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Stability and pH sensitivity of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles

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Abstract

The bilayer stabilization effect of sulfatide and the pH sensitivity of sulfatide-containing 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) small unilamellar vesicles were examined by light scattering and the release of entrapped calcein. At 30 mol% sulfatide, stable DOPE/sulfatide vesicles were formed at the physiological pH and their stability was preserved in the presence of human plasma. These vesicles were found to be pH-sensitive and became leaky at pH 6.0 or when there was a pH-gradient across the membrane bilayer. Under such conditions, the amount of calcein released after 24 h incubation at 37°C was increased by one-fold compared to that found at pH 7.4. Our results suggest that the hydration and partial dehydration of the headgroup of sulfatide upon changing pH play an essential role in determining the pH sensitivity of DOPE/sulfatide vesicles, while the importance of the condensing effect of the glycolipid on membrane bilayer is less significant.

Keywords: Sulfatide; pH-Sensitive liposome; Fluorescence; Phosphatidylethanolamine

1. Introduction

The use of pH-sensitive liposomes has been extensively studied as vehicles for the cytoplasmic delivery of drugs [1,2], enzymes [3], protein toxins [4] and nucleic acids [5,6]. It is now clear that endocytosis is the principal pathway by which liposomes enter the cell. Earlier studies had suggested that the pH of the lumen of endocytic vesicles is mildly acidic [7]. Thus, it is possible for the pH-sensitive liposomes to destabilize or fuse with the endosome membrane, thereby releasing their entrapped contents into the cytoplasm before they are destroyed in lysosome [8].

In general, pH-sensitive liposomes are prepared in such a way that phosphatidylethanolamine (PE) is the predominant component [9]. Other amphiphiles, such as oleic acid [10,11], cholesteryl hemisuccinate [12], gangliosides [13,14] and diacylsuccinylglycerols [15], are required as stabilizers for the PE bilayer phase. These amphipathic additives share some common structural features such as possession of bulky, hydratable, polar and/or negatively charged headgroups. Protonation and/or dehydration of these headgroups by a reduction in the pH would cause the liposomes to become destabilized as the PE component reverts to the hexagonal (H_{II}) phase [16–18]. Applications for therapeutic drug delivery require that the PE-based liposomes should possess significant pH sensitivity and be relatively stable during storage and in the blood circulation.

In addition to their instability upon exposure to serum or plasma [19], phosphatidylcholine (PC)-based liposomes are generally considered to be non-pH-sensitive and hence unsuitable for cytoplasmic delivery. However, there are exceptions [20,21]. Recently, Viani et al. [22] have demonstrated that at pH 7.4 the plasma stability of a liposome composed of egg PC is enhanced considerably by the presence of bovine brain sulfatide. It has been suggested that, by their ability to rigidify acyl chains of the membrane lipids, sulfatides are able to increase the stability of egg PC vesicles at the physiological pH [23], while the destabilization at the acidic pH is probably due to the plasma-induced formation of sulfatide-rich domains in the liposomes [22].

Sulfatide is an acidic glycolipid consisting of a hydrophobic ceramide and a hydratable galactose residue sulfated at the C(3) position. It appears to possess the basic

Abbreviations: PE, phosphatidylethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DNS-Lys, N^{ϵ}-dansyl-L-Lys; PC, phosphatidylcholine

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characteristics of a stabilizer for the PE bilayer structure. It would therefore be interesting to examine how the stability of PE liposomes can be affected by the presence of sulfatides. We had also been led to study the sulfatide-containing PE liposomes by the fact that this glycolipid seems to be able to assist liposomes in passing through the blood-brain barrier [24–28], implying that targeting delivery of sulfatide-containing liposomes to certain tissues such as brain may be achieved by simply manipulating their lipid compositions.

In the present study, we examined particularly the effect of sulfatides on the stability of DOPE small unilamellar vesicles in human plasma. The stability of liposomes was estimated in terms of the leakage of an entrapped fluorescent marker, calcein. Unlike carboxyfluorescein, the fluorescence intensity of calcein is independent of pH in the range employed in this study [29]. Our results show that the small unilamellar vesicles of DOPE/sulfatide (70:30, mol/mol) are pH-sensitive and stable in plasma at the physiological pH. The possible mechanisms for sulfatides to stabilize the DOPE bilayer vesicles at the physiological pH and the subsequent destabilization of such vesicles at acidic pH are discussed.

2. Material and methods

2.1. Materials

DOPE (~ 99%), calcein, human blood plasma and sulfatides from bovine brain were purchased from Sigma (St. Louis, MO). The amount of nonhydroxy and 2-hydroxy fatty acyl chains of the sulfatides is 69.5% and 30.5%, respectively, as determined by thin-layer chromatography using solvent system of chloroform-methanol-acetone-acetic acid-water (8:2:4:2:1, v/v). *n*-(9-Anthroyloxy) fatty acids ($n = 2,7,16$) and N^{ϵ}-dansyl-L-Lys (DNS-Lys) were from Molecular Probes (Eugene, OR). All other reagents were of Analytical Reagent grade and deionized water was used for all the experiments.

2.2. Preparation of bilayer vesicles

DOPE small unilamellar vesicles containing up to 50 mol% sulfatides were prepared by sonication under nitrogen as described by Thulborn and Sawyer [30]. Briefly, appropriate amounts of DOPE and sulfatides were transferred from their stock solutions into glass tubes and dried by evaporation under nitrogen stream. The samples were then stored under vacuum for 24 h at 4°C. The thin lipid film formed on the wall of glass tubes was hydrated with aqueous buffers (2 mM TES, 2 mM histidine, 150 mM NaCl, pH 6.0 or 7.4) and sonicated under nitrogen for 10 min (30 s on and 30 s off for each cycle) with a Heat Systems Sonicator XL at maximum power output. Temperature was controlled with an ice water bath and no lipid degradation was found as examined by thin-layer chromatography. The total lipid concentration was 0.2 mM. For samples used in liposome stability assay, calcein was dissolved in water at an appropriate pH and its final concentration was 175 mM. A 30 min sonication procedure was adopted because a higher initial lipid concentration (1 mM) was used. Calcein loaded in lipid vesicles was separated from its free form by gel filtration on Sephadex G-50 column using the above buffers as eluents [31]. A trace amount of nonexchangeable fluorescent phospholipid marker, 2-(3-(diphenylhexatrienyl) propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine, was included to calibrate the concentration of membrane lipids. The final total lipid concentration of the calcein-loaded liposomes was adjusted to 0.2 mM and the leakage experiments were started immediately thereafter. The formation of stable bilayer vesicles was shown by the 90° light scattering measured at 660 nm using a Perkin-Elmer LS-50B spectrofluorometer [32]. A pH-gradient across the lipid bilayer was generated by adding HCl to vesicles pre-formed at the physiological pH and the external pH of the bilayer vesicles was measured with a Beckman Φ50 pH meter.

2.3. Vesicle stability assay

The stability of DOPE bilayer vesicles containing up to 30 mol% sulfatides was studied by determining the leakage of entrapped calcein. The change in fluorescence intensity was measured with the Perkin-Elmer LS-50B spectrofluorometer. Excitation and emission wavelengths were 495 nm and 516 nm, respectively. The total fluorescence intensity was obtained after the vesicles were lysed by the addition of Triton X-100 (final concentration: 1%). Release of the entrapped calcein from the bilayer vesicles was calculated with the equation:

$$\% \text{ release} = 100 \times (F - F_o)/(F_t - F_o)$$

where F is the calcein fluorescence intensity at any given time, F_o is the initial fluorescence intensity and F_t is the total fluorescence intensity measured after the lysis of liposomes in the presence of 1% Triton X-100.

2.4. Effect of plasma on vesicle stability

DOPE/sulfatide bilayer vesicles were mixed with equal volume of human plasma at the same pH to give a final lipid concentration of 0.1 mM. The increase in calcein fluorescence intensity was then monitored with the Perkin-Elmer LS-50B spectrofluorometer. All measurements were corrected for light scattering caused by the plasma.

2.5. Fluorescence anisotropy

Steady-state fluorescence anisotropies of the *n*-anthroyloxy fatty acids ($n = 2,7,16$) in DOPE/sulfatide vesicles

were measured with the same spectrofluorometer. Excitation and emission wavelengths were 360 nm and 440 nm, respectively. Stock solutions (~ 1 mM in methanol) of the fluorescent probes were added as small aliquots to vesicle suspensions so that the final probe:lipid ratio was 1:200. The samples were then incubated for 2 h at room temperature in the dark. The total lipid concentration was 0.1 mM.

2.6. Emission spectra of DNS-Lys

DOPE/sulfatide (70:30, mol/mol) vesicles (0.2 mM) were prepared in the presence of 2 mol% DNS-Lys. The excitation wavelength was 330 nm and the emission spectrum was measured from 400 to 580 nm with a scan rate of 60 nm/min. The excitation and emission slit widths were 5 nm. Temperature was controlled using a circulating water bath and measured with a thermocouple immersed in the sample cuvette.

3. Results

3.1. DOPE vesicles stabilized by sulfatides

The instability of PE vesicles at physiological pH in isotonic buffers has been well documented. Stable bilayers, however, could be formed under similar conditions in the presence of a second component to PE. Thus, formation of DOPE small unilamellar vesicles with the incorporation of sulfatides was initially studied by measuring the 90° light scattering of the vesicle suspension. As shown in Fig. 1, light scattering at either pH 7.4 or pH 6.0 was substantially reduced upon increasing the content of sulfatides in DOPE/sulfatide vesicles, suggesting that sulfatides promoted the formation of stable bilayer vesicles [32]. However, the amount of sulfatides required to stabilize the DOPE bilayer vesicles was found to be pH-dependent. The minimal mol% of sulfatides needed to obtain stable DOPE bilayer vesicles was approx. 25 mol% at pH 7.4 and 50 mol% at pH 6.0, respectively.

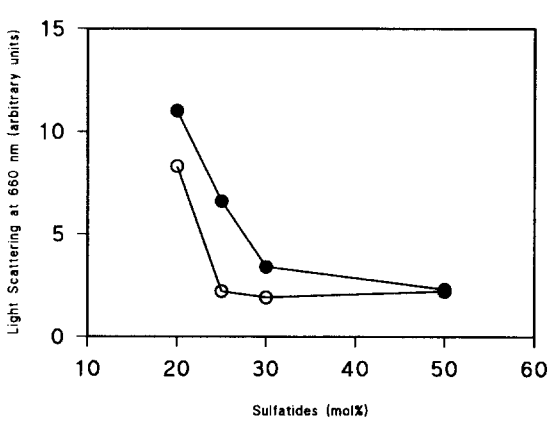

Fig. 1. Stability of DOPE/sulfatide small unilamellar vesicles as determined by 90° light scattering at pH 6.0 (●) and 7.4 (○), respectively. The experiments were conducted at 25°C.

3.2. Effects of plasma on vesicle stability

Calcein-loaded vesicles were used to study the effects of sulfatides on the stability and pH sensitivity of the DOPE/sulfatide bilayer structure. It was previously reported that the effect of a full blood serum on the stability of phospholipid liposomes could be observed in a 25% plasma [31,33]. Thus, human plasma with the appropriate pH value was directly added to a magnetically stirred cuvette containing the lipid vesicles to a final concentration of 50%. Fig. 2 shows the time-courses of calcein release from various DOPE/sulfatide small unilamellar vesicles at 25°C. Under most circumstances, the kinetics of calcein release was a biphasic function with an initial fast leakage in the first 5 min, followed by a prolonged slow release of the fluorescent dye. It is clear that the incorporation of sulfatides into the DOPE vesicles would lead to a marked dose-dependent decrease in calcein release. At pH 7.4, for example, the amount of calcein released from DOPE/sulfatide (90:10, mol/mol) vesicles was more than 4-fold higher than that from the bilayer vesicles containing 30 mol% sulfatides (Fig. 2, panel A). On the other hand, the amount of calcein released from DOPE/sulfatide (70:30, mol/mol) vesicles in the presence of plasma was similar to that released in plasma-free buffer (data not shown). In other words, the DOPE/sulfatide (70:30, mol/mol) vesicles were virtually insensitive to the presence of plasma at the physiological pH.

Fig. 2 also shows that a reduction in the pH of the aqueous medium would result in a marked destabilization of the DOPE/sulfatide vesicles (panel B). For instance, the amount of calcein released from DOPE/sulfatide (70:30, mol/mol) vesicles at pH 6.0 was approx. 3-fold higher than that released at pH 7.4, suggesting that significant pH-sensitivity was retained by the sulfatide-containing DOPE liposomes, particularly by those with higher sulfatide content (e.g., 30 mol%). In order to mimic the acidic environment experienced by liposomes in the endocytic pathway, a pH-gradient was generated across the membrane bilayer by adding HCl solution into the liposome suspension [34]. In this way, a pH of 6.0 in the external medium was obtained while the pH of the internal vesicle cavity remained at 7.4. Fig. 2 (panel C) shows the effect of sulfatides on the kinetics of calcein leakage from DOPE vesicles under the stress of pH-gradient. Both the kinetic characteristics and the extent of calcein leakage from vesicles in the presence of pH-gradient were found to be very similar to those at pH 6.0 (Fig. 2, panel B), suggesting that the DOPE/sulfatide bilayer structure could also be destabilized by the pH-gradient.

The effect of plasma (50%) on calcein release from DOPE/sulfatide (70:30, mol/mol) vesicles was also monitored at 37°C for 24 h (Fig. 3). Similar to what had been

found at 25°C, the kinetics of calcein release was again characterized by a biphasic function. There was a fast increase in calcein fluorescence in the first 2 h of incubation. After that, the DOPE/sulfatide vesicles appeared rather stable at the physiological pH, with a total of 17% of the entrapped calcein being released after 24 h at 37°C. In contrast, the DOPE/sulfatide vesicles at pH 6.0 remained leaky to the fluorescent dye after the initial 2 h incubation, albeit at a much slower rate. Again, the kinetics and extent of calcein release in the presence of pH-gradient were similar to those at pH 6.0, with approx. 37% of the entrapped calcein released after 24 h at 37°C in both cases. Thus, the amount of calcein released from DOPE/sulfatide (70:30, mol/mol) vesicles at the acidic pH or in the presence of the pH-gradient doubled that from the same vesicles at the physiological pH (Fig. 3).

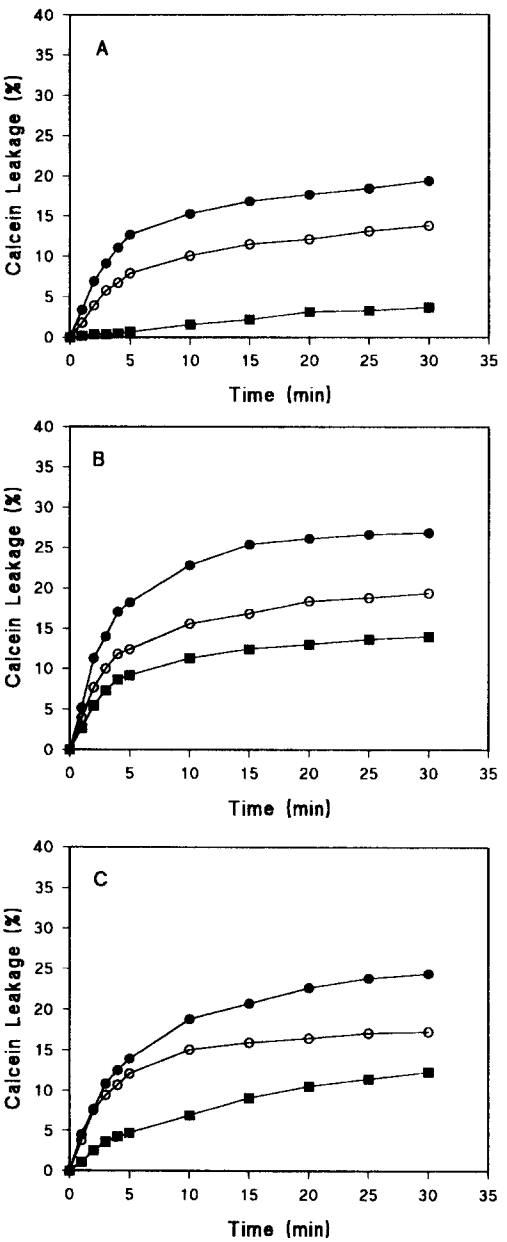

Fig. 2. Stability of DOPE/sulfatide small unilamellar vesicles in 50% plasma as followed by the release of calcein at 25°C. The experiments were conducted at (A) pH 7.4, (B) pH 6.0 or (C) in the presence of a pH-gradient generated as described in Section 2. The levels of sulfatides relative to the total lipids were: (●), 10 mol%; (○), 20 mol%; (■), 30 mol%.

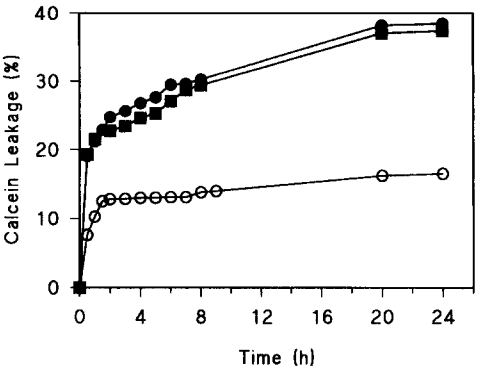

Fig. 3. pH dependence of the stability of DOPE/sulfatide (70:30, mol/mol) small unilamellar vesicles in 50% plasma as followed by the release of calcein at 37°C. (●), pH 6.0; (○), pH 7.4; (■) in the presence of a pH-gradient (refer to Section 2 for details).

3.3. Fluorescence anisotropy studies

The steady-state fluorescence anisotropy of *n*-(9-anthroyloxy) fatty acids has been widely used as a means of estimating membrane fluidity gradient from the surface to the core of a bilayer structure [35,36]. In order to determine if the membrane stability was related to its fluidity, the steady-state fluorescence anisotropies of three *n*-(9-anthroyloxy) fatty acids ($n = 2,7,16$) in DOPE/sulfatide (70:30, mol/mol) bilayer vesicles were measured at either pH 6.0 or pH 7.4. Overall, there was no difference between the anisotropies of the series of probes measured at both pH levels and the anisotropy decreased gradually as the fluorophore was moved towards the terminal methyl group of the acyl chain. Typical values of the steady-state fluorescence anisotropy of the *n*-(9-anthroyloxy) fatty acids ($n = 2,7,16$) were 0.179 ± 0.006, 0.147 ± 0.002 and 0.063 ± 0.003 (mean ± S.D.), respectively. Since the anisotropy of the anthroyloxy fatty acid probes is determined largely by the rate of rotational motion of the fluorophore and by the degree of restriction of its anisotropic motion as reflected in an order parameter, the results suggest that these physical properties of the DOPE/sulfatide vesicles were not affected by a change in pH of the medium.

3.4. The bilayer(L_α)-hexagonal(H_{II}) phase transition

The emission of DNS-Lys undergoes a marked red-shift when a membrane bilayer, in which the fluorescent dye is located, is heated close to the L_α-H_{II} transition temperature [37]. In order to study the effect of sulfatide on the L_α-H_{II} transition of DOPE at various pH, the temperature depen-

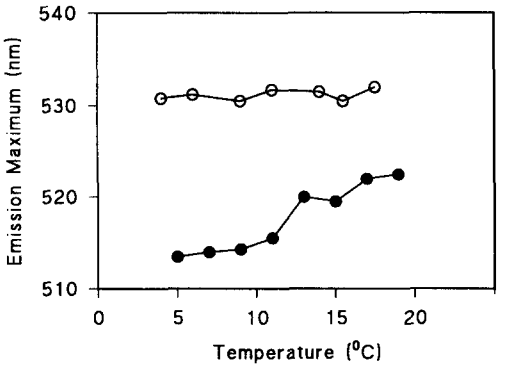

Fig. 4. Temperature dependence of DNS-Lys emission in DOPE/sulfatide (70:30, mol/mol) small unilamellar vesicles. (●), pH 6.0; (○), pH 7.4.

dence of DNS-Lys emission was therefore monitored with DOPE/sulfatide (70:30, mol/mol) bilayer vesicles at pH 6.0 and pH 7.4, respectively. At pH 7.4, there was no well defined L_α-H_{II} phase transition in the temperature range studied, indicating that the incorporation of sulfatides at the physiological pH had greatly reduced the cooperativity of the DOPE L_α-H_{II} phase transition (Fig. 4). In other words, the effect of broadening and/or abolition of the DOPE L_α-H_{II} phase transition brought about by the presence of sulfatide suggests that the glycolipid is indeed an excellent stabilizer for the L_α phase of DOPE vesicles at the physiological pH. When the pH was reduced to 6.0, the stabilization effect of sulfatide on the L_α phase of DOPE seemed to be diminished and a red-shift of the DNS-Lys emission was observed at above 10°C, a temperature range known for the DOPE L_α-H_{II} phase transition to take place [38]. Apparently, the L_α-H_{II} phase transition in DOPE/sulfatide vesicles was a pH-dependent event, with the acidic pH favouring the formation of the hexagonal phase.

Under certain assumptions, the fluorescence emission of the dansyl group of DNS-Lys can also be used to probe the hydrophobicity of its environment [37]. Fig. 4 shows that a reduction of pH from 7.4 to 6.0 below the predicted DOPE L_α-H_{II} phase transition resulted in a large blue-shift of the emission maximum of DNS-Lys, implying that the chromophore of the fluorescent probe was in a more hydrophobic environment at the acidic pH. As a matter of fact, the hydrophobicity probed by the dansyl group of DNS-Lys is determined by the molecular packing and hydration status of the membrane lipid-water interface. The former was known to be affected by the L_α-H_{II} phase transition, which changed the depth of water penetration into the bilayer and would be reflected by a red-shift of the DNS-Lys emission. Therefore, the blue-shift of DNS-Lys fluorescence emission was most likely due to the dehydration at the membrane lipid-water interface, brought about by the protonation of the formerly negatively charged sulfate group of sulfatides. It should be pointed out that the fluorescence intensity of DNS-Lys in DOPE/sulfatide vesicles was more than 50% higher at pH 6.0 than at pH 7.4, whereas the effect of pH on the emission of the same probe in aqueous solution was negligible, suggesting that the environment of DNS-Lys became more hydrophobic at the acidic pH.

4. Discussion

The therapeutic application of pH-sensitive liposomes as a targetable drug delivery system in vivo would require that such vehicles remain stable in the blood circulation until binding and subsequent endocytosis by the target cells. In this study we have presented some interesting properties of the DOPE/sulfatide bilayer vesicles which appear to fulfil such a purpose. For example, at 30 mol% sulfatide and neutral pH, the DOPE/sulfatide bilayer vesicles are stable in buffer (Fig. 1) and in the presence of human plasma (Fig. 2A). These bilayer vesicles retain substantial pH sensitivity and become destabilized at mildly acidic pH (Fig. 2B) and in the presence of a pH-gradient (Fig. 2C). Although the leakage of calcein was increased at higher temperature, its overnight release from the vesicles in plasma at 37°C and pH 7.4 was about 17% of the entrapped dye (Fig. 3), comparable to the best known results obtained for pH-sensitive liposomes [15]. It should be pointed out that Collins et al. [15] and Leventis et al. [39] had previously characterized a series of synthetic double-chain amphiphiles-containing PE liposomes which were stable in plasma and retained various pH sensitivity. Since the inclusion of sulfatides may render the liposomes some unique functions in targeting delivery [24–28], these liposomes should be useful in the design of drug delivery systems for different aims of targeting.

The correlation between membrane fluidity and the stability of liposomes has been noticed by Noda et al. [23] in sufatide-containing PC liposomes. Electron spin resonance study shows that the condensing effect of sulfatide is most obvious at above 10 mol% [40], possibly due to the participation of the amide and hydroxyl groups of the glycolipid and the carbonyl groups of the phospholipid in forming extensive intra- and intermolecular hydrogen bonding network [41,42]. Similar condensing effect of sulfatides in DOPE/sulfatide bilayer vesicles has been demonstrated in our fluorescence anisotropy studies by using anthroloxy fatty acids as probes (data not shown). However, the membrane fluidity in the presence of sulfatides was not affected by the difference in pH, implying that the destabilization of DOPE/sulfatide vesicles at lowering pH could not be explained by the modulation of membrane fluidity. Other factors such as hydration and electrostatic interactions should be considered.

Although the hydration number of the headgroup of sulfatide is not yet available, its galactose moiety should resemble that of corresponding galactocerebrosides in terms of hydration capacity [43]. It has been previously found

that the hydration number of galactose residues is rather small and not much different from that of PE molecules [43–45]. Therefore, the contribution of these residues to the stabilization of PE bilayer structure at the physiological pH should be very limited. In agreement with this, Park and Huang [43] had demonstrated that synthetic glycophospholipid containing a single galactose residue exhibited the poorest activity in stabilizing DOPE bilayer vesicles, as compared with others containing more saccharide units or carrying net negative charge. Since the hydration intensity of a glycolipid is directly related to its ability to stabilize PE bilayer structure [14,43], the additional hydration numbers provided by the negatively charged sulfate group are thus considered to be essential for the stabilization effect of sulfatide. In other words, it should be reasonable to propose that the repulsive force for preventing the destabilization of DOPE/sulfatide vesicles under physiological conditions is provided by the interfacial hydration and electrostatic interaction, brought about mainly by the negatively charged sulfate groups of sulfatide molecules [46–48].

The reduction in pH results in the protonation of the sulfate group of sulfatide. This would be followed by two direct consequences: (1) the negative charge carried by the sulfate group is neutralized and this in turn eliminates the electrostatic interactions between the membrane lipids; (2) partial dehydration at the membrane surface takes place due to the loss of charge. Both should lead to the destabilization of the DOPE/sulfatide bilayer vesicles as a reduction in pH from 7.4 to 6.0 gave rise to a significant increase in calcein release (Figs. 2 and 3). Moreover, the predicted DOPE L_α-H_{II} phase transition was not detectable in the presence of 30 mol% sulfatides at pH 7.4 (Fig. 4), implying that the hydrated sulfatides could interact strongly with DOPE, thus preventing the latter to revert to the H_{II} phase. In fact, the L_α-H_{II} phase transition was not found even up to 37°C (data not shown). When the pH was sufficiently lowered, the blue-shift of DNS-Lys emission (Fig. 4) and the concurrent increase of its fluorescence intensity would suggest that a proton-induced partial dehydration had occurred at the lipid-water interface. The protonation and partial dehydration of sulfatides appeared to enhance the ability of PE to form H_{II} phase and the transition was clearly registered by a red-shift of DNS-Lys emission at increasing temperature (Fig. 4). Our results are therefore consistent with the observation that partial dehydration at the lipid-water interface is essential for the L_α-H_{II} phase transition to occur [16–18]. On the other hand, as a control experiment, DOPE vesicles were also prepared at pH 6.0 and these liposomes were apparently stable below their L_α-H_{II} phase transition temperature. It is particularly interesting to note that the red-shift of DNS-Lys emission in DOPE vesicles (data not shown) occured in the same temperature range as that in DOPE/sulfatide vesicle (Fig. 4). It is therefore highly likely that, at the acidic pH, the association of protonated sulfatides with DOPE is less energetically favoured than the segregation of sulfatides from DOPE and thus the latter is allowed to revert to the H_{II} phase. This explanation is in agreement with the suggestion that differential hydration of various membrane components may induce their lateral phase separation during dehydration [17,49].

In summary, our results show that the ability of sulfatides to form stable pH-sensitive liposomes with DOPE in the presence of plasma. It is believed that the hydration and partial dehydration of the sulfate headgroup play an important role in the stabilization and destabilization of the DOPE/sulfatide vesicles. Formation of sulfatide-rich and DOPE-rich domains probably occurs after the partial dehydration of the headgroups of sulfatides. Since the internalized liposomes will encounter a continuously acidifying compartment during endocytosis, these pH-sensitive liposomes seem quite promising to be used as vehicles to deliver drugs into the cell. In addition, the sulfatide-containing liposomes might also be able to cross the blood-brain barrier to reach the brain [24–28]. These potential applications warrant further explorations.

Acknowledgements

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