.50$ – 3.60 ppm (H from $\text{N}-\text{CH}_2-\text{CH}_2-\text{N}$ from PEI (h)); $\delta \sim 5.10$ ppm (H from $\text{ph}-\text{CH}_2-\text{O}$ from CBz (p)); $\delta \sim 7.35$ ppm (H from Ph – from CBz (q)). Only one cholesterol was conjugated to the secondary amines of each PEI chain. From Figure 2c, the molar ratio of “p”-hydrogen (methylene hydrogen on CBz) to “h”-hydrogen is approximately $1/7$, which indicates the primary amines on each PEI repeating units have already been blocked by CBz. From Figure 2d, the molar ratio of “h”-hydrogen to the hydrogen on cholesteryl group is approximately $164/45$, and the molecular weight of cholesteryl group is approximately 480, which indicates that only one molecule of cholesteryl group was grafted onto a 1800 Da PEI chain. Since all the primary amines on PEI were blocked by CBz, the location of cholesterol grafting should be on the secondary amine of PEI.

The molecular weight of PEI–Chol was determined using MALDI–TOF mass spectrometry. The MALDI–TOF mass

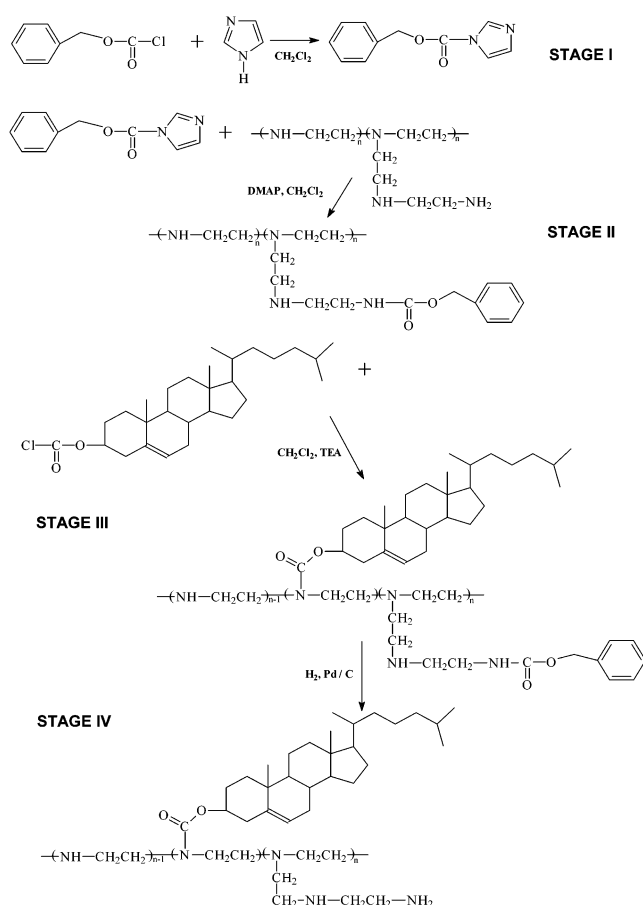


Figure 1. The synthesis of PEI–Chol with CBz blocking method. Stage I: Synthesis of CBz protection reagent. Stage II: PEI primary amine protection with CBz. Stage III: Grafting of cholesterol group. Stage IV: CBz deblocking.

spectrum of PEI 1800 (Figure 3a) was used as a control to estimate the results obtained for PEI–Chol 1800 conjugate. From Figure 3b, the m/z value of PEI–Chol 1800 was around 2000–2150; from Figure 3c, the interval of the isotope peaks is 1. The z value was measured as $(\text{isotope-peak-interval})^{-1}$ and was equal to 1. Therefore, the molecular weight of the product is around 2000–2150.

To study the effect of the cationic headgroup orientation in relation to the lipid anchor, cholesteryl chloroformate was also directly linked to the primary amines of PEI 1800 as reported before.¹⁶ To study the effect of the molecular mass of the cationic headgroup on transfection efficiency, branched PEI of 10000 Da was also used for synthesis and the product was abbreviated as PEI–Chol 10000. NMR spectrum of PEI–Chol 10000 is shown in Figure 2e and the conjugation ratio of PEI and cholesterol was calculated as follows: peak area, PEI–hydrogen ~ 92 and cholesterol–hydrogen ~ 5.6 ; constant cholesterol–hydrogen number 45; PEI–hydrogen number relative to one molecule of cholesterol, $45 \times 92/5.6 = 739.3$; constant PEI repeating unit, hydrogen number 16, M_w 172; PEI M_w relative to one molecule of cholesterol, $172 \times 739.3/16 = \sim 7950$ Da; constant PEI M_w 10K; PEI/cholesterol (mol/mol) $1/1.26$, $\sim 1:1$.

Critical Micellar Concentration (Cmc). The PEI–Chol lipopolymer is amphiphilic in nature because PEI is hydrophilic and water-soluble, while cholesterol is hydrophobic. With the increase in its concentration, PEI–Chol may form

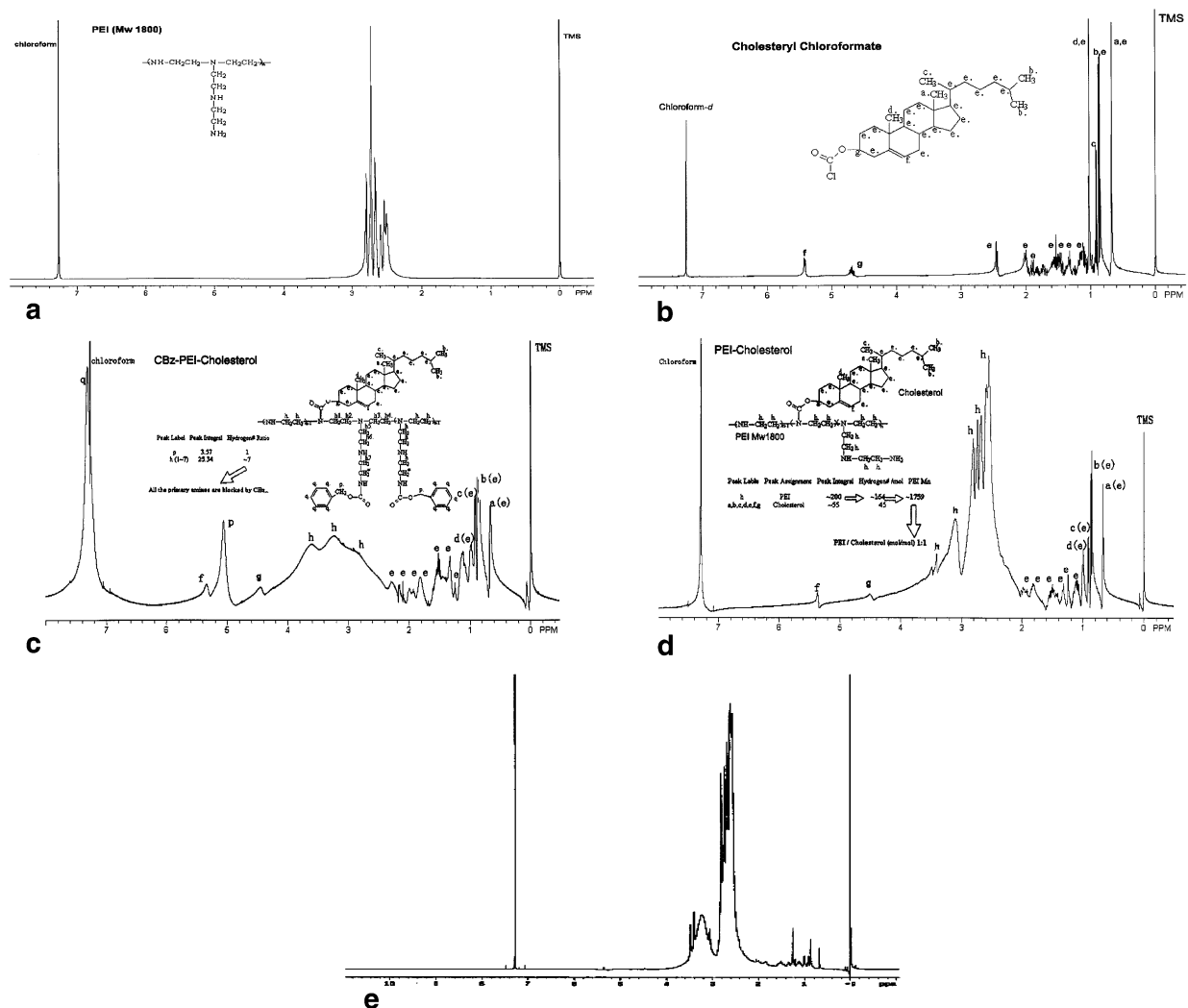


Figure 2. ¹H NMR characterization for PEI–Chol synthesis: (a) PEI 1800 (material); (b) cholesteryl chloroformate (material); (c) CBz-protected PEI–Chol 1800; (d) PEI–Chol 1800, final product; (e) PEI–Chol 10000 (final product). From Figure 2c, the molar ratio of “p”-hydrogen (methylene hydrogen on CBz) to “h”-hydrogen is approximately 1/7, which indicates the primary amine on each PEI repeating units has already been blocked by CBz; from Figure 2d, the molar ratio of “h”-hydrogen to the hydrogen on cholesteryl group is approximately 164/45, and the molecule weight of cholesteryl group is approximately 480, which indicates that every molecule of cholesteryl group has been grafted onto one PEI chain with the *M_w* ~ 1800. Since the molecule weight of the material PEI is just around 1800, the conclusion is that one molecule of cholesteryl group has been grafted onto one PEI chain. Similarly, the conjugation ratio of PEI 10000 and cholesterol was calculated as follows: peak area, PEI-hydrogen ~92 and cholesterol–hydrogen ~5.6; constant cholesterol–hydrogen number 45; PEI–hydrogen number relative to one molecule of cholesterol, 45 × 92/5.6 = 739.3; constant PEI repeating unit, hydrogen number 16, *M_w* 172; PEI *M_w* relative to one molecule of cholesterol, 172 × 739.3/16 = ~7950 Da; constant PEI *M_w* 10K; PEI/cholesterol (mol/mol) 1/1.26, ~1:1.

multimolecular micelles or micellar aggregates in water. The micellar property of PEI–Chol depends on the hydrophilic–hydrophobic balance between the cationic headgroup and the lipid tail. DPH is a hydrophobic dye, which is sparingly soluble in water and has a significant UV absorbance at 356 nm. DPH can be solubilized by micelles due to its preferential partitioning into the core of the micelles, resulting in an increase in absorbance in the aqueous media.¹⁸ From Figure 4, the cmc of PEI–Chol 1800 was 496.6 μg/mL and that of PEI–Chol 10000 was 1330.5 μg/mL. The relatively higher cmc of PEI–Chol 10000 is due to the larger ratio of the hydrophilic moiety (PEI).

Buffer Capacity. Our aim in this experiment was to assess the capacity of these lipopolymers to buffer the drop in pH. The experiment was done in a manner similar to what is expected to happen following cellular uptake in the endosome, i.e., the drop in pH of the environment from the

physiological level to the acidic side. Hence, we did not present the titration data from the fully protonated state toward the alkaline pH side. Although it may be a good idea to talk about the degree of protonation rather than the amount of acid used for titration, our purpose really is to only show that the conjugation of cholesterol to PEI has little effect on the buffering capacity of PEI. It is a known fact that PEI contains all three kinds, primary, secondary and tertiary, of amines whose *pK_a* values cannot be assigned easily because of the effects of local groups, leading to different ionization constants for every amino group.^{21,22} Furthermore, the determination of protonation capacity using the Langmuir isotherm or the Ising models, for example, would make the otherwise simple data pretty complex for interpretation and comparison with different papers; like Bens et al.¹⁹ where the buffering capacity of cationic polymers was measured and interpreted in the same manner.

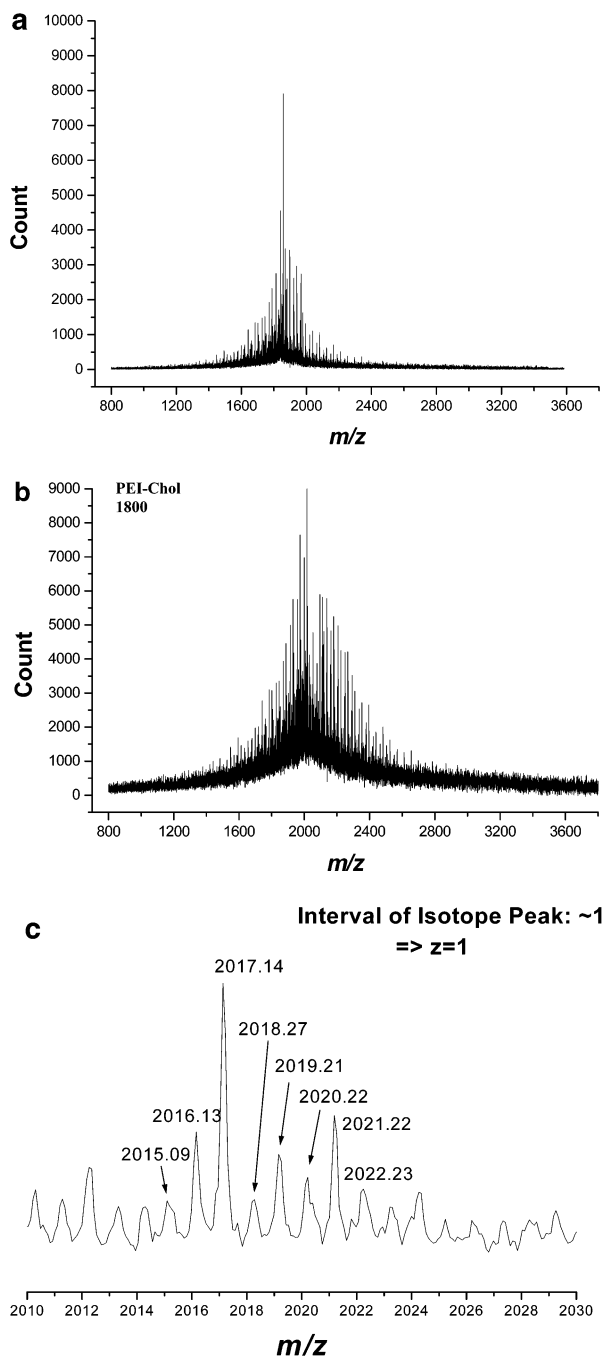


Figure 3. MALDI-TOF mass spectrometry of PEI 1800 and PEI-Chol 1800: (a) MALDI-TOF spectra of PEI 1800; (b) MALDI-TOF spectra of PEI-Chol 1800; (c) MALDI-TOF intervals of isotope peaks. The m/z value of PEI-Chol 1800 was around 2000–2150, and the interval of the isotope peaks is 1, which means that the z value is 1. Therefore, the molecular weight of the product is around 2000–2150.

The acid–base titration profiles obtained for PEI-Chol 1800, PEI-Chol 10000, and PEI 10000, are shown in Figure 5. The buffer capacity of these lipopolymers comes from the different pK_a values of primary, secondary, and tertiary amines on the PEI backbone, especially from the tertiary amine whose pK_a value is around 6–7 (primary amine around 9; secondary amine around 8). The results in Figure 5 indicated that at a pH range from 7 to 4, PEI-Chol possesses efficient buffer capacity for endosomal release of pDNA into the cytoplasm. For PEI-Chol 1800, the buffer capacity originates from the amine groups on PEI.

Band Retardation Assay. Both PEI-Chol 1800 and 10000 efficiently condensed pDNA at the N/P charge ratio of 2.5/1 and above, as no mobility of the pDNA was observed at these charge ratios (data not shown).

Circular Dichroism. CD spectra of naked pDNA, PEI-Chol 1800, and PEI-Chol 10000 and PEI-Chol/pDNA complexes are shown in Figure 6. The CD spectra of free PEI-Chol 1800 and PEI-Chol 10000 indicated that these pure lipopolymers would give little CD signal. The CD spectrum of naked pDNA presented a typical B-DNA curve. Observable changes took place upon lipopolymer-pDNA interaction. The absolute intensity ($\Delta\epsilon$) of both the positive peaks (280–300 nm) and the negative peaks (240–250 nm) varied on each spectrum of the PEI-Chol 1800/pDNA complexes in comparison with that of the naked pDNA. When the N/P ratios of PEI-Chol 1800/pDNA and PEI-Chol 10000/pDNA complexes was increased from 0:1, to 2.5:1, to 5:1, then to 10:1, and finally to 20:1, the absolute intensity of negative peaks varied from 5.6 (naked pDNA), to 6.7, to 3.4, to 5.3, and finally to 3.2 on PEI-Chol 1800/pDNA spectra; and from 3.7 (naked pDNA), to 4.3, to 3.2, to 4.1, and finally to 5.6 on PEI-Chol 10000/pDNA spectra. Similarly, on PEI-Chol 1800/pDNA spectra the positive peak intensity also varied from 4.4 (naked pDNA), to 2.2, to 0.9, to 1.2, and finally to 2.2; and on PEI-Chol 10000/pDNA spectra it varied from 4.5 (naked pDNA), to 0.2, to 0.5, to 0.9, and finally to 2.1. The largely negative “tail” of the CD curve could be observed when the N/P ratio was increased from 2.5/1 to 5/1 and higher on the spectra of the complexes prepared using these lipopolymers and pEGFP. The detailed data are as follows. PEI-Chol1800 negative peaks (approximate location of the peak center, increasing order of PEI-Chol/pDNA charge ratio, N/P): 239.8 (naked pDNA), 239.9, 240.1, 246.7, and 240.9 nm. Positive peaks: 286.2 (naked pDNA), 294.1, 294.5, 296.2, and 293.9 nm. PEI-Chol 10000 negative peaks: 243.2 (naked pDNA), 242.8, 242.9, 242.9, and 237.2 nm. Positive peaks: 285.1 (naked pDNA), 292.1, 292.5, 292.2, and 290.0 nm (Figure 7).

Particle Size and ζ Potential. We determined the particle size and ζ potential of PEI-Chol/pDNA and PEI/pDNA complexes by dynamic light scattering using a Malvern Zetasizer (Southborough, MA 01772) as described by Mahato et al.²⁰ The mean particle size of these complexes was in the range of 110 to 205 nm, except the complexes prepared using free PEI of 1800 kDa, which had the mean particle size of 384 ± 300 nm. The ζ potential of DNA complexes prepared using PEI-Chol and PEI at the N/P ratio of 15/1 was in the range 23.2–38 mV, and increased with an increase in the N/P ratios. The ζ potential was 27.3 mV for PEI-Chol 1800/pDNA complexes when prepared at the N/P ratio of 5/1, but increased to 38.8 mV at the N/P ratios of 20/1. The ζ potential of naked DNA was $-47/+9.2$ mV.

In Vitro Transfection and Cell Viability. We next determined the effect of N/P ratios and the orientation and molecular weight of the cationic headgroup on transfection efficiency of PEI-Chol/pCMS-EGFP complexes in murine Jurkat T-cell lines. Flow cytometry results showed the percentage of both GFP positive and viable cells. As shown

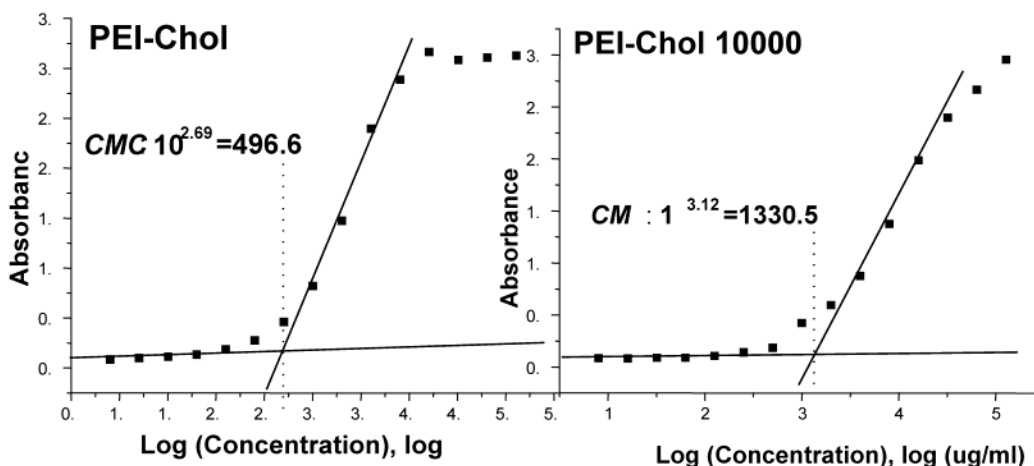


Figure 4. Determination of critical micellar concentration (cmc) of PEI-Chol by the dye solubilization method: (a) PEI-Chol 1800; (b) PEI-Chol 10000. The cmc values of PEI-Chol 1800 and PEI-Chol 10000 are 496.6 and 1330.5 $\mu\text{g/mL}$, respectively.

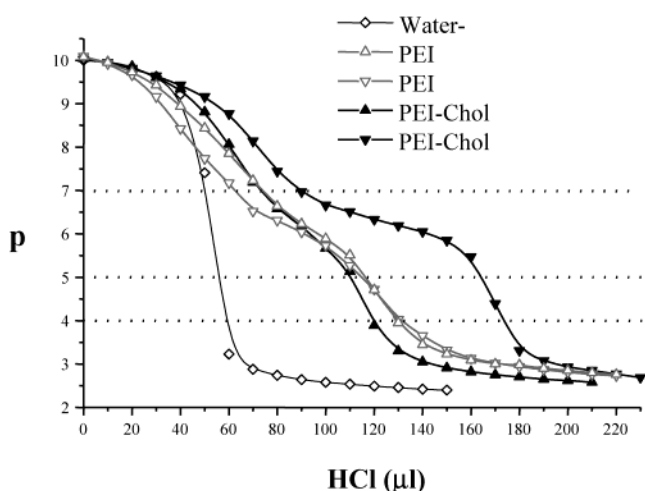


Figure 5. Determination of the buffer capacity of PEI 1800, PEI 10000, PEI-Chol 1800, and PEI-Chol 10000 by acid-base titration. A polymer solution was prepared, pH raised to 10 and divided into small aliquots into each of which was added a different volume of HCl and pH of all the solutions was measured at the same time at room temperature. The concentration of the polymer used was 0.2 mg/mL, and each data point was collected at 10 min after addition of the acid.

in Figure 8, there was increase in GFP positive cells with increase in the N/P ratios of PEI-Chol 1800/pDNA complexes. This could be attributed to the increase in overall positive charges of the complex, which helps in transient cell membrane destabilization, and permeation of the complex into the cells. There was decrease in cell viability with an increase in the N/P ratios. There is, hence, a fine balance between the transfection efficiency and cytotoxicity.

The molecular weight of a polymeric gene carrier plays an important role on its transfection efficiency. Therefore, we repeated our transfection experiments using PEI-Chol 10000/pDNA complexes. We also used plasmid DNA complexed with PEI of 1800, 10000, and 25000 Da as positive controls. Unlike PEI-Chol 1800-based formulations, the transfection efficiency of PEI-Chol 10000/pDNA complexes was similar to that of PEI 10000/pDNA complexes (Figure 9). There was increase in transfection efficiency with increase in the N/P ratios. In the case of unmodified PEIs, there was increase in both transfection efficiency and cytotoxicity with the increase in their molecular weights.

PEI25000/pDNA complexes showed the highest transfection and cytotoxicity when the complexes were prepared at the N/P ratios of 15/1 and used at a dose of 2 μg of pDNA equivalent per 2 million Jurkat cells (Figure 9). The fact that the PEI25000/pDNA complexes yielded higher expression implies that the larger PEI molecules afford either better protection to the plasmids or better cellular uptake of the complexes.

Discussion

We have sought to develop novel lipid and polymeric gene carriers based on a set of predefined design criteria. For efficient transfection and possible clinical application, synthetic gene carriers should meet the following criteria: (1) DNA condensation capability, (2) endosomolytic property, (3) biocompatibility to minimize potential toxic effects on cells and tissue, and (4) facile synthesis and purification to allow large-scale commercial manufacture.²³ On the basis of these design criteria, we have synthesized novel water-soluble cationic lipopolymers (WSLP) using low molecular weight branched poly(ethylenimine) (PEI) and cholesteryl chloroformate. The cholesterol moiety was used as a lipophilic portion grafted onto the branched PEI, which serves as a hydrophilic headgroup due its ionized primary amino groups in the aqueous environment. The cationic headgroup, PEI, has particular advantages over conventional cationic DNA condensing agents, such as (i) it can effectively condense pDNA into colloidal particles, (ii) it can enhance cellular uptake of pDNA by the nonspecific adsorptive mechanisms, and (iii) it can enhance endosomal release of DNA due to its proton-sponge effect.^{24–26} As a hydrophobic anchor group, cholesterol can form a stable micellar complex with the hydrophilic headgroup in the aqueous environment and may provide shielding effect to PEI-Chol/pDNA complexes against erythrocytes and plasma proteins at lower charge ratios.

We have previously synthesized water-soluble lipopolymer (WSLP) using branched PEI of 1800 Da and cholesteryl chloroformate for gene delivery.^{16,17} However, anchoring of cholesterol moiety on the PEI amino groups was nonspecific and utilized the primary amines of PEI. This is undesirable

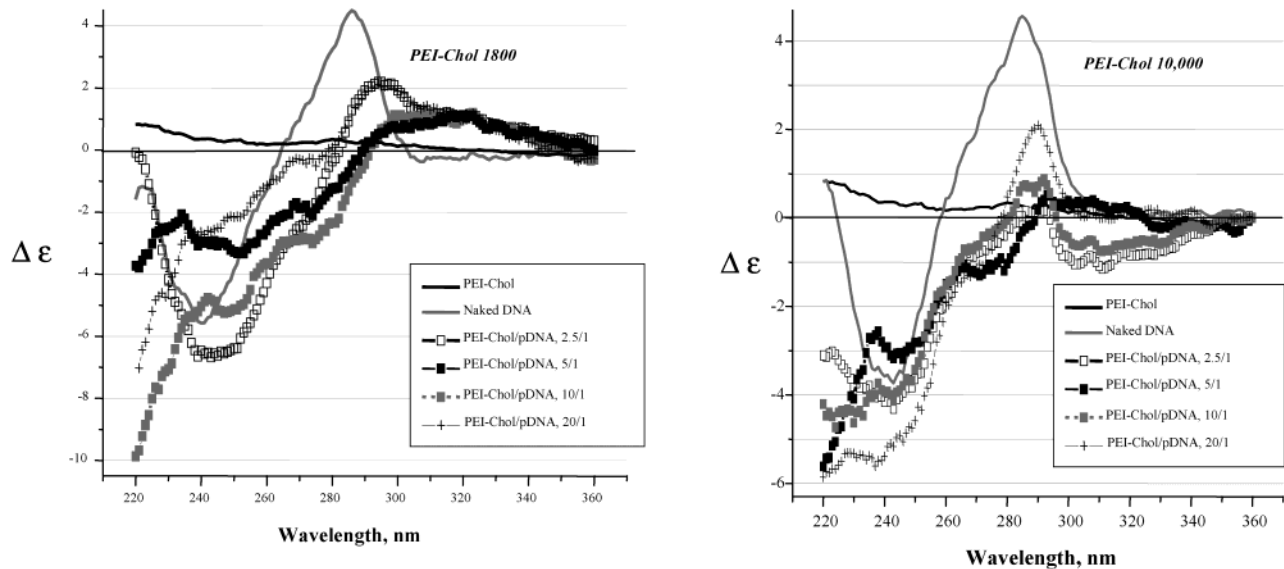


Figure 6. CD spectra of naked pDNA, PEI-Chol 1800, and PEI-Chol 1800/pDNA complexes.

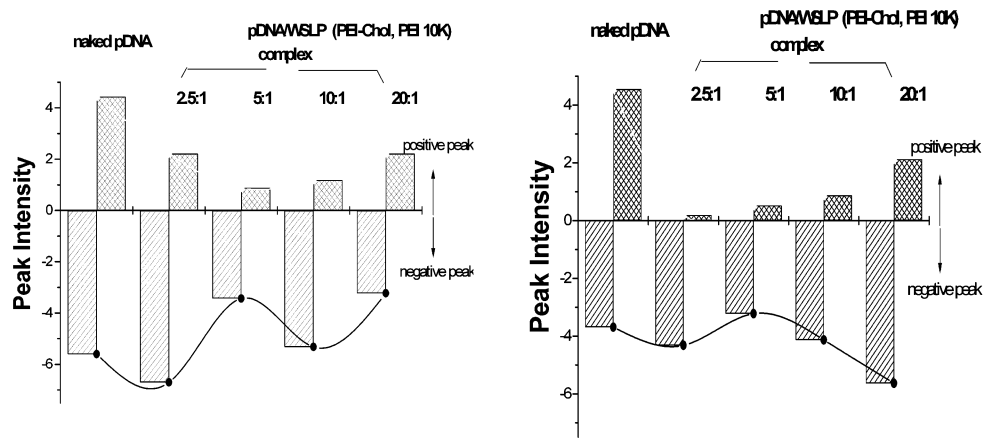


Figure 7. Effect of N/P ratios on the negative and positive CD peak amplitudes.

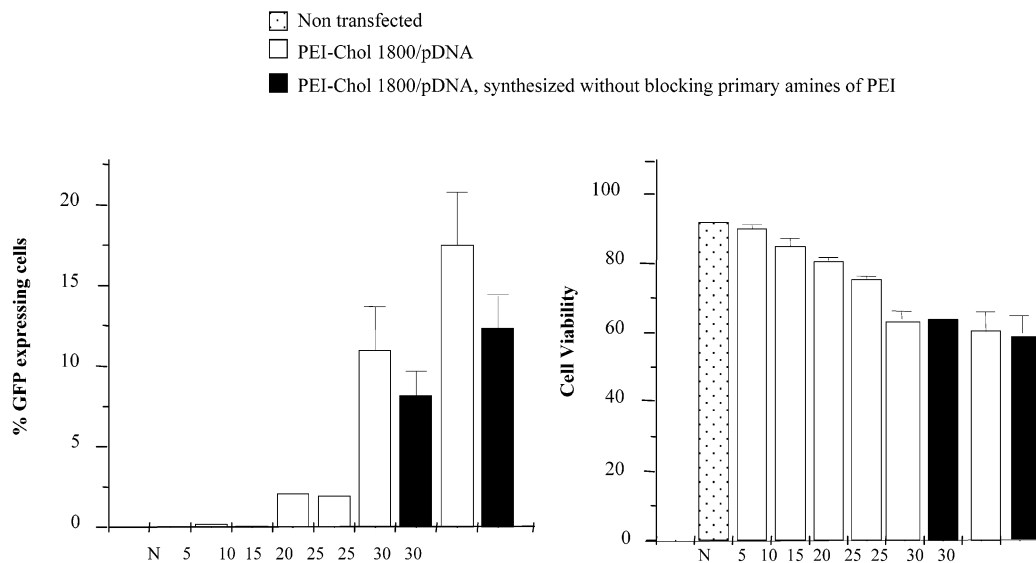


Figure 8. Effect of N/P ratio on in vitro transfection and cell viability of PEI-Chol 1800/pDNA complexes into murine Jurkat T cells. The results are expressed as the mean \pm the standard deviation of the % GFP positive cells determined using flow cytometry. Two micrograms of pDNA was used per two million Jurkat cells. PEI-Chol 1800 was synthesized by conjugating cholesterol to the primary amines of PEI or first blocking the primary amines with CBz so that cholesterol can be conjugated to the secondary amines of PEI. "Unblocked" means the PEI-Chol, which was synthesized without blocking the primary amines of PEI using CBz.

since the primary amino groups are positively charged at physiological pH and contribute to DNA condensation. Also,

grafting of more than one cholesterol molecule onto the PEI would result in enhanced lipophilic surface of the condensing

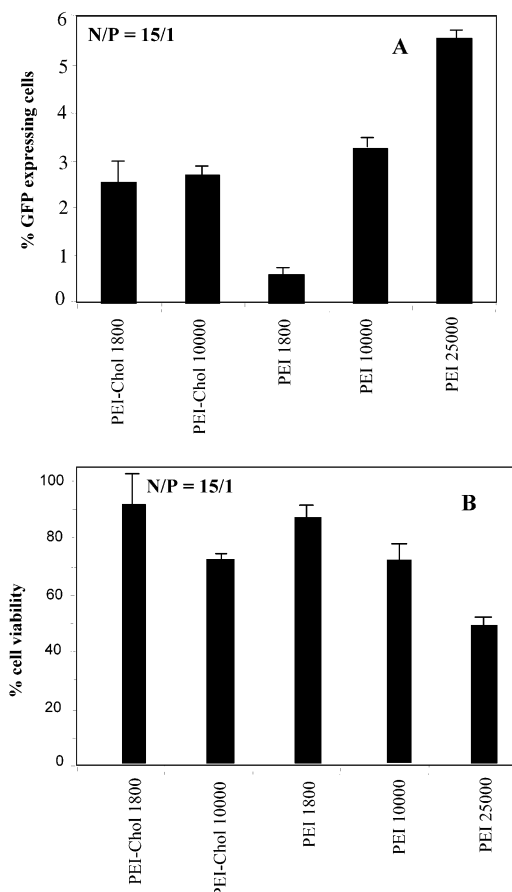


Figure 9. Effect of molecular weight on in vitro transfection and cell viability of PEI/pDNA and PEI-Chol/pDNA complexes into murine Jurkat T cells. PEI/pDNA and PEI-Chol/pDNA complexes were prepared at the N/P ratios of 15/1 and 2 μ g of DNA equivalent per 2 million cells were used for transfection. The results are expressed as the mean \pm the standard deviation of the percent GFP positive cells determined using flow cytometry.

copolymer leading to reduced aqueous solubility, DNA condensation ability and endosomal release. In the present study, we have utilized a novel design whereby only one cholesterol moiety is attached to each PEI molecule and conjugation is specifically to the secondary amine. This was achieved by blocking the primary amines using benzenyloxycarbonyl (CBz) before cholesterol conjugation to PEI. Since the NMR spectra exhibit only the statistical average degree of the product composition, from this result (Figure 2) alone, we cannot confirm that only one cholesterol moiety was grafted onto one PEI chain. Therefore, we determined the molecular weights of PEI-Chol 1800 and unmodified PEI 1800 using MALDI-TOF mass spectrometry (Figure 3, parts a and b). The mass spectra of PEI-Chol 1800 showed narrow distribution around 2100–2200 m/z , which is expected since the molecular mass of cholesterol is 386.67 Da and thus the molecular mass of PEI-Chol 1800 should be around 2200 Da if only one cholesterol was conjugated per 1800 Da PEI molecule. Furthermore, the mass spectra of PEI 1800 were concentrated around 1800 m/z (Figure 3a), suggesting that the conjugation of cholesterol to PEI 1800 was at 1:1 ratio of PEI/Chol. If there were a lot of PEI fractions without any cholesterol moiety and/or with more than one cholesterol moieties, the overall molecular mass distribution of the products would inevitably be much larger

than 2200 Da. However, the conjugation of cholesterol to the secondary amino group in the branch segments of PEI cannot be ruled out. We consider that the cholesterol moiety grafted onto the PEI secondary amine on the backbone instead of the one on the branch segments is most likely because of the steric hindrance of CBz group. Furthermore, the grafting location on different secondary amines would have little influence on the physicochemical properties and transfection efficiency of PEI-Chol.

Plasmid was condensed at DNA concentration of 0.1 mg/mL and the polymer concentrations of 0.016–0.472 mg/mL, which are below the critical micellar concentration (cmc) of these lipopolymers. The cmcs of PEI-Chol 1.8 and 10 kDa were 0.497 and 1.33 mg/mL, respectively (Figure 4). Therefore, the presence of micellar structures in the formulation was relatively few. At high N/P ratios, it is likely that some lipopolymers are present in the suspension of PEI-Chol/pDNA complexes in the free forms and can affect transfection and cytotoxicity.

PEI molecules exist either as a linear or branched form, and have very high charge-potential with every third atom as an amino nitrogen that can be protonated. According to the pK profile, PEI molecules exhibit considerable buffer capacity over almost the entire pH range.^{24,27,28} The pH at which protonation take place is believed to be a function of the pK_a values of the primary, secondary, and tertiary amines. However, in the case of PEI, the local environment for all the amino groups is different from each other, resulting in different pK_a values for different amino groups. Transfection properties of PEI rely on its protonation profile, which increases from 20 to 45% between pH 7 and 5, thus making the molecule a virtual “proton sponge”. This not only tends to inhibit the action of the endosomal nucleases, but also alters the osmolality of the endosome, thus leading to osmotic swelling and eventually rupture of the vesicle.²⁹ The buffering capacity of the lipopolymer is therefore measured in order to predict the endosomal release of pDNA to the cytoplasm. As shown in Figure 5, both PEI and PEI-Chol showed similar acid-base titration curve, indicating that cholesterol conjugation to PEI did not affect the buffering capacity of PEI. Linear PEI of 22 kDa (ExGen 500) has also shown high transfection in the lung epithelia after intratumoral injection of ExGen 500/pDNA complexes into the rabbit. The authors suggested that ExGen 500’s high transfection efficiency might be explained by the “proton sponge” effect similar to that of branched PEI. Coll et al.³⁰ demonstrated high transfection efficiency when ExGen 500/pDNA complexes were slowly delivered into the solid tumor of the nude mice using a micropump.

The CD spectra of pDNA change when pDNA interacts with cationic lipids or polymers. As shown in Figure 6, naked DNA had a typical B-type secondary conformation, which possesses the symmetrical positive peak around 280–290 nm and negative peak around 240–250 nm. This B geometry has 10 bases per turn, the base plane is perpendicular to the helix axis and the rotation per residue is 30 degrees.³¹ After the addition of PEI-Chol 1800 at an N/P ratio of 2.5/1, the B-type helical conformation began to collapse because of the formation of ion pairs between PEI-Chol and pDNA.

Accordingly, the intensity of the positive peak decreased on the CD spectra.³² This suggests that pDNA within the complex prepared at the N/P of 2.5/1 turned into a C-type geometric conformation, which was similar to the B conformation with about 9.3 bases per turn. When the N/P ratio increased to 5/1, the helical assembly collapsed and a largely negative “tail” came into being on the CD curve around 220 nm. This distortion implied the construction of Ψ^- DNA that was a highly organized left-handed chiral assembly (tertiary conformation) of DNA.³² This liquid-cholesteric-crystal structure of the pDNA assembly “within” the complex was strengthened when the N/P ratio was enhanced to 10/1, which was demonstrated by the dramatic increase of the negative peak and the Ψ^- DNA “tail” on the spectrum (Figures 6 and 7). Nonetheless, when the N/P ratio was further enhanced to 20/1, the Ψ^- -tertiary conformation was finally melted, and the DNA assembly was turned into a stack (tertiary) conformation.³³ The corresponding variation on CD spectra was the enhancement of positive peak and flat of negative peak. For PEI-Chol 10000/pDNA complexes, along with the increase in N/P ratio, the pDNA turned from B-type secondary conformation (naked DNA) to C-type at the N/P ratio of 2.5/1, and then Ψ^- -tertiary conformation came into being at the N/P of 5/1 and higher (Figure 6). Simultaneously, the stack conformation was formed at the N/P of 10/1 and higher, which was indicated by the increase of corresponding positive peaks (Figure 7).

Our results of transfection experiments in Jurkat T-cells indicated that increased transfection efficiency and cytotoxicity are associated with increase in positive charges of the complexes prepared using PEI-Chol 1800 (Figure 8). Our transfection data for unmodified PEI/pDNA complexes are consistent with the work performed by Godbey et al.,²² who compared the transfection efficiency of PEIs of 70, 10, and 1.8 kDa and demonstrated increased transfection with increase in PEI molecular masses. Since one cholesterol molecule was conjugated to PEI of 10000 Da, we think the influence of cholesterol on hydrophobicity and complex stability would be minimal. This may be the main reason, unlike for PEI-Chol 1800, there was no increase in transfection when cholesterol was conjugated to PEI of 10000 Da (Figure 9). Increase in the net positive charges promotes the cellular uptake of the complexes, but also results in destabilization and loss of integrity of the cell membranes especially at higher concentration of the complexes, high charge ratios as well as high molecular weight of the PEI moiety—all of which have a common result, viz. an increase in the positive charge density received by the cells being transfected.

The purpose of grafting cholesterol group onto the secondary amine was simultaneously keeping the primary amine for more effective DNA condensation and producing another tertiary amine for more effective endosomal escaping. However, our results showed only modest (1.5-fold) increase in transfection when T-shape grafted PEI-Chol 1800 was used for transfection than the PEI-Chol 1800 synthesized by direct conjugation of cholesterol to the primary amines of PEIs (Figure 8). The transfection efficiency of our lipopolymers is not as high as we would like to have.

However, PEI-Chol is water-soluble, so it avoids the undesirable use of organic solvent in the subsequent grafting reactions.

We also studied the effect of fetal bovine serum on the transfection efficiency of PEI-Chol and PEI. As expected, the presence of 10% serum in the transfection media significantly diminished the transfection efficiency of PEI and PEI-Chol, resulting in less than 1% GFP positive cells. However, the cell viability was significantly higher when Jurkat cells were incubated with the PEI/pDNA complexes in the presence of 10% serum. Our results are in good agreement with most of the cationic lipids and polymers currently used for gene delivery.^{34–36}

In summary, we designed a water-soluble lipopolymer using the PEI secondary amines for cholesterol conjugation. While we have achieved significant advantage with respect to making a water-soluble lipopolymer for gene delivery without the use of organic solvents with enhanced efficiency of transfection, further strides in nonviral gene therapy will come only through the use of a mechanism for cell membrane adhesion of the complexes and enhanced cellular permeability. Effect of lipid tail (cholesterol vs fatty acids) and fusogenic peptides on the transfection efficiency of these lipopolymers will be tested.

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References and Notes

- (1) Mahato, R. I.; Smith, L. C.; Rolland, A. *Adv. Genet.* **1999**, *41*, 95–156.
- (2) Han, S. O.; Mahato, R. I.; Sung, Y. K.; Kim, S. W. *Mol. Ther.* **2000**, *2*, 302–317.
- (3) Miller, A. D. *Angew. Chem., Int. Ed.* **1998**, *37*, 1768–1785.
- (4) Koltover, I.; Salditt, T.; Radler, J. O.; Safinya, C. R. *Science* **1998**, *281* (5373), 78–81.
- (5) Felgner, P. L.; Gaderer, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413–7417.
- (6) Gao, X.; Huang, L. A novel cationic liposome reagent for efficient vectors for the transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 280–285.
- (7) Mahato, R. I.; Anwer, K.; Tagliaferri, F.; Meaney, C.; Leonard, P.; Wadhwa, M. S.; Logan, M.; French, M.; Rolland, A. *Hum. Gene Ther.* **1998**, *9*, 2083–2099.
- (8) Nguyen, H.-K.; Lemieux, P.; Vinogradov, S. P.; Gebhart, C. L.; Guerin, N.; Paradis, G.; Bronich, T. K.; Alakhov, V. Y.; Kabanov, A. V. *Gene Ther.* **2000**, *7*, 126–138.
- (9) Mendiratta, S. K.; Quezada, A.; Matar, M.; Wang, J.; Hebel, H. L.; Long, S.; Nordstrom, J. L.; Pericle, F. *Gene Ther.* **1999**, *6*, 833–839.
- (10) Lemieux, P.; Guerin, N.; Paradis, G.; Proulx, R.; Chistyakova, L.; Kabanov, A.; Alakhov, V. *Gene Ther.* **2000**, *7*, 986–991.
- (11) Mahato, R. I.; Rolland, A.; Tomlison, E. *Pharm. Res.* **1997**, *14*, 853–859.
- (12) Lee, E. D.; Marshall, J.; Siegel, C. S.; Jiang, C.; Yiew, N. S.; Nicholas, M. R.; Nietupski, J. B.; Ziegler, R. J.; Lane, M. B.; Wang, K. X.; Wan, N. C.; Scheule, R. K.; Harris, D. J.; Smith, A. E.; Cheng, S. H. *Hum. Gene Ther.* **1996**, *7*, 1701–1717.
- (13) Vigneron, J. P.; Qudrhiri, N.; Fauquet, M.; Vergely, L.; Bradely, J. C.; Basseville, M.; Lehn, P.; Lehn, J.-M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9682–9686.

- (14) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297–7301.
- (15) Behr, J.-P. *Chimia* **1997**, *15*, 34–36.
- (16) Han, S. O.; Mahato, R. I.; Kim, S. W. *Bioconjugate Chem.* **2001**, *12*, 337–345.
- (17) Mahato, R. I.; Lee, M. Y.; Han, S. O.; Manheshwari, A.; Kim, S. W. *Mol. Ther.* **2001**, *4*, 130–138.
- (18) Jeong, B.; Lee, D. S.; Shon, J. I.; Bae, Y. H.; Kim, S. W. *J. Polym. Sci.: Polym. Chem.* **1999**, *37*, 751–760.
- (19) Bennis, J. M.; Choi, J. S.; Mahato, R. I.; Park, J. S.; Kim, S. W. *Bioconjugate Chem.* **2000**, *11*, 637–645.
- (20) Mahato, R. I.; Kawabata, K.; Nomura, T.; Takakura, Y.; Hashida, M. *J. Pharm. Sci.* **1995**, *84*, 1267–1271.
- (21) Suh, J.; Paik, H. J.; Hwang, B. K. *Bioorg. Med. Chem. Lett.* **1994**, *8*, 1331–1336.
- (22) Godbey, W. T.; Wu, K. K.; Mikos, A. G. *J. Biomed. Mater. Res.* **1999**, *45*, 268–275.
- (23) Pack, D. W.; Putnam, D.; Langer, R. *Biotechnol. Bioeng.* **2000**, *67*, 217–223.
- (24) Tang, M. X.; Szoka, F. C. *Gene Ther.* **1997**, *4*, 823–832.
- (25) von Harpe, A.; Petersen, H.; Li, Y. X.; Kissel, T. *J. Controlled Release* **2000**, *69*, 309–322.
- (26) Boletta, A.; Benigni, A.; Lutz, J.; Remuzzi, G.; Soria, M. R.; Monaco, L. *Hum. Gene Ther.* **1997**, *8*, 1243–1251.
- (27) Remy-Kristensen, A.; Clamme, J.-P.; Vuilleumier, C.; Kuhry, J.-G.; Mely, Y. *Biochim. Biophys. Acta* **2001**, *1514*, 21–32.
- (28) Kichler, A.; Leborgne, C.; Coeytaux, E.; Danos, O. *J. Gene Med.* **2001**, *3*, 135–144.
- (29) Ferrari, S.; Pettenazzo, A.; Garbati, N.; Zacchello, F.; Behr, J.-P.; Scarpa, M. *Biochim. Biophys. Acta* **1999**, *1447*, 219–225.
- (30) Coll, J.-L.; Chollet, P.; Brambilla, E.; Desplanques, D.; Behr, J.-P.; Favrot, M. *Hum. Gene Ther.* **1999**, *10*, 1659–1666.
- (31) Tinoco, I.; Bustamante, C.; Mastro, M. F. *Annu. Rev. Biophys. Bioeng.* **1980**, *9*, 107–141.
- (32) Simberg, D.; Danino, D.; Talmon, Y.; Minsky, A.; Ferrari, M. E.; Wheeler, C. J.; Barenholz, Y. *J. Biol. Chem.* **2001**, *276*, 47453–47459.
- (33) Sakurai, K.; Mizu, M.; Shinkai, S. *Biomacromolecules* **2001**, *2*, 641–650.
- (34) Zelphati, O.; Uyechi, L. S.; Barron, L. G.; Szoka, F. C. *Biochim. Biophys. Acta* **1998**, *1390*, 119–133.
- (35) Yang, J.-P.; Huang, L. *Gene Ther.* **1998**, *5*, 380–387.
- (36) Escriou, V.; Ciolina, C.; Lacroix, F.; Byk, G.; Scherman, D.; Wils, P. *Biochim. Biophys. Acta* **1998**, *1368*, 278–288.

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