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Cytoplasmic Delivery of Liposomal Contents Mediated by an Acid-Labile Cholesterol-Vinyl Ether-PEG Conjugate

Jeremy A. Boomer[#], Marquita M. Qualls[†], H. Dorota Inerowicz, Robert H. Haynes, G.V. Srilakshmi Patri, Jong-Mok Kim, and David H. Thompson^{*}

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907-1393

Abstract

An acid-cleavable PEG lipid, 1'-(4'-cholesteryloxy-3'-butenyl)- ω -methoxy-polyethylene[112] glycolate (CVEP), has been developed that produces stable liposomes when dispersed as a minor component (0.5–5 mol%) in 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). Cleavage of CVEP at mildly acidic pH's results in dePEGylation of the latently fusogenic DOPE liposomes, thereby triggering the onset of contents release. This paper describes the synthesis of CVEP via a six step sequence starting from the readily available precursors 1,4-butanediol, cholesterol, and mPEG acid. The hydrolysis rates and release kinetics from CVEP:DOPE liposome dispersions as a function of CVEP loading, as well as the cryogenic transmission electron microscopy and pH-dependent monolayer properties of 9:91 CVEP:DOPE mixtures, also are reported. When folate-receptor positive KB cells were exposed to calcein-loaded 5:95 CVEP:DOPE liposomes containing 0.1 mol% folate-modified 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-polyethylene[76] glycolamide (folate-PEG-DSPE), efficient delivery of the calcein cargo to the cytoplasm of the cells was observed as determined by fluorescence microscopy and flow cytometry. Fluorescence resonance energy transfer analysis of lipid mixing in these cells was consistent with membrane-membrane fusion between the liposome and endosomal membranes.

INTRODUCTION

Liposomes are clinically important drug delivery vehicles that can encapsulate a wide variety of low molecular weight drugs (1) as well as biopharmaceutical agents such as peptides, oligonucleotides, and plasmids (2–5). Major advantages are often provided by this class of drug carrier system due to their ability to encapsulate cargo by exploiting transmembrane chemical gradients, so called “remote loading” (6,7) and passivate it toward biodegradation *in vivo* during its transport to the target tissue. The performance of these carriers *in vivo* can be further enhanced by passive targeting (8,9) via the enhanced permeability and retention effect (10), typically achieved in liposome systems by incorporation of polyethyleneglycol-lipid conjugates (e.g. mPEG-DSPE) within the bilayer membrane. The primary effect of the PEG layer is to reduce and/or mask opsinization (11), thereby improving blood circulation lifetimes by sterically-stabilizing the liposomes against clearance by the reticuloendothelial system and promoting their deposition at sites of porous and poorly formed vasculature such as those found in tumor tissues and sites of inflammation (12–14). Once deposited at these sites, however, drug retention by PEGylated liposomes can limit the bioavailability of the active agent, even when they have been internalized via receptor-mediated endocytosis pathways using actively-targeted liposomal constructs (15). These observations suggest that the

*Corresponding author. FAX: 765-496-2592; Email: E-mail: davethom@purdue.edu.

[#]Current address: Abbott Laboratories, Abbott Park IL

[†]Current address: GlaxoSmithKline, Collegeville, PA

development of triggerable liposome formulations bearing site-specific targeting ligands could produce greatly increased drug efficacy when exposed to the appropriate stimulus (16–22). Previous work from this laboratory (23,24) suggests that this is indeed the case, however, the slow release kinetics in these systems provided the impetus to develop improved triggerable liposome systems.

Several groups have developed lipid-based carrier systems that employ triggering mechanisms activated by pH (25–35), disulfide exchange (36–43), enzymes (44–46), heat (47–49), and photochemical reactions (50,51) for deploying the encapsulated contents in a site- and/or time-specific manner. The presence of low pH environments within ischemic tissues and endosomal compartments during receptor-mediated endocytosis and vesicle trafficking (Figure 1) suggests that acid-triggering approaches should be a widely-applicable activation strategy since they would not require an external stimulus to initiate liposomal contents release. This has motivated the development of triggering strategies in which the latent fusogenicity of DOPE liposome formulations containing cleavable PEG-lipids is restored upon removal of the PEG corona via dePEGylation (Figure 2). Several fusogenic and/or targeted liposome constructs stabilized by cleavable (25,27,28,33,36,37,41), exchangeable (52–58) or polymerizable (59) PEG conjugates have been designed and investigated, however, a broadly applicable system for *in vivo* application has yet to emerge.

Our laboratory previously reported a novel acid-labile PEG-lipid conjugate, (R)-1,2-di-*O*-(1'-Z,9'-Z-octadecadienyl)-glyceryl-3-(ω -methoxy-poly(ethyleneglycol[112])ate (BVEP, Figure 3), that displays pH-dependent contents release properties when dispersed as a minor component (i.e., 1–10 mol%) in BVEP:DOPE liposome formulations (27). The contents release and membrane fusion kinetics of this system, however, are too slow for many envisioned *in vivo* applications. We anticipated that there were three possible reasons why the observed release kinetics are slow: 1) the acid-labile vinyl ether linkages are buried too deep in the membrane to permit rapid protonation of the vinyl ether β -carbon, the rate determining step in vinyl ether hydrolysis (60)¹; 2) a side reaction of partially-hydrolyzed BVEP occurs to produce a dioxolane-based PEG-lipid conjugate that stabilizes the DOPE membrane via binding of the single aliphatic chain; or 3) the PEG remains weakly adsorbed to the membrane even after it is fully hydrolyzed, thereby preventing the expected lamellar (L_α) to hexagonal (H_{II}) phase transition.

We have developed a cleavable PEG lipid, 1'-(4'-cholesteryloxy-3'-butenyl)- ω -methoxy-polyethylene[112] glycolate (CVEP, Figure 4), to circumvent these limitations. This lipopolymer target was designed because a single hydrolysis event would cleave the PEG headgroup from CVEP, thereby accelerating the rate of dePEGylation and the subsequent contents release and/or fusion processes. Another integral part of this design was the relocation of the vinyl ether linkage closer to the aqueous region where the rate of hydrolysis could be enhanced by placing the acid-sensitive linkage within a region of higher proton activity. This paper describes the synthesis of CVEP, the physical characterization of CVEP:DOPE

¹Since PEG is known to osmotically deplete waters of hydration at membrane and protein surfaces ((61) Kenworthy, A. K., Simon, S. A., McIntosh, T. J. (1995) Structure and phase behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol). *Biophys. J.* 68, 1903–1920, (62) Kuhl, T., Guo, Y. Q., Alderfer, J. L., Berman, A. D., Leckband, D., Israelachvili, J., Hui, S. W. (1996) Direct measurement of polyethylene glycol induced depletion attraction between lipid bilayers. *Langmuir* 12, 3003–3014, (63) Reid, C., Rand, R. P. (1997) Probing protein hydration and conformational states in solution. *Biophys. J.* 72, 1022–1030, (64) Kuhl, T. L., Majewski, J., Wong, J. Y., Steinberg, S., Leckband, D. E., Israelachvili, J. N., Smith, G. S. (1998) A neutron reflectivity study of polymer-modified phospholipid monolayers at the solid-solution interface: Polyethylene glycol-lipids on silane-modified substrates. *Biophys. J.* 75, 2352–2362, (65) Malinin, V. S., Frederik, P., Lentz, B. R. (2002) Osmotic and curvature stress affect PEG-induced fusion of lipid vesicles but not mixing of their lipids. *Biophys. J.* 82, 2090–2100, (66) Hansen, P. L., Cohen, J. A., Podgornik, R., Parsegian, V. A. (2003) Osmotic properties of poly(ethylene glycols): Quantitative features of brush and bulk scaling laws. *Biophys. J.* 84, 350–355.), it is also reasonable to expect that the local proton activity will be lower at this water-depleted interface—an effect that could impact either the first or second steps (or both) in the hydrolysis pathway (Figure 3). This would be especially problematic for BVEP since complete separation of PEG from the lipid anchor requires two sequential hydrolysis events.

liposomes, and their performance with respect to intracellular delivery of liposomal cargo in folate-receptor positive KB cell culture using folate-targeted CVEP:DOPE formulations.

EXPERIMENTAL METHODS

Synthesis of CVEP

2,2'-Dipyridylcarbonate was purchased from TCI-America. MPEG-acid was purchased from Shearwater Polymers. All other compounds were purchased from Aldrich and used without further purification unless otherwise stated. Triethylamine and diisopropylamine were distilled from CaH_2 . All solvents used were spectrophotometric grade, dried over an appropriate desiccant and distilled. Reactions were performed under an argon atmosphere. $\text{Pd(PPh}_3)_4$ was obtained from Strem. Column chromatography was typically performed with 230–400 mesh silica gel using high grade solvents to elute the compounds. Thin layer chromatography was performed using Baker-flex IB-F plates and visualized using UV, I_2 adsorption, KMnO_4 /heat, and/or molybdic acid/heat. $^1\text{H-NMR}$, and $^{13}\text{C-NMR}$ spectra were recorded on a 4.7 T spectrometer. Chemical shifts are reported in ppm relative to the residual solvent peaks as the internal standard. Attached proton test (APT) results are reported as odd (O, one or three protons attached) or even (E, zero or two protons attached) after the corresponding ^{13}C chemical shifts.

4-*t*-Butyldimethylsilyloxybutan-1-ol (1)(67)—*t*-Butyldimethylsilyl chloride (8.65 g, 57.4 mmol) in dry THF (25 mL) was added dropwise to a solution of 1,4-butanediol (22.0 g, 244 mmol; dried 2 h *in vacuo*) and imidazole (5.1 g, 70.5 mmol) in THF (100 mL) at 0 °C over a ~20 min period. The reaction, which turned cloudy after 10 min, was stirred for 1 h, warmed to room temperature, and ether (300 mL) added. The solution was then washed with saturated NH_4Cl (2 × 200 mL) and saturated NaCl (1 × 200 mL). The ether layer was dried over MgSO_4 , filtered, evaporated and dried *in vacuo* yielding a pale yellow oil (11.4 g, 97% yield). NMR analysis indicated that the product was sufficiently pure to utilize in the ensuing step without additional purification. TLC (4:1 hexane:ether, KMnO_4): R_f = 0.35. $^1\text{H-NMR}$ (CDCl_3): 0.05 (s, 6H), 0.85 (s, 9H), 1.60 (m, 4H), 2.60 (bs, 1H), 3.65 (q, 4H). $^{13}\text{C-NMR/APT}$ (CDCl_3): -5.54 (O), 18.15 (E), 25.76 (O), 29.60 (E), 29.79 (E), 62.33 (E), 63.18 (E).

4-*t*-Butyldimethylsilyloxybutanoic acid (2)(68)— KMnO_4 (3.2 g, 20.2 mmol) was dissolved in water (50 mL), stirred vigorously for 10 min, then cooled to 0 °C. A solution of 4-*t*-butyldimethylsilyloxybutan-1-ol (**1**, 1.98 g, 9.7 mmol) and tetraoctylammonium bromide (TOAB, 812 mg, 1.5 mmol) in benzene (40 mL) was added dropwise via addition funnel over a ~20 min period. The solution was then warmed to room temperature and stirred for 3 h. The remaining KMnO_4 was quenched with NaHSO_3 , producing a two-phase mixture with a colorless organic layer. Benzene (20 mL) and 50% $\text{CH}_3\text{CO}_2\text{H}$ (50 mL) were added and the mixture extracted. The benzene layer was then washed with saturated NaCl (50 mL), dried over MgSO_4 , filtered, evaporated, and dried *in vacuo*. The remaining TOAB was removed via elution through a small silica plug with 2:1 hexane:ether mobile phase. The solvent was evaporated and the sample dried *in vacuo* giving a colorless oil (1.65 g, 78% yield). TLC (1:1 hexane:ether, Bromphenol blue): R_f = 0.65. $^1\text{H-NMR}$ (CDCl_3): 0.05 (s, 6H), 0.85 (s, 9H), 1.83 (p, J = 7 Hz, 2H), 2.44 (t, J = 7 Hz, 2H), 3.65 (t, J = 7 Hz, 2H), 11.09 (bs, 1H). $^{13}\text{C-NMR/APT}$ (CDCl_3): -5.47 (O), 18.24 (E), 25.85 (O), 27.57 (E), 30.71 (E), 61.95 (E), 179.96 (E).

Cholesteryl-4-*t*-butyldimethylsilyloxybutanoate (3)—Cholesterol (609 mg, 1.58 mmol), 4-*t*-butyldimethylsilyloxy butanoic acid (**2**, 325 mg, 1.49 mmol), and DMAP (20 mg, 0.14 mmol) were dissolved in CH_2Cl_2 (15 mL) and the solution cooled to 0 °C. EDCI (284 mg, 1.49 mmol) was added, the mixture warmed to room temperature, and stirred for 10 d. The reaction mixture was poured into H_2O (75 mL) and extracted with CH_2Cl_2 (3 × 75 mL). The combined CH_2Cl_2 layers were dried over MgSO_4 , filtered, evaporated, dried *in vacuo*, and the

crude product purified via chromatography (230–400 mesh silica, 9:1 hexane:ether, 1.5 cm diameter $\times$ 5 cm height) to give a white solid (780 mg, 90% yield). TLC (2:1 hexane:ether, acid char): R_f = 0.52. MP: 133–135 °C. $^1\text{H-NMR}$ (CDCl_3): 0.05 (s, 6H), 0.65 (s, 3H), 0.8–2.05 (m, 49H), 2.25–2.4 (m, 4H), 3.65 (t, J = 7 Hz, 2H), 4.6 (m, 1H), 5.35 (d, J = 5 Hz, 1H). $^{13}\text{C-NMR}$ (CDCl_3): -4.8, 12.4, 18.8, 19.1, 19.3, 19.7, 19.9, 21.5, 23.0, 23.1, 23.2, 23.3, 24.3, 24.8, 26.4, 26.5, 28.3, 28.5, 28.7, 31.5, 32.4, 36.3, 36.7, 37.1, 37.5, 38.6, 40.0, 40.2, 40.3, 42.8, 50.5, 56.6, 57.2, 62.5, 123.1, 140.2, 173.5. MS (CI): m/z = 587 ($M + H$).

4-*t*-Butyldimethylsilyloxy-1-cholesteryloxy-1-diethylphosphoryloxy-1-butene

(4)—*n*-Butyl lithium (795 μL , 1.94 mmol, 2.4M in hexane) was added dropwise, via syringe, to diisopropylamine (453 μL , 3.23 mmol) in THF (250 μL) at -78 °C and the solution stirred for 30 min. Cholesteryl-4-*t*-butyldimethylsilyloxy butanoate (**3**, 758 mg, 1.29 mmol) in THF (3 mL) was then slowly added dropwise, via syringe. The solution was stirred for an additional hour, followed by the addition of diethylchlorophosphate (373 μL , 2.58 mmol) in HMPA (4.9 mL) in three aliquots. The solution turned orange/brown and gellated. It was thawed enough to resume stirring, then returned to -78 °C for 10 min. The reaction was then warmed to room temperature and stirred for an additional hour. Ether (50 mL) was added and the reaction mixture filtered through a small silica plug. The solvent was evaporated, the oil redissolved in ether (75 mL), and the organic layer washed with 5% NaHCO_3 (3×75 mL). The ether layer was dried over MgSO_4 , filtered, evaporated, and dried briefly *in vacuo*. The crude product was purified via flash chromatography (230–400 mesh silica, 6:1 hexane:ether, 2 cm diameter $\times$ 16 cm height) to give a white solid (750 mg, 81% yield). The product was used immediately without additional characterization. TLC (2:1 hexane: ether, I_2): R_f = 0.35.

4-*t*-Butyldimethylsilyloxy-1-cholesteryloxy-1-butene (5)—Tetrakis

(triphenylphosphine) palladium (15 mg, 12.4 μmol) and 4-*t*-butyldimethylsilyloxy-1-cholesteryloxy-1-diethylphosphoryloxy-1-butene (**4**, 270 mg, 373 μmol) were added to CH_2Cl_2 (8 mL) and cooled to 0 °C. Triethyl aluminum (653 μL , 653 μmol ; 1.0 M in hexane) was then added dropwise via syringe. The solution was stirred at 0 °C for 1 h before warming to 23 °C and stirring for an additional 3 h. The crude mixture was purified via flash chromatography (230–400 mesh silica, 16:1 hexane:ether, 1.5 cm diameter $\times$ 20 cm height) to give a 90:10 *cis*:*trans* mixture as a white solid (66 mg, 31% yield). TLC (16:1 hexane:ether, *p*-anisaldehyde): R_f = 0.75 (*cis*), 0.71 (*trans*). $^1\text{H-NMR}$ (C_6D_6 , 90:10 *cis*:*trans* mixture): 0.13 (s, 6H), 0.68 (s, 3H), 0.8–2.75 (m, 51H), 3.4 (m, 1H), 3.75 (t, J = 12 Hz, 2H), 4.6 (dt, J_1 = 8 Hz, J_2 = 7 Hz, 0.9H), 5.15 (dt, J_1 = 13 Hz, J_2 = 7 Hz, 0.1H), 5.35 (m, 1H), 6.04 (dt, J_1 = 7 Hz, J_2 = 2 Hz, 0.9H), 6.15 (dt, J_1 = 13 Hz, J_2 = 2 Hz, 0.1H). $^{13}\text{C-NMR/APT}$ (C_6D_6 , 90:10 *cis*:*trans* mixture): -4.8 (O), 12.2 (O), 19.1 (O), 19.5 (O), 19.6 (O), 21.5 (E), 22.8 (O), 23.1 (O), 24.3 (E), 24.6 (E), 26.2 (O), 28.5 (O), 28.8 (E), 28.9 (E), 29.2 (E), 29.6 (E), 29.7 (E), 30.3 (E), 30.6 (E), 32.2 (O), 32.4 (E), 36.2 (O), 36.8 (E), 37.0 (E), 37.3 (E), 37.6 (E), 39.9 (E), 40.0 (E), 40.2 (E), 40.3 (E), 42.6 (E), 51.5 (O), 56.8 (O), 57.0 (O), 63.8 (E), 81.3 (O), 103.0 (O), 106.5 (O), 122.1 (O), 140.5 (E), 141 (O), 145.2 (O).

4-Cholesteryloxy-3-buten-1-ol (6)—TBAF (210 μL , 210 μmol , 1.0 M in THF) and 1.0 mL 40 % TBAH were added via syringe to 4-*t*-butyldimethylsilyloxy-1-cholesteryloxy-1-butene (**5**, 40 mg, 70 μmol) in THF (3 mL). The reaction was run for 12 h at 23 °C, then filtered through a small silica plug with ether and the solvent evaporated. The mixture was purified via flash chromatography (230–400 mesh silica, 8:1 hexane:ether, 1.5 cm diameter $\times$ 15 cm height) to give *cis*-4-cholesteryloxy-3-buten-1-ol (29 mg, 91% yield) and *trans*-4-cholesteryloxy-3-buten-1-ol (1.5 mg, 4% yield) as white solids (95% combined yield). TLC (2:1 hexane:ether, acid char): R_f = 0.18 (*cis*), 0.14 (*trans*). $^1\text{H-NMR}$ (C_6D_6 , *cis*): 0.65 (s, 3H), 0.8–2.05 (m, 39H), 2.3–2.53 (m, 4H), 3.35 (m, 1H), 3.58 (t, J = 6 Hz, 2H), 4.4 (dt, J_1 = 7 Hz, J_2 = 7 Hz, 1H), 5.35 (m, 1H), 5.98 (dt, J_1 = 6 Hz, J_2 = 1 Hz, 1H). $^1\text{H-NMR}$ (C_6D_6 , *trans*): 0.65 (s, 3H), 0.8–2.05

(m, 41H), 2.4–2.5 (m, 2H), 3.35 (t, $J = 7$ Hz, 2H), 3.5 (m, 1H), 4.95 (dt, $J_1 = 13$ Hz, $J_2 = 6$ Hz, 1H), 5.35 (m, 1H), 6.15 (dt, $J_1 = 12$ Hz, $J_2 = 1$ Hz, 1H). ^{13}C -NMR/APT (C_6D_6 , 70:30 cis:trans): 12.06 (O), 12.40 (O), 14.40 (O), 19.04 (O), 19.4 (O), 19.5 (O), 21.0 (E), 21.4 (E), 22.8 (O), 23.0 (O), 24.4 (E), 24.6 (E), 25.5 (E), 28.4 (O), 28.6 (E), 28.7 (E), 29.1 (E), 29.5 (E), 30.0 (E), 32.1 (O), 32.3 (E), 36.2 (O), 36.7 (E), 36.9 (E), 37.3 (E), 37.5 (E), 39.8 (E), 39.9 (E), 40.2 (E), 42.6 (E), 50.5 (O), 50.6 (O), 54.3 (E), 56.6 (O), 57.0 (O), 62.7 (E), 63.0 (E), 75.9 (O), 79.8 (O), 81.2 (O), 102.0 (O), 102.9 (O), 106.2 (O), 122.1 (O), 122.3 (O), 140.4 (E), 140.8 (E), 145.6 (O). MS (CI): $m/z = 457$ (M+H).

1'-(4'-Cholesteryloxy-3'-butenyl)- ω -methoxy-poly(ethylene[112]glycol)ate (CVEP, 7)—4-Cholesteryloxy-3-buten-1-ol (**6**, 44 mg, 96 μmol), mPEG5000-acid (435 mg, 87.0 μmol), DCC (21.6 mg, 104 μmol), and DMAP (10 mg, 81 μmol) were added to CH_2Cl_2 (7 mL) and stirred for 4 d. The reaction mixture was then cooled to 0 °C and the DCU removed via filtration (21 mg). The solvent was concentrated to 2 mL, dripped into cold ether (20 mL), and the ether solution centrifuged at 4000 rpm for 10 min. The ether was carefully decanted and the pellet triturated with fresh ether (20 mL). This process was repeated 5 times. The pellet was then dissolved in CH_2Cl_2 , evaporated to a dry film, dissolved in 2 mL 18 M Ω Millipore H_2O , and lyophilized to give 420 mg PEG containing product (86% yield based on recovered DCU). TLC (90:10:1 CHCl_3 :MeOH: H_2O , I_2): $R_f = 0.75$. ^1H -NMR (CDCl_3 , cis): 0.65 (m, 3H), 0.75–2.5 (m, 50H + H_2O), 3.2–4.5 (m, >120H), 5.35 (m, 1H), 6.1 (m, 1H). ^1H -NMR (CDCl_3 , 65:35 cis:trans): 0.65 (m, 3H), 0.75–2.5 (m, 50H + H_2O), 3.2–4.5 (m, >120H), 5.35 (m, 1H), 6.08 (m, 0.65H), 6.15 (m, 0.35H).

Hydrolysis Kinetics

HPLC solvents were spectrophotometric grade and filtered through a 450 nm FP VericelTM membrane (Gelman Sciences, Ann Arbor, MI) immediately prior to use. Reverse phase HPLC analysis was performed with a Spherisorb C_{18} column (4.6 $\times$ 150 mm, 5 μm particles; Waters Corp., Milford, MA) protected with a C_{18} Guard-PakTM prefilter (Waters). Peak detection was achieved with a Sedex 55 evaporative light scattering detector (Sedere, France). Pump control and data analysis were performed using EZChromTM ver. 6.8 (Scientific Software, Pleasanton, CA). The following gradient elution was performed with a flow rate of 1.5 mL/min: 0–29 min, 6:4 MeOH: H_2O ; 30–50 min, MeOH; 51–65 min, 6:4 MeOH: H_2O . Chromatographic peaks assigned to mPEG-aldehyde ($R_T = 14$ min) and mPEG-acid ($R_T = 10$ –11 min) were verified by comparison with retention times determined for standard samples. The identities of the peaks assigned to CVEP ($R_T = 35.2 \pm 0.2$ min) and cholesterol ($R_T = 36.3$ min) were confirmed by comparing the NMR and mass spectra of lyophilized HPLC fractions with reference spectra.

Preparation of Liposomes

DOPE and EPC were purchased from Avanti Polar Lipids (Alabaster, AL) as either lyophilized powders or chloroform stock solutions. Calcein was purchased from Sigma (St Louis, MO). A 50 mM calcein stock solution was prepared in 20 mM HEPES buffered saline (HBS, 150 mM NaCl, pH 8.0) or 20 mM TRIS (150 mM NaCl, pH 7.4). Low pH hydrolysis was performed in 20 mM citrate buffered saline (CBS, 150 mM NaCl, pH 4.5), or 20 mM oxalate buffer saline (OBS, 150 mM NaCl, pH 2.0).

Liposomes were prepared by the thin film method. A chloroform solution of the desired lipids (5 μmol) was carefully evaporated under nitrogen flow, producing an even, thin film. The film was placed under a 50 μm Hg vacuum for at least 3 h to remove trace solvent impurities. This film was then hydrated in 1 mL 50mM calcein buffer stock buffer solution via 10 freeze-thaw-vortex cycles. The resulting multilamellar liposome solution was then extruded ten times through two stacked 100 nm polycarbonate filters at 50–60 °C with ~200–300 psi nitrogen. After extrusion, the extraliposomal calcein buffer was exchanged with either 150 mM NaCl

or 20 mM HBS buffer via a 0.5×25 -cm Sephadex G-50 column. This exchange process typically resulted in a 2- to 2.5-fold dilution of the liposome solution. The liposomes were always used immediately, but appeared stable for ~1 week when stored at 4 °C (as monitored by calcein leakage and light scattering).

EPC sink liposomes were prepared in the same manner as other liposomes at a 10-fold higher concentration (100mg/mL) in 0.9% NaCl. These liposomes appeared to be stable for at least 3 weeks when stored at 4 °C.

Calcein Release Assay

All solutions were equilibrated at 37 °C for 10 min prior to mixing. An aliquot of liposome stock solution (100 μ L) was added to 1.3 mL of HBS, CBS, OBS, TRIS or 20 mM FD-DMEM containing 10% heat inactivated fetal calf serum, and 0.5% penicillin/streptomycin. Then, 100 μ L 150 mM NaCl or 100 μ L of EPC stock liposomes (sink experiments) were added. This solution was maintained at 37 °C for the duration of the experiment. At each time point, 100 μ L aliquots were removed and diluted with 2 mL HBS. The fluorescence ($\lambda_{\text{ex}}=490$ nm, $\lambda_{\text{em}}=518$ nm) was then measured before and after the addition of two drops of 10% Triton X-100 (solution gently shaken to ensure complete liposome disruption). Percent release was then quantified using the ratio method: % release = $100 \times [(T-T_0)/(1-T_0)]$, where T = Triton ratio = (fluorescence before Triton/ fluorescence after Triton) at time T and T_0 = Triton ratio at T = 0 min(26).

Cytoplasmic Delivery of Calcein

Folate deficient KB cells were plated at 100,000 cells/well in 33 mm dishes with 2.0 mL of FD-DMEM. Two hundred microliters of 5:95:0.5 CVEP:DOPE:folate-PEG-DSPE (targeted) or 5:95 CVEP:DOPE (non-targeted) liposomes (5 μ M total lipid containing 50 mM entrapped calcein) were incubated at 37 °C for 2 h, followed by three thorough washes with PBS buffer ($-\text{Mg}^{2+}$, $-\text{Ca}^{2+}$) to remove any unbound liposomes. Fresh FD-DMEM (2 mL) was added and the cells incubated for an additional 2 h before qualitative fluorescence microscopy analysis of localization and total cellular fluorescence intensity. Fluorescence micrographs were taken approximately 6 h after introduction of liposomes. Endosomal pH inhibition was achieved by preincubating the cells in FD-DMEM containing 50 mM chloroquine 30 min prior to addition of liposomes.

Quantitative evaluation of endosomal release of calcein was determined by flow cytometry. Media from the cell culture dishes was removed and collected in 1.5 mL centrifuge tubes to assure inclusion of all cells. Cell dissociation solution (100 μ L) was added to the wells and incubated for 20 min. This fraction was then added to the collected media and centrifuged. An average of 5000 cells were then analyzed on a Coulter Epics XL flow cytometer using 488 nm excitation and 525 nm/620 nm emission filters.

Lipid Mixing Assay.(69)

NBD-PE (1%), Rh-PE (1%) and folate-PEG3350-DSPE (0.5%) was added to a benzene:methanol (95:5) solution of the CVEP:DOPE dispersion to be studied (5 μ mol total lipid). The solution was vortexed thoroughly, frozen in liquid nitrogen, and then lyophilized overnight. The resulting lipid powder was hydrated with HEPES buffer, pH 7.4, and extruded as described above.

Cells were plated at 100,000 cells/well in Labtek 4-chambered coverslips that had been previously treated with fibronectin (4 μ g/mL). KB cells were incubated for 2 h with 100 μ L of the dual labeled NBD-Rh liposome solution. Cells were then washed three times with liposome-free media, replenished with fresh media, and imaged at various intervals using a

BioRad MRC 1024 laser confocal microscope (488 nm excitation). A 585 nm LP filter was used to collect the rhodamine fluorescence and a 535 nm filter was used to collect the NBD fluorescence.

Light Scattering

Liposome size was determined by quasi-elastic light scattering on a Coulter N4-Plus instrument and distributions calculated using the manufacturer's supplied software (version 1.1). Samples were prepared by diluting 25 μ L liposome stock with 2 mL HBS. Experiments were run in triplicate and the size distributions averaged for these runs.

Transmission Electron Microscopy

Negatively stained EM samples were prepared with 1% $\text{UO}_2(\text{OAc})_2$ using the poststaining method on formvar-coated copper grids and analyzed using a Phillips TEM operating at 40 kV.

RESULTS AND DISCUSSION

Synthesis of CVEP (7)

Initial attempts at the synthesis of CVEP via transformation of a cholesterol ester linkage to a vinyl ether linkage (70–72) in the presence of the PEG functionality proved unsuccessful. This outcome is likely due to the presence of water that is tightly bound by PEG (even after vacuum drying at 50 μ m Hg in the presence of P_2O_5 for two days), which rapidly quenches the LDA reagent during the vinyl phosphate transformation. This approach was abandoned when it became clear that the same interference would occur during the subsequent $\text{Et}_3\text{Al}/\text{Pd}(\text{PPh}_3)_4$ reduction steps. Compounding this problem is the fact that the resulting product mixture would be too difficult to purify on large scale due to the presence of the PEG moiety.

These problems prompted the development of a new synthetic route to CVEP (Figure 5) wherein the water-soluble polymer could be attached to the cholesterol membrane anchoring group during the final step, after installation of the vinyl ether functionality. In this strategy, the PEG headgroup and cholesterol lipid anchor are conjugated via a four carbon, vinyl ether-containing linker derived from 4-hydroxybutanoic acid. The three key advantages provided by this approach are: 1) the PEG headgroup is added in the final step; 2) the Z-vinyl ether transformation step is easily accommodated; and 3) the route allows facile incorporation of a wide variety of alcohol-bearing lipid anchor substituents.

The CVEP synthesis sequence begins with the production of cholesteryl 4-*t*-butyldimethylsilyloxybutanoate (**3**) from 1,4-butanediol in a three step process. Monoprotection of 1,4-butanediol with *t*-butyldimethylsilyl chloride in THF afforded 4-*t*-butyldimethylsilyloxybutane-1-ol (**1**) in 97% yield after extraction of the excess 1,4-butanediol. The presence of the TBDMS ether necessitated the use of neutral oxidation conditions in the subsequent step. Initial attempts using pyridinium dichromate under neutral conditions were successful, however, yields were typically low and the purification difficult. Alternatively, alcohol **1** was readily converted in 78% yield to the corresponding carboxylic acid **2** via a two-phase potassium permanganate oxidation, mediated by tetraoctylammonium bromide as phase transfer catalyst. EDCI/DMAP-mediated coupling of acid **2** and cholesterol afforded **3** in 90% yield.²

²DCC/DMAP conditions were also employed to afford ester **3**, however, the use of EDCI is preferred since product isolation is easier when this dehydrating agent is employed.

The vinyl ether bond was then installed via a two step ester reduction procedure. The kinetic enolate of ester **3** was *O*-trapped with diethylchlorophosphate in the presence of HMPA to afford a vinyl phosphate intermediate (**4**) in 81% yield after chromatographic purification. The normally facile $\text{Et}_3\text{Al}/\text{Pd}(\text{PPh}_3)_4$ -mediated reduction of vinyl phosphates in hexane, however, failed to produce 4-*t*-butyldimethylsilyloxy-1-cholesteryloxy-1-butene (**5**); instead, a mixture of ester **3** and vinyl phosphate **4** was recovered. The use of CH_2Cl_2 as solvent provided vinyl ether **5** in 31% isolated yield, well below the 70+% yields typically observed for this transformation. Unfortunately, use of this more polar solvent produced scrambled vinyl ether stereochemistry (9:1 *Z*:*E*). Two other reduction methodologies, $\text{Li}/\text{NH}_3/t$ -butanol and Bu_3SnH were also attempted, however, these transformations either failed or regenerated ester **3**.

The final stages of CVEP synthesis involved deprotection of vinyl ether **5**, followed by esterification with ω -methoxy-poly(ethylene [112]glycol)-carboxymethyl (mPEG-acid). Desilylation of **5** with tetrabutylammonium fluoride afforded alcohol **6** in 95% overall yield. Addition of small amounts of tetrabutylammonium hydroxide (TBAH, 30% in water) significantly increased the reaction rate and ensured a basic solution, thereby preventing hydrolytic decomposition of the vinyl ether species (occasionally observed in its absence). Careful chromatographic separation of the *E* and *Z* stereoisomers of **6**, followed by a DCC/DMAP-mediated coupling reaction with mPEG-acid produced CVEP (**7**) in 86% yield.³ The seven step sequence in Figure 5 afforded the pure *cis* form of CVEP in 20% overall yield. All subsequent experiments utilized the pure *Z* CVEP isomer, however, no significant differences in release or hydrolysis rates were observed when 2:1 *Z*:*E* mixtures of CVEP were used.

CVEP Hydrolysis Kinetics

CVEP hydrolysis kinetics at 25°C were determined via HPLC-ELSD by monitoring both the time-dependent disappearance of the CVEP peak ($R_T = 34\text{--}35$ min) at different pH's, as well as the appearance times of the degradation products mPEG-aldehyde, mPEG-acid, and cholesterol. Chromatographic peaks for CVEP and its hydrolysis products—cholesterol, mPEG-alcohol and mPEG-acid—were verified by injection of known standards as well as by NMR and MS characterization of all collected fractions, except for mPEG-aldehyde (which used mPEG-alcohol as a retention time standard due to the instability of this material). Under all conditions studied, decreasing CVEP peak areas were accompanied by proportional increases in the peak areas corresponding to mPEG-aldehyde, an intermediate product in the hydrolysis of CVEP (Figure 4). CVEP hydrolysis rates were very fast at pH 1 (Figure 6A), with less than 20% of CVEP remaining and maximal levels of mPEG-aldehyde and cholesterol existing after 15 min exposure. The disappearance rate for CVEP and the appearance rate for mPEG aldehyde were identical. As the reaction time increased, mPEG-acid slowly increased at the expense of both CVEP and mPEG-aldehyde, consistent with the reaction pathway shown in Figure 4. After 15 h reaction time only two HPLC peaks were observed. ^1H NMR analysis of the material eluting between 32–37 min corresponded to cholesterol, whereas the lower R_T species ($R_T = 10\text{--}11$ min) was identified as mPEG-acid.

At pH 3 (Figure 6B), the rates for CVEP disappearance and mPEG-aldehyde appearance also occurred on similar timescales. Unlike the pH 1 experiments, however, the CVEP peak stopped decreasing in relative area after ~35 min and began to increase slightly with time. The peak for cholesterol also began decreasing at the same time as this apparent increase in CVEP concentration. We infer from these observations that a cholesterol-mPEG complex is formed at pH 3.0 (possibly the cholesterol-mPEG hemiacetal), which displays a similar retention time to the CVEP starting material at 35.5 min. Physical mixtures of unmodified cholesterol and mPEG5000 at either pH 1 or 3 (control experiments) showed that only the CVEP sample treated

³EDCI/DMAP coupling also provided CVEP, although the urea side product proved difficult to remove via the ether precipitation method.

at pH 3.0 produced a HPLC peak at this retention time. These observations support the hypothesis that mPEG-cholesterol complexation accounts for the peculiar increase in CVEP peak area at long reaction times at pH 3.0. The data in Figure 6C, showing loss of the vinyl ether α -proton resonance upon exposure to pH 1.0 provides further support of the reaction pathway proposed in Figure 4.

Acid-Triggered Contents Release From CVEP:DOPE Liposomes

Liposomes were prepared via the thin film/extrusion method at CVEP:DOPE ratios ranging from 0.5:99.5 to 5:95. These liposomes were stable for approximately one week, except for the 0.5:99.5 dispersions where lipid aggregation was observed within 2 days. Liposomes prepared in this manner had diameters ranging between 150 and 220 nm as observed via light scattering analysis and electron microscopy.

Contents release from CVEP:DOPE liposomes was examined using the calcein fluorescence dequenching assay. Calcein, encapsulated at self-quenching concentrations (50 mM), undergoes fluorescence dequenching as it leaks into the extraliposomal solution, producing an increase in calcein fluorescence at 520 nm. The calcein release percentage at each time point was then calculated using the ratio method (26). Figure 7 shows the calcein release rates from liposomes comprised of different CVEP:DOPE ratios that were incubated at pH 2.0 and 37 °C. These data show that increased CVEP molar ratios in the CVEP:DOPE liposomal membrane caused a reduction in the observed acid-triggered calcein release rate. We conclude from these results that a critical concentration of CVEP is required to maintain a lamellar phase of DOPE, such that release rates are governed by the extent of displacement from that concentration. In other words, since the rate of CVEP hydrolysis is constant at a given temperature and pH, the rate of contents release is controlled by the amount of CVEP present in the DOPE membrane above the minimal amount required to form a stable lamellar phase. Consequently, when the CVEP content is low, acid-catalyzed degradation of CVEP quickly reaches this critical concentration to produce a rapid release rate. As the CVEP concentration increases, however, more time is required for CVEP hydrolysis to reach this critical point such that slower contents release rates are observed. Comparison of the $t_{50\% \text{ release}}$ values for the 0.5–5% loadings revealed an approximately 15-fold decrease in leakage rates as the CVEP ratio increased, suggesting that contents release rates scale in a roughly linear fashion with PEG loadings over this range. The small observed differences in release rates observed for 0.5:99.5 and 1:99 CVEP:DOPE formulations suggest that liposome formulations containing such low molar ratios may already be near the critical lamellar-hexagonal II (L_{α} -H_{II}) phase boundary.

Contents release rates from CVEP:DOPE liposomes were also examined at pH 4.5, a value that has been reported for late endosomal compartments within KB cells(73). The observed rates for 1%, 3%, and 5% CVEP loadings are shown in Figures 8A, 8B, and 8C, respectively. The contents release rates of pure CVEP:DOPE dispersions at pH 4.5 were surprisingly slow, typically only ~5–10% higher than that observed at pH 7.4 and reaching a maximum of $\leq 25\%$ after 24 h, regardless of the CVEP loading. We attempted to improve the acid-triggered release rates of these CVEP:DOPE dispersions by adding a second population of “sink” egg phosphatidylcholine (EPC) liposomes at a 25-fold molar excess, since Holland *et al.* have previously demonstrated that DOPE liposome dePEGylation could be enhanced by addition of a sink EPC liposome population into which their PEG-lipid conjugates could exchange from the host DOPE membrane.(53,54) Inclusion of sink liposomes in our CVEP:DOPE system was expected to increase the rate of contents release at both pH 4.5 and 7.4 due to a similar PEG-lipid exchange process⁴, however, substantial increases in release rate were only observed at

⁴DePEGylation induced by sink liposomes is believed to proceed via collision-mediated surfactant exchange of PEG-lipid conjugate from the host DOPE liposome membrane to the sink membrane pool. PEG-lipid exchange via the bulk aqueous phase, however, may also occur when the PEG substituents are large such that they tip the hydrophilic-lipophilic balance toward increased water solubility.

pH 4.5 (i.e., sink liposome-induced increases were modest at pH 7.4—typically only 5–10% over a 24 h period). In the 1:99 CVEP:DOPE case (Figure 8A), the combination of pH 4.5 and sink liposomes produced 100% contents release within 550 min, an ~80% increase over the pH 4.5 alone and pH 7.4 + sink liposome cases. The overall magnitude of the sink liposome effect, however, was much less pronounced at the 3% and 5% loading levels, yet $\geq 70\%$ release occurred over a 24 h period. Taken together, the pH 4.5 results are generally consistent with the observations made at pH 2.0—i.e., that increased CVEP loading leads to slower release kinetics from CVEP:DOPE dispersions. We can further infer from these results that contact between the CVEP:DOPE and sink EPC liposomal membranes promotes PEG adsorption to the polymer-free surface. If this occurs with EPC and hydrolyzed CVEP:DOPE liposomes, a net exchange of PEG onto the sink liposome surface can result, giving rise to an accelerated L_{α} -H_{II} transition and contents leakage response. An alternative explanation is that PEG adsorption onto sink EPC during EPC-CVEP:DOPE contact simply improves the proton accessibility to the CVEP vinyl ether linkage, either by rehydrating the interface (due to a net reduction in the local oxoethylene monomer concentration at the CVEP:DOPE surface) or by physically displacing the cleavable site toward the bulk aqueous environment. Although additional experiments are necessary to clarify this issue, we favor the PEG desorption interpretation as the most likely pathway in this system.

Electron Microscopy of CVEP:DOPE Liposomes Before and After Acidification

Negative stain electron microscopy images collected before and after treatment of a 8:92 CVEP:DOPE dispersion at pH 4.5 for 24 h revealed a transformation from vesicular structures with average diameters of ~150 nm (Figure 9A) to electron-dense lipidic material indicative of hexagonal II phase lipid with periodic features of 3.6 nm repeat distances (Figure 9B). Transformation of vesicular structures at pH 8 into aggregated and electron opaque objects after acidification to pH 4 was observed for 8:92 CVEP:DOPE dispersions. The collapsed vesicle structures were similar to the hexagonal phase structures previously observed in acidified BVEP:DOPE(27) and DHCVEP:DOPE(28) phases. These observations support the involvement of the acid-triggered L_{α} -H_{II} phase transition in this system.

Behavior of 1:9 CVEP:DOPE Monolayers Before and After Acidification

Langmuir monolayer hysteresis experiments were performed at pH 7 and 3.5 on 1:9 CVEP:DOPE monolayers to gain further insight into the adsorption behavior of PEG onto DOPE interfaces. As the pH 7 data in Figure 10A show, the presence of PEG introduces a weakly positive surface pressure at large molecular areas relative to 1:9 chol:DOPE (used as a model of a fully-hydrolyzed 1:9 CVEP:DOPE monolayer) as has been shown for PEG adsorbed to DPPC monolayers(74). Upon further compression of the monolayers, the CVEP:DOPE isotherm is displaced by the same increment in molecular area at any given surface pressure due to the presence of the PEG headgroups at the interface. When the surface pressure is relaxed, the monolayer films re-expand with no apparent hysteresis. We infer from these findings that the neighboring PEG units in CVEP:DOPE monolayers interact with one another in the so-called mushroom regime(75) to produce a modest increase in surface pressure. The absence of hysteresis during surface pressure relaxation suggests, however, that there is little interaction between the PEG chains at low areal densities and the DOPE monolayer under these conditions.

CVEP:DOPE monolayers display substantially different behavior at pH 3.7 (Figure 10B). At large molecular areas, the CVEP:DOPE monolayers display low surface pressure as they did at pH 7, but unlike the pH 7 case, the isotherm is gradually displaced toward the chol:DOPE isotherm at small molecular areas. Most significantly, however, is the hysteretic behavior of the film upon re-expansion—essentially retracing the chol:DOPE film out to large molecular areas. Furthermore, the initially low surface pressure upon re-expansion to 150 Å²/molecule

was found to gradually increase to ~10 mN/m over a ten minute period. Since PEG hydrolysis occurs rapidly at this pH, we conclude from these observations that the hydrolyzed PEG remains adsorbed to the chol:DOPE surface until high surface pressures are applied (Figure 10C), when it is then 'squeezed' out of the interface as has been reported for Poloxamers in DPPC and DPPG monolayers(76). Although the surface pressure in CVEP:DOPE liposomes is not known, the implications of these observations are that PEG adsorption to the hydrolyzed CVEP:DOPE liposome interface is the primary inhibition toward rapid contents release and membrane fusion via inhibition of membrane-membrane contact. It also suggests a reason why sink liposomes are needed to exchange the adsorbed PEG from the hydrolyzed CVEP:DOPE liposome surface to another (sink) membrane surface so that L_{α} -H_{II} phase transition and membrane-membrane contact can occur for contents leakage and membrane fusion, respectively.

Cytoplasmic Delivery of Calcein to Folate-Deficient KB Cells

We next explored whether KB cells produced endosomes that were sufficiently acidic for triggering the L_{α} -H_{II} phase transition of internalized CVEP:DOPE liposomes. Our hypothesis was that folate-targeted CVEP:DOPE liposomes could exploit the receptor-mediated endocytosis pathway to deliver their cargo directly to the cytoplasm via acid-triggered dePEGylation and liposome-endosomal membrane fusion (i.e. Figure 1, steps a, d, & e). It was first necessary to probe the stability of CVEP:DOPE liposomes in KB cell growth media by incubating them at 37 °C in either FD-DMEM (folate-deficient Duplecco's modified Eagle media) or pH 7.4 TRIS buffer (control). Even though the release kinetics of 0.5:99.5 CVEP:DOPE formulations were the most favorable in cell free suspensions, these low CVEP loadings produced liposomes that were too unstable in serum for *in vitro* experimentation. We found that 5:95 CVEP:DOPE liposomes were stable in serum, releasing less than 20% of their calcein payload over 24h, most of which occurred within the first hour. This formulation was used for all subsequent cell culture experiments.

The cytoplasmic distribution of calcein within KB cells under various conditions is shown in Figure 11. In these experiments, the cells were incubated for 2 hours with media containing liposomes, washed thoroughly to remove all extracellular liposomes, then incubated for an additional 2 hours in liposome free media prior to examination via fluorescence microscopy. Bright field images are shown on the left and the corresponding fluorescence images on the right. Without folate-targeting (Figure 11 A, D), very low levels of fluorescence were visible. Since KB cells are known to accumulate materials via non-specific pathways, the low levels of fluorescence observed in these experiments were likely the result of either autofluorescence or non-selective uptake of calcein-loaded liposomes from the media. Incorporation of 0.5 mol % folate-PEG-DSPE conjugate as a targeting agent within the 5:95 CVEP:DOPE formulation, however, produced a large increase in cellular fluorescence that appeared diffuse, indicative of cytoplasmic calcein delivery of calcein (Figure 11 B & E). KB cells pretreated for 30 min with chloroquine, an inhibitor of endosomal acidification, exhibited strong punctate fluorescence and significantly reduced cytoplasmic staining, indicative of endosomal trapping (Figure 11 C & F), which suggests that inhibition of acidification reduces the capacity for endosomal escape by blocking the acid-catalyzed dePEGylation reaction. Flow cytometric analysis of these cultures showed a nearly forty-fold increase in average calcein fluorescence when KB cells were incubated with 0.5:5:95 folate-PEG-DSPE:CVEP:DOPE//calcein compared to 5:95 CVEP:DOPE//calcein (non-targeted) controls, indicating that the calcein cargo is efficiently released from CVEP:DOPE liposomes that have been targeted to acidic endosomes (Supplementary Information). Co-incubation of KB cells with 0.5:5:95 folate-PEG-DSPE:CVEP:DOPE//calcein and excess folic acid (i.e., folate blockade with 2 μ M folic acid) reduced the cell-associated calcein fluorescence by more than an order of magnitude. These observations are generally consistent with the findings of Baba *et al.* (77) who have

reported that clathrin-dependent and clathrin-independent endocytosis is not appreciably affected by the presence of <10% surface coverage of non-degradable cholesterol-PEG2200, although internalization is affected at higher (>20%) chol-PEG concentrations in the plasma membrane.

Attempts to identify the mechanism of cytoplasmic delivery via FRET dequenching experiments were inconclusive. Incubation of KB cells with folate-targeted 5:95 CVEP:DOPE liposomes containing 1% each Rh-PE and NBD-PE produced cells that exhibited signs of both Rh and NBD fluorescence, suggesting that some liposomes had undergone significant demixing of the FRET probes, presumably via liposome-endosome membrane fusion, while Rh fluorescence was still observed in other regions, suggesting that the probes were still intimately mixed and engaged in FRET. Whether the latter case is the result of fusion inhibition by adsorbed PEG on hydrolyzed CVEP:DOPE liposomes or their incomplete hydrolysis/dePEGylation due to the presence of modestly acidic and/or early endosomes is not clear at this time. No obvious signs of cellular toxicity were observed in these experiments. Taken together, these results suggest that the combination of endosomal-targeting and acid triggered contents release represents a viable route for the cytoplasmic delivery of liposomal contents to KB cells, with the possible involvement of liposome-endosome membrane fusion as the mode of delivery to the cytosol.

CONCLUSIONS

An acid-labile lipid for triggering fusogenic liposome dePEGylation has been synthesized and tested in solution and cell culture assays. The synthetic route developed for CVEP is readily amenable to a wide variety of alcohol-containing lipid anchor modifications as well as to varied PEG chain lengths and architectures. This cleavable PEG lipid was designed to both increase the rate of contents release by requiring only a single vinyl ether cleavage event. The observed release kinetics in solution phase experiments, however, were significantly slower than those of the previously-reported PEG lipid, BVEP. This suggests the possibility that the liposome structure and interactions between DOPE and CVEP (or its hydrolysis products) are such that the lamellar phase of DOPE is stabilized relative to BVEP:DOPE.

The inclusion of a second population of “sink” liposomes was found to substantially increase release from CVEP:DOPE liposomes at pH 4.5 without inducing significant leakage at pH 7.4. Based on monolayer experiments, we propose that the role of the sink liposomes is to provide a polymer-free interface which facilitates PEG desorption from the DOPE liposome surface via exchange. Since the endosomal membrane may be conceptually viewed as a large sink liposome, the necessity for sink liposomes *in vitro* was not expected to diminish the ability of these liposomes to elicit cytoplasmic delivery. In fact, CVEP:DOPE liposomes targeted to KB cells exhibited significant cytoplasmic delivery of calcein as evidenced by diffuse cellular fluorescence. Neutralization of the endosomal pH with chloroquine also produced strong, punctate fluorescence, indicative of endosomal trapping of the calcein that is retained by unhydrolyzed CVEP:DOPE liposomes. This result also suggests that exchange of unhydrolyzed CVEP into endosomal membranes must be kinetically slower than the rate of CVEP hydrolysis and PEG desorption. Without targeting, only small amounts of diffuse cellular fluorescence were observed due to non-specific cellular uptake processes. In summary, the dePEGylative triggering strategy appears to be a promising route for the site-specific cytoplasmic delivery of liposomal contents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATION

BVEP, (R)-1,2-di-*O*-(1'*Z*,9'*Z*-octadecadienyl)-glyceryl-3-(ω -methoxy-poly(ethyleneglycol [112]))ate
 CBS, citrate buffered saline
 CVEP, 1'-(4'-cholesteryloxy-3'-butenyl)- ω -methoxy-polyethylene[112] glycolate
 DCC, dicyclohexylcarbodiimide
 DMAP, 4-*N,N*-dimethylaminopyridine
 DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
 DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
 DPPG, 1,2- dipalmitoyl-*sn*-glycero-3-phosphoglycerol
 EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
 EPC, egg phosphatidylcholine
 FD-DMEM, folate-deficient Dulbecco's modified Eagle's medium
 folate-PEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(ω -1'-folate-poly(ethylene [76] glycol))amide
 H_{II}, hexagonal II phase
 HBS, HEPES buffered saline
 HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)
 HMPA, hexamethylphosphoramide
 HPLC-ELSD, HPLC with evaporative light scattering detection
 L _{α} , lamellar phase
 LDA, lithium diisopropylamide
 KB cells, a carcinoma cell line of nasopharyngeal origin
 mPEG5000, derivatives of ω -methoxy-poly(ethylene [112] glycol)
 OBS, oxalate buffered saline
 mPEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(ω -methoxy poly(ethylene [112] glycol))amide
 TBAF, tetrabutylammonium fluoride
 TBAH, tetrabutylammonium hydroxide
 THF, tetrahydrofuran
 TOAB, tetraoctylammonium bromide
 TRIS, tris(hydroxymethyl)aminomethane

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Figure 1.

Conceptual diagram of receptor-mediated endocytosis pathway. Step a: Endosome formation. Step b: Endosome acidification and liposome degradation with endosomal trapping. Step c: Endosome-lysosome fusion. Step d: Liposome-endosome fusion. Step e: Lipid-mediated endosomolysis.

Figure 2.

Schematic of lamellar $\rightarrow$ hexagonal II (L_{α} - H_{II}) phase transition driven by dePEGylation of cleavable PEG:DOPE liposomes (left & center). Detachment of PEG induces a change in the local membrane curvature leading to the formation of H_{II} domains within the bilayer. Contact of these sites with other lamellar phases can promote membrane-membrane fusion and transmembrane contents transport (top right). Absence of membrane contact due to low membrane concentrations or steric repulsion results in L_{α} phase collapse and contents release into the surrounding solution (bottom right).

Figure 3.

Acid-catalyzed hydrolysis pathway for BVEP. Mechanistic details of the rate-determining β -carbon protonation, oxonium ion hydration, and hemiacetal cleavage steps are not shown. The protonated intermediate of lyso-BVEP has two possible fates—trapping by water to give the desired products (bottom right) or intramolecular trapping to give the L_α -phase stabilizing species, BVEP acetal (bottom left).

Figure 4. Acid-catalyzed hydrolysis pathway for CVEP. (i) Vinyl ether protonation and hydration; (ii) hemiacetal cleavage; (iii) subsequent hydrolysis of mPEG-aldehyde to produce mPEG-acid.

Figure 5.

Synthesis pathway for CVEP. a: TBDMSCl, imidazole/THF, 0 °C, 1 h. b: KMnO₄, TOAB/C₆H₆/H₂O, 25 °C, 3 h. c: Cholesterol, DMAP, EDCI/CH₂Cl₂, 25 °C, 10 h. d: 1) LDA/HMPA, -78 °C, 1 h; 2) (EtO)₂POCl, -78 °C → 25 °C, 1 h. e: Pd(PPh₃)₄, Et₃Al/CH₂Cl₂, 0 °C → 25 °C, 1 h → 3 h. f: TBAF, TBAH/THF, 25 °C, 12 h. g: mPEG5000-acid, DCC, DMAP/CH₂Cl₂, 25 °C, 4 d.

Figure 6.

CVEP hydrolysis at 25 °C in acidic aqueous solution as detected by HPLC-ELSD (A & B) and ^1H NMR (C).

A: CVEP hydrolysis kinetics at pH 1.0. Top: CVEP disappearance rate. Middle: mPEG-aldehyde appearance/disappearance rate. Bottom: mPEG-acid formation rate.

B: CVEP hydrolysis kinetics at pH 3.0. Top: CVEP disappearance rate. Bottom: mPEG-aldehyde appearance/disappearance rate.

C: NMR characterization of CVEP hydrolysis at pH 1.0. HPLC fractions eluting between $R_T = 32\text{--}37$ min were collected and lyophilized before NMR analysis. Top: CVEP before acid treatment, showing α -H vinyl ether signal (6.12 ppm) and cholesterol vinyl protons (5.38 ppm). Middle: Same CVEP sample after 15 min at pH 1.0. Bottom: Cholesterol standard.

Figure 7.
Calcein release kinetics at pH 2.0 as a function of CVEP molar ratio in extruded DOPE liposomes. CVEP:DOPE ratios: ■, 0.5:99.5; ◇, 1:99; ▲, 2:98; □, 3:97; ◆, 4:96; △, 5:95.

Figure 8.

Calcein release kinetics at pH 4.5 as a function of CVEP loading and sink EPC liposomes.

●, pH 4.5; ■, pH 4.5 with sink EPC; ○, pH 7.4; □, with sink EPC. A, 1:99 CVEP:DOPE; B, 3:97 CVEP:DOPE; C, 5:95 CVEP:DOPE.

Figure 9. Negative stain electron micrographs of CVEP:DOPE dispersions before (left) and after (right) 12 h exposure at pH 4.5. Samples were imaged with a FEI Tecnai F20 TEM operating at 4.5 kV using energy filtered detection for bright-field and selected area diffraction analysis.

Figure 10.

Compression cycles of DOPE, 1:9 chol:DOPE, and 1:9 CVEP:DOPE monolayers. A, pH 7.0; B, pH 3.7; C, Conceptual model showing that PEG is displaced from the chol:DOPE surface at high surface pressure in pH 3.7 solution (where CVEP is rapidly hydrolyzed), but remains adsorbed at low surface pressure and/or when CVEP is intact (pH 7.0). See text for experimental details.

Figure 11.

Phase contrast (A, B, C) and fluorescence (D, E, F) micrographs of KB cells treated for 2 h at 37 °C with 5:95 CVEP:DOPE (A & D), 0.5:5:95 folate-PEG-DSPE:CVEP:DOPE (B & E), and 0.5:5:95 folate-PEG-DSPE:CVEP:DOPE with chloroquine inhibition (C & F), rinsed and incubated with liposome-free media for 2 h prior to imaging. The fluorescence apparent in panel D is due to non-selective uptake of calcein-loaded liposomes from the media.

Figure 12.

Confocal microscope images of KB cells treated as in Figure 11 with 0.5:5:95 folate-PEG-DSPE:CVEP:DOPE liposomes containing 1% Rh-PE and 1% NBD-PE. $\lambda_{ex} = 488$ nm; $\lambda_{em}(\text{Rh}) = 585$ nm; $\lambda_{em}(\text{NBD}) = 535$ nm. The areas of yellow fluorescence in the merged image to the right indicate cellular regions where both Rh and NBD fluorescence are apparent.