Triggered release of doxorubicin following mixing of cationic and anionic liposomes

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Abstract

In many applications, an ability of liposomes to retain drug and then rapidly release it at some later time would be of benefit. In this work, we investigate the ability of cationic large unilamellar vesicles (LUV) to promote rapid release of doxorubicin from anionic LUV. It is shown that the addition of cationic liposomes containing cholesterol, dioleoylphosphatidylethanolamine (DOPE), distearoylphosphatidylcholine (DSPC) and the cationic lipid N,N-dioleyl-N,N-dimethylammonium chloride (DODAC) to doxorubicin-containing LUV composed of cholesterol, DOPE, DSPC and the anionic lipid dioleoyphosphatidylglycerol (DOPG) can result in release of more than 90% of the drug in times of 30 s or less. Further, it is shown that these release characteristics are exquisitely dependent on the presence of DOPE and cholesterol. In the absence of DOPE, much slower release rates are observed, with maximum release levels of 50% after a 2-h incubation at 20 °C. Remarkably, threshold levels of more than 10 mol% cholesterol are required before any appreciable release is observed. [^{31}P]NMR spectroscopy and freeze-fracture electron microscopy studies reveal that systems giving rise to rapid release of doxorubicin exhibit limited formation of inverted hexagonal (H$_{II}$) phase, suggesting that these lipids facilitate drug release by formation of local regions of non-bilayer structure. It is concluded that drug release triggered by mixing anionic and cationic liposomes could be of utility in drug delivery applications.
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1. Introduction

Liposomes that exhibit regulated release properties have considerable potential for drug delivery. Interest in this area has resulted in construction of liposomes that are sensitive to temperature, light, pH and other stimuli [1]. In most cases, sensitization of liposomes was achieved by modification of the liposomal surface with pH-sensitive or thermosensitive polymers [2,3] or by inclusion of ionizable lipids in the liposomal bilayer [3–7].

In previous works, we and others [8–14] have noted that addition of cationic LUV to anionic LUV results in disruption of the LUV structure as evidenced by the formation of larger structures, lipid mixing phenomena [10,12,13] and, in some cases, formation of non-bilayer structures [7,14]. These studies suggest that if the anionic or cationic LUV contained encapsulated drug, considerable drug release would be expected on addition of the oppositely charged LUV population. In the present work, we test this hypothesis, paying particular attention to the influence of dioleoylphosphatidylethanolamine (DOPE) and cholesterol, two lipids that have been shown to favour or facilitate formation of non-bilayer structures such as the inverted hexagonal (H$_{II}$) phase [15,16]. Employing anionic doxorubicin-containing LUV, we show that rapid release of encapsulated doxorubicin can be achieved on addition of cationic LUV and that this rapid release correlates with formation of non-bilayer lipid structures demonstrated by [^{31}P]NMR and freeze-fracture techniques. Further, we show that the presence of both DOPE and cholesterol is essential for rapid release to be observed. These results are discussed in terms of the potential utility of these systems for triggered release applications.

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2. Materials and methods

2.1. Materials

N,N-dimethyl-N,N-di-9-cis-octadecenylammonium chloride (DODAC) was provided by Dr. J. Gaucheron, University of British Columbia (Vancouver, BC, Canada). 1,2-Dioleoyl-sn-glycero-3-phosphoglycerol (DOPG), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol) and doxorubicin hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). 1-O-(2'-(ω-methoxypolyethylene-glycol)succinoyl)-2-N-myristoyl-sphyngosine (PEG-CerC$_{14}$) was obtained from Inex Pharmaceuticals (Burnaby, BC, Canada). All other reagents used in this study were of analytical grade.

2.2. Vesicle preparation

Aqueous dispersions of lipids were obtained by hydrating a lipid film, which was prepared by co-dissolving lipids to desired molar ratios and drying under a nitrogen stream followed by the removal of residual solvent under high vacuum. Dried lipid films were hydrated with 300 mM aqueous solution of ammonium sulfate or 150 mM saline with five freeze–thaw cycles (liquid N$_2$/60 °C) to give multilamellar vesicle (MLV) suspensions. Large unilamellar vesicles (LUV) were generated by extrusion of MLVs through two stacked Nuclepore polycarbonate filters with a pore size of 100 nm (10 passes) using an extrusion device obtained from Lipex Biomembranes (Vancouver, BC, Canada). The mean diameter of LUVs was determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing Inc., Santa Barbara, CA) and found 117 ± 25 nm. Phospholipid concentrations were determined by established methods [17].

2.3. Doxorubicin loading and leakage assay

LUVs dispersed in ammonium sulfate solution were used for loading with doxorubicin [18]. An ammonium sulfate gradient was generated by exchanging the extravesicular solution with 150 mM NaCl, pH 7.4 on Sephadex G-50 spin columns [19]. Doxorubicin hydrochloride was then added to the LUVs (5 mM total lipid) to give a molar drug-to-lipid ratio of 0.05. The samples were incubated at 60 °C for 1 h to provide optimal loading conditions. Unentrapped doxorubicin was removed by running the samples over Sephadex G-50 spin columns prior to detection of liposomally entrapped drug.

To estimate the percentage of doxorubicin leakage from liposomes, an aliquot of liposomal doxorubicin was diluted 500-fold by injection into a cuvette containing 150 mM saline and the fluorescence intensity of doxorubicin was measured (excitation and emission wavelengths 480 and 590 nm, respectively) with a Perkin-Elmer LS50 fluorimeter (Perkin-Elmer, Norwalk, CT). The value for 100% leakage was obtained by addition of 10% Triton X-100 to a final concentration of 0.5%. The percentage of leakage of doxorubicin at a given time was calculated according to:

$$\text{\%leakage} = (F - F_0)/(F_{100} - F_0) \times 100$$

where F is the fluorescence intensity of the sample at a given time, and F_0 and F_{100} are intensities of samples before inducing leakage and after addition of Triton X-100, respectively.

2.4. Freeze-fracture electron microscopy

Samples were cryofixed in the presence of 33% glycerol by plunging them into liquid Freon 22 cooled by liquid nitrogen. The fractured surface was shadowed unidirectionally with platinum (45°) and coated with carbon (90°) employing a Balzers freeze-etching system BAF 400D (Balzers, Liechtenstein). Replicas were analysed using a JEOL model JEM 1200EX electron microscope (Soquelec, Montreal, Canada).

2.5. [^{31}P]NMR spectroscopy

Proton decoupled [^{31}P]NMR spectra were obtained using a Bruker MSL-200 spectrometer operating at 81.02 MHz. Acquisition parameters included 3.0-μs 60° pulse, a spectral width of 25 kHz with 4 k data points and a 1-s interpulse delay time. The sample temperature (± 1 °C) was regulated using a Bruker VT-100 temperature controller. If not otherwise indicated, the temperature was maintained at 25 °C. An exponential multiplication corresponding to 50 Hz line broadening was applied to the free induction decays prior to Fourier transformation. The chemical shift was referenced to external 85% phosphoric acid (H$_3$PO$_4$).

3. Results

3.1. Characterization of charged liposomal systems

LUVs composed of DSPC, DOPE, cholesterol and charged lipids (anionic, DOPG or cationic, DODAC) at varying ratios were used throughout these studies. Both DOPG and DODAC are permanently charged and are lipids that preferentially adopt the bilayer phase upon hydration. The presence of DOPE (which facilitates the formation of the H$_{II}$ phase) at levels up to 17 mol% did not destabilize the lipid bilayer of charged LUVs at room temperature. Maintaining the phospholipids/cholesterol (1:1) ratio while adding increased levels of DOPE was achieved by appropriate adjustment of DSPC levels. To evaluate the effect of a poly(ethylene)glycol (PEG) coating on the interaction of

oppositely charged vesicles, PEG-CerC$_{14}$ was included in some liposomal preparations.

Doxorubicin was loaded into anionic and cationic LUVs (DSPC/DOPE/charged lipid/Chol at varying mole ratios) in response to a pH gradient induced by ammonium sulfate. We found that this method could be used for loading doxorubicin into both cationic and anionic liposomes; however, the anionic liposomes were slightly more stable and thus doxorubicin was loaded into the anionic LUV in all the experiments conducted here. Efficiency of encapsulation with initial doxorubicin-to-lipid ratio = 0.05 was close to 100%. No leakage of entrapped doxorubicin from anionic LUVs was detected over a 1-h incubation time.

3.2. Effect of charged lipid content on doxorubicin release

Initial studies were conducted to determine whether release of encapsulated doxorubicin upon mixing of charged LUVs that did not contain DOPE could be achieved by increasing the charged lipid content in liposome preparations. Mixing of equal volumes of doxorubicin-containing DSPC/DOPG/Chol (34:17:50) LUV and DSPC/DODAC/Chol (34:17:50) LUV (4 mM total lipid in both populations) resulted in rapid aggregation that appeared as a transformation of the translucent LUV preparations to turbid, milky dispersions. Quasi-elastic light scattering measurements revealed formation of large structures with a broad size distribution (400–600 nm). As shown in Fig. 1, these systems show some leakage of doxorubicin upon mixing where the initial rate increased with the charged lipid content. However, the leakage did not exceed 50% of the total encapsulated doxorubicin after a 2-h incubation at 20 °C even at 40 mol% charged lipid content.

Fig. 2. Effect of DOPE on the rate of doxorubicin release from anionic LUVs after mixing with equimolar amounts of cationic LUVs. Lipid compositions: (■) DSPC/charged lipid/Chol (34:17:50), (●) DSPC/DOPE/charged lipid/Chol (29:4:17:50), (▲) DSPC/DOPE/charged lipid/Chol (25:8:17:50), (▼) DSPC/DOPE/charged lipid/Chol (17:17:17:50). DOPG and DODAC were used as charged components for anionic and cationic LUVs, respectively. The arrow indicates the moment of mixing.

3.3. Effect of DOPE and cholesterol on triggered release properties of LUVs

Both DOPE and cholesterol promote the formation of non-bilayer structures such as the H$_{II}$ phase in mixed lipid systems [14–16,20–22]. If the mechanism of release involves formation of H$_{II}$ phase or related non-bilayer structures, it would be expected that inclusion of DOPE and/or cholesterol into the bilayer of the cationic and anionic liposomes should promote more extensive bilayer destabilization upon interaction, resulting in faster release.

Fig. 2 shows time courses of doxorubicin leakage from DSPC/DOPE/DOPG/Chol liposomes induced by mixing with DSPC/DOPE/DODAC/Chol liposomes at varying DOPE content. Comparing these data to those shown in Fig. 1, it is apparent that the presence of DOPE remarkably enhances both the rate and the amount of doxorubicin leakage. At 17 mol% DOPE, release of 90% of the encapsulated doxorubicin was observed within 30 s after mixing. It may be noted that the interactions of the DOPE-containing LUVs giving rise to maximum doxorubicin leakage gave rise to aggregation that produced particles larger than could be measured by light scattering.

The effect of cholesterol on the leakage of doxorubicin from anionic DSPC/DOPE/DOPG/Chol LUVs following mixing with cationic DSPC/DOPE/DODAC/Chol LUVs is illustrated in Fig. 3. Remarkably, as the cholesterol content was increased from 10 to 17 mol%, the doxorubicin leakage increased from less than 5% over 2 h to more than 90% within 30 s. It may be concluded that cholesterol is absolutely required to achieve the rapid release rates suitable for "triggered release" applications.
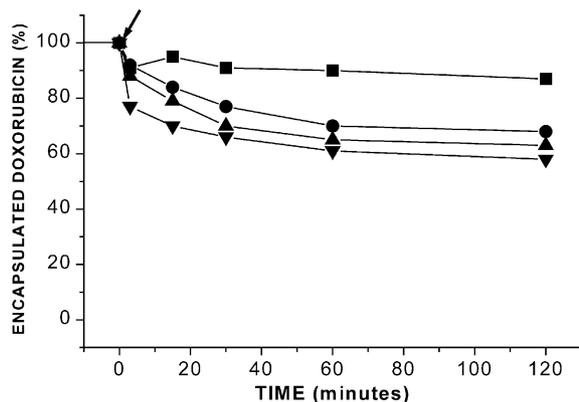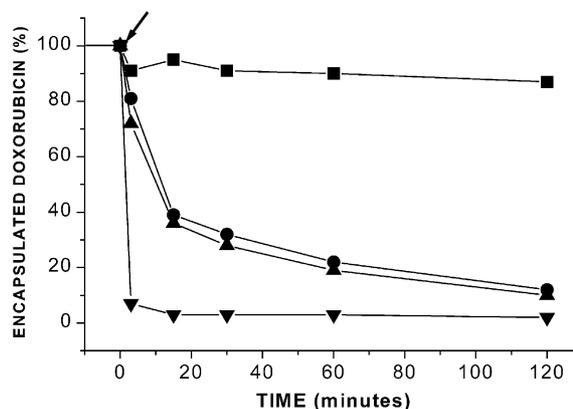

Fig. 1. Release of doxorubicin from anionic LUVs after mixing with equimolar amounts of cationic LUVs. Lipid compositions: (■) DSPC/charged lipid/Chol (34:17:50), (●) DSPC/charged lipid/Chol (25:25:50), (▲) DSPC/charged lipid/Chol (30:30:40), (▼) DSPC/charged lipid/Chol (20:40:40). DOPG and DODAC were used as charged components for anionic and cationic LUVs, respectively. The arrow indicates the moment of mixing.
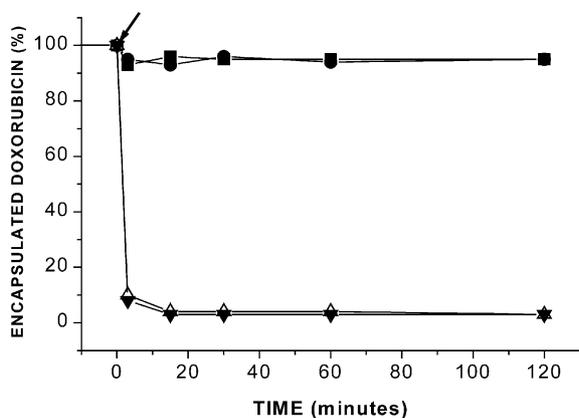
Fig. 3. Effect of cholesterol on the rate of doxorubicin release from anionic LUVs after mixing with equimolar amounts of cationic LUVs. Lipid compositions: (■) DSPC/DOPE/charged lipid (66:17:17), (●) DSPC/DOPE/charged lipid/Chol (56:17:17:10), (△) DSPC/DOPE/charged lipid/Chol (50:17:17:17), (▼) DSPC/DOPE/charged lipid/Chol (17:17:17:50). DOPG and DODAC were used as charged components for anionic and cationic LUVs, respectively. The arrow indicates the moment of mixing.

3.4. Structural properties of the lipid dispersions produced on mixing cationic and anionic LUV

It is well established [23] that phospholipids organized in (large) bilayer structures give rise to [^{31}P]NMR spectra with a characteristic asymmetric line shape with a high field peak and a low field shoulder, displaying a residual chemical shift anisotropy of approximately -40 ppm for lipids in the liquid crystalline state. In contrast, phospholipids such as DOPE that adopt the inverted hexagonal phase in isolation exhibit reversed asymmetry compared to the "bilayer" line shape with a chemical shift anisotropy that is a factor of two smaller than observed for bilayer spectra.

As shown in Fig. 4A–B, the lipid dispersion produced on mixing equimolar amounts of DSPC/DOPG/Chol (34:17:50) and DSPC/DODAC/Chol (34:17:50) LUV reveals the [^{31}P]NMR line shape associated with bilayer structure. Incorporation of DOPE (in the absence of cholesterol) also resulted in a bilayer line shape (Fig. 4C,D). However, a mixture of DSPC/DOPE/DOPG/Chol (17:17:17:50) and DSPC/DOPE/DODAC/Chol (17:17:17:50) LUVs gave rise to a spectrum in which a weak H$_{II}$ phase signal was superimposed on a bilayer signal (Fig. 4E), suggesting that part of lipid molecules adopted the H$_{II}$ phase upon mixing. When doxorubicin was present in the anionic LUVs, a significant increase in the size of the H$_{II}$ component was observed (Fig. 4F). In order to unambiguously establish that the superimposed [^{31}P]NMR resonance corresponded to H$_{II}$ phase lipid and not a narrow "isotropic" peak arising from small vesicles, the chemical shift of the superimposed peak was compared to that arising from inorganic phosphate (see Fig. 4G). The chemical shift difference of 6.8 ppm is consistent with the superimposed peak arising from H$_{II}$ structure. In summary, these data show that, in systems that exhibit rapid

release, the lipid dispersions obtained on mixing the anionic and cationic LUV exhibit limited H$_{II}$ phase structure. In addition, these results suggest that doxorubicin itself exerts an additional disordering effect on the lipid bilayer structure, inducing formation of larger H$_{II}$ phase domains and therefore promoting its own release.

The structures of the dispersions formed on mixing of oppositely charged LUVs were also examined by freeze-fracture electron microscopy. It was found that mixed anionic DSPC/DOPE/DOPG/Chol (17:17:17:50) LUV (that did not contain doxorubicin) and cationic DSPC/DOPE/DODAC/Chol (17:17:17:50) LUVs predominantly form aggregates (Fig. 5B). However, mixing of anionic doxorubicin-loaded LUVs with cationic vesicles resulted in formation of significantly bigger aggregates and some very large particles (Fig. 5C). Fragments of some structures reveal the characteristic striated pattern attributed to lipids
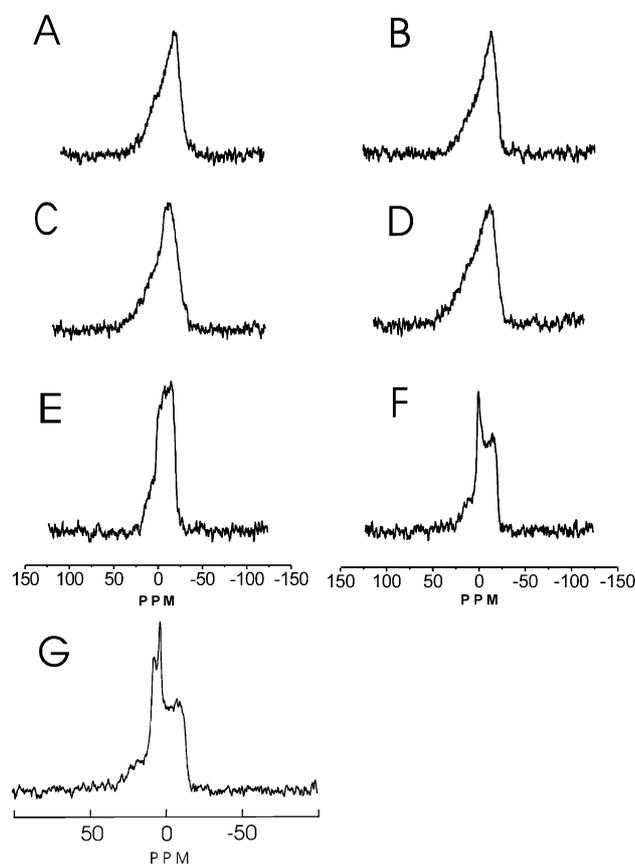

Fig. 4. [^{31}P]NMR spectra obtained from dispersions resulting from mixing of cationic and anionic LUVs. The LUVs were composed of: (A) DSPC/charged lipid/Chol (34:17:50), (B) DSPC/charged lipid/Chol (34:17:50) with doxorubicin loaded into anionic LUVs, (C) DSPC/DOPE/charged lipid (66:17:17), (D) DSPC/DOPE/charged lipid (66:17:17) with doxorubicin loaded into the anionic LUVs, (E) DSPC/DOPE/charged lipid/Chol (17:17:17:50), (F) DSPC/DOPE/charged lipid/Chol (17:17:17:50) with doxorubicin loaded into the anionic LUVs, (G) DSPC/DOPE/charged lipid/Chol (17:17:17:50) with doxorubicin loaded into anionic LUVs and inorganic phosphate present in the extraliposomal media. DOPG and DODAC were used as charged components for the anionic and cationic LUVs, respectively.
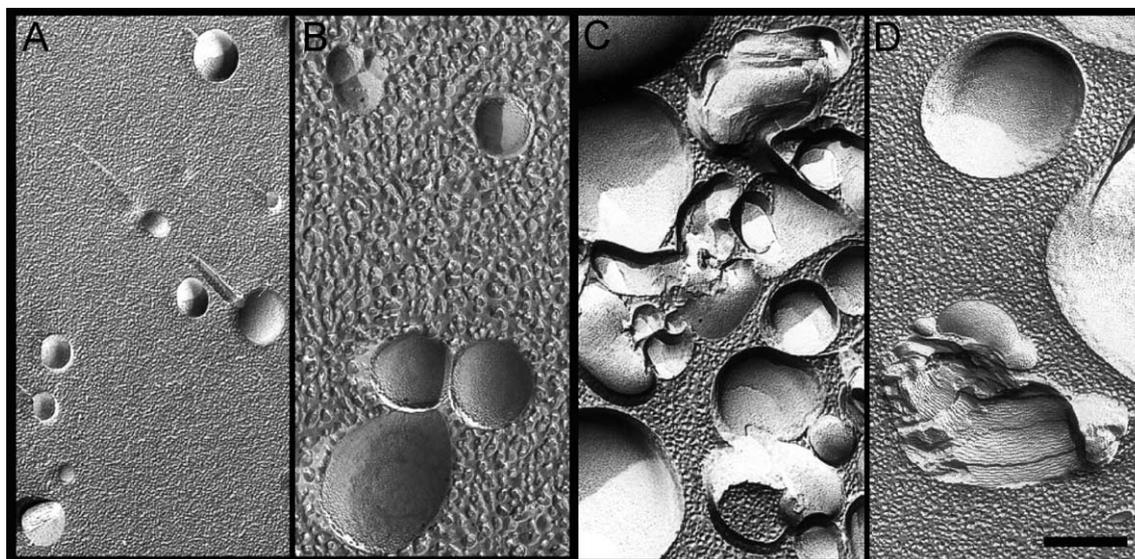
Fig. 5. Freeze-fracture electron micrographs of liposome preparations. (A) DSPC/DOPE/DOPG/Chol (17:17:17:50) LUV formed by extrusion through two stacked filters with 0.1 μm pore size, (B) equimolar mixture of oppositely charged DSPC/DOPE/charged lipid/Chol (17:17:17:50) LUVs, (C) equimolar mixture of oppositely charged DSPC/DOPE/charged lipid/Chol (17:17:17:50) LUVs with doxorubicin loaded into the anionic LUVs, (D) formation of H_{II} phase structures in mixtures of DSPC/DOPE/charged lipid/Chol (17:17:17:50) LUVs in the presence of doxorubicin. Original magnification was 20 000 ×, and the bar represents 200 nm.

organized in the hexagonal H_{II} phase (Fig. 5D). In agreement with the results obtained by [^{31}P]NMR, these data support the presence of non-bilayer H_{II} phase structure in the lipid dispersions resulting from mixing cationic and anionic LUV that give rise to rapid release of encapsulated doxorubicin.

3.5. Incorporation of PEG-CerC$_{14}$ prevents doxorubicin release

Previous studies have shown that a PEG coating can inhibit calcium-induced fusion between LUVs [12]. It was therefore of interest to determine whether incorporation of a PEG-lipid could prevent release of contents upon mixing of oppositely charged LUVs. It was found that inclusion of 7.5 mol% of PEG-CerC$_{14}$ into both the cationic and anionic vesicles prevents virtually any leakage of encapsulated doxorubicin following mixing (Fig. 6). However, when the PEG-CerC$_{14}$ content was decreased to 5 mol%, release of about 22% of liposomal doxorubicin occurred within 30 s after mixing. Interestingly, during the next 15 min of incubation, quenching of doxorubicin fluorescence was observed. This effect cannot be attributed to the self-association of doxorubicin because of the low doxorubicin concentration in the extraliposomal media. Rather, it suggests that a considerable fraction of PEG-coated doxorubicin-containing LUVs maintains a transmembrane pH gradient sufficient to reload doxorubicin released from destabilized vesicles. Dynamic light scattering did not reveal an increase of particle size after mixing of LUVs containing 7.5% or 5% PEG. When the PEG content was diminished to 2.5 mol%, a

similar "reloading" effect was observed, but, in this case, released doxorubicin was not completely reencapsulated and the liposome size and polydispersity increased from 117 ± 30 to 155 ± 36 nm. It should be noted that inhibition of doxorubicin release upon mixing was also achieved when 10 mol% PEG-CerC$_{14}$ was present only in the doxorubicin-loaded LUVs (data not shown).
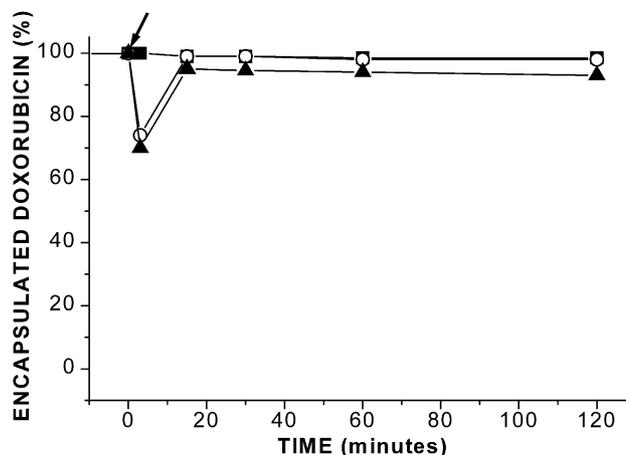

Fig. 6. Effect of PEG-CerC$_{14}$ coating on the rate of doxorubicin release from the anionic LUVs after mixing with equimolar amounts of cationic LUVs. Lipid compositions: (■) DSPC/DOPE/charged lipid/Chol/PEG-CerC$_{14}$ (17:17:17:50:7.5), (○) DSPC/DOPE/charged lipid/Chol/PEG-CerC$_{14}$ (17:17:17:50:5), (▲) DSPC/DOPE/charged lipid/Chol/PEG-CerC$_{14}$ (17:17:17:50:2.5). DOPG and DODAC were used as the charged components for anionic and cationic LUVs, respectively. The arrow indicates the moment of mixing.

4. Discussion

The results of this study demonstrate that relatively immediate and complete release of doxorubicin encapsulated in anionic LUV can be achieved on mixing with cationic LUV and that this release is dependent on the presence of DOPE and cholesterol. Three interesting aspects of this work concern the mechanism of drug release, the role of lipids such as DOPE and cholesterol and the potential applications of these systems for triggered release of drug in vivo. We discuss these areas in turn.

The mechanism whereby the addition of cationic LUV to doxorubicin-containing anionic LUV induces drug release is clearly not just due to aggregation. It may be noted that for all the systems investigated here, considerable aggregation as evidenced by an increased particle size as determined by QELS occurred on addition of the cationic LUV to the anionic, drug-containing LUV. However, in the absence of DOPE and/or cholesterol little or no release of drug is observed, indicating that aggregation alone does not result in particularly rapid or complete release. Equally, however, the release cannot be correlated with fusion. The [^{31}P]NMR spectra resulting from the dispersions of cationic and anionic LUV prepared in the absence of DOPE or cholesterol are classical "bilayer" spectra consistent with lipid structures of 200 nm diameter or larger [24], much larger than the 100 nm LUV that these structures originated from. These structures must be fused, rather than stacked, as stacked 100 nm diameter systems would be expected to give rise to considerable narrower spectra due to motional averaging effects arising from lateral diffusion processes [24]. The parameter that appears to correlate most closely with the ability of cationic LUV to cause rapid and complete release of doxorubicin entrapped in anionic LUV is the appearance of an H_{II} phase component in the resulting lipid dispersion. That the presence of a local region of H_{II} structure should give to rapid leakage is perhaps not unexpected, given that it represents a relatively massive and potentially long-lived perturbation of local bilayer structure.

The requirement for DOPE and cholesterol in order to achieve rapid release of doxorubicin can be readily understood in terms of a need for local regions of non-bilayer structure. DOPE assumes the H_{II} phase in isolation and progressively promotes non-bilayer organization when incorporated in bilayer systems [16]. Similarly, cholesterol has long been noted to induce H_{II} structure in mixed DOPE–DOPC systems [21]. Assuming complete mixing of lipids following addition of the cationic DSPC/DOPE/DODAC/Chol (17:17:17:50) LUV to the anionic DSPC/DOPE/DOPG/Chol (17:17:17:50) LUV, the lipid composition of the dispersions produced is DSPC/DOPE/DODAC/DOPG/cholesterol (1:1:0.5:0.5:3). Given that mixtures of DODAC and DOPG assume the H_{II} phase organization [14], the ratios of bilayer lipid, H_{II} phase-preferring lipid and cholesterol are then 1:2:3. Previous studies have dem-onstrated appreciable formation of H_{II} phase structure in mixed lipid systems where the ratio of bilayer lipid, H_{II} phase lipid and cholesterol are 1:2:1 [21].

There are several ways in which triggered release of encapsulated drug arising from mixing cationic and anionic LUV could be of utility for in vivo drug delivery applications, all of which involve the use of PEG-coated LUV systems. Briefly, the results presented here demonstrate that the interactions between the LUV and subsequent release of drug can be inhibited by the presence of a PEG coating. In previous work [25], we have demonstrated that the dissociation rates of PEG-ceramides can be modulated by varying the length of the acyl chain associated with the ceramide anchor. For example, the dissociation rates increase from < 1 min for PEG-CerC$_8$ to 1.2 h for PEG-CerC$_{14}$ and to more than 13 days for PEG-CerC$_{20}$. Two circumstances leading to time-dependent triggered release can be envisaged. The first involves co-administering PEG-coated populations of cationic and anionic LUV, one or both of which contains the drug, and relying on the dissociation of the PEG-lipid to render the systems vulnerable to interaction and drug release. Alternatively, the drug could be encapsulated in PEG-coated cationic LUV and drug release triggered by interaction with the negatively charged constituents associated with the membranes of cells in vivo, following dissociation of the PEG coating. This would require that rapid release can occur when only one of the membranes contains phosphatidylethanolamine on the outer monolayer, a circumstance that is not characterized here. These and other possibilities are currently being investigated.

Acknowledgements

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