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Bilayer stabilization of phosphatidylethanolamine by *N*-biotinylphosphatidylethanolamine

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We have examined the ability of biotinylated phosphatidylethanolamine and similar lipids to stabilize the bilayer phase of polymorphic dioleoylphosphatidylethanolamine (DOPE). Sonicated lipid mixtures were characterized in terms of their aggregation state, size and ability to encapsulate and retain the fluorescent dye, calcein. Titration of DOPE with *N*-biotinyl-PE indicated that stable liposomes could be produced by sonication of DOPE based dispersions containing *N*-biotinyl-PE at concentrations greater than 8 mol%. These liposomes were relatively small, could efficiently encapsulate calcein, and showed minimal leakage upon prolonged storage at 4°C. Maleimido-4-(*p*-phenylbutyrate)-PE (MPB-PE) was equally effective at stabilizing the bilayer phase of DOPE whereas *N*-dinitrophenyl-PE and *N*-(dinitrophenyl-caproyl)-PE were relatively poor stabilizers, requiring at least 15 mol% for stabilization at pH 7.4. Differential scanning calorimetry of dielaidoylphosphatidylethanolamine (DEPE)/*N*-biotinyl-PE mixtures indicated that stabilizer concentrations as low as 2 mol% could abolish the L_α/H_{II} phase transition of DEPE.

Introduction

Unlike aqueous dispersions of most phospholipids which assume a lamellar structure in the form of lipid vesicles, PE assembles into nonlamellar phases through mechanisms which are incompletely understood. The ability of diunsaturated species of PE to adopt nonlamellar phases is favored by the relatively small molecular volume of the hydrated head group as compared to the larger volume of the fluid acyl chains. This results in a molecule which has been described by molecular shape theory [1] as a cone in contrast to the inverted cone shape displayed by lysolipids or a cylindrical shape assumed by most other phospholipids which have larger hydrated head groups such as the choline species.

Many different types of compounds have been found to be effective structural stabilizers of unsaturated PE bilayers. These include diacylphospholipids [2,14,15], lysolipids [3,4], gangliosides [5–7], haptenated lipids [8], fatty acids [9,10], cholesterol [11], cholesterol derivatives [16], detergents [4], membrane glycoproteins [8,12] and palmitylated antibody [13]. The amount of additive needed for bilayer formation strongly depends upon the nature of the membrane stabilizer and ranges from greater than 30 mol% for cholesterol [11] to 0.5 mol% for glycophorin [12]. Those which are the most efficient stabilizers in binary mixtures tend to have bulky and/or highly charged headgroups which, when packed in with PE, promote bilayer formation.

We have previously examined several different agents for their ability to facilitate PE-based liposome formation [2,3,6–10]. DOPE/TPE mixtures could be stabilized by 5 mol% of the ganglioside GM_1 but not by its degalactosylated product GM_2 [7]. In addition, the GM_1-stabilized liposomes could be catalytically lysed by treatment with β-galactosidase which converts GM_1 to GM_2 [7]. The strict requirement for specific carbohydrate content of a stabilizer has also been demonstrated for glycophorin A [19]. These observa-

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Abbreviations: DOPC, dioleoylphosphatidylcholine; PE, phosphatidylethanolamine; DOPE, dioleoyl-PE; DEPE, dielaidoyl-PE; DPPE, dipalmitoyl-PE; MPB, maleimido-4-(*p*-phenylbutyrate); DNP, dinitrophenyl; TNP, trinitrophenyl; DMPG, dimyristoylphosphatidylglycerol; DSC, differential scanning calorimetry; B-PE, *N*-biotinyl-PE; L_α, lamellar phase; T_H, bilayer to hexagonal phase transition temperature; H_{II}, hexagonal phase; Hepes, hydroxyethylpiperizineethanesulfonic acid; TPE, transphosphatidylated egg PE.

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tions indicate that the polar portion of the stabilizer is critical for the formation and preservation of the PE-based bilayer.

Target-sensitive immunoliposomes are also two component liposomes based on unsaturated PE but are both stabilized and targeted by using either haptenated lipid [8] or fatty acid derivatized antibody [13]. The haptenated liposomes become destabilized upon binding immobilized antibody and the liposomes which have antibody incorporated at its surface become destabilized simply upon binding with the target antigen. If the antigen is a cell surface protein then these liposomes can be used for site specific pericellular release of drugs [22]. This type of controlled release is especially important for delivery to cells which have low endocytic activity and are thus poor targets for the pH-sensitive liposomes. Antibody is incorporated into these target-sensitive immunoliposomes through hydrophobic interactions of antibody which has been covalently modified with fatty acid. Others have demonstrated that antibody can also be attached to liposomes through covalent or non-covalent attachment to derivatized membrane phospholipids [17]. Such approaches include conjugation of thiolated antibody to preformed liposomes containing MPB-PE [26] or of biotinylated antibody to liposomes containing *N*-biotinyl-PE using a streptavidin bridge [30]. These methods of derivatization to preformed liposomes facilitated the proper orientation of the antibody and avoids the use of detergent which is employed with acylated antibody. Conjugation of antibody to PE-based liposomes using these stratagies may also produce target-sensitive immunoliposomes composed of only three primary components: DOPE, a stabilizer such as derivatized PE, and antibody or other targeting ligand.

In the current study, we have examined the ability of several head group modified PE's to stabilize the bilayer phase and thus serve to mediate the formation of targetable and stable DOPE-based liposomes.

Materials and Methods

Materials

All phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL) in chloroform and used without further purification. Their purity was checked periodically by TLC using a solvent system of chloroform/methanol/water, (70:30:5, v/v). Lipid quantitation was determined by a phosphate assay [20]. All other chemicals were purchased from Sigma (St. Louis, MO). Hexadecyl [^{3}H]cholestanyl ether was prepared as described previously [21].

Differential scanning calorimetry

Ten mmol of lipid was dried down and placed in vacuo for at least 30 min to remove any residual solvent. Lipids were then hydrated with 2 ml of 10 mM Hepes buffer, pH 7.5 at 50°C for at least 6 h. The lipids were dispersed by vortex mixing and then allowed to cool to room temperature for at least 3 h prior to degassing and loading into the calorimeter. A MC-2 high-sensitivity differential scanning calorimeter (Microcal Co., Amherst, MA) was used in these studies. Samples were heated from 15°C to 85°C at a scan rate of 20°C/h.

Determination of PE stabilization

We have used two assays to evaluate the formation of stable lipid vesicles resulting from the admixture of DOPE and various head group-modified phosphatidylethanolamines. 90° light scattering was used to quantitate the aggregation state of the sample and entrapment of a self-quenching fluorescent dye was used to monitor the formation and stability of intact vesicles. For either assay, DOPE was first mixed with 3–15 mol% of the functionalized PE and dried as a thin film in a test tube. Samples were vacuum desiccated for at least 30 min to remove residual solvent. The lipid (1 mmol) was hydrated with 0.2 ml of 30 mM Hepes, 50 mM calcein, 2 mM EGTA, 0.02% NaN_3 (pH 7.4) with an osmolarity of 200 mosM. Unless stated otherwise the samples were allowed to hydrate 12–20 h at 25% under nitrogen atmosphere. Lipid was initially dispersed by vortex mixing and then sonicated in a bath type sonicator (Laboratory Supplies, Hicksville, NY) for 3 min and again for 2 min following a 5 h interval between sonications. Aliquots of the sample were removed within 5 h after the last sonication for light scattering analysis or subjected to gel filtration for quantitation of calcein entrapment.

Ninety degree light scattering measurements were performed on a Perkin-Elmer LS-5 fluorescence spectrophotometer equipped with a stirred and water-jacketed cuvette holder. The excitation and emission wavelengths were both set at 660 nm with slit widths of 10 nm and 3 nm, respectively. Samples were taken directly from the sonicated mixture and diluted 100-fold with 10 mM Hepes, 100 mM NaCl, 1 mM EGTA, 0.02% NaN_3 which had been filtered and was isotonic with the hydration buffer. Measurements were taken with a stirred cell at 25%. Quasi-elastic light scattering was employed to determine the size of liposomes using a Coulter N4SD submicron particle size analyzer (Hialeah, FL).

The efficiency of calcein encapsulation was determined fluorometrically following the removal of unencapsulated dye by gel filtration over Bio-Gel A 0.5 M columns (0.5 × 22 cm). Void-volume fractions containing lipids were diluted 1000-fold with 10 mM Hepes, 100 mM NaCl, 1 mM EGTA, 0.02% NaN_3 (pH 7.4; elution buffer) and calcein fluorescence was deter-

mined with the Perkin-Elmer LS-5 Self-quenching of calcein was evaluated as

$$\% \text{ quenching} = (1 - F_0/F_t)100$$

where F_0 and F_t are the fluorescence intensities before and after addition of 5% deoxycholate to a final concentration of 0.15% (w/v). Fluorescence was evaluated at an excitation wavelength of 490 nm (5 nm slit width) and emission of 520 nm (3 nm slit width) at a temperature of 25°C.

Liposome leakage measurements

The spontaneous release of calcein was monitored by diluting liposomes to a final concentration of 0.1 mM with 10 mM Hepes, 100 mM NaCl, 1 mM EDTA, 0.02% NaN_3 (pH 7.4) and incubating at the indicated temperature. After the given time period, approx. 1 nmol lipid was diluted into 2 ml of 10 mM Hepes, 100 mM NaCl, 1 mM EDTA, 0.02% NaN_3 (pH 7.4) at room temperature for fluorescence measurements as described above.

Calcein release was calculated as:

$$\% \text{ Release} = \frac{(F_t/F)(F_x - F_0)}{F_s - F_0}$$

where F_0 is the fluorescence intensity of the liposomes in HBS at time zero. F_x is the fluorescence intensity after a given time x. F_t and F are the total intensities after deoxycholate treatments of the original or incubated samples, respectively. F_t/F is a correction term which is used to minimize the pipeting and other systematic errors between measurements.

Results

L_α-phase stabilization of DEPE

High-sensitivity differential scanning calorimetry was used to monitor the phase transition of DEPE. Long chain unsaturated species of PE exhibit polymorphism of which three phases can be readily detected by calorimetry. Pure DEPE (di 18:1, *trans*) exhibited a chain melting transition (T_c) at 37.9 ± 0.3°C ($n = 5$) and an L_α to hexagonal phase (H_{II}) transition (T_{II}) at 67.4 ± 1.0°C ($n = 5$). Addition of *N*-biotinyl-egg-PE (B-PE) progressively increases the T_{II} and decreases the enthalpy of the transition until it is abolished at concentrations of this stabilizer greater than 2 mol% (Fig. 1). No significant effect on T_c was observed at these concentrations. We have also examined other PE derivatives for their capacity to prevent the transition of DEPE into the non-bilayer hexagonal phase. DEPE with three mol% maleimido-4-(*p*-phenylbutyrate) egg PE (MPB-PE) or *N*-(trinitrophenyl-caproyl)-egg PE (TNPcapPE) failed to exhibit any L_α/H_{II} transition at

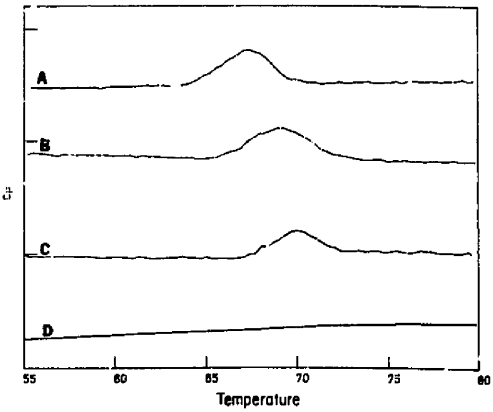

Fig. 1. DSC of the L_α to H_{II} transition of DEPE. Samples of 5 mM DEPE in 10 mM Hepes (pH 7.4) were heated at a rate of 20 C°/h after prolonged hydration at 50°C. The samples contained (A) 0, (B) 0.5, (C) 1.0 or (D) 2.0% B-PE which was mixed with DEPE in the organic phase prior to liposome formation. Samples with 2.0 mol% B-PE gave no detectable transition (not shown). The chain-melting transition occurred around 38°C for all samples.

the temperature range tested (not shown) and thus also served to stabilize the bilayer phase of the lipids.

Comparative stabilization of DOPE liposomes

DOPE at neutral pH and ambient temperature adopts a nonbilayer hexagonal phase unless secondary components (other than water) exhibiting shape complementarity are included [1]. The bilayer to hexagonal phase transition for DOPE is 8–10°C [1] which makes it a good base lipid for evaluating its stability and potential destabilization at ambient or physiological temperatures, in contrast to DEPE. We have tested several PE's having a modified polar head group for their ability to stabilize DOPE. Liposome stabilization potential was monitored by light scattering analysis of lipid mixtures following dispersion by sonication. Light scattering is dependent upon both the size and number of particles. Lipids in the H_{II} phase form a small number of large aggregates and scatter much less light than the highly turbid samples seen at low mole fractions of the putative stabilizers. Sonication of truly stable lipid mixtures produces small homogeneous vesicles that scatter little light as seen with stabilizer concentrations exceeding 10 mol% (Fig. 2). At low mol% stabilizer turbid suspensions were formed by sonication which were unstable and over time would eventually coalesce into large aggregates. Of the PE derivatives examined, DNPcapPE, TNPcapPE, MPE-PE, and B-PE, only DNPcapPE failed to stabilize DOPE-based liposomes in the 8–12 mol% range (not shown). Only at 15 mol% DNPcapPE were stable transluscent vesicles formed. DNP-PE without the caproyl spacer was

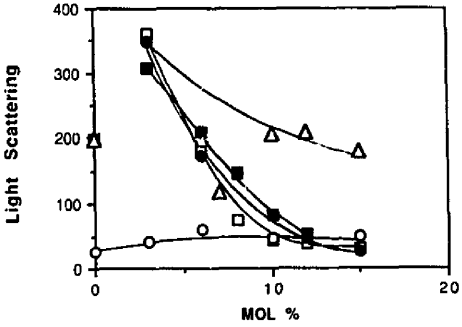

Fig. 2. Stabilization of DOPE with headgroup derivatives of PE. 90° light scattering was measured for DOPE with various mol% B-PE (■), Bcap-PE (□), MPB-PE (●), or TNP-caproyl-PE (△) or for DOPC with MPB-PE (○).

relatively ineffective even at 15 mol% (not shown). The trinitrophenyl derivative of capPE was significantly more effective at DOPE stabilization but was still not as efficient as those with the other head group modifications. The maleimide and two biotin derivatives all showed comparable stabilization potential. DOPC forms stable bilayers alone and addition of MPB-PE has no effect on the light scattering by these liposomes (Fig. 2).

Bilayer stabilizing capacity was also evaluated by monitoring dye encapsulation efficiency. Calcein fluorescence is self-quenching at high concentrations of the dye. For example, calcein entrapped in DOPC liposomes is approx. 80% quenched (see Methods) when prepared in a buffer containing 50 mM calcein. Liposomes having equally high encapsulation efficiencies can also be prepared with DOPE when it is mixed with an effective structural stabilizer. Fig. 3 illustrates the relative encapsulation efficiency for five stabilizers

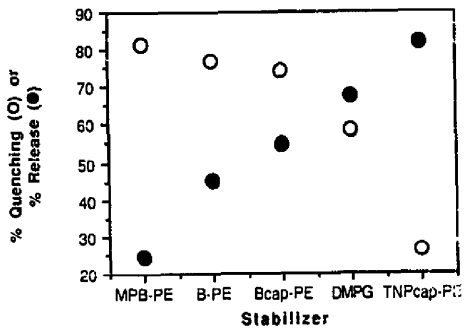

Fig. 3. Effect of stabilizer on dye entrapment and leakage in DOPE liposomes. DOPE was mixed with 8 mol% of the given stabilizing lipid and dye entrapment efficiency (% calcein quenching) was determined as described in Methods (○). Leakage was determined by evaluating the % of dye released following a 12 day incubation at room temperature (●)

mixed with DOPE at a concentration of 8 mol% MPB-PE and the biotin derivatives produce vesicles with high calcein entrapment as determined by the high quench values. The stabilizing capacity of DMPG has been previously demonstrated at 10 mol% and above [3]. At 8 mol% it was substantially better than TNPcapPE but not as effective as the others. Available acyl chain variants of the TNPcapPE derivative were tested for their liposome-stabilization capacity. When mixed with DOPE at 10 mol%, the dipalmitoyl derivative gave higher quench values than did the dioleoyl or egg species (data not shown). Thus the stabilization potential depended upon both the nature of the head group and the acyl chain composition.

The long term structural stability of these liposomes was followed by monitoring their leakage rate over a 12 day period at 25°C. The release rate of calcein was inversely proportional to the initial quench value with TNPcapPE the most leaky and the MPB-PE liposomes the least (Fig. 3). This indicates that those samples which were most efficient at entrapping dye were also the most efficient at retaining it over long periods. The biotin-PE derivatives, although initially having nearly identical quench values as MPB-PE are more leaky than the maleimide derivative at ambient temperature. Dye leakage from the liposomes was temperature dependent. Liposomes prepared with DOPE/B-PE (9:1, mol/mol) showed virtually no leakage over a one month period when stored at 4°C. However, upon incubation at 25°C for 24 h the percent quenching dropped to 70% (from an initial value of 77%) and was only 51% after 24 h at 37°C.

Stabilization by N-biotinyl-PE

B-PE was used as the primary stabilizer in all subsequent studies designed to evaluate the optimal parameters for DOPE bilayer stabilization. Calcein quenching and liposome size were monitored to determine the mole fraction of B-PE needed for maximal bilayer stability. Dye entrapment had maximized by 5–6 mol% although this formulation was still quite leaky over a 5-day period compared to vesicles containing 8 mol% (Fig. 4B). When liposome size was examined by dynamic light scattering as a function of the stabilizer concentration it was found that the liposomes had also reached a minimal size plateau at 8 mol% (Fig. 4A). Thus stable DOPE liposomes can be formed with the inclusion of as little as 8 mol% B-PE which makes this one of the most potent lipid stabilizers tested to date. Similar results were found with MPB-PE as the stabilizer.

Vesicle parameters were monitored as a function of hydration time and temperature for mixtures of DOPE/B-PE (9:1, mol/mol). Dry lipid films were hydrated with buffer at 2 or 25°C and then incubated at the indicated time and temperature prior to a 5 min

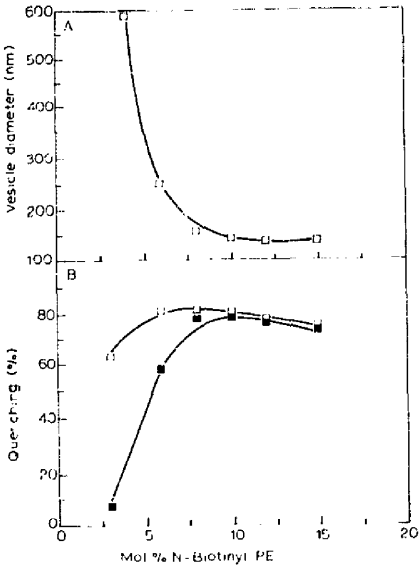

Fig. 4. Stabilization of DOPE with B-PE. In panel A, the average size of the liposomes from the corresponding samples was followed by dynamic light scattering. In panel B, calcein entrapment was followed as a function of stabilizer concentration. Free dye was removed by gel filtration after sonication and quenching was determined on samples immediately (□) or 5 days later (■).

sonication. Free calcein was then immediately removed by gel filtration and the quench value determined as described in Methods. Although DOPE alone is known to be poorly hydrated [23], the DOPE/B-PE mixtures hydrates almost instantaneously as determined through a functional measurement of dye entrapment (Fig. 5). The ability to entrap dye was equally efficient when hydrated at either 2 or 25°C which is below or above the T_{II} for pure DOPE (8–10°C). The average size of the liposomes was relatively independent of hydration conditions. When the longer term stability of liposomes which had been hydrated for up to 8 h was examined after an eleven day incubation at room temperature then differences in hydration conditions did become evident. The longer the samples had been hydrated the more leaky they had become. The samples which had been hydrated at 25°C were also somewhat more stable than those which had been hydrated at the lower temperature. Increasing the B-PE concentration to 12 mol% did not significantly alter the initial level of dye entrapment although these liposomes were slightly smaller and less leaky than those with 10 mol% (data not shown). In addition, if the liposomes were sonicated for an additional 15 min (20 min total) then the resulting liposomes were smaller but they were also much more leaky over a 10 day period.

During the process of bilayer formation DOPE-based lipid mixtures undergoes several structural changes. For example, liposomes composed of DOPE stabilized with oleic acid have recently been observed to increase in size as the result of spontaneous fusion following sonication to form SUV's of various sizes (Liu, D., personal communication). Likewise, we have monitored the characteristics of the sonicated DOPE/B-PE mixtures to determine if these liposomes with a double-chain stabilizer exhibit similar growth kinetics. Well hydrated lipid films were sonicated and fractionated at the various times following sonication to remove unencapsulated calcein. Calcein quenching and size were monitored immediately after the gel filtration. As illustrated in Fig. 6 the B-PE-stabilized liposomes are also unstable for a period following sonication. During this time both the size and percent quenching increase and then level off at about 24 h.

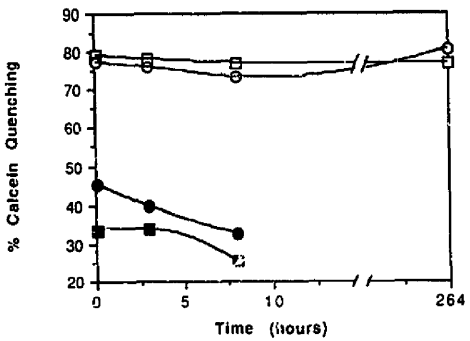

Fig. 5. Effect of hydration time and temperature on dye entrapment and leakage. Films of DOPE/B-PE (92/8) were hydrated at 2°C (□, ■) or 25°C (○, ●). After the indicated interval the mixtures were sonicated and immediately fractionated and assayed for dye entrapment (□, ○) Stability was determined by reevaluating dye quenching after an 11 day incubation at room temperature (■, ●).

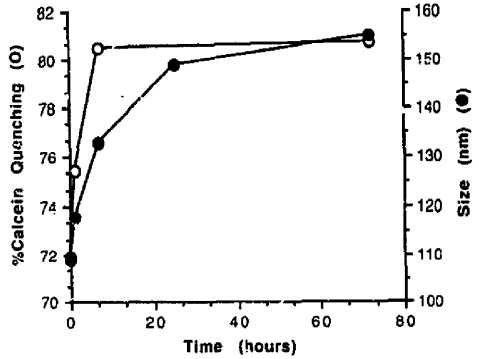

Fig. 6. Liposome changes with time post-sonication. DOPE/B-PE (9:1, mol/mol) were hydrated at room temperature. The mixtures were sonicated for 5 min and after the indicated interval they were fractionated on Bio-Gel A-1.5 and assayed for size (●) and dye entrapment (○).

The size and dye entrapment increase at similar rates with a net increase in size of 40% and a 12% increase in calcein quenching.

The growth in liposome size following sonication could be the result of simple aggregation or fusion of two or more liposomes. Although we have not yet experimentally differentiated the two possibilities, the degree of growth suggests that fusion between two liposomes could be occurring. A doubling of the surface area of a spherical liposome should result in a 41% increase in the diameter of the sphere which is very close to what we observed. The PE/OA liposomes which also exhibit a net size increase following sonication appeared to be fusion products rather than aggregates by electron microscopy [34]. In addition, PE-based liposomes are known to fuse much more rapidly under conditions where the stabilizer has been rendered ineffective [9,35]. The corresponding increase in calcein quenching with time post-sonication is not due to more dye becoming entrapped prior to fractionation but rather is probably due to reduced leakage of the liposomes during the interval between column fractionation and the fluorescence measurements. This change as well as the size increase is potentially due to a reannealing process in which the lipids are rearrranging into a more stable interaction [37].

Discussion

The lipid derivatives which were tested in this study for their ability to facilitate PE bilayer formation. All had bulky, polar head groups relative to native PE. The degree of charge on the lipid influences its overall shape in lipid mixtures through its effects on degree of hydration and hydrogen bonding. Although none of the introduced functional groups are themselves charged, the PE derivatives have a net negative charge since the now functionalized amine is no longer protonatable. For example, incorporation of 2.4 mol% MPB-PE into a liposome formulation increases the zeta potential (more negative) by 73% [24]. However, the active maleimide group of MPB [25] and MPB-PE [26] is labile, especially at pH values greater than 7, but the structure of the resulting thiol-unreactive product is unknown. One potential mechanism for maleimide inactivation is ring hydration and opening to become carboxylated which could produce a second negative charge on the molecule. The more rapid inactivation of maleimidobenzoic acid esters compared to the carboxy-cyclohexylmethyl-maleimide esters is consistent with this mechanism [25]. Thus the MPB-PE stabilizer may have a net charge of -2 under these conditions. Other negatively charged phospholipids such as phosphatidylserine have also been shown to stabilize DOPE bilayers although at higher levels than which is needed for MPB-PE [32]. Negatively charged lipophilic anticancer drugs such as araCDP-diglyceride and methotrexate-DMPE can also stabilize DOPE for vesicle formation (Constantinides, P. and Wright, S., unpublished observations). MPB-PE (egg PE or DOPE derivative) is among the most potent liposome stabilizers of any PE derivative which we have yet tested. This compound produces stable nonleaky liposomes at concentrations as low as 8 mol% which is equivalent to a stabilizer/PE ratio of 1:12. This is in contrast to the phenolic derivatives (DNP and TNP-PE) which, although having an aromatic ring like MPB-PE, lacked the potentially charged maleimide and were relatively poor stabilizers at these concentrations.

The ability to form stable liposomes composed of DOPE and *N*-biotinyl-PE immediately after buffer addition indicates that the lipid mixture is hydrated much more rapidly than DOPE alone. It is well known that lipids which favor the inverted hexagonal structure, especially PE, are very poorly hydrated compared to bilayer-forming lipids such as PC (Ref. 28 and refences therein). There is some evidence which suggests that it is not the head group size which determines the phase preference but rather the extent of head group hydration [28].

The similar stabilization potency of *N*-biotinyl-PE and *N*-(biotinyl)-caproyl-PE suggests that the proximity of the functional biotin moiety to the bilayer surface is not crucial for bilayer stabilization. In fact, the ability of liposomes containing B-PE to bind exogenous avidin or streptavidin [29] indicates that even the derivative without the caproyl spacer has an extended structure which is capable of protein binding. Avidin-coated liposomes can then be coated with biotinylated antibody to form immunoliposomes [30]. Such immunoliposomes may be potentially target sensitive [13,22] if they are composed primarily of PE rather than PC. In fact we have found that B-PE-stabilized DOPE liposomes which have acylated antibody incorporated in its membrane can be bound and lysed upon incubation with anti-mouse antibody-coated particles [31]. These liposomes may be useful for diagnostic immunoassays or for drug delivery in formats where liposome contents release upon antigen binding is desirable.

Acknowledgments

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