JOURNAL OF LIPOSOME RESEARCH
Vol. 14, Nos. 3 & 4, pp. 155–173, 2004

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Distal Cationic Poly(Ethylene Glycol) Lipid Conjugates in Large Unilamellar Vesicles Prepared by Extrusion Enhance Liposomal Cellular Uptake

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ABSTRACT

Cationic poly(ethylene glycol)-lipid conjugates (CPLs), a class of lipid designed to enhance the interaction of liposomes with cells, possess the following architectural features: 1) a hydrophobic lipid anchor of distearoylphosphatidylethanolamine (DSPE); 2) a hydrophilic spacer of poly(ethylene glycol); and 3) a cationic head group prepared with 0, 1, 3, or 7 lysine residues located at the distal end of the PEG chain, giving rise to CPL possessing 1, 2, 4, or 8 positive charges, respectively (CPL_1 to CPL_8). Previously we have described the synthesis of CPL, have characterized the postinsertion of CPL into PEG-containing LUVs and SPLP (stabilized plasmid-lipid particles), have shown significant increases in the binding of CPL-LUV to cells, and have observed dramatically enhanced transfection (up to a million-fold) of cells with CPL-SPLP in the presence of calcium [Chen et al. (2000) Bioconjugate Chem. 11,

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DOI: 10.1081/LPR-200033437
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433–437; Fenske et al. (2001) Biochim. Biophys. Acta 1512, 259–272; Palmer et al. (2003) Biochim. Biophys. Acta 1611, 204–216]. In the present study, we examine a variety of CPL properties (such as polarity and CMC) and characterize CPL-vesicular systems formed by extrusion and examine their interaction with cells. While CPL polarity was observed to increase dramatically with increasing charge number, CMC values were all found to be low, in the range of other PEGylated lipids, and exhibited only a small increase, going from CPL_1 (1.3 μM) to CPL_8 (2 μM). The CPLs were almost quantitatively incorporated into large unilamellar vesicles (LUVs) prepared by the extrusion method and were evenly distributed across the lipid bilayer. Lower levels of incorporation were obtained when CPLs were incubated with preformed liposomes (DSPC/Chol, 55:45) at 60°C. The binding of CPL-LUVs to BHK cells in vitro was found to be dependent on the distal charge density of the CPL rather than total surface charge. Liposomes possessing CPL_4 or CPL_8 were observed to bind efficiently to cell surfaces and enhance cellular uptake in BHK cells (as observed with both lipid and aqueous content markers), whereas those possessing CPL_1 or CPL_2 exhibited little or no binding. These results suggest new directions for the design of liposomal systems capable of in vivo delivery of both conventional and genetic (plasmid and antisense) drugs.

Key Words: Drug delivery; Large unilamellar vesicles; Cationic PEG lipids; Liposome-cell binding; Critical micellar concentration.

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INTRODUCTION

There has been a strong impetus for developing efficient lipid-based systems capable of systemic delivery of both conventional and genetic drugs (1–8). Tremendous effort has been devoted to the design of stealth liposomes, which possess long circulation lifetimes, and can be used for systemic delivery (9–14). However, it has become increasingly clear that stealth liposomes are also comparatively inefficient at facilitating cellular uptake and therefore therapeutic efficacy is reduced (15–18).

The mechanism of longevity of stealth liposomes in vivo can be attributed to steric hindrance (19) and the mobility of polar hydrophilic surface polymer barriers (20). Polymer barriers prevent or reduce the rate of macromolecular adsorption in blood and sterically inhibit both electrostatic and hydrophobic interactions between liposomes and blood components. The first step for the cellular uptake of liposomes by host cells is the binding of the liposomes to the cell surface, followed by adsorptive endocytosis. Therefore, an increase in liposomal binding to the cell surface would certainly enhance cellular uptake of liposomes into the cells. Since cell surfaces are negatively charged, we hypothesize that stealth liposomes possessing a distal positively charged polymer coating may efficiently bind to host cell surfaces and enhance cellular uptake. Liposomal circulation half-lives may not be drastically affected by including a small amount of such distally charged polymers, since the mobility of the remaining neutral surface polymer barrier would reduce or prevent interactions with a variety of blood components. In a recent study, Zalipsky and coworkers reported that liposomes containing a monovalent cationic amino-PEG-PE as the only polymer exhibited stealth circulation properties (21). Interactions of the distal charge at the end of the polymer with blood components could also be avoided by varying the polymer length of the

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distal-charged PEG-lipids. Distal charges could be hidden or screened by the surrounding neutral coating polymers if the chain length of the charged polymers was shorter. After removal of the coating polymers by the blood components during circulation, charges at the distal end of polymers would be exposed for electrostatic interactions with cell surfaces. Such programmable polymer-coating liposomal drug delivery systems have been reported (12,22–24).

We have previously shown that the binding of LUVs to cells is enhanced some 50-fold by the presence of 5 mol% of CPL_4 (25), and that similar increases in cell binding occur when CPL_4 is inserted into the external leaflet of preformed PEG-containing vesicles (26,27). Most striking, though, is the 10^6-fold increase in in vitro transfection potency observed in SPLP systems containing CPL_4 in the presence of Ca^{2+} (27), a result which highlights the potential of this approach. In the present study we partially characterize certain physical properties of the CPLs (polarity and CMC) in order to gain greater insight into their behavior in liposomal systems. We focus on their incorporation into LUVs, both conventional and PEG-containing, formed by the extrusion technique (28), and examine their ability to enhance the cellular uptake of liposomes. In analyzing these compounds, a structure-function relationship was sought so that factors which enhance cellular uptake could be identified. Thus, in addition to CPL_1, CPL_2, and CPL_4 (26), we present binding data for CPL_8 as well. The results demonstrate the importance of distal chain charge density in determining liposome uptake, and reveal enhanced delivery of both lipid and liposomal contents to BHK cells in vitro, both in the absence and presence of serum.

MATERIALS AND METHODS

Chemicals and Reagents

tBoc-NH-PEG_{3400}-CO_2NHS was obtained from Shearwater Polymers, Inc (Huntsville, AL). Nα,Nε-di-tBoc-L-lysine-NHS, triethylamine and cholesterol were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON). Trifluoroacetic acid, ethyl ether and chloroform were obtained from Fisher Scientific (Vancouver, BC). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine and 1,2-distearoyl-sn-glycero-3-phosphocholine were obtained from Northern Lipids (Vancouver, BC). 1,2-distearoyl-3-phosphatidylethanolamine-mono-methoxy-PEG_{2000} ($MPEG_{2000}$-DSPE) was obtained from Genzyme (Cambridge, MA).

Synthesis of CPLs

The distal cationic PEG-lipid conjugates (CPLs) were synthesized using the procedure described previously (25) without the fluorescence-labeling step involving incorporation of the dansyl moiety. Briefly, a hydrophobic DSPE anchor was conjugated to a hydrophilic poly(ethylene glycol) (PEG) spacer by coupling of tBoc-NH-PEG-CO_2NHS onto the amino group of DSPE. The first compound of a series of CPLs, CPL_1, containing one primary amino group, was obtained after removing the tBoc protection group by hydrolysis with trifluoroacetic acid. CPLs with higher numbers of amino groups (2, 4, and 8 as shown in Fig. 1) were subsequently made by repeat coupling with tBoc-protected lysine followed by deprotection.

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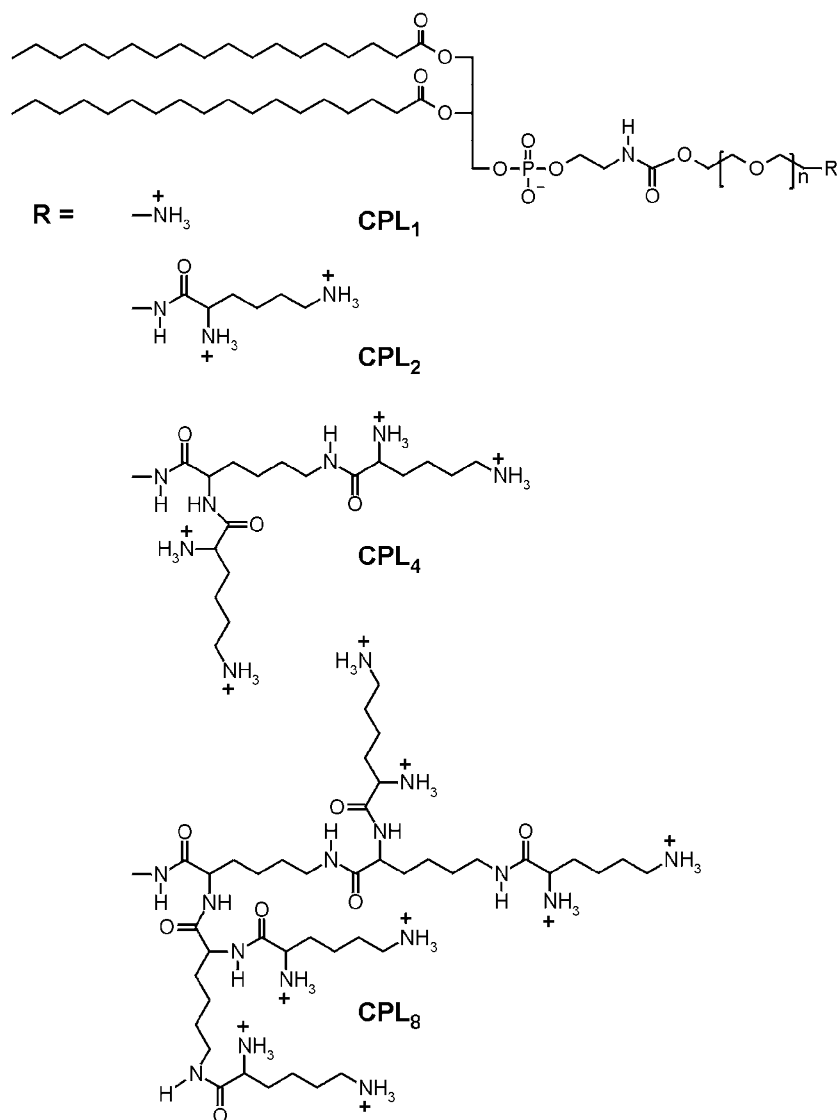

Figure 1. Chemical structures of the cationic PEG-lipid conjugates. Each CPL consists of a DSPE anchor, the PEG_{3400} spacer chain, and a cationic headgroup (R) consisting of an amino group (CPL_1), a lysine residue (CPL_2), or linked lysine residues (CPL_4 and CPL_8). The number of the CPL refers to the number of positive charges in the headgroup at physiological pH.

Determination of the Critical Micellar Concentration (CMC)

The CMCs of the CPLs were determined using the NPN assay as previously reported by Brito and Vaz (29). A series of different concentrations of CPLs were prepared in HBS buffer (25 mM Hepes, 150 mM NaCl, pH 7.4). 5 μM of NPN (from a

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stock NPN solution in 95% ethanol) was added into the above CPL solutions. After incubation of the mixtures at room temperature for 30 min, fluorescence intensities were measured at $\lambda_{em} = 410$ nm and $\lambda_{ex} = 356$ nm using a Perkin Elmer LS 50 luminescence spectrometer.

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Liposome Preparation: CPL-LUVs Prepared by the Hydration-Extrusion Method

Large unilamellar vesicles (LUV) were prepared by extrusion as described by Hope et al. (28). Appropriate amounts of lipids (DSPC/Chol, 55:45 mol/mol) containing trace amounts of Rh-PE with or without CPLs in chloroform, were dried under a stream of nitrogen gas to form a homogeneous lipid film. The lipid film was hydrated in HBS with or without HPTS (50 mM) by vortex mixing. The resulting multilamellar vesicles (MLVs) were extruded 10 times through two stacked 100 nm polycarbonate filters using an extruder (Lipex Biomembranes, Inc., Vancouver, BC, Canada) at 65°C. Free CPL and, in some cases, free HPTS were removed by gel filtration on a column of Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO) equilibrated with HBS.

Liposome Preparation: CPL-LUVs Prepared by the Post-Insertion Method

Preformed LUVs containing DSPC/Chol (55:45 mol/mol) with a trace amount of [^{3}H]CHE were prepared as described above. Appropriate amounts of CPL and preformed LUV were mixed and incubated in a water bath at 60°C for 2 h. After cooling to room temperature, the uninserted CPL was separated from CPL-LUVs by gel filtration on a column of Sepharose CL-6B (Sigma Chemical Co., St. Louis, MO) equilibrated with HBS.

Determination of Liposome Size

The diameters of the liposomes with or without CPL incorporation were determined by quasielastic light scattering (QELS) using a Nicomp 370 submicorm particle sizer (Santa Barbara, CA).

Quantification of CPLs Using a Fluorescamine Assay

Quantitatively, the levels of CPLs were determined using a fluorescamine assay. Fluorescamine is intrinsically nonfluorescent but reacts in milliseconds with primary aliphatic amines to yield fluorescent derivatives. Excess reagent is rapidly converted to a nonfluorescent product by reaction with water (29–31). CPL-containing samples were dissolved in 2 mL of borate buffer (0.2 M, pH 8). For liposomal samples, 10 μL of 10% Triton X-100 was added to solubilize the membranes. The amount of 20 μL of fluorescamine (10 mg/mL) in ethanol was then added dropwise to the vortexed CPL sample solutions. After incubating for 20 min at room temperature, the fluorescence of the solutions was measured at $\lambda_{em} = 475$ nm with $\lambda_{ex} = 397$ nm using a Perkin Elmer

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LS 50 Luminescence spectrometer. A series of diluted free CPLs were used as standards for quantification of the exact amount of CPLs in the CPL-LUV samples.

For studies on the distribution of CPL between the inner and outer membrane leaflets, fluorescence at low temperature was performed, where fluorescamine is unable to penetrate the membrane bilayer. The determination of CPL in the outer leaflet was as follows: an appropriate amount of sample was diluted with 2 mL of Borate buffer (pH 8.5) and cooled in ice water. A total of 20 μl of cooled fluorescamine (10 mg/mL) in ethanol was slowly added into the vortexed solutions. After 20 min, the fluorescent emission spectrum was scanned from 450 to 500 nm with a constant excitation at $\lambda_{ex} = 397$ nm using a Perkin Elmer LS 50 luminescence spectrometer. The determination of total CPL was as follows: 10 μl of 10% Triton X-100 was added to the above sample solution to solubilize the membrane, and then an additional 20 μl of cooled fluorescamine (10 mg/mL) in ethanol was added. The fluorescence spectrum was recorded again, as just described.

Cell Culture and Cellular Uptake

For the uptake studies, 1×10^5 BHK cells per well on 12-well plates were grown overnight in 2 mL of DMEM with or without 10% FBS at 37°C in 5% CO_2. A total of 20 nmol of the 2, 3, and 4 mol% CPL-LUV samples containing 0.5 mol% rhodamine-PE were mixed with HBS to a final volume of 200 μL, and this was added to the top of the cells followed by the addition of 800 μL of media. The samples were incubated on top of the cells for 1, 2, and 4 h. At the appropriate time points, cells were washed three times with PBS and lysed by adding 600 μL of 0.1% Triton X-100 in PBS, pH 8.0. Rhodamine and BCA assays were performed in order to determine the levels of lipid uptake and cellular protein, respectively. The rhodamine fluorescence of the lysate was measured in a 1.0 mL microcuvette on a Perkin-Elmer LS50 luminescence spectrophotometer using a λ_{ex} of 560 nm and a λ_{em} of 600 nm with slit widths of 10 and 20 nm, respectively. An emission filter of 430 nm was also used. Lipid uptake was determined by comparison of the fluorescence in the lysate to that of a lipid standard and normalized to the total amount of protein in the cells, as determined by the BCA protein assay (Pierce, Rockford, IL).

Fluorescence micrographs were also taken prior to cell lysis on an Axiovert 100 Zeiss Fluorescent microscope (Carl Zeiss Jena GmbH) using a rhodamine filter from Omega Opticals (Brattleboro, VT) with the following specifications: $\lambda_{ex} = 560 \pm 20$ nm, 600 nm LP, and DC 590 nm.

RESULTS

Synthesis and Characterization of CPLs

A simple and straightforward synthetic approach was used to prepare the CPLs as described previously (25), except that incorporation of the fluorescent dansyl label was omitted. Primary amine and phosphate contents of the resulting CPLs were determined by fluorescamine (29–31) and phosphorus (32) assays, as described. As listed in Table 1, the ratio of NH_2/P for CPL_1, CPL_2, CPL_4, and CPL_8 were very close to the

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Table 1. Partial physicochemical properties of cationic-PEG-lipid-conjugates.

Sample	NH_2/P ratio[a]	R_f[b]	CMC (μM)[c]
DPPE-PEG_{2000}M	–	–	2.8
DSPE-PEG_{2000}M	–	–	1.9
DSPE-CPL-1	0.96	0.83	1.3
DSPE-CPL-2	2.07	0.70	1.5
DSPE-CPL-4	3.90	0.15	1.9
DSPE-CPL-8	7.88	0.04	2.0

[a]The ratios of amine and phosphate were measured by fluorescamine and Fiske/Subbarow assays, respectively, as described in the text.
[b]The R_f was calculated from TLC analysis in a solvent system ($CHCl_3$:MeOH:Et_3N:H_2O, 60:37:0.5:2.5).
[c]CMC was determined by NPN assay.

theoretical values (0.96, 2.07, 3.90, and 7.88 respectively). These data confirm that the resulting CPLs bear the correct number of primary amino groups.

The intended goal of this study was to enhance the cellular uptake of liposomes through the incorporation of CPLs within vesicles. The number of potential charges on the CPLs and their critical micellar concentrations (CMCs) may have implications for the action of CPLs in both vesicle formulation and cell binding. The CMCs of the CPLs would be an index indicating their propensity to be retained in a lipid bilayer, as well as possibly indicating the postinsertion efficiency of CPLs in preformed vesicles (26).

CMCs, as measured by the NPN assay, were very similar for all four CPLs (1.3–2.0 μM) although there was a trend of increasing CMC as the number of amines at the PEG terminus increased (Table 1). The number of primary amines significantly affected the polarities (as indicated by R_f values from TLC analysis) but not the CMCs of the CPLs. The reasonably low CMCs values explain the ease by which CPL insert into preformed vesicles without disrupting the liposomal membrane, as confirmed by postinsertion of CPLs into empty vesicles and vesicles possessing encapsulated DNA (26,33).

Table 2. Characterization of conventional liposomes containing DSPE-CPLs prepared by hydration-extrusion method.

Entry	Lipid composition	Size (nm)	CPL incorporation(%)
1	DSPC/Chol(60:40)	110	–
2	DSPC/Chol/CPL-1(58.7:40:1.3)	120	98.5
3	DSPC/Chol/CPL-2(58.7:40:1.3)	122	94.5
4	DSPC/Chol/CPL-4(58.7:40:1.3)	122	98.1
5	DSPC/Chol/CPL-8(58.7:40:1.3)	122	97.6
6	DSPC/Chol/CPL-1(57.5:40:2.5)	120	98.5
7	DSPC/Chol/CPL-2(57.5:40:2.5)	122	94.5
8	DSPC/Chol/CPL-4(57.5:40:2.5)	122	98.1
9	DSPC/Chol/CPL-8(57.5:40:2.5)	122	97.6

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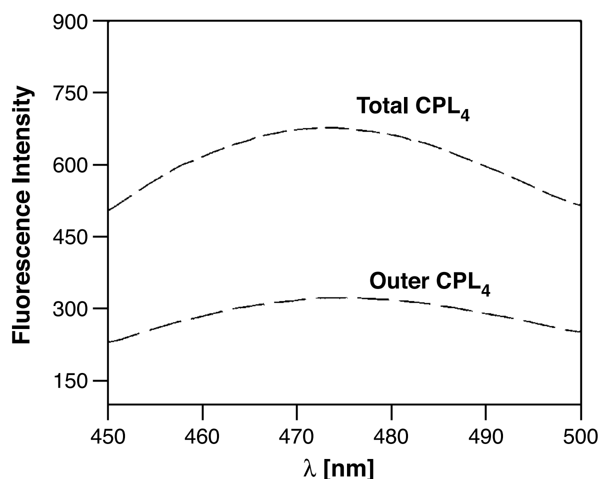

Figure 2. Distribution of CPL_4 between the inner and outer leaflets of the liposomal membrane. CPL_4-LUVs (DSPC/Chol/CPL_4, 55:40:5) were prepared by the extrusion method, following which the distribution of CPLs was quantified by the fluorescamine assay, as described in the text.

Incorporation of CPLs into Conventional Liposomes Using the Hydration-Extrusion Method

Liposomes were formulated with CPLs as one of the lipid components. Although the polarity of CPLs was strongly influenced by the number of primary amines, the ability to incorporate into liposomes during extrusion was not affected. As listed in Table 2, CPLs were almost quantitatively incorporated into the liposomes for CPL concentrations of 2.5 mol% or less. The high incorporation rate may be due to the relatively high bilayer affinity of the DSPE anchor in the CPLs. No significant influence of the number of charges and buffer pH was observed upon CPL incorporation into liposomes. Fluorescamine labeling studies indicated that the incorporated CPLs were equally distributed between the inner and outer leaflets of the liposomal membrane (Fig. 2). It was interesting to note that a pH gradient could be held across the membrane of the CPL-containing LUVs (data not shown), suggesting that weakly basic drugs could be loaded within these CPL-containing LUVs.

Table 3. Characterization of the stealth liposomes containing DSPE-CPLs prepared by hydration-extrusion method.

Entry	Lipid composition	Size (nm)	CPL incorporation(%)
1	DSPC/Chol/PEG-PE(56:40:4)	128	–
2	DSPC/Chol/PEG-PE/CPL-1(56:40:2:2)	130	96.7
3	DSPC/Chol/PEG-PE/CPL-2(56:40:2:2)	130	101
4	DSPC/Chol/PEG-PE/CPL-4(56:40:2:2)	130	104
5	DSPC/Chol/PEG-PE/CPL-8(56:40:2:2)	130	105

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Incorporation of CPLs into Long-Circulating Liposomes by Hydration-Extrusion Method

CPLs were incorporated into liposomes containing 2 mol% PEG-PE, a lipid commonly used to increase circulation lifetimes of liposomes. Table 3 lists the formulations prepared with equivalent amounts of CPL and PEG-PE. Similar to the previous liposomal formulation, the number of primary amines in the CPLs did not affect their incorporation into the stealth liposomes. Furthermore, all the CPLs tested in these experiments were nearly quantitatively incorporated into the PEG-PE containing liposomes. It is interesting to note that the size of the CPL-containing stealth liposomes is larger (~130 nm) than the conventional CPL-containing vesicles (~120 nm). We

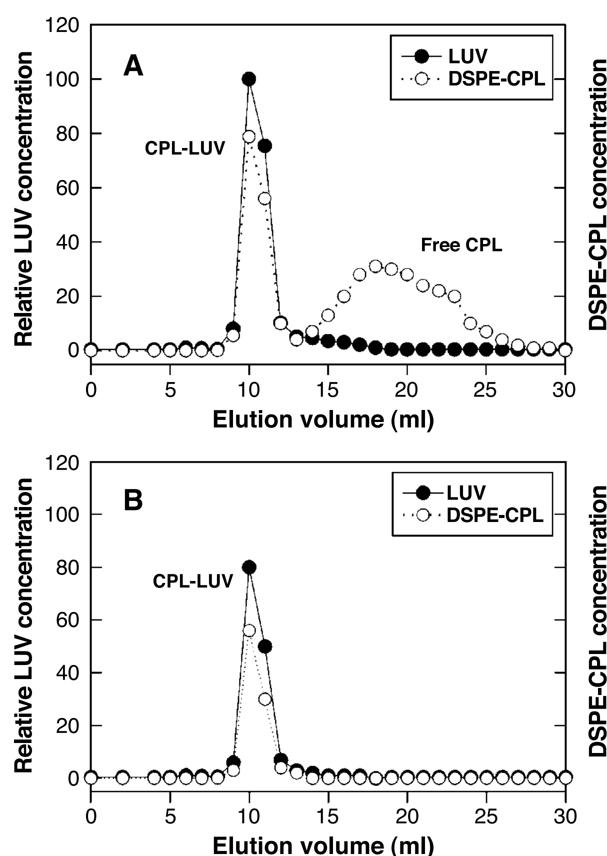

Figure 3. (A) Insertion of CPL_4 into DSPC/Chol (55:45) LUVs containing a trace amount of $[^3H]$CHE. LUVs (5 μmol lipid) were incubated with 0.355 μmol CPL_4 at 60°C for 2 h, following which the sample was applied to a column of Sepharose CL-4B equilibrated in HEPES-buffered saline. 1 mL fractions were collected and assayed for CPL (○) and lipid (●) as described. (B) Retention of CPL_4 in DSPC/Chol (55:45) LUVs. The main LUV fraction from Fig. 3A was reapplied to a column of Sepharose CL-4B equilibrated in HEPES-buffered saline. 1 mL fractions were collected as above.

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suggest that the neutral PEG coating could prevent the CPL from folding back toward the liposomal membrane, thus resulting in an apparent size increase. In a similar manner, the insertion of CPL_4 into LUVs containing PEG_{2000}-Cer was previously found to increase vesicle diameter by 10–20 nm (26).

Incorporation of CPLs into Liposomes by the Postinsertion Method

As described previously (26), CPLs can also be inserted into preformed LUVs (containing either DOPC or DOPE) using a simple postinsertion method. The postinsertion method also works for LUVs formed from highly saturated lipids such as DSPC. As shown in Fig. 3A, some 60% of the initial CPL was incorporated into the outer leaflet of DSPC/Chol LUVs using the postinsertion method. The CPL was

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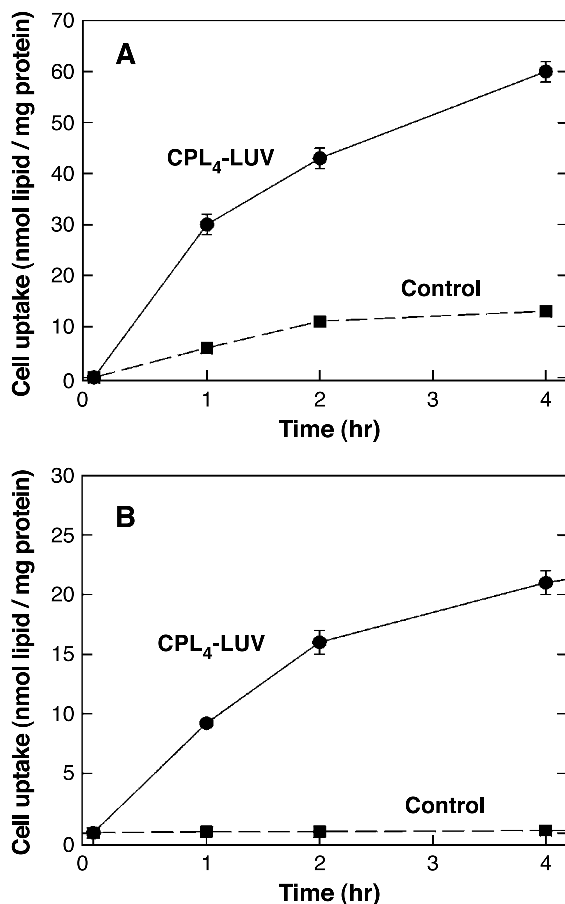

Figure 4. Cell uptake of CPL_4-LUVs in BHK cells in (A) PBS-CMG and (B) DMEM (10% FBS). Control LUVs (DSPC/Chol, 60:40) and CPL_4-LUVs (DSPC/Chol/CPL_4, 58.7:40:1.3) containing 0.5 mol% rhodamine-PE were prepared by extrusion as described in the text.

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retained when the CPL-LUV was re-eluted on a column of Sepharose CL-4B (Fig. 3B). These results demonstrate the potential of preparing CPL-LUVs without altering existing drug encapsulation procedures.

In Vitro Cellular Uptake of Conventional CPL-Liposomes

The in vitro cellular uptake of CPL-containing liposomes was studied employing baby hamster kidney (BHK) cells. The fluorescent-labeled lipid rhodamine-DOPE (Rh-PE) was used to determine lipid uptake. BHK cells were incubated with liposomes with or without inserted CPL using either PBS-CMG or serum-containing medium. As shown in Fig. 4A and 4B, CPL_4 significantly enhances the cellular uptake of liposomes when compared to control samples (non-CPL containing LUV). The time-dependent uptake of CPL-LUVs is still increasing after 4 h. Note that the binding of both CPL_4-LUVs and control LUVs are reduced in the presence of DMEM containing 10% FBS. Figure 5 summarizes the cellular uptake of the different CPL-containing vesicles after a 4 h incubation. Compared to the control formulation, reduced cellular uptake was observed for liposomes containing CPL_1, a moderate increase for CPL_2 (2-fold), and a large increase for both CPL_4 and CPL_8-containing systems (5-fold). The similar degree of increase resulting from CPL_4 and CPL_8 indicates that a minimum charge density of four on the CPLs satisfies the requirement for achieving maximum enhanced cellular uptake.

In Vitro Cellular Uptake of Sterically Stabilized CPL-Liposomes

It was of interest to determine whether incorporation of CPL into sterically stabilized liposomes also enhanced their cellular uptake efficiencies. Liposomes containing 2 mol% each of neutral PEG_{2000}-PE and CPL were prepared using the

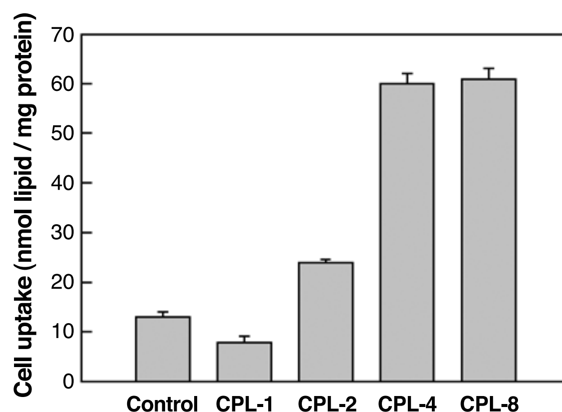

Figure 5. Cell uptake of conventional liposomes containing CPLs in BHK cells in PBS-CMG after 4 h incubation. Control LUVs (DSPC/Chol, 60:40) and CPL-LUVs (DSPC/Chol/DSPE-CPL, 58.7:40:1.3) containing 0.5 mol% rhodamine-PE were prepared by extrusion as described in the text.

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extrusion method. Liposomes with or without CPL were incubated on cells for 1, 2, and 4 h and cellular uptake levels were determined by measuring the Rh-PE fluoresence. Although the presence of additional neutral PEG-PE reduced the cellular binding of liposomes (Fig. 6A, B), especially in the presence of media containing 10% serum, enhanced cellular uptake was still observed for LUVs containing CPL_4 and CPL_8 (Fig. 6B). Interestingly, in the presence of DMEM containing 10% FBS, the binding of CPL_8-LUVs was approximately twice that of CPL_4-LUVs (Fig. 6B). In addition, it was found that an entrapped fluorescent aqueous marker (HPTS) could be delivered into cells in association with the liposomes (data not shown). This suggests that CPL insertion had little (if any) effect on membrane properties such as permeability.

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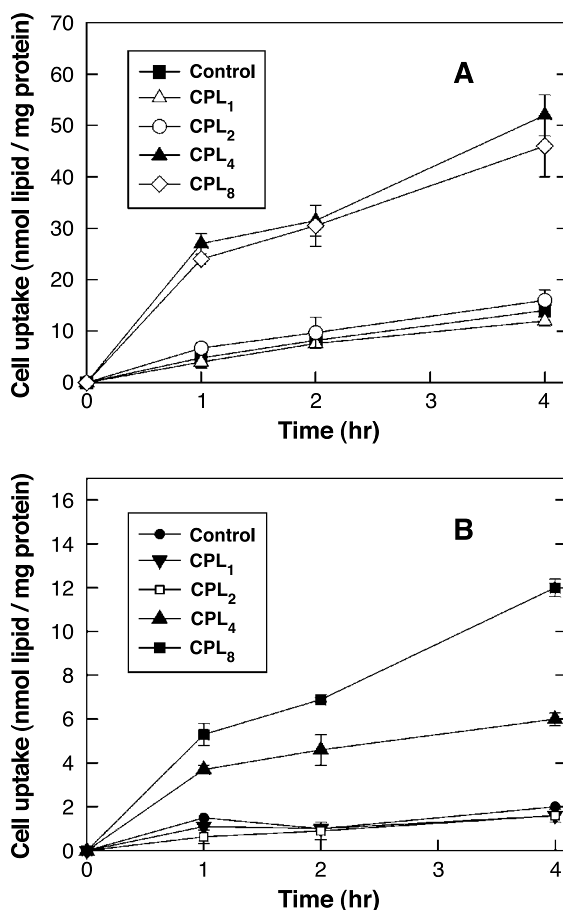

Figure 6. Cell uptake of PEG-containing liposomes containing CPLs in BHK cells in (A) PBS-CMG and (B) DMEM (10% FBS). Control LUVs (DSPC/Chol/PEG-PE, 56:40:4) and CPL-LUVs (DSPC/Chol/PEG-PE/CPL, 55.5:40:2:2) containing 0.5 mol% rhodamine-PE were prepared by extrusion as described in the text.

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DISCUSSION

The present paper and those preceding it (25–27) represent an attempt to design a liposomal delivery system that can take advantage of disease site targeting (the passive accumulation of liposomes at tumors or sites of inflammation resulting from sluggish, leaky vasculature), and which exhibits enhanced cellular uptake resulting from nonspecific electrostatic interactions (increased binding and uptake resulting from CPL). It is becoming increasingly clear that systemic delivery properties will only be realized for liposomal systems exhibiting long-circulation lifetimes (on the order of 6–7 h). While most PEG-containing liposomes have long circulation lifetimes after i.v. injection, the PEG-lipid used in their construction is neutral and therefore inhibits binding to cellular membranes and subsequent cellular uptake of liposomes. In order to overcome the reduced cellular uptake problem, we have attempted to design a new type of long-circulating liposome containing a distal positively charged PEG as a portion of the polymer coating. This should facilitate liposomal binding to cell surfaces, resulting in enhanced cellular uptake.

The design of the distal cationic-poly(ethylene glycol)-lipid conjugates (CPLs), illustrated in Fig. 1, involves the separation of a positively charged headgroup from the lipid anchor by means of a long hydrophilic spacer chain. By varying the length of PEG chain, 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. Poly(ethylene glycol) was chosen as the spacer as it possesses a number of properties that render it suitable for pharmaceutical applications. PEG polymers are nontoxic, nonionic, water soluble, highly flexible, and exhibit low immunogenicity. PEG lipids have been used in the design of long-circulating liposomes for systemic drug and gene delivery (9–11,14,15) and also for conjugating ligand moieties to the distal end of PEG chains (14,32,34–38). In order to optimize CPL-liposomes for systemic delivery applications, the ideal spacer length in the CPL should be shorter than the normal PEG chains (M.W. 2000–5000 Da) used for long-circulating liposomes. This would hide the distal charges of the CPL within the normal PEG exclusion barrier, allowing retention of long circulation lifetimes while at the same time extending the positive charges away from the liposomal surface to enhance interactions between liposomes and target cells. The use of different-sized PEG chains in the CPLs and the PEG-lipids used to modulate vesicle circulation and cellular uptake may represent a step toward the design of a new generation of long-circulating liposomes as drug carriers. The optimized PEG spacer length in CPL may vary with the specific conditions: in vitro or in vivo, local or systemic administration, and different formulations. It must be stressed that the current systems have been designed solely for in vitro applications, and that further development will be required for systemic use.

As depicted in Fig. 1, a stable lipid anchor, DSPE, was used to maximize CPL incorporation into the liposome and to minimize the dissociation from liposomes during circulation. The propensity of the CPLs to remain in a lipid bilayer are demonstrated by their low CMC values (1.3–2.0 μM), which are less than or comparable to those of PEG-PEs (1.9–2.8 μM) (Table 1). The results of CPL-LUVs prepared by different methods (Tables 2 and 3) indicate that CPLs are almost completely incorporated within liposomes formed by extrusion and are not removed from the bilayer following a

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second pass through a gel-filtration column (Fig. 3). It has been reported that dissociation rates of phospholipid-anchored macromolecules from vesicles are primarily dependent on the fatty acid chain length of the lipid anchors, and also influenced to some degree by the properties of the lipid headgroups and the host liposome composition (21,22). In general, the rate of intervesicle transfer increases roughly 6.3-fold when each acyl chain in the lipid anchor is decreased by one methylene unit (21). Based on this calculation, we predict that the neutral coating PEG-DPPE with dipalmitoylphospholipid anchor (C16 for each acyl chain) will dissociate 12-fold faster than CPL (with C18 acyl chains). The presence of different lipid anchors (C18 for CPL vs C16 for PEG-lipids) and the different lengthened PEGs will certainly further enhance differences in their dissociation rates from liposomes. After removal of a certain amount of normal PEG-lipid during circulation, the distal charges would be exposed and become available for binding to the target cell surface. This would allow for extended circulation times sufficiently long to permit liposome accumulation at a site of disease, such as a tumor, where the vasculature is leaky and blood flow is reduced, followed by cell binding and uptake resulting from PEG-PE dissociation. The cellular uptake of CPL-vesicles was demonstrated in this study by using PEG_{3400} in the CPL, which is relatively longer than the PEG_{2000} in the PEG-DSPE.

In the present study, we have characterized the properties of highly saturated LUVs into which distal cationic poly(ethylene glycol) lipid conjugates have been incorporated using both extrusion (28) and postinsertion (26) techniques and evaluated for enhanced liposomal cellular uptake. Our previous studies focused on post-insertion of CPLs into conventional and PEG-containing neutral and cationic LUVs containing unsaturated phospholipids (26,27).

The ionic nature of the various CPLs differs due to the abundance of charges at the termini of the PEG chains (see Fig. 1). However, we found that the different CPL polarities did not affect their incorporation into liposomes. Tables 2 and 3 demonstrate that the various CPLs were incorporated quantitatively into both conventional and PEG-containing liposomes when included in the initial lipid mixture prior to extrusion. This method of formation results in a symmetric transmembrane distribution of CPLs in the LUVs (Fig. 2). The low CMCs of the CPLs and the high affinity of the DSPE anchor also ensure easy insertion into preformed vesicles. When CPL was incubated with preformed liposomes (DSPC/Chol, 55:45 mol:mol) at 60°C for 2 h, 60% of the CPL was inserted into the external vesicle leaflet as shown in Fig. 3. The retention of CPL following postinsertion was confirmed by column chromatography of the purified CPL-LUV (Fig. 3). This indicates that the observed insertion was a true incorporation rather than a nonspecific association with the liposomes. Furthermore, this method demonstrates the potential for preparing CPL-containing vesicles without altering established drug-loading procedures. Studies of this nature are currently in progress.

An amino acid, *L*-lysine ($H_2N(CH_2)_4CH(NH_2)CO_2H$), was used in the synthesis to produce distal cationic groups with two types of protonable primary amines whose charge potential is dependent on the microenvironmental pH. In its free form as amino acid or oligopeptide, the pK_a of the ε-NH_2 is 10.5–11.1 and 6.8–8.0 for α-NH_2. The percentage of charged amine in the CPL would, therefore, vary according to the acidity of the surrounding microenvironment. It may be possible that CPL-bearing liposomes would be more positively charged inside the endocytic compartment where higher

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acidity (pH 4–6) is encountered. The increased positive charge may help liposomal interactions such as fusion with endocytic membranes and aid with the release of their contents into the cytoplasm (39). Cell binding studies illustrated that the potency of cellular uptake is primarily dependent on the density of the distal positive charges on the CPLs. In order to achieve a significant enhancement in cellular uptake, at least four charges at the distal end of the PEG chain are required (Figs. 4–6). Normalizing the total net charge in a liposome by using increasing amounts of CPL_1 or CPL_2 did not result in the same degree of uptake as an equivalent charge ratio of CPL_4 or CPL_8 (data not shown). The high cellular uptake seen in the present study using serum-containing media further demonstrates the potential utility of the CPL for enhancing cellular uptake of liposomes. The present CPL design allows for a further increase in the distal charge density by using either ornithine ($H_2N(CH_2)_3CH(NH_2)CO_2H$) or 2,3-diamino-propionic acid ($H_2NCH_2CH(NH_2)CO_2H$) instead of lysine in the CPL synthesis. The distance between the ε and α-amines for these three amino acids decreases in the order of lysine (7.5 Å) > ornithine (6.0 Å) > 2,3-diaminopropionic acid (3.0 Å). Therefore, the charge density of CPLs made with the above amino acids would be significantly different although they would all have the same number of amines. The correlation of CPL charge density with cellular uptake potential of the CPL-LUVs will be studied and discussed in the future.

As a measure of liposomal retention, fluorescence of Rh-PE and HPTS after a 4 h incubation shows that the HPTS remains associated with the liposomes within endosomes (data not shown).

To summarize, we have demonstrated that CPLs bearing between 1 and 8 positive charges on the distal end of a PEG spacer chain differ considerably in polarity but exhibit similar CMC values. CPLs are quantitatively incorporated into vesicles when CPL-LUVs are formed by extrusion; lower levels of incorporation can be achieved by insertion of CPL into preformed vesicles. CPL-LUVs formed by extrusion exhibit enhanced binding and uptake on BHK cells when the CPL possess four or more positive charges on the distal end of the PEG spacer. This is also observed when PEG polymers are present on the vesicle surface. While the current system is designed for in vitro applications, approaches are outlined for increasing the charge density of CPL constructs, and for solving the contradictory requirements of an inert stable particle possessing a long circulation lifetime which converts into an active entity capable of binding to cells following arrival at a target site. By balancing the length of the PEG chains between CPL and stabilizing PEG, and modulating the dissociation rates of both PEG lipid species, programmable drug carriers could be designed that satisfy the above requirement. Although the utility of CPL in different formulations and their application in vivo require further investigation and system optimization, a new strategic approach in developing liposomes for systemic applications has clearly been illustrated.

ABBREVIATIONS

BHK	baby hamster kidney
tBoc	*tert*-butyloxycarbonyl
tBoc-NH-PEG_{3400}-CO_2-NHS	tBoc protected and NHS activated PEG_{3400}

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$CHCl_3$	chloroform
Chol	cholesterol
CPL	cationic poly(ethylene glycol) lipid conjugate
CPL_1	CPL with 1 positive charge
CPL_2	CPL with 2 positive charges
CPL_4	CPL with 4 positive charges
CPL_8	CPL with 8 positive charges
DCC	N,N′-dicyclohexyl-carbodiimide
DCU	dicyclohexyl urea
di-tBoc-lysine-NHS	N_α,N_ε-di-tBoc-L-lysine-N-hydroxysuccinimide ester
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE	1,2-distearoyl-sn-glycero-3-phospho-ethanolamine
DSPE-PEG_{2000}	1,2-distearoyl-3-phosphatidylethanolamine-PEG_{2000}
Et_3N	triethylamine
LUV	large unilamellar vesicle
NHS	N-hydroxysuccinimide
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

ACKNOWLEDGMENTS

The authors thank Dr. N. Maurer and I. M. Hafez for helpful discussions. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and Inex Pharmaceuticals Corporation.

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