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Acid-Triggered Release from Sterically Stabilized Fusogenic Liposomes via a Hydrolytic DePEGylation Strategy†

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A novel acid-labile poly(ethylene glycol) (PEG)-conjugated lipid, (*R*)-1,2-di-*O*-(1′*Z*,9′*Z*-octadecadienyl)-glyceryl-3-(*ω*-methoxy-poly(ethylene glycolate, MW5000) (BVEP), a neutral PEG-derivatized analogue of diplasmenylcholine, has been used at low molar ratios to disperse the nonlamellar, fusogenic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) as unilamellar liposomes. It was anticipated that acid-catalyzed hydrolysis of the vinyl ether linkages would destabilize BVEP/DOPE liposomes by removal of the water-soluble, sterically stabilizing PEG layer, thereby promoting contents release and membrane−membrane fusion. This paper describes the hydrolysis rates, contents release rates, and fusion kinetics of BVEP-stabilized DOPE liposomes at 1:99, 3:97, and 5:95 molar ratios of BVEP/DOPE. Calcein leakage kinetics indicate that 3:97 BVEP/DOPE liposomes offer the best stability at pH 7.4 while retaining favorable leakage properties at pH 4.5 ($t_{50\%release} \approx 4$ h). *N*-Rhodamine phosphatidylethanolamine/*N*-nitrobenzoxadiazole phosphatidylethanolamine lipid mixing assays show that membrane fusion occurs on a much slower time scale than leakage in these systems, with ∼12% lipid mixing occurring over a 24 h time period at pH 2.0. No appreciable membrane fusion occurred in these liposomes at either pH 7.4 or 4.5 when monitored for up to 3 days. ^{31}P NMR spectra at pH 7.4 contain a single isotropic line shape, consistent with the presence of large liposomes. The ^{31}P NMR line shape did not change significantly even after long exposure times at pH 4.0; however, Mn^{2+} addition experiments with acid-treated samples produced line-broadened spectra, indicating that all the phosphorus sites were continuous with the bulk water phase. Time-dependent cryogenic transmission electron microscopy experiments indicate that extensive liposome collapse to give small dense aggregates occurs over a 1−4 h period when 3:97 BVEP/DOPE liposomes are acidified to pH 4.5. Taken together, these results suggest that acid-catalyzed hydrolysis of BVEP/DOPE liposomes does result in dePEGylative triggering; however, the primary outcome of this cleavage process is contents leakage and liposome collapse to give <100 nm particles that are presumed to be inverted hexagonal phase structures, with membrane lipid mixing occurring on a kinetically slower time scale.

Introduction

Liposomes have been widely studied as drug delivery vehicles. This interest stems from their ability to encapsulate many different classes of therapeutic agents, including small molecules, peptides, oligonucleotides, and plasmids, within a biocompatible and processible bilayer shell.[1−5] Once encapsulated, most therapeutic agents adopt the pharmacokinetic profile of the host liposome.[4] Commercial liposomal doxorubicin formulations, for example, utilize this property to reduce acute toxicity and improve localization at the tumor site, effectively enhancing the therapeutic index of this potent antitumor agent. Passive tumor targeting is typically achieved in these systems via incorporation of poly(ethylene glycol)−lipid conjugates (e.g., DSPE−PEG), which confer enhanced circulation lifetimes by sterically stabilizing the liposomes against opsonization, thereby avoiding clearance by the mononuclear phagocytic system.[6,7] In addition, such PEGylated liposomes typically display improved extravasation from highly fenestrated tumor vasculature.[8−12]

Once deposited at the target sight, however, cargo retention by PEGylated liposomes ultimately limits the bioavailability and efficacy of the encapsulated agent. Likewise, efficient cargo escape from the endosomal compartment prior to lysosomal degradation appears to be a limiting factor in several actively targeted (folate, antibody, or low-density lipoprotein) liposomal formulations, despite efficient cellular uptake via receptor-mediated endocytosis.[3,13−16] These observations suggest,

† Dedicated to the memory of David F. O'Brien, deceased July 2002.

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therefore, that the development of triggerable liposome formulations could produce increased drug efficacy when exposed to the appropriate stimulus at their target site. To this end, several systems employing pH, enzymatic, thermal, and photochemical triggering have been described.[17−23]

The intrinsic low pH within endosomal and ischemic tissue environments makes acid-triggerable carrier systems very attractive alternatives for the controlled delivery of drugs.[1] (The primary advantage of these, and other chemically responsive carrier systems, is that prior knowledge of the target tissue site and/or application of an external triggering stimulus is not required to elicit site-specific cargo deposition as is needed for light or thermally activated systems. The main disadvantage of acid-triggered liposomal systems, however, is that weakly basic, low molecular weight cargo such as doxorubicin is often encapsulated using remote loading techniques that employ solutions that are either acidic or possess high Ca^{2+} concentrations. Consequently, utilization of acid-labile liposomes for doxorubicin and other weakly basic drugs may reduce the options available for their efficient encapsulation via remote loading methods.) This interest arises from the fact that the acid-labile carrier is conceptually similar to a prodrug, except that chemical activation can lead to the release of many thousands of cargo molecules instead of single entities as in conventional prodrug strategies. Several different acid-triggering approaches—employing either cosurfactant-stabilized 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE),[23−25] polymers,[26−28] or viral fusion proteins[29,30] as the pH-sensitive switch—have been developed. Cosurfactant-stabilized DOPE systems have been the most widely studied due to the well-known capacity of DOPE to promote membrane fusion.[31] Unfortunately, a lack of serum stability of these formulations has greatly limited their potential application in vivo. Incorporation of a sterically stabilizing PEG coating improves their serum stability; however, this is achieved at the expense of diminished delivery efficiency and fusogenic capacity.[25] These limitations have motivated the search for triggering strategies

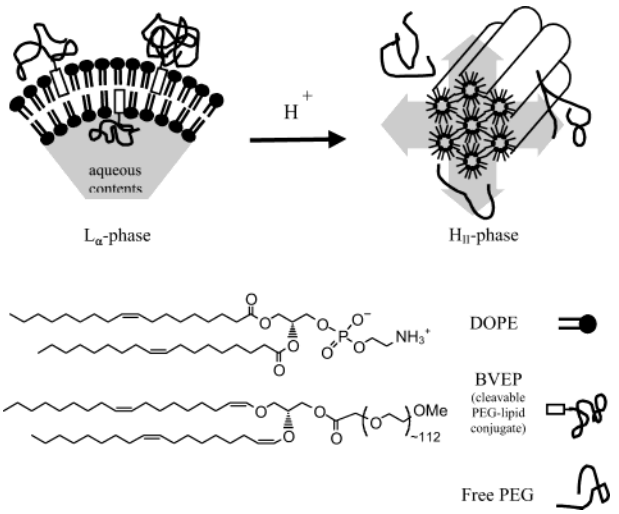

Figure 1. Schematic of the acid-catalyzed dePEGylation strategy showing the lamellar (L_α) to hexagonal (H_{II}) phase conversion of DOPE upon hydrolytic cleavage of BVEP and removal of the sterically stabilizing PEG corona.

in which the latent DOPE fusogenicity is restored upon removal of the PEG corona (i.e., dePEGylation).

In principle, there are two feasible routes to accomplishing dePEGylation—passive removal via lipid exchange processes[32] and active removal via chemical or enzymatic transformation of a suitably designed PEG−lipid conjugate. Cullis and co-workers demonstrated that 1−5% of a PEG−lipid conjugate could stabilize the lamellar (L_α) phase of PE and PE/PS liposomes.[33,34] These liposomes could be induced to fuse by incubating in the presence of a second population of nonfusogenic, "sink" liposomes in 12−36-fold molar excess. The kinetics of spontaneous exchange of the PEG conjugates into the sink liposome population was observed to be inversely proportional to the chain length of the anchoring lipid (increased hydrophobicity) and directly proportional to the molecular weight of the PEG headgroup (increased hydrophilicity). These investigators have also shown that careful regulation of the PEG conjugate parameters allows for the production of transiently serum-stable liposomes with programmable in vivo lifetimes.[35] Zalipsky and co-workers have described a chemical activation route that utilizes cleavage of disulfide-linked PEG−lipid conjugates to trigger dePEGylation. DOPE liposomes stabilized with 2−6 mol % mPEG−DTP−DSPE[36] or mPEG−DTB−DSPE[37] were shown to be serum stable yet, became fusogenic and released their contents when exposed to 10 mM 1,4-dithiothreitol or 15 μM to 1.5 mM cysteine, respectively. Contents release and membrane fusion rates were proportional to the concentration of reducing agent present. O'Brien and co-workers have described an intriguing strategy in which the PEG corona is not removed but rather sequestered into discrete domains upon photoinduced polymerization of 1,2-bis[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphocholine lipo-

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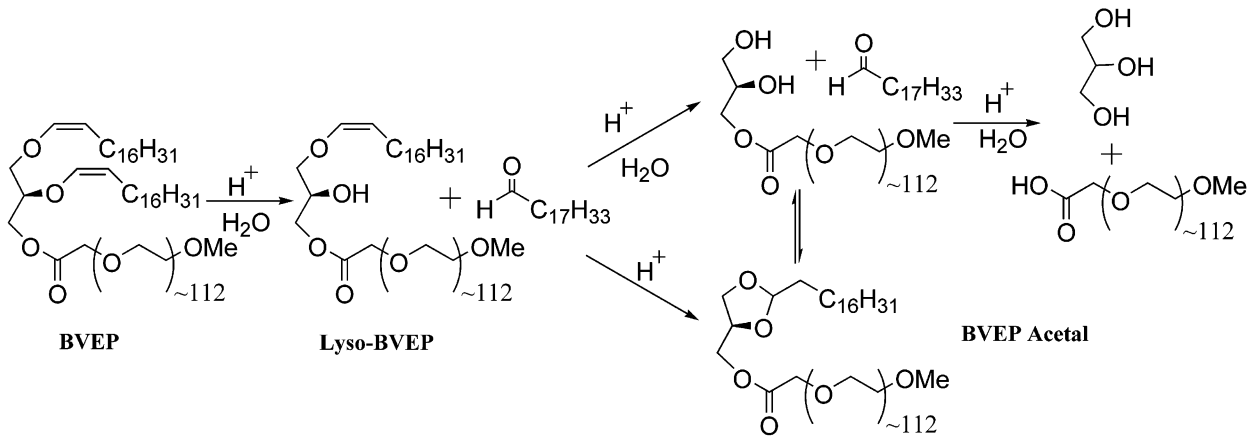

Figure 2. Proposed hydrolysis pathway for BVEP indicating the sequential cleavage of the two alkenyl chains.

somes.[38] These liposomes exhibited 85% contents release after only 8 min of irradiation. This system has also been activated with visible light in HeLa cell culture.[39] Szoka and co-workers have also reported a ortho-ester-linked PEG lipid that undergoes rapid hydrolysis in acidic media to produce lipid mixing and liposome release when formulated with PE.[40,41]

We have previously described acid-sensitive liposome formulations utilizing the vinyl ether lipids plasmenylcholine and diplasmenylcholine.[13,15,17,42,43] These formulations were shown to efficiently release their contents when exposed to a low pH environment. The rate of contents release was found to be inversely proportional to pH; however, cholesterol incorporation increased liposome stability even at low pH, giving rise to cholesterol concentration dependent reductions in release rates. Folate-targeted diplasmenylcholine liposomes exhibited a 6000-fold increase in the efficacy of the potent antimetabolite cytosine-β-D-arabinofuranoside (Ara-C), relative to free drug.[13] Cationic derivatives of this material have also shown enhanced transgene expression in vitro[44] relative to acid-insensitive controls. Despite this demonstrated utility, the difficulty and prohibitive cost of synthesizing diplasmenylcholine necessitated the development of an alternative approach which would effect cytoplasmic delivery of liposomal contents but require only small quantities of synthetic vinyl ether lipids.

We now report a strategy utilizing (*R*)-1,2-di-*O*-(1′*Z*,9′*Z*-octadecadienyl)-glyceryl-3-(ω-methoxy-poly(ethylene glycolate, MW5000) (BVEP, Figure 1), an acid-labile, poly(ethylene glycol)-derivatized analogue of diplasmenylcholine, to disperse DOPE as unilamellar liposomes (Figure 1). It was anticipated that acid-catalyzed hydrolysis of the vinyl ether linkages within acidic media would destabilize BVEP/DOPE liposomes (Figure 2) in a manner similar to that reported for other vinyl ether linked PEG lipids.[45,46] This destabilization would then be expected to promote rapid contents release and membrane−membrane fusion. If a receptor-mediated endocytosis mechanism for drug internalization were employed with BVEP/DOPE formulations, acid-triggered dePEGylation should promote the $L_\alpha - H_{II}$ phase transition, leading to liposome−endosome fusion and cytoplasmic delivery of the liposomal contents. This paper describes the acid-catalyzed hydrolysis rates, contents release rates, and fusion kinetics of BVEP-stabilized DOPE liposomes to evaluate their potential as efficient carrier systems for intracellular drug delivery based on this dePEGylative phase transition concept.

Experimental Section

BVEP was synthesized in 26% overall yield from (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol.[47] DOPE and eggPC were purchased from Avanti Polar Lipids (Alabaster, AL) as either lyophilized powders or chloroform solutions. NBD-PE and Rh-PE were purchased from Molecular Probes (Eugene, OR) as lyophilized powders. 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). Low pH hydrolysis was performed in either 20 mM citrate-buffered saline (CBS, 150 mM NaCl, pH 4.5) or 20 mM oxalate-buffered saline (OBS, 150 mM NaCl, pH 2.0). Benzene/methanol (95:5) lipid stock solutions (5 mg/mL) were prepared from lyophilized powders or thin films and stored at −40 °C. No appreciable decomposition was observed for at least 1 week under these conditions.

HPLC solvents were spectrophotometric grade and filtered through a 450 nm FP Vericel membrane (Gelman Sciences, Ann Arbor, MI) immediately prior to use. Reverse-phase HPLC analysis was performed with a Spherisorb C_{18} column (4.6 × 150 mm, 5 μm particles; Waters Corp., Milford, MA) protected with a C_{18} Guard-Pak prefilter (Waters).[48] Peak detection was achieved with a Sedex 55 evaporative light scattering detector (Sedere, France). Pump control and data analysis were performed using EZChrom ver. 6.8 (Scientific Software, Pleasanton, CA). Gradient elution (0−30 min; from 6:4 MeOH:H_2O to 100% MeOH), followed by isocratic elution (30−50 min; 100% MeOH), was performed with a 1.5 mL/min flow rate.

BVEP Hydrolysis. A 400−500 μL aliquot of BVEP stock was placed in a glass screw cap vial and the solvent removed via N_2 flow and residual solvent removed under vacuum (30 min, 200 μ). The film was then dissolved in 400−500 μL of 0.1 M HCl (pH 1.0) or 0.1 mM HCl (pH 4.0). At each time point, 50 μL aliquots were removed, frozen with liquid nitrogen, and lyophilized. The dried sample was redissolved in 50 μL of water and analyzed in duplicate via HPLC (15 μL injections). Retention times were as

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follows: MPEG-CO_2H = 9.9 min, MPEG-OH = 13.4 min, and BVEP = 39−40 min. MPEG-OH and MPEG-CO_2H peak identities were verified via injection of standards; BVEP and decomposition product peaks were verified by semipreparative scale HPLC fractionation, isolation, and spectroscopic characterization via NMR and MALDI-MS.

Liposome Preparation. Benzene/methanol (95:5) lipid stock solutions were prepared from lyophilized powders. The lipid stocks were combined at the desired ratios (5 μmol of total lipid) and vortexed thoroughly (at least 1 min) until the lipids were fully dissolved. The total volume was raised to 1 mL with benzene/methanol (95:5), prior to freezing in liquid nitrogen and lyophilization for 8 h. The lyophilized powder was then hydrated in 1 mL of 50 mM calcein buffer stock solution via 10 freeze−thaw−vortex cycles. This MLV solution was then extruded 10 times through two stacked 100 nm polycarbonate filters at 50−60 °C with ∼200−300 psi N_2. 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 as determined by phosphate analysis. Light scattering analysis typically suggested mean population sizes between 150 and 250 nm. 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).

Calcein Release Assay. All solutions were equilibrated at 37 °C for 10 min prior to mixing. An aliquot of the liposome stock solution (100 μL) was added to 1.4 mL of 20 mM HBS (pH 7.4) or 20 mM CBS (pH 4.5). This solution was maintained at 37 °C for the duration of the experiment. At each time point, 100 μL aliquots were removed and diluted with 2 mL of HBS. The fluorescence (λ_{ex} = 490 nm, λ_{em} = 518 nm) was then measured before and after the addition of 2 drops of 10% Triton X-100. Percent release was then quantified using the ratio method: % release = $100[(T - T_0)/(1 - T_0)]$, where T = Triton ratio = (fluorescence before Triton/fluorescence after Triton) at time T and T_0 = Triton ratio at T = 0 min.

Lipid Mixing Assay. All manipulations were performed under minimal light conditions to avoid photobleaching of the fluorophores. Labeled liposome stock solutions containing 1 mol % each of NBD-PE and Rh-PE were prepared at the desired BVEP/DOPE ratio, except that they were hydrated in 20 mM HBS. A second set of stock solutions containing unlabeled liposomes was prepared at identical lipid compositions and concentrations. The membrane fusion test solutions were prepared by adding 25 μL of labeled liposome stock and 75 μL of unlabeled liposome stock to 1.4 mL of CBS, HBS, or OBS buffer (all solutions were incubated at 37 °C for 10 min prior to mixing). This solution was maintained at 37 °C for the duration of the experiment. At each time point, 100 μL aliquots were removed and diluted with 2 mL of HBS. The fluorescence intensity (λ_{ex} = 468 nm) was then measured at 530 nm (F_{530}) and 590 nm (F_{590}), and the ratio $R = F_{530}/F_{590}$ was calculated for each time point. The 100% lipid mixing (R_{100}) value was obtained by treating one of the aliquots with either probe tip sonication for 5 min or 2 drops of 10% Triton X-100 solution. The extent of membrane fusion was then obtained with the following formula: % fusion = $100(R - R_0)/(R_{100} - R_0)$, where R_0 represents the time zero F_{530}/F_{590} ratio.

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

^{31}P NMR Spectroscopy. Lipid dispersions were prepared by probe-type sonication of 50 mg of 3:97 BVEP/DOPE in 1 mL of 20 mM HBS at 0 °C until a homogeneous solution was produced. The neutral pH samples were warmed to 25 °C prior to spectral acquisition. Low pH samples were initially dispersed in 0.5 mL of 5 mM HBS, prior to addition of 0.5 mL of CBS and incubation at 37 °C. A 0.1 mL aliquot of D_2O was introduced into the samples prior to spectral acquisition. In some cases, a 200 mM $MgCl_2$ solution was added as line broadening agent to achieve a final Mg^{2+} concentration of 5 mM. ^{31}P NMR spectra were acquired over a 24 h period at 121 MHz and 25 °C using a proton decoupling sequence. In each case, approximately 800 scans were collected with a 3 s interpulse delay and processed using 100 Hz linebroading. Chemical shifts are reported relative to sonicated egg phosphatidylcholine liposomes at 0 ppm.

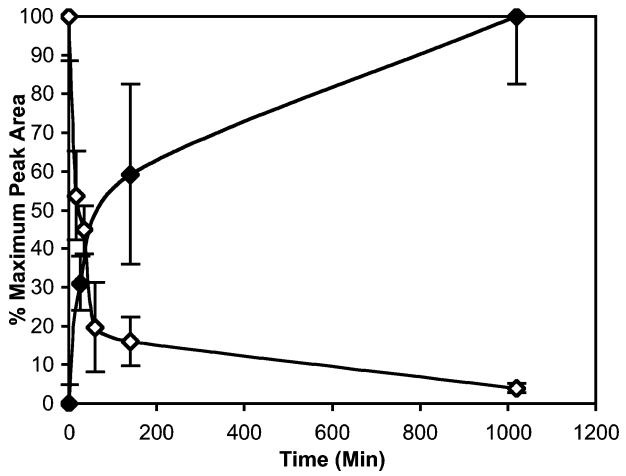

Figure 3. BVEP hydrolysis rate at pH 4.0 (HCl) monitored via HPLC-ELSD: ◇, BVEP disappearance kinetics; ◆, MPEG−glycerol formation kinetics reported as mean % maximum area ± standard deviation.

Cryogenic TEM (c-TEM). The technique used for c-TEM has been described in detail elsewhere.[49] Briefly, this consisted of placing a small drop of sample solution on a copper grid covered by a holey polymer film. Excess liquid was blotted away with filter paper in a custom-built controlled temperature and humidity environmental chamber to produce thin (20−500 nm) sample films spanning the holes in the polymer film. The films were vitrified by quick freezing in liquid ethane and transferred to a Zeiss EM 902 transmission electron microscope for examination. The specimens were kept below 108 K during the transfer and viewing procedures to prevent sample perturbation and ice crystal formation. All observations were made in zero loss bright-field mode at an accelerating voltage of 80 kV.

Results

Hydrolysis Kinetics. BVEP hydrolysis kinetics (Figure 3) were monitored using HPLC-ELSD analysis to determine the time-dependent degradation of BVEP (R_T = 39 min) exposed to aqueous solutions of hydrochloric acid (pH 1.0 or 4.0). Decreases in BVEP peak area were accompanied by corresponding increases in a degradation product identified as 1-methoxypoly(ethylene glycol), MW5000 (MPEG-OH), by injection of standard samples. The hydrolysis is very rapid at pH 1.0, with less than 3% of the BVEP starting material remaining after 50 min ($t_{1/2}^{1.0}$ = 10 min). These experiments indicate that MPEG-OH appears on the same time scale as BVEP disappearance; however, an additional product, identified as 1-methoxypoly(ethyleneglycol)ic acid, MW 5000 (MPEG-CO_2H, R_T = 9.9 min), by co-injection of a commercial sample of MPEG-CO_2H, is formed on a slower time scale. BVEP hydrolysis is significantly slower at pH 4.0, with ∼20% of the material remaining after 60 min ($t_{1/2}^{4.0}$ = 25 min). Formation of MPEG-OH and MPEG-CO_2H was also found to be slower under these conditions. TLC analysis of BVEP/DOPE liposomes exposed to pH 4.0 showed the disappearance of BVEP and the simultaneous appearance of two new lower R_f spots attributed to MPEG-OH and MPEG-CO_2H, in qualitative agreement with our HPLC-ELSD results (data not shown).

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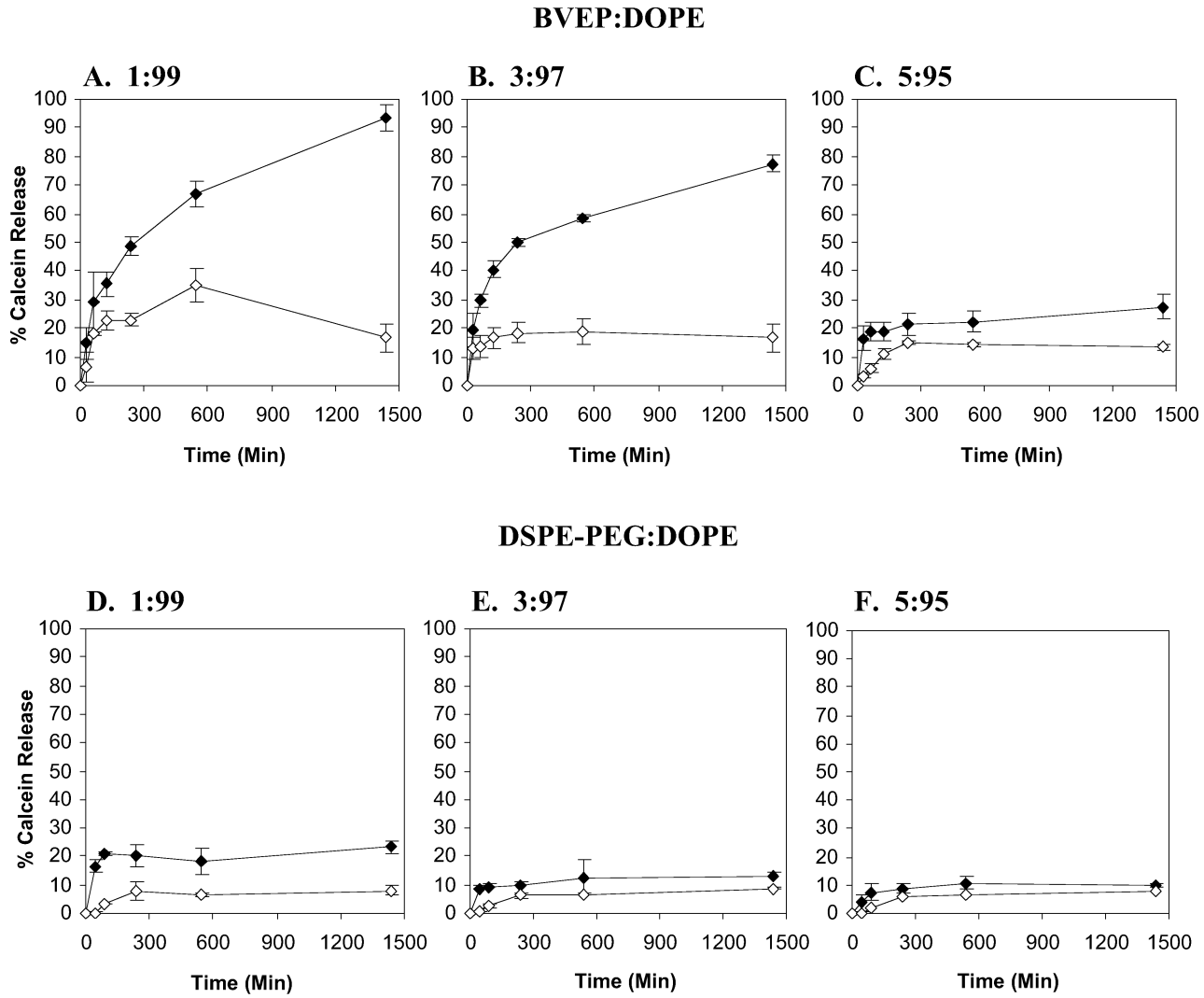

Figure 4. Calcein release kinetics from 1:99 (A), 3:97 (B), and 5:95 (C) BVEP/DOPE liposomes and 1:99 (D), 3:97 (E), and 5:95 (F) DSPE-PEG/DOPE liposomes pH 4.5 (◆) and 7.4 (◇). Reported as mean release ± standard deviation.

Contents Release Kinetics. The kinetics of contents release from BVEP/DOPE liposomes were examined via fluorescence dequenching experiments using encapsulated calcein. Figure 4 shows kinetic plots for calcein release from BVEP/DOPE (A, B, and C) and acid-insensitive DSPE-PEG (D, E, and F) at PEG-conjugate:DOPE ratios of 1:99, 3:97, and 5:95, respectively. The 1:99 BVEP liposomes exhibited over 90% release of entrapped calcein after 24 h, with nearly 50% release occurring within 240 min. Conversely, incubation at pH 7.4 produced only 25% release after 24 h, with approximately 20% leakage occurring within the first 120 min. Slower release profiles were observed as the PEG loading on the surface of the liposome increased. In the case of 3:97 BVEP/DOPE liposomes, pH 4.5 release of calcein reached 78% after 24 h, even though the initial rate was similar to the 1:99 BVEP/DOPE formulation. The kinetic profile for 3:97 BVEP/DOPE at pH 7.4 exhibited 18% release within the first 120 min and remained constant, within experimental error, over the 24 h period of observation. Increasing the PEG content to 5%, however, substantially reduced the overall release kinetics to 28% after 24 h. It is noteworthy that the pH 4.5 release profile for the 5:95 formulation was nearly the same as that observed at pH 7.4.

Acid-induced release under the same conditions was monitored for DOPE liposomes stabilized with 1, 3, and 5 mol % of the acid-insensitive lipid, DSPE-PEG. The liposomes were prepared in a similar fashion, and their stability was monitored via fluorescence dequenching experiments. At all lipid ratios examined, pH 4.5 release never exceeded 25% and pH 7.4 release never exceeded 10%. As observed in the BVEP/DOPE formulations, increased PEG loading stabilizes the liposomes and reduces their contents release rates.

Liposome−Liposome Fusion. The extent of acid-triggered liposome−liposome fusion was quantified using a lipid-mixing assay based on fluorescence resonance energy transfer (FRET) from NBD to Rhodamine. Labeled 3:97 BVEP/DOPE liposomes containing 1% NBD-PE and 1% rhodamine-PE (Rh-PE) were mixed at a 1:3 ratio with unlabeled 3:97 BVEP/DOPE liposomes in HBS buffer and incubated at 37 °C. Samples were excited at 468 nm, and their fluorescence emissions at $\lambda_{em} = 530$ nm (NBD) and $\lambda_{em} = 590$ nm (Rh) were recorded. Dilution of the probe lipids into the unlabeled liposomes as a result of membrane fusion produces a decrease in Rh fluorescence and a concomitant increase in NBD fluorescence. Figure 5 shows the fusion kinetics at pH 7.4, 4.5, and 2.0. Incubation at pH 4.5 and pH 7.4 both produced only 5% fusion over a 72 h period; however, the rate of fusion increased significantly when the pH was decreased to 2.0.

Lipid Phase Behavior. The kinetics of the anticipated $L_\alpha - H_{II}$ phase change were also explored via ^{31}P NMR spectroscopy and cryogenic transmission electron micros-

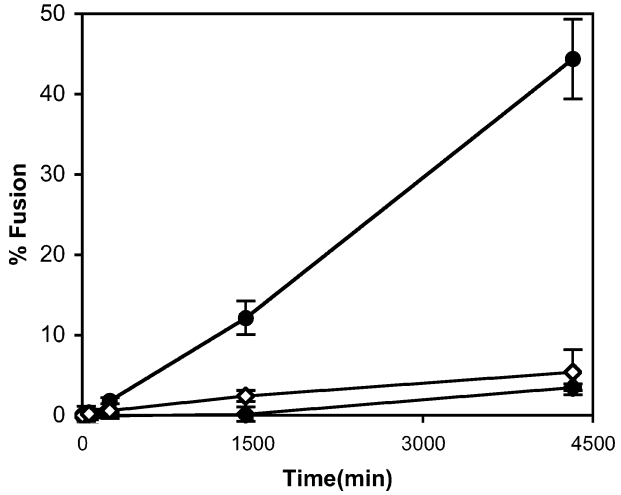

Figure 5. Kinetics of membrane fusion in BVEP/DOPE (3:97) liposomes at pH 2.0 (●), 4.5 (◆), and 7.4 (◇). Reported as mean % fluorescence recovery ± standard deviation.

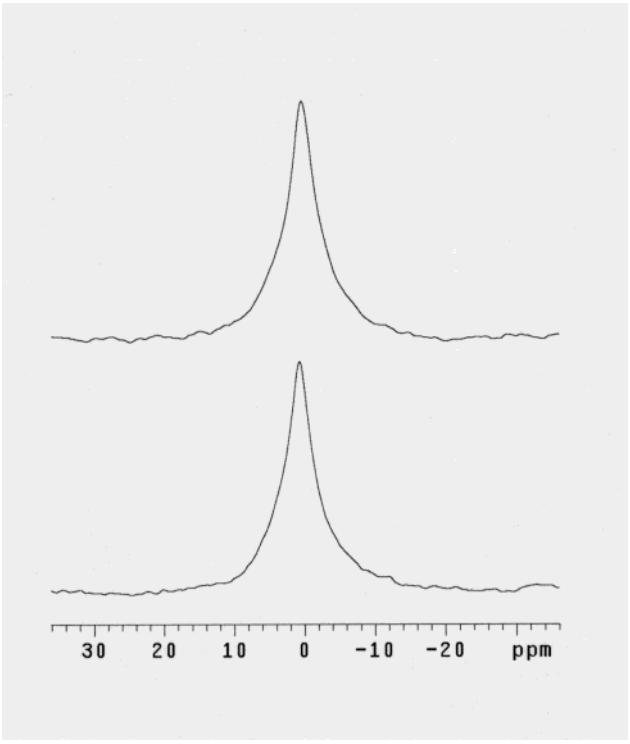

Figure 6. ^{31}P NMR spectra of 3:97 BVEP/DOPE dispersions (50 mg of phospholipid/mL): top, after incubation in 20 mM HEPES, pH 7.4, 25 °C; bottom, after incubation for 24 h in 20 mM CBS, pH 4.5, 37 °C. Mn^{2+} addition experiments left ~45% of the ^{31}P peak intensity remaining at pH 7.4; however, the ^{31}P line was completely broadened after 24 h exposure at pH 4.5.

copy (c-TEM). ^{31}P NMR spectra of 3:97 BVEP/DOPE liposomes displayed a single, isotropic peak at pH 7.4, indicative of phosphorus headgroups that are undergoing rapid tumbling on the NMR time scale (Figure 6). Addition of Mn^{2+} to these samples produced line broadened spectra with approximately 45% of the original ^{31}P line intensity remaining (data not shown). Exposure of these liposomes to pH 4.5 for 24 h, however, produced ^{31}P NMR spectra that were essentially unchanged relative to the pH 7.4 samples, leading one to assume that the lamellar morphology of the liposomes may have remained intact. The ^{31}P line shape was completely broadened after Mn^{2+} addition to these samples (data not shown), indicating

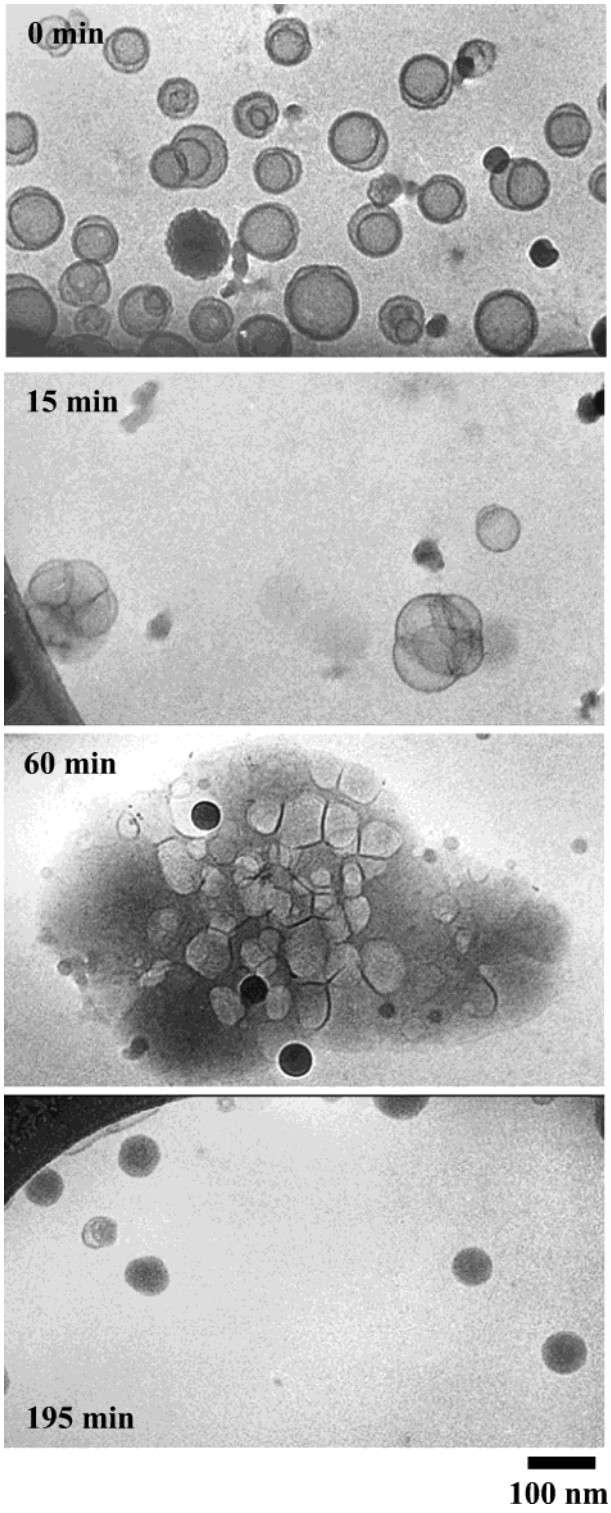

Figure 7. Time-dependent cryogenic transmission electron microscopy of 3:97 BVEP/DOPE liposomes at pH 4.5.

that the phosphorus sites in these rapidly tumbling aggregates are continuous with the bulk water phase. c-TEM experiments, however, confirm that marked changes in liposome morphology and aggregation behavior do occur in 3:97 BVEP/DOPE formulations that have been incubated at pH 4.5 (Figure 7). Within 15 min, the liposomes began to aggregate into small clusters; these intermediates formed larger clusters of partially collapsed liposomes by 60 min. After 195 min, these large clusters had converted to dark 80−100 nm structures that we attribute to inverse hexagonal tubes having an inner

aqueous phase that is continuous with bulk water (i.e., an H_{II}-like phase). We infer from these observations that the liposome → inverted hexagonal phase transition also occurs in the ^{31}P NMR experiments; however, the inverted hexagonal structures produced are sufficiently small that they still undergo rapid tumbling on the NMR time scale. These results, together with the membrane fusion data (Figure 5), suggest that the inverted hexagonal phase particles do not undergo appreciable membrane fusion on this time scale, otherwise an anisotropic line shape would have been observed. This conclusion was confirmed by negative stain TEM analysis of the NMR samples before and after acid treatment; the initially formed liposomes were converted to small densely stained lipidic structures after exposure to pH 4.5.

Discussion

BVEP/DOPE liposomes containing low molar ratios of BVEP were found to be stable for up to 24 h at 37 °C in pH 7.4 HBS. These formulations release entrapped calcein when incubated at 37 °C in pH 4.5 CBS with rates that are inversely proportional to the BVEP content in the membrane (i.e., surface loading of PEG). Control experiments with a nonhydrolyzable DSPE-PEG analogue, however, show that very little release occurs from DSPE-PEG/DOPE liposomes, even upon exposure to pH 4.5 solutions. Comparison of the various BVEP/DOPE formulations indicates that the differential release rates (i.e., release kinetics at pH 4.5 vs 7.4) are highest for 1:99 BVEP/DOPE; however, these formulations may have limited stability in vivo. It is worth noting that 5:95 BVEP/DOPE formulations produced very little contents leakage at pH 4.5 over 24 h, suggesting that this composition undergoes slow dePEGylation under these conditions. Taken together, these results suggest that the 3:97 BVEP/DOPE formulation would be the best suited for further development.

Time-resolved c-TEM results show—in direct contrast to the slow leakage kinetics—that significant liposome collapse into dense particles (attributed to inverted hexagonal phase structures) occurs within 3 h at 37 °C for 3:97 BVEP/DOPE formulations that have been exposed to pH 4.5. Despite this rapid phase change, only low levels of membrane fusion (5% after 72 h at pH 4.5) were detected using the conventional NBD-Rh lipid mixing assay. Reduction of the pH to 2.0, however, increased the extent of fusion to 50% over the same time period. The molecular basis for these slow fusion kinetics is unclear at this time (see below). Nontheless, it is clear that dePEGylative triggering of membrane fusion using BVEP/DOPE formulations will be slow under physiologically relevant conditions.

Although these results clearly demonstrate that acid-triggered dePEGylation of BVEP/DOPE formulations is a viable route for liposomal contents release, the kinetics and acid sensitivity must be improved for in vivo applications. There are several possible explanations for the observed slow response of the system: (1) slow intrinsic rate of vinyl ether hydrolysis, (2) altered microenvironment in the vicinity of the vinyl ether bond such that the degree of hydration is lower and/or the pH is higher than that present in the bulk solution, or (3) inhibition of the L_α–H_{II} phase transition due to (a) surface adsorption of hydrolyzed PEG onto the DOPE surface, or (b) involvement of a secondary reaction (e.g., BVEP acetal in Figure 2) that retains a PEG amphiphile on the DOPE liposome. At present, we are only able to argue against the possible involvement of a secondary reaction (i.e., point 3b above) for the following reasons. If acid-catalyzed hydrolysis of BVEP occurs in a stepwise manner as one would expect, cleavage of the first vinyl ether bond would produce a reactive alcohol intermediate. Assuming that cleavage of the second chain proceeds via the same mechanism as the first chain, it is conceivable that an internal trapping reaction of the protonated reactive alcohol intermediate by the proximal hydroxyl group could produce a cyclic acetal (BVEP acetal). Since this cyclic acetal species would possess one alkyl chain, it might still be capable of stabilizing the DOPE lamellar phase by retaining the PEG at the membrane interface. Acid-catalyzed cleavage of this acetal would occur at a much slower rate at these pH values, thereby accounting for the slow leakage and fusion kinetics. Our HPLC-ELSD and TLC data, however, provide no evidence of a degradation byproduct with intermediate mobility (as would be expected for the partially hydrolyzed intermediate, BVEP acetal). If such a species is generated and not detectable under our experimental conditions, it suggests that very low loadings of PEG lipid (i.e., <0.5 mol %) are capable of stabilizing DOPE liposomes at low pH.

Another possible explanation for these data is that the PEG is being cleaved, but remains associated with the DOPE membrane. Such a situation could make the liposomes more leaky, but not permit the close membrane contact necessary for membrane fusion. A further possibility is that rapid aggregation, observed via c-TEM, impedes the L_α–H_{II} phase transition due to entrapment of interbilayer PEG, thereby resulting in decreased rates of contents release and fusion. The most likely explanation, however, is that a combination of slow BVEP vinyl ether hydrolysis kinetics and PEG association with the DOPE membrane accounts for the slow membrane fusion rates observed under these conditions. Comparative studies with neutral and negatively charged vinyl ether PEG lipids suggest that interfacial charge and/or hydration can have a significant impact on leakage kinetics.[46] The reports of Kresge and co-workers further show that vinyl ether hydrolysis rates are strongly dependent on the stereoelectronic properties of the vinyl ether itself.[50] Taken together, these findings suggest that the rate of vinyl ether hydrolysis must be increased to fully realize the potential of dePEGylative triggering from vinyl ether-linked PEG lipid/DOPE formulations.

Conclusions

A novel acid-triggered dePEGylation strategy has been described using binary mixtures of BVEP, a first-generation acid-cleavable vinyl ether PEG–lipid conjugate, and the fusogenic lipid, DOPE. BVEP can stabilize the DOPE lamellar phase when incorporated at 1–5 mol % in the liposomal membrane. Exposure of these BVEP/DOPE liposomes to acidic media triggers the release of water-soluble contents with rates that depend on the BVEP molar ratio in the liposomal membrane. Membrane fusion of these liposomes also occurs upon acidification; however, the rates of this process are significantly slower than for contents leakage.

Acid-catalyzed dePEGylation is a promising mechanism for efficient site-specific delivery of liposomal contents. The contents release and membrane fusion kinetics of BVEP/DOPE formulations, however, are not rapid enough for most in vivo applications. This limitation has prompted the development of second-generation pH sensitive PEG–

(50) Keeffe, J. R.; Kresge, A. J. *The Chemistry of Enols*; Rappoport, E., Ed.; Wiley: New York, 1990; pp 399–483.

lipid conjugates. The release and fusion characteristics of these conjugates are currently under investigation.

Nomenclature

BVEP = (*R*)-1,2-di-*O*-(1′*Z*,9′*Z*-octadecadienyl)-glyceryl-3-(*ω*-methoxy-poly(ethylene glycolate, MW5000)
CBS = citrate-buffered saline
c-TEM = cryogenic tranmission electron microscopy
diplasmenylcholine = 1,2-di-*O*-(1′-alkenyl)-*sn*-glycero-3-phosphocholine
DOPE = 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DSPE-PEG = 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (*ω*-methoxypoly(ethylene glycol)amide, MW5000)
H_{II} = hexagonal II phase
HBS = HEPES-buffered saline
HEPES = hydroxyethylpiperidine ethylsulfonate
HPLC-ELSD = high-pressure liquid chromatography with evaporative light scattering detection
L_α = lamellar phase
MLV = multilamellar vesicles
MPEG-CO_2H = *ω*-methoxypoly(ethylene glycol)ic acid, MW5000
MPEG-OH = *ω*-methoxypoly(ethylene glycol), MW5000
NBD-PE = *N*-nitrobenzoxadiazole phosphatidylethanolamine
OBS = oxalate-buffered saline
PE = phosphatidylethanolamine
PS = phosphatidylserine
Rh-PE = *N*-rhodamine phosphatidylethanolamine

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