Novel Water Insoluble Lipoparticulates for Gene Delivery

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Purpose. The objective was to design and prepare water insoluble lipoparticulates (ISLPs) for efficient gene delivery to lung tissue.
Methods. Nona{(ethylenimine)-*co*-[(2-aminoethyl)-*N*-choleseteryl-oxycarbonyl-ethylenimine]} (NEACE-T) was synthesized in both its free-base and chloride salt-forms using linear polyethylenimine (PEI, M_w 423) as a headgroup and cholesteryl chloroformate as a hydrophobic lipid anchor resulting in a T-shaped lipononamer. Semitelechelic *N*α-cholesteryloxycarbonyl nona(ethylenimine) (st-NCNEI-L) was synthesized similarly resulting in a linear lipononamer. As confirmed by ^{1}H-NMR, the site of conjugation was either a primary amine resulting in a linear configuration (st-NCNEI-L) or a secondary amine resulting in a T-shaped configuration (NEACE-T). ISLPs were prepared by combining NEACE-T or st-NCNEI-L with a colipid, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) at 1/1, 1/2, and 2/1 molar ratios and the lipoparticulates were hydrated and filtered. ISLP/p2CMVmIL-12 complexes were characterized for particle size, zeta potential, surface morphology, cytotoxicity, and *in vitro* transfection efficiency.
Results. Transgene expression was dependent on the site of cholesterol conjugation, lipononamer:colipid molar ratio, and ISLP/p2CMVmIL-12 charge ratios. ISLP/p2CMVmIL-12 complexes were nontoxic to murine colon adenocarcinoma (CT-26) cells at 9/1 (±) or lower, had a mean particle size of 330–400 nm while the ζ potential varied from 36–39 mV. Atomic force microscopy (AFM) showed the surface morphology to be that of an oblate spheroid with a size comparable to that determined by dynamic light scattering. ISLP/p2CMVmIL-12 complexes prepared using free-base NEACE-T:DOPE (1/2) at charge ratios of 3/1 and 5/1 (±) provided the highest levels of transgene expression, 18 times more than the levels provided by the salt-form. Secreted levels of mIL-12 p70 were 75 times higher for ISLP/p2CMVmIL-12 complexes than naked p2CMVmIL-12 and nearly 4 times higher than PEI 25 kDa/p2CMVmIL-12 complexes.
Conclusions. The transfection efficiency of the ISLPs was dependent on the site of cholesterol conjugation, amount of colipid, and charge ratio. The highest levels of transgene expression were provided by NEACE-T:DOPE (1/2)/p2CMVmIL-12 at a 3/1 (±) charge ratio.

KEY WORDS: insoluble; lipononamer; lipoparticulates; cytokine; transgene.

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INTRODUCTION

Over the last decade there have been scores of attempts to design a non-viral gene delivery system which could achieve the level of gene expression and specificity offered by viral vectors while maintaining the flexible characteristics of cDNA size, immune response bypass, and safety. One of the most successful nonviral gene delivery systems has been cationic lipids. Early advances in cationic lipid-mediated gene delivery showed that the chemistry of a cationic lipid-based gene carrier is dependent on three areas (1–4). First, a polar cationic headgroup is necessary for condensation of polyanionic DNA, which is necessary for DNA protection and efficient delivery. In addition, the cationic headgroup is able to interact with the anionic lipids of the cellular membrane, thus enhancing the cellular uptake. A single positive charge per molecule (1) has been transcended by multi-charged cationic headgroups (5–7), which provide superior electrostatic levels for DNA condensation (8,9). The cationic headgroup has also been modified to increase the ability of DNA condensation. Both branched and linear polyethylenimine (PEI) have been reported to be effective for gene delivery because of enhanced endosomal release of DNA to the cytoplasm due to the proton-sponge effect of PEI (10,11). However, PEI of 25 kDa or above is toxic to cells and PEI/pDNA complexes are prone to aggregation. Unlike high molecular weight branched PEI, linear PEI of 423 Da is nontoxic and its structure is similar to that of spermine (except in the number of secondary amines), which has been used as a cationic headgroup in various cationic lipids. Second, it was found that a hydrophobic lipid anchor is essential for enhanced DNA stability in the bloodstream and cellular uptake. Cellular uptake is enhanced by the favorable interaction between the hydrophobic groups of the gene carrier and the cellular membrane. Cholesterol is a naturally occurring lipid and metabolized in the body, making it highly biocompatible; therefore, cholesterol has been widely used as a hydrophobic lipid anchor (1,12–18). Finally, the linker between the hydrophobic anchor and the polar headgroup determines the chemical stability and biodegradability of cationic lipids. These linker groups should be biodegradable yet strong enough to sustain *in vivo* (4).

Therefore, in this study, we designed novel lipononamers using linear PEI of 423 Da and cholesteryl chloroformate in both a T-shaped (NEACE-T) and linear (st-NCNEI-L) configuration, mixed with a colipid to prepare insoluble lipoparticulates (ISLPs), formed complexes with p2CMVmIL-12, and characterized in terms of particle size, zeta potential, surface morphology, cytotoxicity, and cellular uptake and gene expression.

EXPERIMENTAL PROCEDURES

Materials

Linear polyethylenimine (PEI, M_w 423), benzyl chloroformate (CBZ-Cl), palladium on activated carbon (10% w/w Pd), Celite 521, and cholesteryl chloroformate were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL).

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Anhydrous methylene chloride, chloroform, dimethyl sulfoxide (DMSO), acetone, and formic acid were purchased from Sigma Chemical Company (St. Louis, MO, USA). Hydrochloric acid was purchased from Mallinckrodt (Hazelwood, MO, USA). RQ1 RNase-Free DNase was purchased from Promega (Madison, WI, USA). DH5α competent cells were purchased from Promega (Madison, WI, USA). DNA grade high-melting agarose and ethidium bromide were purchased from ISC Bioexpress (Kaysville, UT, USA). Rosewell Park Memorial Institute (RPMI 1640) medium and heat-inactivated fetal bovine serum were purchased from Hyclone (Logan,UT, USA). Trypsin-EDTA was purchased from Gibco-BRL (Gaithersburg, MD, USA). QIAGEN endofree maxi plasmid purification kits were purchased from QIAGEN (Valencia, CA, USA). Ethidium monoazide bromide (EMA) was purchased from Molecular Probes (Eugene, OR, USA). BDOptEIA ELISA kits for murine interleukin-12 (mIL-12) p70 were purchased from Pharmingen (San Diego, CA, USA). Other materials were of commercial grade and were used without further purification.

Methods

Synthesis and Characterization of Insoluble Liponomers

Nona{(ethylenimine)-*co*-[(2-aminoethyl)-*N*-cholesteryloxycarbonyl-ethylenimine]} (NEACE-T) was synthesized in its free-base and salt-forms using low molecular weight (423 Da) linear polyethylenimine (PEI) as a cationic headgroup and cholesteryl chloroformate as a hydrophobic lipid anchor (Fig. 1). 3.4 g (8 mmol) PEI 423 was added to dry methylene chloride under nitrogen and on ice after which 3.5 g (20 mmol) CBZ-Cl was added dropwise to protect the two primary amine sites. The reaction was allowed to proceed for 8 h resulting in CBZ-PEI-CBZ after removing the methylene chloride. The protected molecule was dissolved in 200 mL of DMSO under nitrogen. 10.8 g (24 mmol) cholesteryl chloroformate was dissolved in 50 mL anhydrous methylene chloride, added dropwise to the CBZ-PEI-CBZ, and reacted under nitrogen and on ice for 12 h resulting in CBZ-PEI-Chol-CBZ, where the cholesterol was conjugated to a secondary amine by a carbamate bond. Deprotection was completed by one of two ways: (a) the addition of 1.0 g 10% w/w Pd under a hydrogen gas balloon resulting in the free-base form of NEACE-T or (b) the addition of 1.0 g 10% w/w Pd to which 14 mL formic acid was slowly added followed by 20 mL 1N HCl resulting in the salt-form of NEACE-T. Both mixtures were filtered through Celite 521 and washed with a small amount of DMSO. This product was purified by solvent precipitation in acetone and vacuum filtered with three acetone and methanol washes to remove CBZ, free cholesterol, and free PEI resulting in the final products: NEACE-T free-base and salt-form. As the products were water insoluble and cationic, HPLC could not be used since the maximum amount of non-aqueous solvent is 50%. The ISLP conjugates were not soluble in 50% aqueous solvent, which also prevented purification by FPLC. The compound was desiccated overnight, analyzed by ^{1}H NMR (Varian Mercury 400, Inc., Palo Alto, CA, USA), and stored at −20°C.

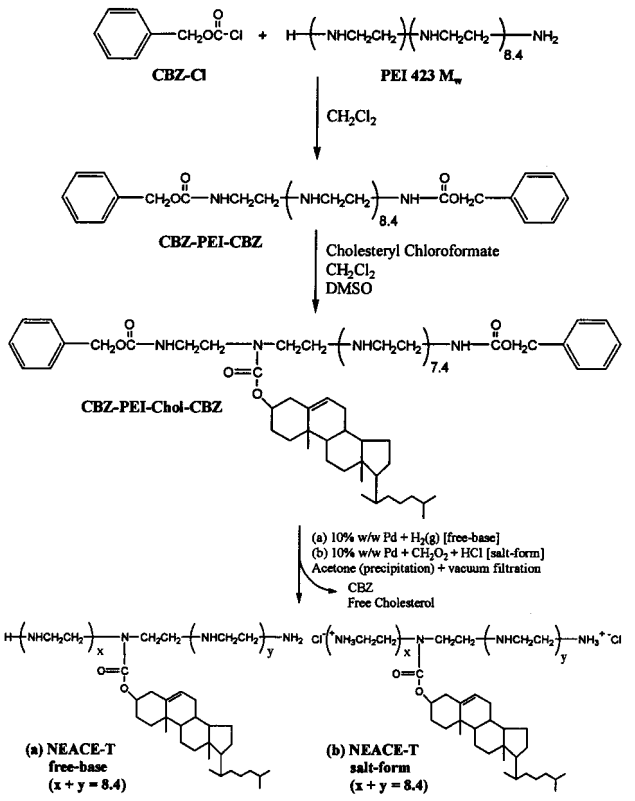

Fig. 1. Synthesis scheme for free-base and chloride salt-form of NEACE-T showing the primary amine protection chemistry and conjugation of cholesterol to a secondary amine.

To study the importance of the orientation of the cationic headgroup in relation to the lipid anchor, semitelechelic *N*α-cholesteryloxycarbonyl nona(ethylenimine) (st-NCNEI-L) was synthesized (synthesis not shown). 3.4 g (8 mmol) PEI 423 was added to dry methylene chloride under nitrogen and on ice to which 10.8 g (24 mmol) cholesteryl chloroformate, dissolved in 50 mL anhydrous methylene chloride, was added dropwise. The reaction was stirred under nitrogen and on ice for 12 h resulting in st-NCNEI-L, where the cholesterol was conjugated to a primary amine. This product was purified by solvent precipitation in acetone and vacuum filtered with three acetone washes to remove free cholesterol. The compound was desiccated overnight, analyzed by ^{1}H NMR, and stored at −20°C.

Preparation of Insoluble Lipoparticulates

To prepare insoluble lipoparticulates (ISLPs), NEACE-T or st-NCNEI-L was dissolved in chloroform and mixed with the colipid DOPE at the molar ratios of 1/1, 1/2, and 2/1. The clear solutions were rotated on a Büchi RE111 rotary evaporator at 30°C for approximately 60 min, resulting in thin, translucent lipid films. The flasks were covered with punctured-parafilm and the lipid films were dried overnight under vacuum to remove residual chloroform. The films were hydrated in 3 mL ultra-pure water to give a final concentration of 5 mM for PEI. The hydrated films were slowly vortexed for 60 min and subsequently filtered through 0.8 μm pore size polycarbonate filters three times followed by filtration through 0.4 μm filters three times using a 10 mL thermobarrel extruder (Lipex Biomembranes, Vancouver, Canada). The filtered films were freeze-dried and rehydrated to give a final concentration of 5 mM showing that by a mass-balance approximately 15% of the material and volume were

lost through extrusion for both the free-base and salt-form samples. The preparation of NEACE-T alone was made according to the same procedure but without the addition of a colipid.

Amplification and Purification of mIL-12 Expression Plasmids

The p2CMVmIL-12 vector was constructed with the p35 and p40 subunits each under the control of a separate CMV promoter as described previously (19). Plasmids were amplified using DH5α competent cells and purified using a QIAGEN endofree maxi plasmid purification kit as per the manufacturer's protocol. The purity, integrity, and concentration of the plasmids were determined by UV spectrophometric assay at 260/280 nm and 1% agarose gel electrophoresis. Purity was greater than 1.8 and a restriction enzyme assay showed proper bands.

Preparation of ISLP/p2CMVmIL-12 Complexes

Both p2CMVmIL-12 and lipoparticulates were diluted separately with 5% glucose to a volume of 200 μl each. The p2CMVmIL-12 solution was added to the lipoparticulates, mixed gently, and complex formation was allowed for 30 min at room temperature. To formulate ISLP/p2CMVmIL-12 complexes of various charge ratios ranging from 1/1 to 9/1 (±), two positive charges per molecule of PEI (M_w 423) at physiological conditions were used to calculate total positive charges and 330 nucleotides per negative charge to calculate the total negative charges (20).

Gel Retardation Assay

The ability of the ISLPs to condense pDNA was evaluated by a gel retardation assay. The ISLP/p2CMVmIL-12 complexes were electrophoresed on a 1% agarose gel pretreated with 0.5 mg/mL ethidium bromide in 1× Tris-boric acid-EDTA (TBE) buffer at 84 V. Naked DNA was used as a control. Gels were analyzed on a UV transilluminator to show the location of the DNA.

DNase Protection Assay

The ability of the ISLPs to protect pDNA from surrounding nucleases was evaluated by a DNase protection assay. ISLP/p2CMVmIL-12 complexes were formed at a final pDNA concentration of 0.1 μg/μl and 300 μl total volume and incubated at ambient conditions for 30 min. Eppendorf tubes were labeled for appropriate DNase incubation stop times, e.g. 0, 2, 5, 15, 30, and 60 mins. 100μl of stop solution (100 mM EDTA, 400 mM NaCL) was added to each tube. 50 μl of complex was removed and added to the 0 min incubation tube with gentle mixing. To remaining complexes, 25 units of Promega RQ1 RNase-Free DNase was added and incubated at 37°C for the extent of the experiment. 50 μl samples were withdrawn at appropriate times and gently mixed with stop solution. Following the final sample, the incubation stop tubes contained 100 μl stop solution plus 50 μl sample. 7.9 μl of 20% SDS was added to each sample and mixed gently (final concentration 1% SDS) to facilitate dissociation of pDNA from ISLPs, and the samples were incubated for 1 h at 65°C. Samples were loaded on a 1% agarose gel and electrophoresed at 84 V.

Particle Size and Zeta Potential

Particle size and zeta potential of cationic lipoparticulates/pDNA complexes were measured as previously described (21). Briefly, ISLP/p2CMVmIL-12 complexes were formulated at several charge ratios and diluted 5 times in a cuvette. Samples were measured for particle size and zeta potential on a Brookhaven Instruments Corp. (Holtsville, NY) ZetaPALS. Experimental conditions were 37°C, pH 7.0, using a 677 nm wavelength at a constant angle of 15°. The zeta potential was calculated from the electrophoretic mobility based on Smoluchowski's formula. Values for the particle size are mean effective diameters.

Atomic Force Microscopy

Methods for determining the surface morphology of ISLP/p2CMVmIL-12 complexes have been previously described (22,23). Briefly, red mica was freshly cleaved as a thin wafer and subsequently soaked in 33 mM magnesium acetate overnight to favor the replacement of potassium ions by divalent magnesium ions for stronger DNA binding. Mica was then sonicated for 30 m in distilled water and the surface was subjected to glow discharge for 15 s in a vacuum between 100–200 mTorr. As soon as the mica surface was exposed to air, 20 μl of 0.1 mg/mL of ISLP/p2CMVmIL-12 complexes were placed on the mica surface and 2 min was allowed for binding. The mica surface was then gently rinsed with deionized water and blown dry with nitrogen. A Digital Instruments Nanoscope II SFM (Santa Barbara, CA) was used for imaging at room temperature in the attractive force regime under 30–60% relative humidity. The microscope was operated using cantilever oscillation frequencies between 12–24 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.

Tumor Cell Lines

CT-26 murine colon adenocarcinoma cell line was a gift from Dr. Charles Tannenbaum of the Cleveland Clinic Foundation (Cleveland, OH) (24). CT-26 cells were grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL streptomycin, and 50 μg/mL gentamycin at 37°C and humidified 5% CO_2.

Cell Viability

The cytotoxicity of ISLP/p2CMVmIL-12 complexes at various charge ratios was evaluated by a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). 5,000 CT-26 cells were seeded on 96-well plates and incubated for 48 h at 37°C and humidified 5% CO_2 until 80% confluent. ISLP/p2CMVmIL-12 complexes were prepared at various charge ratios ranging from 1/1 to 9/1 (±). Cells were transfected with 0.1 μg p2CMVmIL-12 for 4 h at 37°C and humidified 5% CO_2. After 4 h the cell media was changed and the cells were incubated for an additional 20 h at

the same conditions. The manufacturer's instructions for use of the highly water-soluble tetrazolium salt (WST-8) were followed. Briefly, the CCK solution was thawed at room temperature and 10 μl were added to each well. The plates were incubated at 37°C and humidified 5% CO_2 for 3 h after which the absorbance was read at 450 nm with a reference wavelength of 600 nm. The negative control was assigned 100% cell viability and the remaining samples were normalized to this value.

In Vitro Transfection

Five thousand CT-26 cells were seeded on 96-well plates in RPMI 1640 containing 10% FBS and antibiotics. After reaching 80% confluency, they were transfected with ISLP/p2CMVmIL-12 complexes prepared at different charge ratios ranging from 1/1 to 9/1 (±). The total amount of plasmid loaded was 0.1 μg of p2CMVmIL-12 per 100 μl medium in each well. Cells were allowed to incubate for 4 h in the presence of complexes and 10% FBS at 37°C and humidified 5% CO_2 following which the medium was changed and replaced with fresh RPMI 1640 with 10% FBS. Cells were incubated for an additional 20 h at 37°C and humidified 5% CO_2 for a total transfection time of 24 h. As mIL-12 p70 is a secreted protein, the cell media was removed for analysis by ELISA and reported as mIL-12 p70 (pg/mL). Both untreated and naked DNA cultures were used as negative and positive controls, respectively.

ELISA

The measurement of mIL-12 p70 was completed using BDOptEIA™ ELISA sets for mIL-12 p70 (Pharmingen, San Diego, CA) as per the manufacturer's instructions. The mIL-12 p70 concentration was reported in terms of pg/mL.

Cellular Uptake

p2CMVmIL-12 was labeled with ethidium monoazide bromide (EMA) as previously described (25). 200 μg of pDNA was mixed with 5 μg EMA in 2 mL ultra-pure water (50:1 molar ratio of nucleotide to probe) and incubated for 10 min at room temperature. The solution was then exposed to UV light at 302 nm for 3 min and purified on an Äkta FPLC (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with a refractive index detector. For purification a Superdex 75 column was used with sterile PBS as the running buffer. To remove intercalated but not covalently bound EMA, CsCl was added to a final concentration of 1.1 g/mL and gently mixed until dissolved. Sodium citrate saturated isopropanol was added, the solution was mixed and allowed to settle, after which the upper phase, containing unbound EMA, was discarded. The sample was washed with isopropanol and the labeled pDNA in the bottom layer was precipitated overnight at –20°C with 8 volumes of 1:3 TE/absolute ethanol. The solution was then centrifuged at 15,000 × g for 10 min, the supernatant was removed and the pellet redissolved in 500 μl TE buffer. The recovered concentration and purity of the EMA-labeled pDNA was determined by UV at 260 and 280 nm. Three hundred thousand CT-26 cells were seeded on 6-well plates containing 10% FBS RPMI-1640 and incubated at 37°C and humidified 5% CO_2 until 80% confluent. The cells were transfected with ISLP/EMA-labeled p2CMVmIL-12 complexes prepared at charge ratios of 1/1 to 5/1 (±). After 4 h the cell media was changed and the cells were incubated for an additional 20 h at the same conditions. Cell suspensions obtained from the plates were observed under fluorescence microscopy.

RESULTS

Synthesis and Characterization of Insoluble Liponanomers

We synthesized water insoluble liponanomers using linear PEI of 423 Da and cholesteryl chloroformate. Figure 1 illustrates the synthesis scheme for the free-base and chloride salt-forms of NEACE-T. Following synthesis and purification, we determined the structure of these liponanomers using ^{1}H NMR. The NMR results for free-base NEACE-T are as follows (Fig. 2A): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.70 [s, 3.24 H of CH_3 from cholesterol (a)]; δ 0.85 [7.29 H of $(CH_3)_2$ from cholesterol (b)]; δ 0.92 [4.64 H of CH_3 from cholesterol (c)];

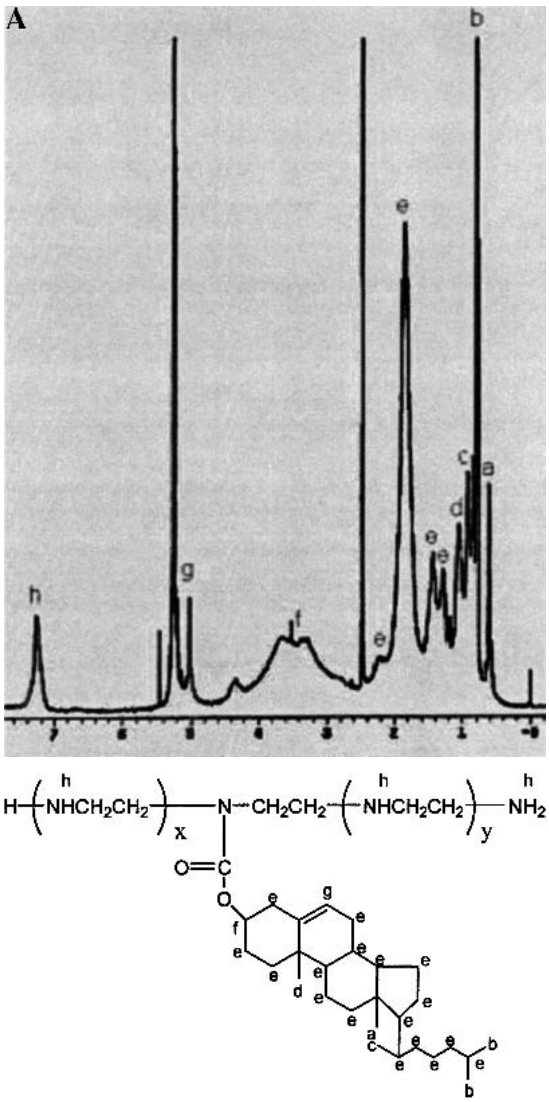

Fig. 2. ^{1}H-NMR spectra and peak assignments for free-base NEACE-T (methylene-d2 chloride): (a) ^{1}H-NMR spectra for free-base NEACE-T (methylene-d2 chloride) and (b) peak assignments for free-base NEACE-T.

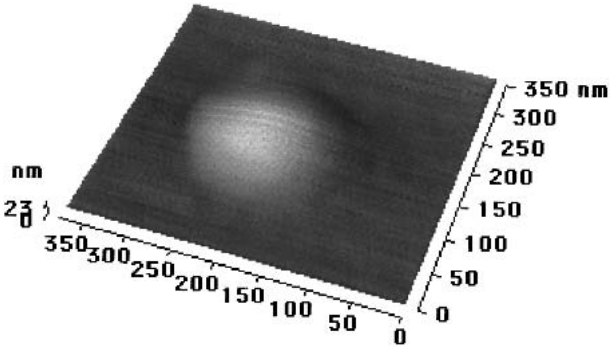

Fig 3. AFM image of ISLP/p2CMVmIL-12 (5/1, ±) showing the oblate spheroid structure of the complex. ISLP = free-base NEACE-T:DOPE (1/1).

δ 1.1 [s, 6.46 H of CH_3 from cholesterol (d)]; δ 1.1–2.3 [(8.61 H), (1.44 H), (4.49 H), (9.13 H), (4.97 H), (7.04 H), and (2.64 H) from CH_2-CH_2 and CH_2CH of cholesterol (e)]; δ 3.4 [5.23 H from cholesterol (f)]; δ 5.3 [1.00 H of $=C=CHC$ from cholesterol (g)]; and δ 7.2 [3.03 H of $NHCH_2CH_2$ (h)] . NMR peaks were assigned based on the data sheet for cholesterol provided by Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). δ 0.68 linewidth at 50% increased from 1.87 for cholesterol to 3.85 (free-base) and 5.09 (salt-form) strongly indicating conjugation between the PEI and cholesterol. NMR showed free-base NEACE-T had ~1.2 cholesterol per PEI.

Physicochemical Properties of ISLP/pDNA Complexes

DNA condensing ability of NEACE-T:DOPE and st-NCNEI-L:DOPE insoluble lipoparticulates was determined by a 1% agarose gel retardation assay. The free-base form of NEACE-T:DOPE (1/2) fully condensed pDNA at a 1/1 (±) charge ratio and above, as no free DNA was seen (data not shown). The salt-form of NEACE-T:DOPE (1/2) fully condensed pDNA at a 7/1 (+/−) charge ratio and above, significantly higher than free-base NEACE-T:DOPE. st-NCNEI-L:DOPE (1/2) fully condensed pDNA at a 5/1 (±) charge ratio and above. Three samples were chosen to evaluate the ability of the lipononamers to protect condensed DNA from nucleases at charge ratios of 3/1 (±): NEACE-T:DOPE (1/1) (free-base and salt-form) and st-NCNEI-L:DOPE (1/1). After incubation with DNase and subsequent enzyme inactivation with EDTA and dissociation with SDS, it was shown that both forms of NEACE-T:DOPE (1/1) effectively protected pDNA up to 60 min (Fig. 4A, B).

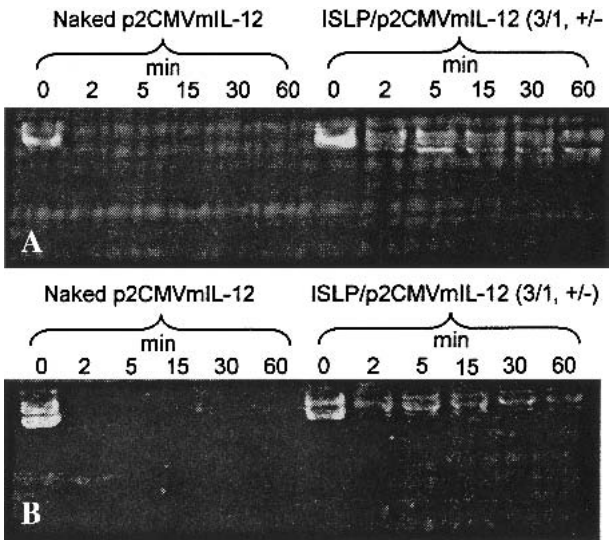

Fig. 4. DNase protection assay of (a) free-base and (b) salt-forms of NEACE-T:DOPE (1/1)/p2CMVmIL-12 (3/1, +/−). Time indicated is duration of exposure (min) to DNase before addition of stop solution.

Particle size distribution of ISLP/pDNA complexes was determined by dynamic light scattering. The mean particle size was in the range of 350–375 nm for the complexes prepared using free-base NEACE-T (Fig. 5A) and approximately 530 nm for the complexes prepared using st-NCNEI-L. Salt-form NEACE-T:DOPE was not evaluated due to its poor gel retardation results. Zeta potential of ISLP:DOPE/p2CMVmIL-12 complexes was 32–35 mV (Fig. 5B).

Surface morphology of NEACE-T:DOPE/p2CMVmIL-12 complexes was determined by atomic force microscopy (AFM) and found to be an oblate spheroid of ~300 nm in diameter. Figure 3 shows an AFM image for NEACE-T:DOPE (1/1)/pDNA (5/1, ±). Although the particle size according to the AFM does not correlate exactly with that measured by laser light scattering, this may be due to the fact that as the cantilever moves across the complex, the surface is

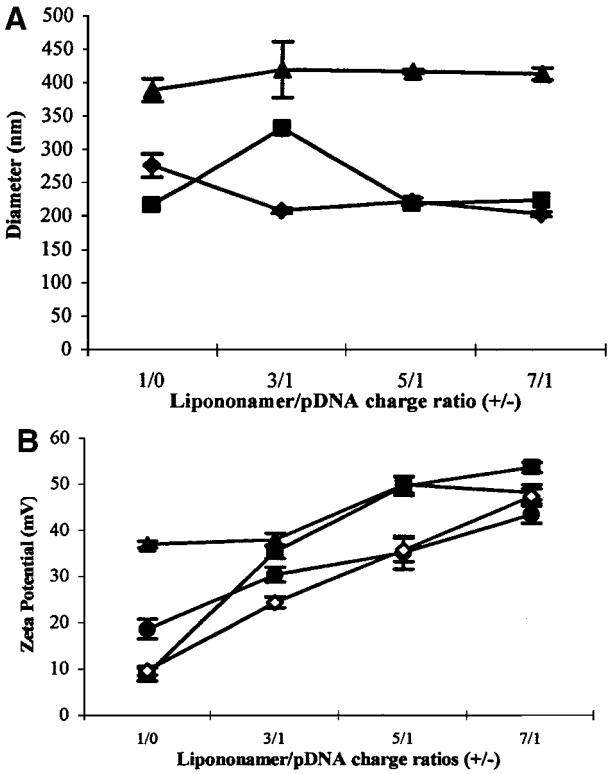

Fig. 5. Particle size and zeta potential data for free-base NEACE-T:DOPE/pDNA: (a) particle size data for NEACE-T:DOPE/pDNA complexes, data reported as mean effective diameter ± S.D., n = 3, (●) NEACE-T:DOPE (2:1), (■) NEACE-T:DOPE (1:1), (▲) NEACE-T:DOPE (1:2), and (b) zeta potential data for NEACE-T:DOPE/pDNA complexes, data reported as mean ± S.D., n = 3, (●) NEACE-T:DOPE (2:1), (■) NEACE-T:DOPE (1:1), (▲) NEACE-T:DOPE (1:2), and (◇) NEACE-T.

compressed; consequently, the dimensions per the AFM measurement are slightly less than that of the particle sizing data.

Cell Viability

The cytotoxicity of ISLP and ISLP/p2CMVmIL-12 complexes after 24 h of incubation at 37°C was determined using a Cell Counting Kit-8 on CT-26 colon adenocarcinoma cells. Cell viability data show that transfected cells were at least 80% viable when incubated with ISLP or ISLP/p2CMVmIL-12 complexes prepared at charge ratios of 9/1 (±) or below, using both free-base and salt-form NEACE-T. As the amount of ISLP was increased, cell viability dropped slightly, as seen with free-base NEACE-T:DOPE (Fig. 6). st-NCNEI-L was not evaluated for cytotoxicity due to its poor *in vitro* transfection results.

In Vitro Transfection

ISLP/p2CMVmIL-12 complexes formulated with different NEACE-T molecules, colipids and ratios of colipids, and at different charge ratios (±) in 5% (w/v) glucose were evaluated for their transfection efficiency in CT-26 colon carcinoma cells. Following transfection, the cell culture supernatants were analyzed by ELISA and the free-base NEACE-T:DOPE results are shown in Fig. 7. The levels of secreted mIL-12 were nearly 75 times higher compared to naked p2CMVmIL-12. NEACE-T:DOPE/p2CMVmIL-12 (5/1, ±) complexes showed nearly 4 times higher transfection than PEI 25 kDa/p2CMVmIL-12 complexes. The T-shaped configuration showed 17 times higher transfection than the linear configuration.

Cellular Uptake

The level of pDNA uptake into CT-26 cells was observed by labeling p2CMVmIL-12 with EMA followed by complex formation with ISLPs at 1/1, 3/1, and 5/1 charge ratios. The relative amounts of pDNA taken up by the cells were proportional to the amount of red fluorescence observed in the cells (Fig. 8A–D). Cells transfected with naked EMA-labeled p2CMVmIL-12 showed very little cellular uptake. Cells transfected with free-base NEACE-T at DOPE ratios of 1/1 and 1/2 with EMA-labeled pDNA showed similar levels to that of naked pDNA at charge ratios of 1/1 and 3/1; however, observable levels of uptake were seen at charge ratios of 5/1.

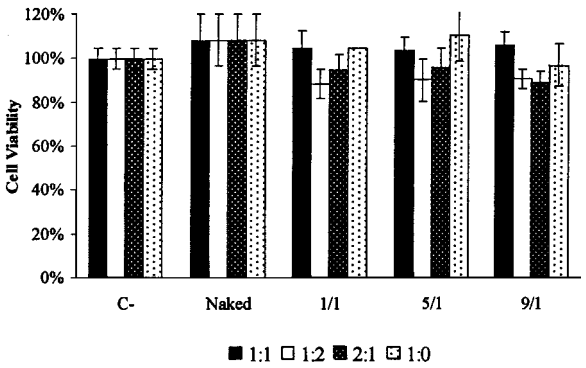

Fig. 6. Cytotoxicity data for ISLP/p2CMVmIL-12 complexes. Free-base NEACE-T/p2CMVmIL-12, data reported as mean ± SD of n = 8. The legend indicates the free-base NEACE-T:DOPE molar ratio.

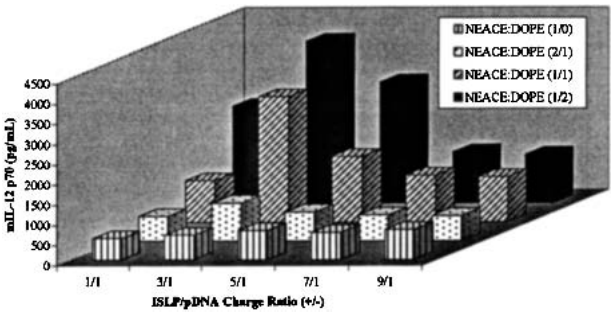

Fig. 7. mIL-12 p70 ELISA data for CT-26 cells transfected with 0.1 μg p2CMVmIL-12 plus free-base NEACE-T at varying molar ratios of DOPE. Data reported as mean protein levels of mIL-12 p70 (pg/mL), n = 8.

Cells transfected with NEACE-T:DOPE (2/1)/EMA-labeled p2CMVmIL-12 at charge ratios of 1/1, 3/1, and 5/1 showed levels higher than that of naked pDNA and increased with the charge ratio. Transfected cells with NEACE-T:DOPE (1/0)/EMA-labeled p2CMVmIL-12 at charge ratios of 1/1, 3/1, and 5/1 showed slightly higher uptake than those transfected with naked pDNA.

DISCUSSION

The early success of 3β-[*N*-(*N*',*N*'-dimethylaminoethane)carbamoyl]-cholesterol (DC-Chol) (1) lipid-based gene delivery systems spurred recent interest in the development of novel cholesterol-based cationic lipids. The levels of gene expression obtained with 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 (1). Among all the basic components of the cationic lipid, such as the hydrophobic lipid anchor, linker, and cationic headgroup, the nature of the headgroup has been shown to have a dominant role in transfection efficiency. Spermine cholesteryl carbamate with its headgroup in the "T-shape" configuration has been proven to be more efficient for *in vivo* gene delivery (2). Cholesteryl chloroformate coupled with spermine via a primary amine to generate a linear structure, rather than via a secondary amine to generate a T-shaped cationic headgroup, has proven to be less effective (8).

In this study, we synthesized cationic lipononamers using linear PEI of M_w 423 Da and cholesteryl chloroformate and investigated the effect of the orientation of the cationic headgroup, protonation state, charge ratios, and cationic lipid:colipid molar ratios on transfection efficiency. There were no significant differences in the cytotoxicity between free-base and salt-form lipononamers after transfection into CT-26 colon adenocarcinoma cell lines. The relative transfection activities were attributed to the structural differences in the headgroup orientation and the choice and amount of colipids used for the preparation of insoluble particulates. Both free-base and salt-form NEACE-T resembles a cognate ligand for a cell-surface receptor and thereby facilitate cellular internalization. In addition, free-base NEACE-T condenses pDNA more efficiently than st-NCNEI-L. The latter scenario is most likely since st-NCNEI-L, which has the linear shaped configuration, carries only one positive charge at the free primary amine. In contrast, NEACE-T has a T-shaped configuration,

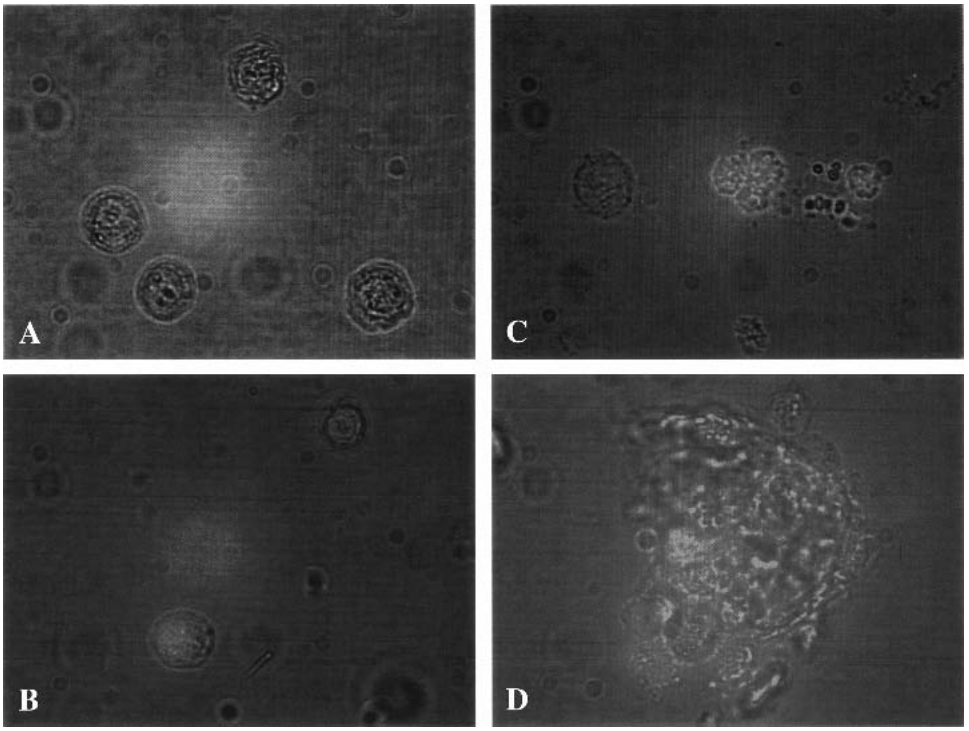

Fig. 8. Cellular uptake of CT-26 cells transfected with EMA-labeled p2CMVmIL-12 plus free-base NEACE-T with increasing molar ratios of DOPE. TRITC epifluorescent microscopy images with 500 msec integration: (A) naked pDNA, (B) free-base NEACE-T:DOPE (2/1)/pDNA (5/1, ±), (C) free-base NEACE-T:DOPE (1/1)/pDNA (5/1, ±), and (D) free-base NEACE-T:DOPE (2/1)/pDNA (5/1, ±).

which carries two positive charges at both primary amines at physiological conditions. The discrepancy between the pDNA condensing ability of free-base and salt-forms of NEACE-T lie in the native protonation state. Although free-base NEACE-T is initially electrostatically neutral, on addition to the pDNA in the aqueous glucose solution, the lipononamer becomes charged and efficiently condenses the pDNA. On the other hand, the salt-form of NEACE-T is initially electrostatically positive and the dissociation of the chloride ions from the primary amines may be hindered in physiological conditions; therefore, the salt-form is not as capable of condensing pDNA as the free-base form.

Several cationic lipids have been shown to efficiently condense pDNA (14,26). For enhanced endosomal release and also to provide stability to the DNA particles in the bloodstream, DOPE was added to these cationic liponomamers to form insoluble lipoparticulates. DOPE undergoes its transition from a linear, bilayer orientation to a hexagonal shape at low pH (27), which destabilizes the endosomal compartment at acidic pH. In addition, linear PEI of 423 Da used as a cationic headgroup may also destabilize endosomes (28).

The cationic lipid/pDNA complexes can passively target lung tissues as a result of transient accumulation of complexes in the lung (26). For efficient gene delivery to the lung, lipid/pDNA complexes should be > 200 nm. Since water soluble lipopolymer condenses pDNA into 40–60 nm particles (29), we decided to synthesize water insoluble lipopolymer using PEI 423 as a headgroup. These insoluble lipopolymers can be used to prepare lipoparticulates with the co-lipid DOPE and form complexes with pDNA, which are likely to passively target lung tissues as a result of transient accumulation of the complex in the lung. The use of PEI 423 as a headgroup was to further explore the benefit of T-shaped headgroups, which has been reported to be superior compared to the linear shape. Spermine as a headgroup will have only two primary amines whereas the use of PEI 423 as a headgroup will have an increase in the number of secondary amines. Moreover, conjugation of cholesterol to one of the secondary amines of PEI 423 will produce a tertiary amine. Both secondary and tertiary amines are expected to provide efficient buffering capacity and endosomal release of pDNA into the cytoplasm. Protonation of primary, secondary, and tertiary amines depends on their different pK_a values and the local pH. The secondary amines are likely to protonate much slower than the primary amines at the physiological pH. Due to the different methods of deprotection, the conjugates resulted in either free-base (without chloride ions) or salt-form (with chloride ions). The condensation of pDNA by free-base NEACE-T or free PEI 423 does not involve the dissociation of chloride ions. However, condensation by the salt-form NEACE-T does involve the dissociation of chloride ions. Free PEI comes from the manufacturer in a free-base form. The cationic lipid with the T-shape configuration and addition of DOPE provided the most effective transgene expression *in vitro*. The effect of charge ratio is unique to each gene carrier, target cell line, and serum concentration, but lipid/pDNA complexes prepared at the charge ratios of 3/1 or 5/1 (±) have been reported to show high *in vivo* gene expression with minimal cytotoxicity (26). In the present study, ISLP/p2CMVmIL-12 complexes prepared using free-base NEACE-T showed high *in vitro* transfection also at charge ratios of 3/1 and 5/1 (±).

Although each of the ISLPs formulated with different lipid/colipid ratios (1/0, 2/1, 1/1, and 1/2) have the same concentration of PEI, as the concentration of DOPE is increased, the surface charge density of the complexes is decreased. To study the cellular uptake of the ISLP/p2CMVmIL-12 complexes, we labeled the plasmid with EMA, transfected CT-26 cells with ISLP/EMA-labeled p2CMVmIL-12 complexes, and observed the cells under epifluorescence microscopy. The greatest cellular uptake was observed with cells transfected with ISLPs formulated with a lipid/colipid ratio of 2/1. These complexes have the greatest relative amount of cationic lipid besides those formulated without the colipid (lipid/colipid 1/0); however, ISLPs without colipid may not be as stable. In addition, there appears to be an increase in cellular uptake with increased charge ratio up to 5/1 (±) in all of the complexes formulated with the colipid. These data are consistent with the theory that endocytosis is increased with greater positive surface charge and surface charge density, as long as the stability of the complexes is not reduced.

Once the complexes are endocytosed, the next crucial step is endosomal release. As the pH of the endosome decreases, DOPE has a phase transition from a lipid bilayer (L_α) formation to a reverse micelle (H_{II}) formation (30). Swelling of endosomes and dispersion of the neutral colipid into the endosomal membrane allows the cationic lipids of the complexes to mix with the anionic lipids of the membrane. The molar ratio of a cationic lipid to colipid has been reported to affect the phase transition of the lipid/plasmid complexes and consequently endosomal release. Gene expression increases as the amount of DOPE increases, likely as a result of enhanced endosomal release. It is interesting to note that cellular uptake is improved with less DOPE because of a charge density effect and endosomal release is improved with additional DOPE because of a phase transition effect. Although these effects oppose each other, they are both important for efficient gene expression. There must be an optimal balance, which we feel we have yet to reach. However, the complexes which have the most efficient endosomal release (those with the greatest amount of DOPE) show the highest levels of gene expression, indicating that endosomal release may be particularly crucial.

In our previous study, we designed a water-soluble lipopolymer (WSLP) for gene delivery has been successful both *in vitro* and *in vivo* (19,29). WSLP/p2CMVmIL-12 complexes increased the *in vivo* levels of interleukin-12 (IL-12), interferon-gamma (IFN-γ), and nitric oxide (NO) after intratumoral injection into BALB/c mice. Consequently, the rate of tumor growth was inhibited and the survival rate was increased. Water insoluble lipoparticulates (ISLPs) were designed for future treatment of pulmonary metastases by systemic administration. Lung tissue may be passively targeted by cationic lipid/pDNA complexes due to the transient accumulation of these complexes in the lung (26); moreover, the lung's large surface area may act as a bioreactor in its ability to produce increased levels of therapeutic proteins for use throughout the body.

In summary, we designed a water insoluble lipononamer and prepared insoluble lipoparticulates. ISLPs efficiently condense and protect plasmid DNA from degradation by nucleases and promote enhanced mIL-12 gene expression into CT-26 colon adenocarcinoma cell lines. The optimum formulation studied was free-base NEACE-T:DOPE (1/2)/p2CMVmIL-12 (3/1, ±).

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