Published in final edited form as:

Langmuir. 2013 June 4; 29(22): 6615–6623. doi:10.1021/la304340e.

Lipid Nature and Their Influence on Opening of Redox-active Liposomes

Martin Loew, Jerimiah C. Forsythe, and Robin L. McCarley*

Department of Chemistry and Center for Biomolecular Multi-scale Systems, Louisiana State University, Baton Rouge, LA 70803-1804

Abstract

The pathway for contents release from reduction-sensitive liposomes based on a quinone-dioleoylphosphatidylethanolamine lipid conjugate (Q-DOPE) was outlined using results from fluorescent dye contents release assays, as well as single- and multiple-angle light scattering. Experimental observations are consistent with a shape/size change of the reduced liposomes prior to their aggregation, with subsequent near-quantitative contents release achieved only when the lipid membrane experiences conditions favorable to a lamellar to an inverted hexagonal phase transition. Addition of poly(ethyleneglycol)-modified DOPE (PEG-DOPE) to the Q-DOPE liposomal formulation results in stabilization of the lipid bilayer, whereas incorporation of DOPE yields faster contents release. At high DOPE concentrations, DOPE/PEG-DOPE/Q-DOPE liposomes exhibit larger contents release, indicating a change in pathway for contents release. The outcomes here provide a better understanding of the underlying principles of triggered liposomal contents release and the potential utility of specific lipid properties for the rational design of drug delivery systems based on the novel Q-DOPE lipid.

INTRODUCTION

Optimization of lipid compositions is a crucial task in the design of liposomal drug delivery systems. To that end, the dichotomous interplay between liposome stabilizing materials and those components that enable efficient contents release at the target site has to be understood. For example, increased blood circulation times can be achieved by stabilizing the structure of pure phospholipid liposomes and minimizing their removal by the reticuloendothelial system¹ through the incorporation of polymer-grafted lipids^{2–4} or certain peptides.⁵ Thus, the liposomes can accumulate efficiently at bodily target sites having leaky vasculature and poor lymphatic drainage via the enhanced permeability and retention (EPR) effect that is exploited in cancer therapy.⁶ However, use of a liposomal carrier system that remains stable to release of its encapsulated molecules interferes with the necessity to reveal the payload in its active form at the target site, unless contents release can be had by environmental stimuli at that site.

The use of poly(ethyleneglycol)-modified phosphatidylethanolamine lipids (PEG-PE) in making liposomes with increased blood circulation times has resulted in clinically successful cancer therapies based on the delivery of Doxorubicin from Doxil liposomes.^{7, 8} Although release of the chemotherapeutic Doxorubicin from the PEG-based liposomes is thought to

*CORRESPONDING AUTHOR: Telephone: (225) 578-3239. Facsimile: (225) 578-3458. tunnel@LSU.edu.

The authors declare no competing financial interest.

SUPPORTING INFORMATION

Additional figures (S1–S4), preparation method of liposomes, and the accompanying text for Figure S4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

result from destruction of the chemical gradient used to load them with the drug, the process is slow.⁹ As a result, the relatively large amount of required liposomal drug results in side effects, such as hand-foot syndrome and mucositis in patients.¹⁰ In addition, clinically-relevant drug release is specific to Doxorubicin.

Triggerable-delivery liposome systems have been under development in the past 20 years, with the premise that payload release will occur upon the liposome sensing specific environments present at its destination. Various non-clinical liposomal formulations can be triggered, for instance, by low pH,^{11–13} the presence of thiolytic agents,^{7, 8} or enzymatic action.^{14–16} Although there are variations in lipid composition and the nature of the triggering mechanism, many liposomal systems share the same underlying release principle, namely use of a triggering event to elicit a change in the phase of the lipids composing the bilayer of the liposome so as to expel the encapsulated contents. For example, a stable liquid-crystalline lamellar phase (L_α) of a lipid under one set of conditions allows for formation of liposomes that can be loaded with target molecules. Under the influence of the specific trigger of choice, the properties of the lipids composing the liposome change to yield a non-lamellar lipid phase, the production of which leads to payload release. This can be achieved—with the exception of several pH-sensitive formulations—by the cleavage of lipid head groups/lipids to generate destabilized membranes typically composed of non-bilayer forming lipids, such as dioleoylphosphatidylethanolamine, DOPE,²⁰ or derivatives of diacylglycerol.^{7, 8} DOPE, or specific mixtures of DOPE or diacylglycerol with bilayer-prone lipids, forms inverted micelle hexagonal phases (H_{II}) above its phase transition temperature T_H .^{21, 22} Therefore, the release of encapsulated molecules is postulated to result from the $L_\alpha \rightarrow H_{II}$ phase transformation.¹² However, this release mechanism is not yet fully understood and most likely is specific to the different liposomal systems that rely on generation of an H_{II} -prone lipid mixture.

For a rational design of liposomal formulations and control of their properties, knowledge about the mechanism for a given triggerable system is required. Then, the influence of specific components on the release mechanism—such as saturated or unsaturated phospholipids, PEGylated lipids, or other materials that alter phase transition temperatures, intrinsic curvature of lipid layers, and fluidity of the lipid membranes—can be evaluated. Following this approach, we report here on investigations of a recently invented, reduction-sensitive liposomal system²⁰ that use light scattering and fluorescence contents release assays. After extracting information about different intermediate stages during the release of liposomal contents, the effect of added DOPE and PEGylated DOPE diluent lipids was then determined. A proposed mechanistic model is used to explain the influence of both diluent lipids on release kinetics and the amount of contents released, thus giving insights in their potential role in drug delivery systems.

EXPERIMENTAL PROCEDURES

Materials and Vesicle Preparation

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, DOPE, and the ammonium salt of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(poly(ethylene glycol))-2000], PEG₂₀₀₀-DOPE (Avanti Polar Lipids; Alabaster, AL) were used without further purification. The reduction-sensitive lipid (Q-DOPE) was synthesized as described previously. Calcein, KCl, K₂HPO₄, KH₂PO₄, Na₂S₂O₄, Triton X-100, and 2-(cyclohexylamino)-ethanesulfonic acid, CHES, were obtained from Sigma-Aldrich (St. Louis, MO). Sephadex G50 fine was from GE Healthcare (Piscataway, NJ). Large unilamellar vesicles (LUVs) were produced by extrusion;²⁴ for details see Supporting Information.

Fluorescence Measurements

All fluorescence measurements were performed at 25 °C using a Perkin Elmer LS55 fluorescence spectrometer (Waltham, MA) with excitation at 490 nm (slit 2.5 nm) and emission at 515 nm (slit 2.5 nm). The total intensity of emitted light was adjusted using neutral density filters from Omega Optical (Brattleboro, VT). For the time-dependent measurements, fluorescence intensity was measured in 1 min intervals.

Contents Release Assay

Calcein release assays were performed using a modified protocol,²⁵ as outlined in the Supporting Information.

Static Light Scattering at 90°

LUVs devoid of calcein were prepared as described above using pH 7.4 and 9.0 buffers. Light scattering (5-s intervals) was measured at 90° with a Perkin Elmer LS55 fluorescence spectrometer (Waltham, MA) following a protocol of Goldenbogen *et al.*⁸ Incident light was 600 nm, scattered light was recorded at 610 nm, and a slit width of 10 nm was used for both the incident and scattered light.

Multiple-angle Light Scattering (MALS)

LUVs devoid of calcein were prepared as described above using pH 7.4 and 9.0 buffers. To examine shape changes induced by reduction, solid sodium dithionite (final concentration ~500 μ M) was added to the sample, followed by gentle mixing prior to injection of the sample into the measuring cell, where it remained under quiescent conditions. MALS was performed using a Dawn Heleos (Wyatt Technology; Santa Clara, CA) equipped with a GaAs Laser operating at a wavelength of 658 nm. Light intensity was recorded at 10 angles (43.2°, 51.9°, 60.2°, 69.5°, 79.8°, 90.0°, 110.5°, 121.0°, 131.9°, 142.0°).

Analysis of MALS Data

Data was analyzed as described previously²⁶ according to the Rayleigh-Gans-Debye (RGD) theory. In brief, intensity values $I(\theta)$ measured at scattering angles θ divided by the intensity measured at 90°, $I(90^\circ)$, were plotted against the scattering angle θ . The data points were fitted to the RGD scattering factor $P(q)$, where $q = (4\pi/\lambda) \sin(\theta/2)$. Here, n is the refractive index of the solvent, and λ is the wavelength of the incident light. For spherical vesicles, $P(q)$ is given by Equation 1:

$$P(q) = \left[\frac{3}{(u^3 + v^3)} \right]^2 \left[u^3 \frac{j_1(qu)}{(qu)} - v^3 \frac{j_1(qv)}{(qv)} \right]^2 \quad (1)$$

With $j_1(x) = \sin x/x^2 - \cos x/x$, u is the outer radius of vesicle, and v is the inner radius of the vesicle.

RESULTS AND DISCUSSION

Contents Release from Reduction-sensitive Liposomes

In a previous study, it was shown that (i) stable unilamellar vesicles can be made using the reduction-sensitive quinone propionic acid lipid, Q-DOPE, **1a** in Scheme 1;²⁰ (ii) small, water-soluble molecules such as calcein dye can be entrapped inside these vesicles; and (iii) contents release can be triggered by dithionite reduction of the quinone head group to the corresponding hydroquinone **1b**, that in turn causes its rapid cyclizative removal to yield DOPE **1c** and the cyclized lactone **1d**.²⁰

In Figure 1A are shown the time-dependent fluorescence emission traces at 515 nm for Q-DOPE vesicles initially containing calcein at concentrations of 40 mM (blue trace) and 80 mM (black trace) at pH 7.4 after addition of dithionite reducing agent. Although calcein concentrations ~ 80 mM are typical for measuring its release,²⁷ ~ 40 mM values are just as well suited for release measurements and even more so for detecting variations of liposome inner volume, because calcein fluorescence dequenching is highly sensitive to concentration changes near 40 mM. However, the less efficient quenching at 40 mM results in a higher initial fluorescence, but this does not impact the outcomes and their explanation for the intensity variations we observe. As seen in Figure 1A, the fluorescence intensity remains almost constant during the first minutes following addition of dithionite reducing agent. About 12 min after addition of reducing agent, the fluorescence intensity first decreases over a 2–3 min time period and then (15–25 min) increases to a level slightly higher than the initial intensity. The decrease in calcein fluorescence at ~ 12 min is interpreted as being caused by an increase in intraliposomal calcein concentration resulting from a deformation or size contraction of the liposomes. Hence, the subsequent (15–25 min) increase in fluorescence corresponds to a dilution of calcein resulting from a volume increase of the LUVs, most likely due to a relaxation of the deformed/contracted vesicles caused by their aggregation and subsequent interliposomal membrane interaction. The hypothesis of a contraction followed by a relaxation step is supported by the “divot” in the fluorescence intensity curves being more pronounced for the 40 mM calcein concentration.

Approximately 28 min after dithionite addition, the observed abrupt and rapid fluorescence increase that occurs over a period of 5 min is the burst-phase release³⁰ of calcein. From here forward, the period of time from dithionite addition until the start of the burst phase will be referred to as the lag time or lag phase. Addition of the detergent Triton X-100 (up arrows in Figure 1A) ensures complete rupture of any possible remaining liposomes, and for the time points denoted in Figure 1A, virtually 100% of the entrapped calcein was released during the 5-min burst phase. Please note, the fluorescence traces of Figure 1A show the relative fluorescence intensities of calcein instead of the calcein release (Figures 1B, 2, and 4) in order to demonstrate the difference between the calcein release at 40 mM and 80 mM. The initial fluorescence of Figure 1A (40 mM calcein encapsulated, pH 7.4) displays a relatively high fluorescence intensity because calcein is not strongly quenched at a concentration of 40 mM compared to values of 80 mM or more;^{28, 29} the fluorescence intensities immediately before and after the addition of dithionite are not significantly different, pointing to a lack of calcein release.

As a control, we added the non-reducing salt NaHSO_3 to the Q-DOPE liposomes instead of $\text{Na}_2\text{S}_2\text{O}_4$; the fluorescence intensity remained constant; neither the fluorescence dip nor any significant release was observed.²⁰ In the absence of any added material, no observable contents release occurred (Figure S3, lower panel, right).

Effect of T_H on Calcein Release

We hypothesized in our previous work²⁰ that burst-phase contents release from reduced Q-DOPE liposomes results from liposomal contact-mediated lysis, with the key event being transition of DOPE lipids from the lamellar L to the H_{II} phase that occurs when a critical concentration of H_{II} phase-competent lipid (DOPE) is formed in the outer leaflet.³¹ In order to test this hypothesis, we investigated possible inhibition of contents release from the reduced Q-DOPE liposomes at experimental temperatures T_{exp} below or near T_H of the generated DOPE in two different ways: first, at room temperature and basic pH,³² the surface of the resulting DOPE outer leaflet will be anionic and the T_H of the DOPE will be well above ambient temperature;³³ second, at near neutral pH and 10 °C, the zwitterionic DOPE will have its T_H near this value.

In Figure 1A is shown the time-dependent fluorescence intensity of calcein for reduced Q-DOPE vesicles at pH 9.0 (red) and pH 7.4 (blue). At pH 9.0, the fluorescence intensity decreases rapidly 3 min after dithionite addition and is immediately followed by a fluorescence increase between 3 and 5 min; this process is assigned as the deformation/contraction phase. Then, an extremely slow efflux of calcein occurs over the next 120 min; only 40% of the contents have been released 15 h later (Figure S1). This behavior is in clear contrast to that noted at pH 7.4, where there is the dominant burst-phase contents release (>95%) that is preceded by the divot in the fluorescence trace associated with contraction/deformation and subsequent relaxation of the liposomes. Similar behavior is noted for reduced Q-DOPE vesicles at 10 °C and 25 °C with pH 7.4 (Figure S2 in the Supporting Information). There is an exceedingly small amount of contents released from the liposomes when the experimental temperature T_{exp} is near the T_{H} of DOPE (10 °C), while prompt observation of near-quantitative, burst-phase calcein release occurs when T_{exp} is above T_{H} .

Our observations can be explained by recognizing the selectivity of leaflet reduction, the impact of pH and T_{exp} on the lactone cyclization (Scheme 1) kinetics, and the influence of experimental temperature on the rate of the $\text{L} \rightarrow \text{H}_{\text{II}}$ phase change. As dithionite does not cross lipid membranes,³⁴ reduction of the quinone head groups and subsequent generation of DOPE takes place in only the outer lipid leaflet. We attribute the monitored liposome contraction to this lipid asymmetry (see Release Pathway discussion below). At pH 9.0 and 25 °C, the cyclization rate for hydroquinone group cleavage is greatly enhanced due to catalysis by base.³⁵ Also, head group cleavage is faster at pH 7.4 and 25 °C versus 10 °C as judged by NMR kinetics.^{20, 36} Therefore, the lipid asymmetry-induced contraction/deformation with its associated fluorescence decrease appears earlier at pH 9.0 than at pH 7.4 with $T_{\text{exp}} = 25$ °C, as is true at pH 7.4 for $T_{\text{exp}} = 25$ °C versus 10 °C. In addition, the expected kinetically stable bilayer structure attained at pH 9.0 and $T_{\text{exp}} = 25$ °C or pH 7.4 and $T_{\text{exp}} = 10$ °C results in exceedingly slow calcein release. These outcomes agree with those predicted by a contact-mediated lysis model of liposome opening,³¹ wherein contact of the H_{II} -competent outer leaflets of apposed bilayers of vesicles at temperatures above T_{H} —not mere aggregation of vesicles—leads to rupture of the bilayers.

Effect of Lipid Concentration on Release Kinetics

To evaluate the interplay between vesicle-vesicle interactions (aggregation) and contents release for Q-DOPE LUVs, we performed calcein release assays at different lipid (liposome) concentrations. It has been shown that contents release from LUVs, caused by cleavage of stabilizing lipids incorporated into LUV membranes composed mainly of DOPE, is accompanied by concentration-dependent aggregation of the vesicles,^{7, 11, 12} an event consistent with a liposome contact-mediated lysis mechanism. In Figure 1B is shown the release of calcein at Q-DOPE lipid concentrations of 12.5–200 μM . The starting point for the fluorescence decrease (beginning of the divot in fluorescence) at roughly 12 min is relatively unaffected by lipid concentration, but the time point for subsequent recovery of fluorescence intensity is delayed at Q-DOPE concentrations below 100 μM . While fluorescence recovery begins at ~14 min for 100 and 200 μM Q-DOPE, it starts at ~16–17 min for 25 and 50 μM Q-DOPE, and 20 min for 12.5 μM Q-DOPE. As a consequence, the depth of the divot in fluorescence intensity is more pronounced at lower lipid concentrations.

The onset of the burst phase is shifted to later times by about 1 min for each lipid concentration step from 200 μM to 25 μM . Importantly, the overall amount of released calcein is unchanged. In contrast, at a Q-DOPE concentration of 12.5 μM , the burst phase starts about 35–40 min after reduction of Q-DOPE, and after 80 min, only about 30% of the calcein is released. In general, faster release kinetics at higher lipid concentrations demonstrate the necessity of inter-vesicle contact for contents release, but do not provide

proof of vesicle aggregation subsequent to liposome opening. Perhaps, mere vesicle collisions could be involved in the recovery of fluorescence before the burst phase, as well as in the release of calcein. To determine the possible role of vesicle aggregation in the triggered release of contents from reduced Q-DOPE LUVs and to test the hypothesis of vesicle deformation/contraction prior to burst-phase release, we performed light scattering measurements.

Size and Shape Changes of Q-DOPE Vesicles Observed by Static Light Scattering

To maintain the experimental conditions of the calcein release assay, we first performed static light scattering at 90° using a fluorescence spectrometer equipped to measure scattered light. In contrast to the constant scattered light intensity observed for the Q-DOPE liposomes at pH 7.4 and pH 9.0, addition of dithionite led to an increase in scattered light intensity roughly 28 min (pH 7.4; Figure 2A, red) and 10 min (pH 9.0; Figure 2B, red) later (in Figure S3, lower left panel, is shown the raw data of scattering at 90° before and after reduction of the LUVs at pH 7.4 and pH 9.). Before the burst phase at pH 7.4 in Figure 2A, a slight decrease in the scattering intensity (red) was observed at ~14 min that is coincidental with the decrease in fluorescence (black) that we have attributed to a size decrease in/deformation of the liposomes due to membrane asymmetry (vide supra). Simultaneous to the burst-phase release of contents at ~28 min, the scattering intensity also increases sharply, indicating significant vesicle aggregation. A similar scattering profile was found at pH 9.0 (Figure 2B, red), but it is shifted to earlier times than the pH 7.4 case, in agreement with the faster base-catalyzed cyclization reaction (vide supra). Although the scattering intensity at pH 9.0 rises sharply about 10 min after dithionite addition (Figure 2B; red), no significant contents release was observed (black).

The light scattering results at pH 7.4 and pH 9.0 demonstrate that aggregation of reduced Q-DOPE vesicles, resulting from generation of DOPE in the proximal leaflets by the lactone cyclization reaction, is not sufficient to induce contents release. At pH 7.4, vesicle aggregation is correlated with the release of encapsulated molecules, whereas calcein remains entrapped in the aggregated vesicles at pH 9.0 because the experimental temperature is below that of the T_H of diacylphosphatidylethanolamines,^{33, 37} thereby inhibiting the $L \rightarrow H_{II}$ phase transition.

To obtain information about the size and shape of the Q-DOPE vesicles at pH 7.4 and pH 9.0 prior to and including the burst-phase release event, the time-dependent intensity of scattered light was measured at 10 different angles (43.2°–142.0°) for vesicles in the presence and absence of dithionite. Form factors derived from the Rayleigh-Gans-Debye, RGD, approximation^{26, 38} were used. Prior to reduction, scattering intensities for the Q-DOPE LUVs at pH 7.4 and pH 9.0 were well fitted to a model based on spherical unilamellar vesicles with a geometric radius of about 40 nm and a 4 -nm membrane thickness,³⁹ Figure 3 (measured data points: solid dots; fit: green line). Upon reduction of Q-DOPE LUVs, the time-dependent, multi-angle intensity traces of scattered light (Figure S3) were similar in shape to those at 90° obtained with the fluorescence spectrometer, Figs. 2A and 2B. To learn about the morphological nature of the contracted/deformed Q-DOPE LUVs formed by dithionite reduction, determinations of the form factor in this time regime of the experiment were carried out. The processed intensity data could not be fitted to form factors based on spherical LUVs (Figure 3, open squares) for either pH, pointing to the presence of non-spherical reduced Q-DOPE vesicles. Attempts to fit the data to a model having either prolate or oblate vesicles were unsuccessful.

Effect of PEG-DOPE on Calcein Release from Reduction-sensitive Liposomes

In order to gain knowledge about the role of lipid composition on contents release from the reduced Q-DOPE liposomes, we examined the behavior of Q-DOPE liposomes containing: i) low mole ratios of a non-protein-fouling lipid, PEG-DOPE, similar to those commonly used in liposomal drug delivery systems; ii) the inverted phase-competent lipid DOPE; and iii) combinations of PEG-DOPE and DOPE.

First, we explored the influence of PEGylated lipids on the release of calcein from Q-DOPE liposomes. To this end, we prepared Q-DOPE LUVs with various amounts of PEGylated DOPE (PEG₂₀₀₀-DOPE; $M_r(\text{PEG}) = 2,000$ Da). In Figure 4A are displayed representative calcein release curves for reduced Q-DOPE liposomes containing 0.0%, 0.1%, 0.5%, and 3.0% PEG₂₀₀₀-DOPE. Liposomes with 0.0% and 0.1% PEG₂₀₀₀-DOPE (blue and black lines) exhibit similar release curves. First, the fluorescence divot appears, as described above. After a lag time of about 30 min, the burst phase for calcein release begins and is more than 90% complete within ~10 min. When Q-DOPE liposomes contain 0.5% PEG₂₀₀₀-DOPE (red line), the lag time and the onset time for the fluorescence divot are virtually unchanged when compared to liposomes containing less PEG₂₀₀₀-DOPE. However, for the 0.5% PEG₂₀₀₀-DOPE/99.5% Q-DOPE LUVs, only 30% of their contents is released after waiting twice as long before lysing them with Triton X-100. We attribute this lower level of contents release to a stabilizing effect resulting from the presence of the PEG-DOPE lipids. For 3% PEG-DOPE/97% Q-DOPE LUVs (green line), a very small amount of the contents are released, although the size/shape change fluorescence divot is still observed during the lag phase.

Influence of DOPE on Calcein Release from Reduction-sensitive Liposomes

The influence of DOPE on the release of calcein from Q-DOPE liposomes was tested with vesicles containing 0.0%, 3.8%, and 10.0% DOPE, Figure 4B. For 3.8% DOPE/96.2% Q-DOPE LUVs (black line), the burst phase appeared 10 min earlier than for 100% Q-DOPE LUVs (blue line), and some calcein release was also observed prior to the burst phase. In LUVs with 10% DOPE (red line), the burst phase started 1–2 min earlier. For fruitful contact-mediated lysis of the reduced Q-DOPE LUVs, a critical concentration of DOPE must first be generated by cyclization of the reduced quinone so that successful apposed bilayer contact and the subsequent L₁ → H_{II} phase transition take place. For the DOPE/Q-DOPE LUVs, this critical DOPE concentration in the membrane is reached earlier, thereby allowing for earlier release of their cargo. Furthermore, the fluorescence profile during the lag phase was changed for the DOPE/Q-DOPE LUVs. When 0% or 3.8% of DOPE is present in the membrane, the fluorescence divot is observed in the first 15 min (for 3.8% DOPE) to 20 min (for 0% DOPE) before the burst phase starts. For 10% DOPE/90% Q-DOPE liposomes, the divot before the burst phase is not readily apparent, as only a slight decrease in calcein fluorescence is observed during the lag phase. This outcome indicates a change in the contents release pathway when moving from vesicles containing 0% or 3.8% DOPE to vesicles with 10% DOPE, as discussed later.

We investigated the release of calcein from Q-DOPE vesicles containing both PEG₂₀₀₀-DOPE and DOPE. For 0.5% PEG₂₀₀₀-DOPE, we found the combined effects of both additional lipids: a decreased amount of contents release caused by PEG₂₀₀₀-DOPE and faster release kinetics, as well as an altered release pathway, due to DOPE. Interestingly, we could partially regain larger release rates applying DOPE concentrations of 10% and higher, that is, when the release pathway is changed as a consequence of the high DOPE concentrations (Figure S4 and accompanying text in the Supporting Information).

Contents Release Pathway

Combining our observations with previously published results, the process of reduction-induced contents release from Q-DOPE liposomes can be posited, Scheme 2. The addition of dithionite leads to rapid reduction of the quinone groups⁴⁰ situated only on the outer leaflet, due to its inability to pass the anionic lipid bilayer.³⁴ As a consequence, DOPE is formed on only the outer leaflet, and thus the outer and inner portions of the bilayer exhibit different lipid compositions (Scheme 2, phase “reduction and cleavage”). When the contraction phase starts at ~12 min, as indicated by the decreased calcein fluorescence and our light scattering data, about 20% of the hydroquinone moieties have been cleaved to yield the critical amount of outer leaflet DOPE to begin the vesicle deformation/size decrease.²⁰

To explain the shape/size change of the LUVs associated with the fluorescence divot, at least two different effects may play a role. First, a change in the spontaneous curvature of the membrane has to be considered.⁴¹ In a simplistic model, the phase behavior,⁴² as well as the curvature of lipid bilayers,⁴³ can be connected to the molecular shape of the lipids. Due to its ability to form stable bilayers, Q-DOPE must exhibit a cylindrical shape. Cleavage of the quinone head group only in the outer leaflet generates DOPE, an inverted cone-shaped lipid, whose presence induces a more negative curvature only in the outer leaflet. The differences in spontaneous curvature of the inner and outer leaflets lead to deformation/contraction of the vesicles. Alternatively, according to the area-difference elasticity (ADE) model,⁴⁴ area differences between lipid membrane leaflets determine their morphology. Heuvingh and Bonneau⁴⁵ recently reported on the induced deformation of giant unilamellar vesicles (GUVs) made of dioleoylphosphatidylcholine. In their work, use of a chemical reaction selective for a given lipid leaflet led to budding of new vesicles inside or outside of the GUVs. Inward budding resulted when the chemical reaction led to a lower total area for the outer leaflet. Analogous to their observations, we propose that reductive cleavage of the bulky quinone head group lowers the area of the outer leaflet, causing deformation of the vesicles. Before the burst-phase release of calcein, the recovery of fluorescence in the divot was shown to be dependent on lipid concentration. Therefore, this phenomenon is connected to interactions between liposomes. Although we cannot give a conclusive explanation at this time, preliminary results suggest that hemifusion of deformed liposomes is involved in relaxation of the shape of the deformed LUVs and, thus, in an increase of the inner volume of the liposomes. This view is supported by experiments showing that hemifusion is favored in phosphatidylethanolamine-containing lipid membranes.⁴³ An upcoming paper will address these new findings regarding hemifusion. Subsequently, aggregation of reduced Q-DOPE vesicles takes place during the burst phase and is necessary for the subsequent contents release at pH 7.4, because the associated release kinetics are strongly reduced at low lipid concentrations, Figure 1B. These observations are in accord with those for pH-sensitive liposomes wherein aggregation was necessary before their contents were released. Suppression of contents release from reduced Q-DOPE LUVs at pH 9.0 ($T_H > \text{ambient}$), along with their demonstrated aggregation, demonstrates that the deformation and aggregation events are not sufficient to cause burst-phase contents release; it is necessary to have a non-bilayer competent lipid composition comprising the outer leaflet. That is to say, not only must there be present in the proximal leaflet a sufficient amount of non-bilayer *prone* lipid, but also the experimental temperature must be above that for the $L \rightarrow H_{II}$ transition. Thus, the cause of the burst phase at pH 7.4 is the structural rearrangement of the lipids during the events leading up to and including the $L \rightarrow H_{II}$ transition for a membrane enriched in DOPE.³² Based on the kinetics of reduced quinone cleavage²⁰ at pH 7.4, the outer leaflet contains roughly 50% DOPE at burst-phase onset.

Some remarks about the vesicle aggregation and leakage at pH 9.0 may complete our thoughts on the release mechanism. When pure diacylphosphatidylethanolamine liposomes are prepared at a high pH (9), usually no time-dependent aggregation or significant

contents release is observed. For instance, Ellens *et al.*³² investigated the destabilization of different diacylphosphatidylethanolamine liposomes caused by temperature, pH, and presence of Ca^{2+} via measurement of aggregation and contents release. At pH 9.5, they found no transient aggregation, but stated that the size of the liposomes exceeds the value for other negatively charged liposomes produced with the same method; they conclude that the liposomes are *initially* oligomeric, that is, preaggregated prior to subsequent triggered release/aggregation measurements. In the case of Q-DOPE vesicles, the LUVs are not preaggregated before the reduction at pH 9.0, as it can be concluded from size measurements via dynamic light scattering; but the generation of DOPE leads to aggregation and an enhanced light scattering. Moreover, the slight release we observe at pH 9.0 is also in agreement with the results of Ellens *et al.*³² At a low pH (<7), a significant release below the T_H was found for all tested PE vesicles;³² at pH 9.5 the release was “less than 1%/min,” similar to our findings. The average leakage from Q-DOPE vesicles 1000 min after reduction was about 0.04%/min. Besides that work, other attempts to encapsulate dyes into PE liposomes have repeatedly failed.^{46, 47} In summary, the leakage from Q-DOPE vesicles at pH 9.0 can be attributed to the destabilization of the membrane caused by the high amounts of DOPE on the outer leaflets although no apparent phase transition occurs. Possibly the strong transverse lipid asymmetry also plays an important role.

We are aware that further experiments, e.g. detailed cryogenic transmission electron microscopy to track the morphology transitions of the vesicles, have to be performed to provide additional support for this hypothesis. Nevertheless, the proposed release pathway does not only fit well to the data presented here, but it also agrees with results from other groups that tested the release mechanism of liposomal systems that rely on the generation of an unstable lipid membrane in the context of the dependency of lipid vesicle aggregation on lipid concentration, as well as the need for a critical amount of stabilizing lipids that have to be cleaved to induce the process of contents release.^{7, 12} Moreover, our model is well suited to explain the dependency of release kinetics and overall release of entrapped molecules on the presence of PEG-DOPE and DOPE in Q-DOPE liposomes, to which we next turn.

Influence of PEG-DOPE and DOPE on Contents Release and Pathway

We found that adding DOPE to Q-DOPE liposomal compositions led to two effects that alter the kinetics of calcein release and the release pathway. In the presence of 3.8% DOPE, it was found that liposome contraction/deformation, relaxation, and burst-phase contents release occur earlier than for cases of lower DOPE concentration, because less DOPE has to be generated in the liposomes to reach the critical amount of DOPE required to induce these processes. These outcomes are in agreement with the model in Scheme 2. When 10% DOPE was incorporated into the membranes, the release kinetics are only slightly altered compared to that found for 3.8% DOPE, but the contraction/deformation and relaxation processes are not apparent. To explain this change in the calcein fluorescence trace for the 10% DOPE liposomes, one has to take into account the proposed release pathway in Scheme 2. The suggested deformation of the liposomes due to lipid asymmetry between the inner and outer membrane leaflets is caused by the formation of DOPE in the outer lipid layer. However, when a defined, sufficient amount of DOPE is also present in the inner leaflet, the difference in spontaneous curvatures of the inner and outer leaflets is not significant enough to lead to a shape change of the membrane, Figure 4B.

In contrast, the kinetics of liposomal contents release were not affected by the presence of PEG₂₀₀₀-DOPE up to the maximum amount investigated, 3.0% (see Figure 4A). Neither the starting point of liposome deformation/contraction, nor the time for the “relaxation” and the burst-phase release were altered significantly. However, the fraction of initially encapsulated calcein that was released was found to decrease from over 90% released (0%, 0.1% PEG₂₀₀₀-DOPE), to 30% (0.5% PEG₂₀₀₀-DOPE), and about 5–15% (3% PEG₂₀₀₀-DOPE).

First, it has to be mentioned that the concentrations of PEG₂₀₀₀-DOPE applied in this study are below the amount necessary for transition from the mushroom to brush regime.⁴⁸ Thus, differences concerning the calcein release are not related to conformational changes of the PEG chains.

The influence of PEGylated lipids on the induced release of molecules from liposomes has been explored in various studies of pH-sensitive liposomes,^{17, 18, 49} where it was found that a decreased amount of contents was released. Other investigations also revealed a decreased level of contents release due to the presence of PEG lipids, particularly for dehydration-induced⁵⁰ and melittin-initiated leakage⁵¹ of liposomes. The lower level of release was attributed to steric effects of the PEGylated lipids that hinder the required liposomal aggregation (dehydration-induced release) and stabilization of lamellar lipid structures (dehydration-induced and melittin-initiated release). In addition, conclusions about the influence of PEG lipids on triggered contents release can be drawn from studies of liposomal fusion. Similar to triggered liposomal release resulting from generation of H_{II}-prone lipids, the mechanism of fusion relies on aggregation of liposomes overcoming the hydration barrier and the ability of the lipid mixture to undergo an L to H_{II} phase transition. Moreover, fusion events are mostly accompanied by leakage. Holland *et al.* observed an inhibition of Ca²⁺-induced fusion of phosphatidylserine-containing liposomes caused by PEGylated dimyristoylphosphatidylethanolamine,⁵² in that study, the effect was attributed to the steric barrier provided by the PEG head group that prevented interliposomal bilayer contact. A study by Basáñez *et al.* gives detailed insights into the influence of PEGylated distearoylphosphatidylethanolamine, DSPE, on phospholipase C-triggered fusion.⁵³ They found that the use of increasing amounts of PEG-DSPE ($M_r(\text{PEG})=2,000$) led to slower rates of aggregation, and lipid and contents mixing. Steric repulsion between apposed PEG head groups hindered aggregation of liposomes containing more than 0.1% PEG-DSPE. Furthermore, lipid and contents mixing—events representing complete liposomal fusion—were inhibited at PEG-DSPE concentrations of 0.1–0.5%, with the decreased lipid/contents mixing being proposed to be due to a stabilization of the lamellar phase.

Stabilization of the lamellar phase and inhibition of liposomal contact formation are two possible explanations for the effects of PEG₂₀₀₀-DOPE on contents release from Q-DOPE/PEG₂₀₀₀-DOPE liposomes. If inhibition of liposome aggregation were the reason, one would expect to see a delay in onset of the burst phase; however, this was not observed in any case in our studies. Therefore, we exclude the possibility of PEG₂₀₀₀-DOPE influencing inter-vesicle contacts with the use of 0.1–3.0% of PEG₂₀₀₀-DOPE in the Q-DOPE LUVs. Importantly, the observed decrease in contents release can be well explained with the known ability of PEG-PE to stabilize the lamellar phase of H_{II}-prone lipid mixtures.⁵⁴ For instance, in mixtures of DOPE and PEG-DSPE ($M_r = 2,000$), a PEG-DSPE content of 8% was sufficient for the formation of a pure lamellar phase at 20 °C.⁵⁵ In contrast, several thiolytic- and pH-sensitive liposomal systems rely on the cleavage of PEG moieties from lipid membranes to generate non-bilayer structures so as to achieve release of encapsulated molecules; it was shown that liposome contents were not released until the amount of PEG lipid in the membrane dropped below 2.3%–3%.^{9, 55} In the work at hand, when triggered contents release from Q-DOPE vesicles containing PEG₂₀₀₀-DOPE occurs, the amount of PEG₂₀₀₀-DOPE in the membrane remains constant. Thus, upon aggregation of these liposomes, PEG₂₀₀₀-DOPE has to be excluded (moved) from liposomal contact areas due to steric arguments, and the lipid bilayer outside of the contact areas becomes enriched in PEGylated lipid. As a result, after a defined, critical concentration of PEG₂₀₀₀-DOPE is attained in this “non-aggregation” membrane area, stable lamellar structures are formed that prevent further efflux of calcein. Our data shows that full inhibition of contents release from reduced Q-DOPE liposomes is achieved when between 0.5% and 3.0% of PEG₂₀₀₀-DOPE is present.

Finally, the kinetics of contents release, and the total amount of contents released, can be controlled by using Q-DOPE formulations containing both PEG₂₀₀₀-DOPE and DOPE. When 0.5% PEG₂₀₀₀-DOPE is present in the vesicles, the total amount of contents released can be substantially augmented by incorporating DOPE in the LUVs, pointing to a possible change in the release pathway. Therefore, the results provide clues for future efforts in the rational design of a drug delivery system that has both long circulation times and an efficient release at the target site.

Impacts on Drug Delivery Systems

For the delivery of drug molecules to targets inside cells, these molecules must pass cellular membranes (plasma membrane or after uptake, the endosomal membrane). To overcome this obstacle, triggerable liposomes can be designed to fuse with the target membrane. For pH-sensitive liposomes, it was shown that they are able to fuse after activation, but only with other pH-sensitive liposomes and not with other (not pH-sensitive) membranes.⁵⁶ Moreover, preliminary studies on reduced Q-DOPE liposomes provide no support for the fusion of these vesicles. To achieve a fusogenic behavior of Q-DOPE membranes, the formulation of the liposomes can be altered. While the generated DOPE promotes hemifusion—an accepted precursor of fusion—cone-shaped lipids such as lysolipids or fatty acids are known to induce full membrane fusion.⁵⁷ An alternative approach may include the use of fusogenic peptides.⁵⁸

Using a PEG₂₀₀₀-PE concentration of 3% or more in Q-DOPE liposomes, a value typical for drug delivery systems so as to suppress their clearance by the reticuloendothelial system, will abolish the redox-triggered release from Q-DOPE vesicles. Even the incorporation of 50% DOPE into Q-DOPE liposomes containing 3% PEG₂₀₀₀-PE did not restore high release values. However, PEGylated PE itself represents a sheddable coating for liposomes: PEG lipids can diffuse out of the membrane and will insert into acceptor membranes if present (see reference 59⁵⁹ and references therein), i.e. liposomes not containing PEG lipids or cellular membranes. The kinetics of this process are dependent on the chain length and saturation level of the lipid anchors. For instance, PEG₂₀₀₀-DOPE, bearing monounsaturated lipid chains, has been proven to exchange into acceptor membranes, while PEG₂₀₀₀-distearoyl-PE having saturated lipids was not extracted from the donor membrane in the observed time frame.⁶⁰ Hence, PEG₂₀₀₀-DOPE might be well suited to shield Q-DOPE liposomes for the time it needs to reach the target while its concentration is lowered during circulation. A suppressed payload release by small amounts of PEG₂₀₀₀-PE remaining in the Q-DOPE vesicles might then be overcome by the incorporation of DOPE into the membrane of the liposome, as we have found for Q-DOPE vesicles containing 0.5% PEG₂₀₀₀-DOPE and 10 to 21% DOPE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Rafael Cueto for assistance with the light scattering measurements. This work was made possible in part by financial support from the US National Science Foundation (CHE-0910845) and the US National Institutes of Health (5R21CA135585).

References

1. Ishida T, Harashima H, Kiwada H. Liposome clearance. *Biosci Rep.* 2002; 22(2):197–224. [PubMed: 12428901]

2. Torchilin V. Multifunctional and stimuli-sensitive pharmaceutical nanocarriers. *Eur J Pharm Biopharm.* 2009; 71(3):431–44. [PubMed: 18977297]
3. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int J Nanomedicine.* 2006; 1(3):297–315. [PubMed: 17717971]
4. Ogawa Y, Epand RM. Effect of N-acyl-phosphatidylethanolamine on the membrane fusion between Sendai virus and liposome. *Biosci Rep.* 1997; 17(4):401–8. [PubMed: 9367055]
5. Metselaar JM, Bruin P, de Boer LW, de Vringer T, Snel C, Oussoren C, Wauben MH, Crommelin DJ, Storm G, Hennink WE. A novel family of L-amino acid-based biodegradable polymer-lipid conjugates for the development of long-circulating liposomes with effective drug-targeting capacity. *Bioconjugate Chem.* 2003; 14(6):1156–64.
6. Maruyama K. Intracellular targeting delivery of liposomal drugs to solid tumors based on EPR effects. *Adv Drug Deliv Rev.* 2011; 63(3):161–9. [PubMed: 20869415]
7. Zalipsky S, Qazen M, Walker JA 2nd, Mullah N, Quinn YP, Huang SK. New detachable poly(ethylene glycol) conjugates: cysteine-cleavable lipopolymers regenerating natural phospholipid, diacyl phosphatidylethanolamine. *Bioconjugate Chem.* 1999; 10(5):703–7.
8. Goldenbogen B, Brodersen N, Gramatica A, Loew M, Liebscher J, Herrmann A, Egger H, Budde B, Arbuzova A. Reduction-sensitive liposomes from a multifunctional lipid conjugate and natural phospholipids: reduction and release kinetics and cellular uptake. *Langmuir.* 2011; 27(17):10820–10829. [PubMed: 21819046]
9. Barenholz Y. Liposome application: problems and prospects. *Curr Opin Colloid In.* 2001; 6:66–77.
10. Aapro M, Bernard-Marty C, Brain EG, Batist G, Erdkamp F, Krzemieniecki K, Leonard R, Lluch A, Monfardini S, Ryberg M, Soubeyran P, Wedding U. Anthracycline cardiotoxicity in the elderly cancer patient: a SIOG expert position paper. *Ann Oncol.* 2011; 22(2):257–67. [PubMed: 20956616]
11. Boomer JA, Inerowicz HD, Zhang ZY, Bergstrand N, Edwards K, Kim JM, Thompson DH. Acid-Triggered Release from Sterically Stabilized Fusiogenic Liposomes via a Hydrolytic DePEGylation Strategy. *Langmuir.* 2003; 19:6408–15.
12. Guo X, MacKay JA, Szoka FC Jr. Mechanism of pH-triggered collapse of phosphatidylethanolamine liposomes stabilized by an ortho ester polyethyleneglycol lipid. *Biophys J.* 2003; 84(3):1784–95. [PubMed: 12609880]
13. Simões S, Moreira JN, Fonseca C, Düzgüneş N, de Lima MC. On the formulation of pH-sensitive liposomes with long circulation times. *Adv Drug Deliv Rev.* 2004; 56(7):947–65. [PubMed: 15066754]
14. Andresen TL, Davidsen J, Begtrup M, Mouritsen OG, Jørgensen K. Enzymatic release of antitumor ether lipids by specific phospholipase A2 activation of liposome-forming prodrugs. *J Med Chem.* 2004; 47(7):1694–703. [PubMed: 15027860]
15. Romberg B, Flesch FM, Hennink WE, Storm G. Enzyme-induced shedding of a poly(amino acid)-coating triggers contents release from dioleoyl phosphatidylethanolamine liposomes. *Int J Pharm.* 2008; 355(1–2):108–13. [PubMed: 18206323]
16. Zhu G, Mock JN, Aljuffali I, Cummings BS, Arnold RD. Secretory phospholipase A responsive liposomes. *J Pharm Sci.* 2011; 100(8):3146–59. [PubMed: 21455978]
17. Roux E, Passirani C, Scheffold S, Benoit JP, Leroux JC. Serum-stable and long-circulating, PEGylated, pH-sensitive liposomes. *J Control Release.* 2004; 94(2–3):447–51. [PubMed: 14976960]
18. Hong MS, Lim SJ, Oh YK, Kim CK. pH-sensitive, serum-stable and long-circulating liposomes as a new drug delivery system. *J Pharm Pharmacol.* 2002; 54(1):51–8. [PubMed: 11829129]
19. Ishida T, Okada Y, Kobayashi T, Kiwada H. Development of pH-sensitive liposomes that efficiently retain encapsulated doxorubicin (DXR) in blood. *Int J Pharm.* 2006; 309(1–2):94–100. [PubMed: 16364578]
20. Ong W, Yang Y, Cruciano AC, McCarley RL. Redox-triggered contents release from liposomes. *J Am Chem Soc.* 2008; 130(44):14739–44. [PubMed: 18841890]
21. Das S, Rand RP. Modification by diacylglycerol of the structure and interaction of various phospholipid bilayer membranes. *Biochemistry.* 1986; 25(10):2882–9. [PubMed: 3718927]

22. Gruner SM. Stability of Lyotropic Phases with Curved Interfaces. *J Phys Chem B*. 1989; 93:7562–7570.
23. Carpino LA, Triolo SA, Berglund RA. Reductive Lactonization of Sterically Methylated Quinone Propionic Acid Esters and Amides. *J Org Chem*. 1989; (54):3303–3310.
24. Hope MJ, Bally MB, Webb G, Cullis PR. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta*. 1985; 812:55–65. [PubMed: 23008845]
25. Allen TM, Cleland LG. Serum-induced leakage of liposome contents. *Biochim Biophys Acta*. 1980; 597(2):418–26. [PubMed: 7370258]
26. Pencer J, White GF, Hallett FR. Osmotically induced shape changes of large unilamellar vesicles measured by dynamic light scattering. *Biophys J*. 2001; 81(5):2716–28. [PubMed: 11606284]
27. Benachir T, Lafleur M. Study of vesicle leakage induced by melittin. *Biochim Biophys Acta*. 1995; 1235(2):452–60. [PubMed: 7756355]
28. Tejuca M, Dalla Serra M, Potrich C, Alvarez C, Menestrina G. Sizing the radius of the pore formed in erythrocytes and lipid vesicles by the toxin sticholysin I from the sea anemone *Stichodactyla helianthus*. *J Membr Biol*. 2001; 183(2):125–35. [PubMed: 11562794]
29. Jayaraman S, Song Y, Verkman AS. Airway surface liquid pH in well-differentiated airway epithelial cell cultures and mouse trachea. *Am J Physiol Cell Physiol*. 2001; 281(5):C1504–11. [PubMed: 11600413]
30. Guo X, Szoka FC Jr. Steric stabilization of fusogenic liposomes by a low-pH sensitive PEG-diortho ester-lipid conjugate. *Bioconjugate Chem*. 2001; 12(2):291–300.
31. Bentz J, Ellens H, Szoka FC. Destabilization of phosphatidylethanolamine-containing liposomes: hexagonal phase and asymmetric membranes. *Biochemistry*. 1987; 26(8):2105–16. [PubMed: 3620441]
32. Ellens H, Bentz J, Szoka FC. Destabilization of phosphatidylethanolamine liposomes at the hexagonal phase transition temperature. *Biochemistry*. 1986; 25(2):285–94. [PubMed: 3954998]
33. Seddon JM, Cevc G, Marsh D. Calorimetric studies of the gel-fluid (L beta-L alpha) and lamellar-inverted hexagonal (L alpha-HII) phase transitions in dialkyl- and diacylphosphatidylethanolamines. *Biochemistry*. 1983; 22(5):1280–9. [PubMed: 6838853]
34. McIntyre JC, Sleight RG. Fluorescence assay for phospholipid membrane asymmetry. *Biochemistry*. 1991; 30(51):11819–27. [PubMed: 1751498]
35. King MM, Cohen LA. Stereopopulation Control. 7. Rate Enhancement in the Lactonization of 3-(o-Hydroxyphenyl)propionic Acids: Dependence on the Size of Aromatic Ring Substituents. *J Am Chem Soc*. 1983; 105:2752–2760.
36. Perera, KLIS. Master's Thesis. Louisiana State University; Baton Rouge: 2012. Kinetic Studies on Lactonization of Quinone Propionic Acid Derivatives for the Development of Redox Responsive Liposomes.
37. Basáñez G, Nieva JL, Rivas E, Alonso A, Goñi FM. Diacylglycerol and the promotion of lamellar-hexagonal and lamellar-isotropic phase transitions in lipids: implications for membrane fusion. *Biophys J*. 1996; 70(5):2299–306. [PubMed: 9172753]
38. van Zanten JH, Monbouquette HG. Characterization of Vesicles by Classical Light Scattering. *J Colloid Interface Sci*. 1991; 146(2):330–336.
39. Huang C, Mason JT. Geometric packing constraints in egg phosphatidylcholine vesicles. *Proc Natl Acad Sci USA*. 1978; 75(1):308–10. [PubMed: 272647]
40. Amsberry KL, Borchardt RT. Amine prodrugs which utilize hydroxy amide lactonization. I. A potential redox-sensitive amide prodrug. *Pharm Res*. 1991; 8(3):323–30. [PubMed: 2052518]
41. Helfrich W. Elastic properties of lipid bilayers: theory and possible experiments. *Z Naturforsch C*. 1973; 28(11):693–703. [PubMed: 4273690]
42. Gruner SM, Cullis PR, Hope MJ, Tilcock CP. Lipid polymorphism: the molecular basis of nonbilayer phases. *Annu Rev Biophys Biophys Chem*. 1985; 14:211–38. [PubMed: 3890880]
43. Chernomordik LV, Kozlov MM. Protein-lipid interplay in fusion and fission of biological membranes. *Annu Rev Biochem*. 2003; 72:175–207. [PubMed: 14527322]

44. Miao L, Seifert U, Wortis M, Dobereiner HG. Budding transitions of fluid-bilayer vesicles: The effect of area-difference elasticity. *Phys Rev E*. 1994; 49(6):5389–5407.
45. Heuvingh J, Bonneau S. Asymmetric oxidation of giant vesicles triggers curvature-associated shape transition and permeabilization. *Biophys J*. 2009; 97(11):2904–12. [PubMed: 19948119]
46. Ralston E, Hjelmeland LM, Klausner RD, Weinstein JN, Blumenthal R. Carboxyfluorescein as a probe for liposome-cell interactions effect of impurities, and purification of the dye. *Biochim Biophys Acta*. 1981; 649(1):133–137. [PubMed: 7284432]
47. Oku N, Kendall DA, MacDonald RC. A simple procedure for the determination of the trapped volume of liposomes. *Biochim Biophys Acta*. 1982; 691(2):332–340.
48. Garbuzenko O, Barenholz Y, Prieve A. Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. *Chem Phys Lipids*. 2005; 135(2):117–29. [PubMed: 15921973]
49. Slepishkin VA, Simões S, Dazin P, Newman MS, Guo LS, Pedroso de Lima MC, Düzgüneş N. Sterically stabilized pH-sensitive liposomes. Intracellular delivery of aqueous contents and prolonged circulation in vivo. *J Biol Chem*. 1997; 272(4):2382–8. [PubMed: 8999949]
50. Käsbaumer M, Lasic DD, Winterhalter M. Polymer induced fusion and leakage of small unilamellar phospholipid vesicles: effect of surface grafted polyethylene-glycol in the presence of free PEG. *Chem Phys Lipids*. 1997; 86:153–159.
51. Rex S, Bian J, Silvius JR, Lafleur M. The presence of PEG-lipids in liposomes does not reduce melittin binding but decreases melittin-induced leakage. *Biochim Biophys Acta*. 2002; 1558(2): 211–21. [PubMed: 11779570]
52. Holland JW, Hui C, Cullis PR, Madden TD. Poly(ethylene glycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry*. 1996; 35(8):2618–24. [PubMed: 8611565]
53. Basáñez G, Goñi FM, Alonso A. Poly(ethylene glycol)-lipid conjugates inhibit phospholipase C-induced lipid hydrolysis, liposome aggregation and fusion through independent mechanisms. *FEBS Lett*. 1997; 411(2–3):281–6. [PubMed: 9271221]
54. Holland JW, Cullis PR, Madden TD. Poly(ethylene glycol)-lipid conjugates promote bilayer formation in mixtures of non-bilayer-forming lipids. *Biochemistry*. 1996; 35(8):2610–7. [PubMed: 8611564]
55. Johnsson M, Edwards K. Phase behavior and aggregate structure in mixtures of dioleoylphosphatidylethanolamine and poly(ethylene glycol)-lipids. *Biophys J*. 2001; 80(1):313–23. [PubMed: 11159404]
56. Bergstrand N, Arfvidsson MC, Kim JM, Thompson DH, Edwards K. Interactions between pH-sensitive liposomes and model membranes. *Biophys Chem*. 2003; 104(1):361–79. [PubMed: 12834854]
57. Chernomordik LV, Kozlov MM. Mechanics of membrane fusion. *Nat Struct Mol Biol*. 2008; 15(7):675–83. [PubMed: 18596814]
58. Torchilin VP, Levchenko TS. TAT-liposomes: a novel intracellular drug carrier. *Curr Protein Pept Sci*. 2003; 4(2):133–40. [PubMed: 12678852]
59. Romberg B, Hennink WE, Storm G. Sheddable coatings for long-circulating nanoparticles. *Pharm Res*. 2008; 25(1):55–71. [PubMed: 17551809]
60. Li WM, Xue L, Mayer LD, Bally MB. Intermembrane transfer of polyethylene glycol-modified phosphatidylethanolamine as a means to reveal surface-associated binding ligands on liposomes. *Biochim Biophys Acta*. 2001; 1513(2):193–206. [PubMed: 11470091]

Figure 1.

(A) Fluorescence of calcein during release from Q-DOPE liposomes at pH 7.4 containing 40 mM (blue) and 80 mM (black) calcein after the addition of sodium dithionite, as well as from liposomes containing 40 mM calcein at pH 9.0 (red). (B) Release of calcein from reduced Q-DOPE liposomes at different lipid concentrations. Measurements were performed at an initial encapsulated calcein concentration of 40 mM. Arrows indicate the addition of Triton X-100 to yield 100% calcein release.

Figure 2. Scattered light (red) and fluorescence intensity (black) for reduced Q-DOPE liposomes containing calcein at (A) pH 7.4 and (B) pH 9.0. Calcein release and light scattering experiments were both performed with the same fluorescence spectrometer and cuvettes.

Figure 3.

Intensities of scattered light of Q-DOPE vesicles measured at different angles divided by the intensity at 90° (squares, circles), as well as the calculated form factor $P(\theta)/P(90^\circ)$ (lines) plotted against scattering angle θ . Measured intensity of Q-DOPE vesicles at pH 9.0 before (filled circles) and 5 min after the addition of dithionite (hollow squares). Applying form factors for spherical unilamellar vesicles, the radius of LUVs could be fitted to about 40 nm (red line). Reduced Q-DOPE vesicles could not be fit to this model.

Figure 4.

(A) Calcein release curves from reduced Q-DOPE LUVs containing 0.0%, 0.1%, 0.5%, and 3.0% PEG₂₀₀₀-DOPE. (B) Calcein release from reduced Q-DOPE liposomes containing 0.0%, 3.8 %, and 10.0% DOPE. Arrows indicate the time when Triton X-100 was added to lyse any possible remaining LUVs.

Scheme 1.

Reduction-initiated cleavage of Q-DOPE **1a**. The hydroquinone intermediate **1b** undergoes an intramolecular cyclization reaction to yield the inverted-phase-forming DOPE **1c** and the cleaved lactone **1d**. $\text{R} = \text{C}_{18}\text{H}_{33}\text{O}$.

Scheme 2.

Proposed pathway for contents release from reduced Q-DOPE liposomes.