Fluorescent-Labeled Poly(ethylene glycol) Lipid Conjugates With Distal Cationic Headgroups

Tao Chen,[†,‡] Kim F. Wong,*[,†] David B. Fenske,[†] Lorne R. Palmer,[†] and Pieter R. Cullis[†,§]

Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3, and Inex Pharmaceuticals Corporation, 100-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC, Canada V5J 5J8. Received December 7, 1999;
Revised Manuscript Received February 11, 2000

The synthesis of a new class of fluorescent cationic poly(ethylene glycol) lipid conjugates (CPLs) is described. These lipids consist of a hydrophobic distearoyl-phosphatidylethanolamine (DSPE) anchor coupled to a highly fluorescent N_ϵ-dansyl lysine moiety, which is attached to a hydrophilic poly(ethylene glycol) (PEG) spacer that is linked to a cationic headgroup made of lysine residues. Introduction of the dansyl moiety allows rapid and accurate quantification of CPLs within lipid bilayers using fluorescence techniques. The synthetic scheme is straightforward, using repeated amino-carboxyl coupling reaction steps, with purification by precipitation. A series of dansylated CPLs was synthesized with zero, one, three, and seven lysine residues located at the distal end of the PEG chain, giving rise to CPLs with one, two, four, and eight distal positive charges, respectively. The structures of the CPLs were confirmed by ^{1}H NMR spectroscopy and chemical analysis. CPLs provide a means of introducing positive charge to a bilayer that is localized some distance from the membrane surface, and are of particular interest for nonviral gene delivery applications. The usefulness of CPLs is demonstrated by the enhanced in vitro cellular binding and uptake of liposomes containing CPL_4.

INTRODUCTION

To optimize the in vivo delivery of drugs using liposomal systems, general methods are required for increasing the interactions of liposomes with cells. To date, attempts to include specific targeting information on the liposome surface, such as antibody (*1*−*3*), vitamin (*4*−*8*), oligopeptide (*9*, *10*), or oligosaccharide (*10*) constructs specific for a particular membrane protein or receptor, have not been successful in achieving this goal, despite promising in vitro results. Many of these studies utilized poly(ethylene glycol) (PEG)[1] lipids with the ligands attached at the distal end of the PEG spacer, thereby physically removing the ligand from the vesicle surface and above any PEG coating present to impart long circulation lifetime characteristics.

This paper describes the synthesis of a new class of cationic lipids designed to enhance *nonspecific* targeting by increasing the electrostatic attraction between liposomes and cells. Such a role for cationic lipids such as DOTMA (*11*) or DODAC (*12*) has been demonstrated in DNA:liposome transfection complexes. Complexes are large aggregates formed as a result of electrostatic interactions between cationic liposomes and plasmid DNA. The presence of a slight excess of cationic lipid (relative to DNA) gives rise to a net positive charge that enhances interactions with cells. The key feature of the cationic poly(ethylene glycol) lipids (CPLs) described in the present paper is the presence of a multivalent cationic headgroup located at the distal end of a PEG spacer chain, which in turn is anchored in the membrane by attachment to a molecule of distearoyl-phosphatidylethanolamine (DSPE). By varying the length of this chain with respect to that of the surface PEG, it should be possible to achieve placement of the positive charge at some distance from the liposome surface, where interactions with cells would be maximized. The potential utility of CPL in the area of drug delivery may be strongly influenced by the type of lipid anchor, the variation in the chain length of the PEG spacer, and the number of positive charges on the headgroup. The former two parameters have been shown to influence the rate at which PEG-lipids will transfer between membranes (*13*), which could influence circulation longevity. To explore the role of the latter parameter, we have synthesized a series of dansylated CPL with zero, one, three, and seven lysine residues in the headgroup, giving rise to CPL with one, two, four, and eight positive charges, respectively. The presence of the highly fluorescent dansyl group

* To whom correspondence should be addressed. Phone: (604) 822-2649. Fax: (604) 822-4843. E-mail: kimwong@interchange.ubc.ca.

† University of British Columbia.

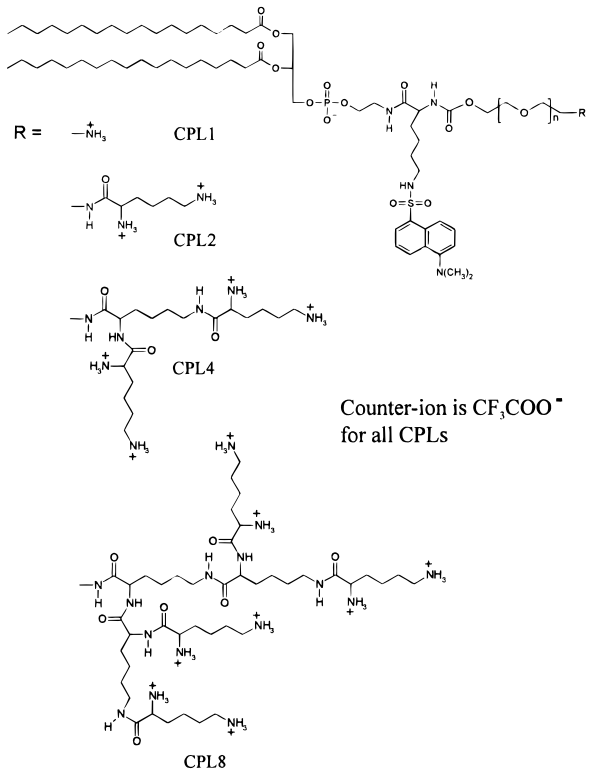
‡ Current address: Theralipids, Inc., 6100 Royalmount Ave., Montreal, PQ, Canada H4P 2R2.

§ Inex Pharmaceuticals Corporation.

[1]Abbreviations: BHK, baby hamster kidney; tBoc, *tert*-butyloxycarbonyl; tBoc-NH-PEG_{3400}-CO_2-NHS, tBoc protected and NHS activated PEG_{3400}; Chol, cholesterol; CPL, cationic poly(ethylene glycol) lipid conjugate; CPL_1, CPL with one positive charge; CPL_2, CPL with two positive charges; CPL_4, CPL with four positive charges; CPL_8, CPL with eight positive charges; DCC, *N*,*N*′-dicyclohexyl-carbodiimide; DCU, dicyclohexyl urea; DMEM, Dulbecco's Modified Eagle Medium; DODAC, *N*,*N*-dioleoyl-*N*,*N*-dimethylammonium chloride; DOTMA, *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylamine; FBS, fetal bovine serum; NHS, *N*-hydroxysuccinimide; di-tBoc-lysine-NHS, N_α,N_ϵ-di-tBoc-L-lysine-*N*-hydroxysuccinimide ester; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine; LUV, large unilamellar vesicle; PBS, phosphate-buffered saline; PEG_{3400}, poly(ethylene glycol) with an average MW of 3400; Rh-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(Lissamine Rhodamine B Sulfonyl); TFA, trifluoroacetic acid.

10.1021/bc990171x CCC: $19.00
Published on Web 03/30/2000

Scheme 1. Structures of the Cationic-PEG-Lipids



allows rapid and accurate quantification of the lipid using fluorescence techniques. All of the lipids contain a DSPE anchor coupled to N_ϵ-dansyl lysine, which is attached to a PEG spacer with a molecular weight of 3400. Aside from CPL_1, which possesses a headgroup made of a single primary amine, all others contain increasing numbers of lysine residues. The chemical structures of the CPLs are shown in Scheme 1. The potential usefulness of CPLs is demonstrated by enhanced in vitro cellular binding and uptake of CPL_4-LUVs.

EXPERIMENTAL PROCEDURES

Materials and Reagents. tBoc-NH-PEG_{3400}-CO_2-NHS was obtained from Shearwater Polymers (Huntsville, AL). N_α,N_ϵ-di-tBoc-L-lysine-*N*-hydroxysuccinimide ester, N_ϵ-dansyl-L-lysine, *N*-hydroxysuccinimide (NHS), and *N*,*N*′-dicyclohexyl-carbodiimide (DCC) were purchased from Sigma-Aldrich Canada (Oakville, ON). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) were obtained from Northern Lipids (Vancouver, BC). Fluorescamine and rhodamine-DSPE (Rh-PE) were obtained from Molecular Probes (Eugene, OR). Cholesterol (Chol) was obtained from Sigma Aldrich Canada (Oakville, ON). Trifluoroacetic acid, diethyl ether, methanol, triethylamine, and chloroform were obtained from Fisher Scientific (Vancouver, BC). All reagents were used without further purification.

General Methods. All reactions were performed in 16 × 100 mm glass test tubes. ^{1}H NMR spectra were obtained employing a Bruker MSL 200 spectrometer operating at 200 MHz. Deuterated chloroform ($CDCl_3$) was used as the solvent in the NMR experiments. Proton chemical shifts (δ) were referenced to $CHCl_3$ set at 7.24 ppm. When signals were reasonably resolved, their intensities were integrated to allow an estimation of the number of protons. The chemical shifts of exchangeable amino group protons, observed between 7 and 8 ppm, are not given. These peaks were assigned on the basis of their removal by a D_2O exchange.

Phosphorus and fluorescamine assays were performed to confirm the ratio of primary amine per phosphate in each CPL as follows.

The phosphate concentration of the CPL was determined using the Fiske-Subarrow phosphorus assay (*14*). The primary amine concentration in the CPL was determined using the fluorophore fluorescamine. A fluorescamine solution (0.6 mg/mL) in acetone was prepared. An aliquot of CPL solution in HBS (2−4 μL) was made up to 250 μL with 200 mM sodium borate, pH 8.0. To this mixture, 50 μL of the fluorescamine solution was added dropwise with vortexing, followed by 1700 μL of water. The fluorescence of this solution was measured using a Perkin-Elmer LS50 Luminescence Spectrometer with λ_{ex} of 397 nm and λ_{em} of 475 nm and excitation and emission slit widths of 10 nm. The primary amine concentration of the CPL was determined from a lysine standard curve.

tBoc-NH-PEG_{3400}-CO_2-(N_ϵ-dansyl)lysine (1). tBoc-NH-PEG_{3400}-CO_2-NHS (500 mg, 147 μmol) in 3 mL of dry chloroform was added slowly to a solution of N_ϵ-dansyl-L-lysine (65 mg, 171 μmol) in 1 mL of methanol and 200 μL of triethylamine. After the reaction mixture was stirred at room temperature for 3 h, the solvent was removed under a N_2 stream and further dried under vacuum. The crude product was washed by first dissolving it in a minimum amount of chloroform with warming and then precipitating it out with the addition of 10 mL of diethyl ether. The ether was added while vortexing. Precipitation of **1** was accelerated by cooling. The precipitate was then pelleted by centrifugation, and the ether was discarded. This chloroform/ether wash and precipitation procedure was repeated. The dry solid was then dissolved in 4 mL of chloroform and cooled in an ice bath for 15 min. Methanol (2 mL) was added if this cooled solution was clear. If a precipitate (excess dansyl lysine) developed, it was filtered off prior to the addition of methanol. The chloroform/methanol solution was washed with 1.2 mL of 0.1 M HCl. The chloroform phase was extracted and dried, and the solid was redissolved in 6 mL of chloroform/methanol (2:1 v/v) and washed with 1.2 mL of distilled water. The chloroform phase was extracted and dried to a thick paste, and tBoc-NH-PEG-CO_2-(N_ϵ-dansyl)lysine (**1**) was precipitated with 10 mL of ether. After centrifugation and the removal of ether, the dried product was a light yellow solid.

Yield: 520 mg (93%). TLC (silica gel) chloroform/methanol (85:15 v/v): R_f 0.56. ^{1}H NMR ($CDCl_3$): δ 1.08 (t), 1.40 (s, 11H), 2.66 (s, 1H), 2.86 (s, 8H), 3.27 (q), 3.50 (t), 3.60 (s, 309H), 3.96 (t), 4.19 [m(broad)], 5.03 [s(broad), 1H], 5.22 (t, 1H), 5.43 (d, 1H), 7.16 (d, 1H), 7.51 (q, 2H), 8.19 (d, 1H), 8.27 (d), 8.50 (d, 1H) ppm.

Dansylated CPL_1-tBoc (3). First, tBoc-NH-PEG_{3400}-CO_2-(N_ϵ-dansyl)lysine-NHS (**2**) was prepared as follows. A solution of tBoc-NH-PEG_{3400}-CO_2-(N_ϵ-dansyl)lysine (**1**) (500 mg, 132 μmol) and NHS (31.5 mg, 274 μmol) in 2 mL of dry chloroform was added to DCC (42.8 mg, 207 μmol) dissolved in 1 mL of dry chloroform. The reaction mixture was stirred for 2 h at room temperature. The byproduct, dicyclohexyl urea (DCU), was filtered using a Pasteur pipet with a cotton plug. The filtrate, containing tBoc-NH-PEG_{3400}-CO_2-(N_ϵ-dansyl)lysine-NHS (**2**), was slowly added to a solution of DSPE (120.6 mg, 161 μmol) in 2 mL of dry chloroform and 200 μL of triethylamine. The dissolution of DSPE in dry chloroform and triethylamine required warming to 65 °C. After the reaction mixture was stirred at room temperature for 3 h, it was

dried, and chloroform/ether washed and precipitated as described earlier until the disappearance of DSPE on TLC as visualized with ninhydrin. This removal of excess DSPE required at least three washings. The product, dansylated CPL_1-tBoc (**3**), was dissolved in chloroform/methanol (2:1), washed with dilute HCl and water, and precipitated using ether as described for (**1**).

Yield: 575 mg (96%). TLC (silica gel) chloroform/methanol (85:15) R_f 0.58. ^{1}H NMR ($CDCl_3$): δ 0.85 (t, 4H), 1.22 (s, 48H), 1.41 (s, 10H), 1.55 (t), 2.27 [m(broad), 6H], 2.90 [m(broad), 6H], 3.04 (s, 8H), 3.27 (q), 3.61 (s, 275H), 4.14 [m(broad)], 4.32 (d), 4.38 (d), 5.05 [s(broad)], 5.23 [s(broad)], 5.58 [m(broad)], 7.37 (d, 1H), 7.49 [s(broad), 1H], 7.59 (t, 2H), 8.24 (d, 1H), 8.50 (d, 1H), 8.59 (d, 1H) ppm.

Dansylated CPL_1 (4). Trifluoroacetic acid (TFA) (2 mL) was added to a solution of dansylated CPL_1-tBoc (**3**) (550 mg, 121 μmol) in 2 mL of chloroform and stirred for 4 h at room temperature. The solution was concentrated to a thick paste and chloroform/ether washed three times. After the removal of ether, the solid was dissolved in 6 mL of chloroform/methanol (2:1) and washed with 1.2 mL of 5% sodium bicarbonate. The chloroform phase was extracted, dried, and redissolved in 6 mL of chloroform/methanol (2:1) and washed with 1.2 mL of distilled water. The chloroform phase was concentrated to a thick paste, and the purified CPL_1 (**4**) was obtained through a chloroform/ether wash and vacuum-dried.

Yield: 535 mg (97%). TLC (silica) chloroform/methanol/water (65:25:4) R_f 0.76. ^{1}H NMR ($CDCl_3$): δ 0.85 (t, 4H), 1.22 (s, 46H), 1.54 [m(broad), 8H], 2.23 (t, 6H), 2.84 (s, 9H), 3.16 [m(broad), 3H], 3.26 (t, 3H), 3.61 (s, 263H), 3.98 (q), 4.17 (t), 4.33 (d), 4.38 (d), 5.19 [s(broad)], 5.93 (d, 1H), 7.13 (d, 1H), 7.46 (t, 1H), 7.52 (t, 1H), 8.15 (d, 1H), 8.43 (t, 2H) ppm.

Dansylated CPL_2-tBoc (5). A solution of N_α,N_ϵ-di-tBoc-L-lysine-*N*-hydroxysuccinimide ester (105 mg, 236 μmol) in 2 mL of dry chloroform was gradually added to a solution of dansylated CPL_1 (**4**) (510 mg, 112 μmol) in 2 mL of chloroform containing 200 μL of triethylamine and stirred at room temperature for 3 h. The completion of the reaction was indicated by the disappearance of primary amine as visualized by ninhydrin assay on TLC. The reaction mixture was concentrated to a thick paste and chloroform/ether washed (~3 times) until the disappearance of excess N_α,N_ϵ-di-tBoc-L-lysine-*N*-hydroxysuccinimide ester as checked by TLC. The product was dissolved in 6 mL of chloroform/methanol (2:1) and washed with 1.2 mL of 0.1 M HCl. The chloroform phase was extracted, dried, redissolved in 6 mL of chloroform/methanol (2:1), and washed with 1.2 mL of distilled water. The chloroform phase was concentrated to a thick paste and the purified compound was obtained through a chloroform/ether wash and vacuum-dried.

Yield: 510 mg (96%). TLC (silica gel) chloroform/methanol (85:15) R_f 0.58. ^{1}H NMR ($CDCl_3$). δ 0.85 (t, 3H), 1.22 (s, 44H), 1.41 (s, 20H), 1.56 [m(broad)], 1.78 [m(broad)], 2.27 (m, 5H), 2.88 (s), 2.91 (s), 2.97 (s), 3.06 (s, 7H), 3.26 (t), 3.44 (t), 3.62 (s, 252H), 3.97 (t), 4.05 (d), 4.13 (m), 4.33 (d), 4.38 (d), 4.68 [s(broad)], 5.22 [s(broad)], 5.51 [s(broad)], 6.57 [t(broad), 1H], 7.39 (d, 1H), 7.51 [s(broad), 1H], 7.60 (t, 2H), 8.26 (d, 1H), 8.53 (d, 1H), 8.61 (d, 1H) ppm.

Dansylated CPL_2 (6). The synthesis of CPL_2 (**6**) was the same as that of CPL_1 (**4**) by deprotecting dansylated CPL_2-tBoc (**5**) (490 mg, 103 μmol).

Yield: 478 mg (97%). TLC (silica) chloroform/methanol/water (65:25:4) R_f 0.63. ^{1}H NMR ($CDCl_3$). δ 0.85 (t, 3H), 1.22 (s, 42H), 1.55 (m, 10H), 1.93 [s(broad), 4H], 2.24 (t, 5H), 2.85 (s, 8H), 3.26 (t, 3H), 3.61 (s, 271H), 3.95 (q), 4.17 (s), 4.34 (s), 5.18 [s(broad), 1H], 6.31 (d, 1H), 6.89 (s, 1H), 7.10 (d, 1H), 7.49 (m, 1H), 8.15 (d, 1H), 8.34 (d, 1H), 8.47 (d, 2H) ppm.

Dansylated CPL_4-tBoc (7). The synthesis of CPL_4-tBoc (**7**) was the same as that of CPL_2-tBoc (**5**) by reacting N_α,N_ϵ-di-tBoc-L-lysine-*N*-hydroxysuccinimide ester (170 mg, 383 μmol) with dansylated CPL_2 (**6**) (455 mg, 95 μmol).

Yield: 475 mg (96%). TLC (silica gel) chloroform/methanol (85:15) R_f 0.58. ^{1}H NMR ($CDCl_3$): δ 0.85 (t, 3H), 1.22 (s, 43H), 1.40 (s, 39H), 1.71 [m(broad), 6H], 2.27 (m, 5H), 2.88 (s), 2.90 (s), 2.95 (s), 3.05 (s, 10H), 3.25 (t, 3H), 3.43 (s), 3.61 (s, 262H), 3.97 (t), 4.05 (d), 4.15 (m), 4.32 (d), 4.37 (d), 4.51 [s(broad)], 4.75 [s(broad)], 4.90 [s(broad)], 5.23 [t(broad), 1H], 5.52 [s(broad)], 5.80 [s(broad), 1H], 7.15 [m(broad), 1H], 7.38 (d, 1H), 7.50 (s, 1H), 7.59 (t, 2H), 8.25 (d, 1H), 8.51 (d, 1H), 8.60 (d, 1H) ppm.

Dansylated CPL_4 (8). The synthesis of CPL_4 (**8**) was the same as that of CPL_1 (**4**) by deprotecting dansylated CPL_4-tBoc (**7**) (450 mg, 86 μmol).

Yield: 440 mg (97%). TLC (silica) chloroform/methanol/water (65:25:4) R_f 0.19. ^{1}H NMR ($CDCl_3$). δ 0.85 (t), 1.22 (s), 1.53 [m(broad)], 2.34 [m(broad)], 2.86 (s), 3.26 (t), 3.62 (s), 3.87 [s(broad)], 3.97 (t), 4.17 [s(broad)], 4.33 (d), 5.18 [s(broad)], 7.15 (d), 7.43 (s), 7.51 (t), 8.15 (d), 8.32 (d), 8.48 (d), 9.05 [s(broad)] ppm.

Dansylated CPL_8-tBoc (9). The synthesis of CPL_8-tBoc (**9**) was the same as that of CPL_2-tBoc (**5**) by reacting N_α,N_ϵ-di-tBoc-L-lysine-*N*-hydroxysuccinimide ester (70 mg, 158 μmol) with dansylated CPL_4 (**8**) (100 mg, 19 μmol).

Yield: 112 mg (96%). TLC (silica gel) chloroform/methanol (85:15) R_f 0.58. ^{1}H NMR ($CDCl_3$): δ 0.84 (t, 3H), 1.08 (s), 1.21 (s, 39H), 1.39 (s, 75H), 1.66 (m [broad]), 2.26 (m, 4H), 2.89 (s, 4H), 3.06 (s, 11H), 3.25 (t, 3H), 3.43 (s), 3.49 (s), 3.60 (s, 248H), 3.96 (t), 4.04 (d), 4.12 (t), 4.31 (d), 4.36 (m), 5.19 [m(broad)], 6.77 [m(broad), 1H], 6.91 [s(broad), 1H], 7.24 ($CHCl_3$), 7.41 (d), 7.50 [s(broad)], 7.60 (t), 8.25 (d, 1H), 8.53 (d, 1H), 8.63 (d, 1H) ppm.

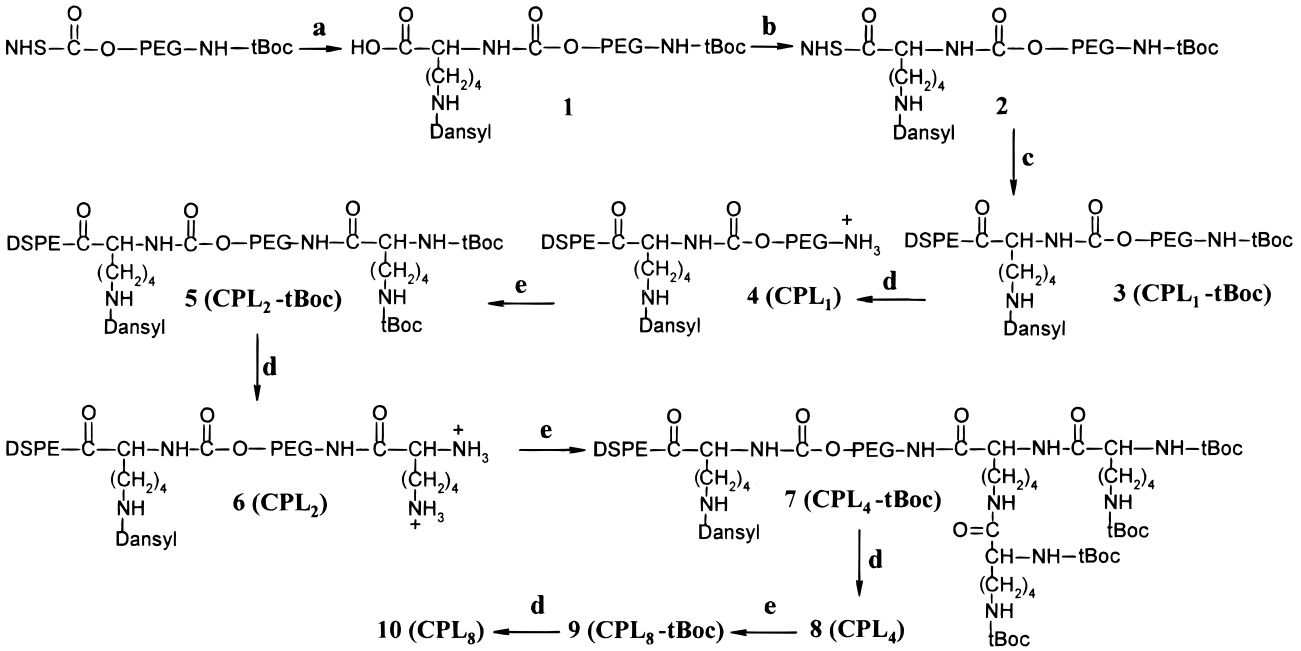
Dansylated CPL_8 (10). The synthesis of CPL_8 (**8**) was the same as that of CPL_1 (**4**) by deprotecting dansylated CPL_8-tBoc (**9**) (50 mg, 8 μmol).

Yield: 48 mg (96%). TLC (silica) chloroform/methanol/water (65:25:4) R_f 0.13. ^{1}H NMR ($CDCl_3$): δ 0.85 (t, 3H), 1.22 (s, 34H), 1.52 [s(broad)], 2.23 [s(broad)], 2.86 (d), 3.27 (d), 3.61 (s, 274H), 3.96 (t), 4.18 [m(broad)], 7.14 [s(broad)], 7.24 ($CHCl_3$), 7.50 [m(broad)], 8.12−8.27 [s(broad)], 8.47 [m(broad)] ppm.

In Vitro Binding and Uptake. Large unilamellar vesicles (100 nm) composed of DSPC/Chol/rhodamine-PE/CPL_4 (49.5:45:0.5:5 mol %) and DSPC/Chol/rhodamine-PE (54.5:45:0.5 mol %) were prepared by extrusion as described by Hope et al. (*15*).

BHK cells, obtained from American Tissue Culture Collection (ATCC CCL-10, Rockville, MD), were plated in 12-well plates at 2.5×10^5 cells/well in DMEM (Gibco BRL, Burlington, ON) + 10% FBS (Gibco BRL, Burlington, ON) and allowed to grow overnight in an incubator at 37 °C with 5% CO_2. Samples (40 nmol) were incubated on the cells in DMEM + 10% FBS for 1, 2, 4, and 6 h. At each time point, the media was removed, the cells were washed twice with PBS, and lysed with 600 μL of lysis buffer (PBS containing 0.1% Triton X-100). Lipid uptake was quantified by measuring the rhodamine fluorescence in the lysate using $\lambda_{ex} = 560$ nm and $\lambda_{em} = 590$ nm with excitation and emission slit widths of 10 and 20 nm, respectively, using a Perkin-Elmer LS-50 Luminescence Spectrometer. Lipid uptake was normalized to the num-

Scheme 2. Sequential Synthesis of the CPLs[a]



[a] Key: (a) N_ϵ-dansyl-L-lysine, triethylamine, methanol, chloroform, 3 h; (b) NHS, DCC, chloroform, 2 h; (c) DSPE, triethylamine, chloroform, 4 h; (d) TFA, chloroform, 4 h; (e) N_α,N_ϵ-di-tBoc-L-lysine-*N*-hydroxy-succinimide ester, triethylamine, chloroform, 3 h.

ber of cells using the BCA protein assay reagent kit (Pierce, Rockford, IL).

RESULTS AND DISCUSSION

The CPLs were synthesized by repeated coupling reaction steps involving amines and NHS-activated carbonate groups as outlined in Scheme 2. This consists of (a) incorporating the dansyl fluorescent label to the hydrophilic PEG spacer, (b) coupling of the DSPE anchor, and (c) attachment of the cationic headgroup to the lipid. The heterobifunctional PEG polymer tBoc-NH-PEG_{3400}-CO_2-NHS (MW 3400) was chosen for two reasons. First, it was commercially available. Second, it is insoluble in ether which provided a very convenient means of purifying its derivatives, **1**−**10**. Other reagents were used in excess to ensure the complete conversion of the PEG polymer to its derivatives. The excess reagents were soluble in ether and, therefore, could be removed by washing in ether during purification.

Incorporation of the fluorescent label, N_ϵ-dansyl lysine, to the PEG polymer by coupling the α-amino group of dansyl lysine with the NHS activated carbonate of PEG gave the lysine derivative **1**. The DSPE anchor was coupled via intermediate **2**, which was formed by the esterification of **1** using NHS and DCC. The resulting PEG lipid, **3**, was deprotected by removing the tBoc to form CPL_1, **4**, with one positive charge. The positive charges in the other CPLs are carried by the amino groups of lysine. Here, the NHS-activated and di-tBoc-protected lysine was attached to the free amino function of CPL_1 to form intermediate **5** which, upon deprotection, yielded CPL_2, **6**, with two positive charges. The attachment of two lysine residues to the amino groups of CPL_2 via intermediate **7** gave CPL_4, **8**, with four positive charges. Thus, CPL_8, **10**, with eight positive charges was synthesized with the attachment of four lysine residues as the headgroup. As can be seen, this provides a very convenient means of synthesizing multivalent CPLs that are of particular interest for nonviral drug delivery applications.

The structures of the purified intermediates and CPL in synthetic Scheme 2 were verified by ^{1}H NMR spectroscopy and chemical analysis. The ^{1}H NMR spectra showed well-resolved resonances for the PEG, tBoc, and acyl chains of DSPE at approximately 3.61, 1.41, and 1.21 ppm, respectively, and for the resonances of the dansyl moiety (aromatic protons at 7.1−8.5 ppm; methyl protons at 2.8−3.0 ppm). From the integrated signal intensities of the former three peaks, it was found that the ratio of tBoc/PEG or tBoc/DSPE was 1.0, 2.1, 4.0, and 8.1 for CPL_1-tBoc, CPL_2-tBoc, CPL_4-tBoc, and CPL_8-tBoc, respectively. As each tBoc is attached to an amino group, this gives the number of amino groups in the headgroup of each CPL relative to the CPL_1. That essentially identical results were obtained using the ratios of tBoc relative to both PEG and DSPE demonstrates the presence of lipid and polymer in correct proportion to the headgroup. The complete cleavage of the tBoc-protecting groups was verified by the loss of tBoc NMR peaks and chemical analysis, which determined the ratio of primary amine to phosphate in each of the CPL by using the fluorescamine and phosphorus assays. The amine/phosphate ratios for CPL_1, CPL_2, CPL_4, and CPL_8 were found to be 1.0, 2.2, 3.7, and 8.0, respectively. These corresponded well with the expected number of positive charge bearing amino groups of the respective CPLs.

A number of PEG-lipids similar to those described in the present paper have been designed to enhance liposomal targeting to cells. While some are intended for specific-targeting purposes, with folate (*4*), oligopeptide (*9*, *10*), or oligosaccharide (*10*) ligands present in the distal headgroups, cationic PEG-lipids have also been described. A nonfluorescent monovalent cationic amino-PEG-DSPE lipid similar to CPL_1 has been prepared previously and liposomes containing this lipid were found to possess prolonged circulation lifetimes (*16*). In addition, a separate study has reported the synthesis of a nonphospholipid anchored PEG with a distal divalent cationic headgroup (*17*). Finally, a dansyl-labeled PEG has previously been synthesized by Pendri et al. (*18*),

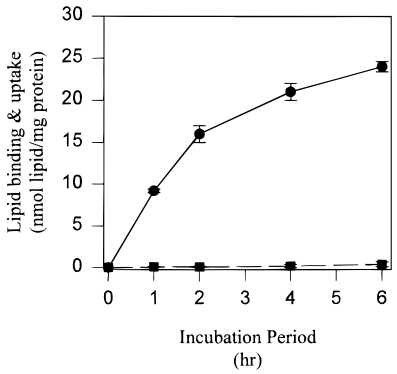


Figure 1. Time course for the binding and uptake of 20 mM DSPC/Chol/Rh-PE (54.5:45:0.5 mol %) LUV (■) and DSPC/Chol/Rh-PE/CPL_4 (49.5:45:0.5:5 mol %) LUV (●) by 1×10^5 BHK cells grown in DMEM + 10% FBS.

while Silvius and Zuckermann reported the synthesis of a fluorescent-labeled methoxyPEG-PE (*13*). The CPLs described here possess several attributes which may increase their usefulness relative to other cationic lipids. First, the phospholipid anchor will readily allow efficient incorporation of CPL into liposomal systems. Second, the dansyl label will permit accurate and convenient quantification of the CPL in the bilayer using fluorescence techniques. Finally, the valency of the cationic headgroup in the CPL can easily be modified using lysine residues.

To demonstrate that CPL enhance the interaction of liposomes with cells, we have incorporated CPL_4 into a common LUV system [DSPC/Chol/Rh-PE (54.5:45:0.5)], and have examined the time course of vesicle binding/uptake in BHK cells (Figure 1). In the presence of CPL_4 the cellular uptake of the liposomes is ~50 times greater than in its absence. These results demonstrate the potential utility of the CPL system. Further studies detailing the preparation of CPL-LUV systems and their application in the delivery of plasmid DNA to cells will be published elsewhere.

ACKNOWLEDGMENT

This work was supported by grants from the Medical Research Council of Canada (MRC).

LITERATURE CITED

(1) Meyer, O., Kirpotin, D., Hong, K., Sternberg, B., Park, J. W., Woodle, M. C., and Papahadjopoulos, D. (1998) Cationic liposomes coated with poly(ethylene glycol) as carriers for oligonucleotides. *J. Biol. Chem. 273*, 15621−15627.

(2) Kao, G. Y., Change, L. J., and Allen, T. M. (1996) Use of targeted cationic liposomes in enhanced DNA delivery to cancer cells. *Cancer Gene Ther. 3*, 250−256.

(3) Hansen, C. B., Kao, G. Y., Moase, E. H., Zalipsky, S., and Allen, T. M. (1995) Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. *Biochim. Biophys. Acta 1239*, 133−144.

(4) Gabizon, A., Horowitz, A. T., Goren, D., Tzemach, D., Mandelbaum-Shavit, F., Qazen, M. M., and Zalipsky, S. (1999) Targeting Folate Receptor with Folate Linked to Extremities of Poly(ethylene glycol)-Grafted Liposomes: In Vitro Studies. *Bioconjugate Chem. 10*, 289−298.

(5) Lee, R. J., and Low, P. S. (1994) Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J. Biol. Chem. 269*, 3198−3204.

(6) Reddy, J. A., and Low, P. S. (1998) Folate-mediated targeting of therapeutic and imaging agents to cancers. *Crit. Rev. Ther. Drug Carrier Syst. 15*, 587−627.

(7) Holladay, S. R., Yang, Z., Kennedy, M. D., Leamon, C. P., Lee, R. J., Jayamani, M., Mason, T., and Low, P. S. (1999) Riboflavin-mediated delivery of a macromolecule into cultured human cells. *Biochim. Biophys. Acta 1426*, 195−204.

(8) Wang, S., and Low, P. S. (1998) Folate-mediated targeting of antineoplastic drugs, imaging agents, and nucleic acids to cancer cells. *J. Controlled Release 53*, 39−48.

(9) Zalipski, S., Puntambekar, B., Boulikas, P., Engbers, C. M., and Woodle, M. C. (1995) Peptide Attachment to Extremities of Liposomal Surface Grafted PEG Chains: Preparation of the Long-Circulating Form of Laminin Pentapeptide, YIGSR. *Bioconjugate Chem. 6*, 705−708.

(10) Zalipsky, S., Mullah N., Harding, J. A., Gittelman, J., Guo, L., and DeFrees, S. A. (1997) Poly(ethylene glycol)-Grafted Liposomes with Oligopeptide or Oligosaccharide Ligands Appended to the Termini of the Polymer Chains. *Bioconjugate Chem. 8*, 111−118.

(11) Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielson, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A. 84*, 7413−7417.

(12) Zhang, Y. P., Sekirov, L., Saravolac, E. G., Tardi, P., Clow, K., Leng, E., Sun, R., Cullis, P. R., and Scherrer P. (1999) Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties. *Gene Ther. 6*, 1438−1447.

(13) Silvius, J. R., and Zuckermann, M. J. (1993) Interbilayer Transfer of Phospholipid-Anchored Macromolecules via Monomer Diffusion. *Biochemistry 32*, 3153−3161.

(14) Fiske, C. H., and Subbarow, Y. (1925) The colorimetric determination of phosphorous. *J. Biol. Chem. 66*, 375−400.

(15) Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume, and ability to maintain a membrane potential. *Biochim. Biophys. Acta 812*, 55−65.

(16) Zalipski, S., Brandeis, E., Newman, M. S., and Woodle, M. C. (1994) Long circulating, cationic liposomes containing amino-PEG-phosphatidylethanolamine. *FEBS Lett. 353*, 71−74.

(17) Schulze, U., Schmidt, H.-W., and Safinya, C. R. (1999) Synthesis of Novel Cationic Poly(ethylene glycol) Containing Lipids. *Bioconjugate Chem. 10*, 548−552.

(18) Pendri, A., Martinez, A., Xa, J., Shorr, R. G. L., and Greenwald, R. B. (1995) Poly(ethylene glycol) Fluorescent Linkers. *Bioconjugate Chem. 6*, 596−598.

BC990171X