Ca^{2+}-induced fusion of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles

Xiaofeng Wu and Qiu-Tian Li[1]

Department of Biochemistry, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260

Abstract **The fusogenic properties of sulfatide-containing 1,2-dioleoyl-3-*sn*-phosphatidylethanolamine (DOPE) small unilamellar vesicles (SUVs) in the presence of $CaCl_2$ were studied by mixing membrane lipids based on an assay of fluorescence resonance energy transfer (FRET). Fusion of the vesicles was also confirmed by mixing aqueous contents with the Tb/dipicolinate (DPA) assay. The half-times of lipid mixing revealed that the fusion rate decreased with increasing molar concentration of sulfatide. This inhibitory effect was more obvious at sulfatide concentrations higher than 30 mol%, where hydration at the membrane surface reached its maximum and the fusion was no longer pH-sensitive in the range of pH 6.0–9.0. Similar inhibitory effect was also observed in Ca^{2+}-induced fusion of DOPE/ganglioside GM_1 vesicles but at a lower concentration of the glycosphingolipid (20 mol%). In contrast, increasing the concentration of phosphatidylserine (PS) in DOPE/PS SUVs resulted in an increase in the rate of Ca^{2+}-induced lipid mixing and the pH sensitivity of this system was not affected. These results are consistent with an increasing steric hindrance to membrane fusion at higher molar concentration and larger headgroup size of the glycosphingolipids. Interestingly, the pH sensitivity of the sulfatide-containing liposomes was retained when they were allowed to fuse with synaptosomes in the absence of Ca^{2+} by a mechanism involving protein mediation.**—Wu, X., and Q-T. Li. **Ca^{2+}-induced fusion of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles.** ***J. Lipid Res.*** **1999.** 40: **1254–1262.**

Supplementary key words pH-sensitive liposomes • fluorescence resonance energy transfer • lipid mixing • headgroup steric hindrance • membrane fusion

It is well documented that pH-sensitive liposomes made of phosphatidylethanolamine (PE), and one bilayer stabilizer possessing a titratable negatively charged headgroup, fuse spontaneously with other lipid bilayers at acidic pH (for review see ref. 1). Alternatively, fusion can also be induced by the presence of physiological stimuli such as Ca^{2+} (2–5). Because endocytosis is regarded as the principal pathway by which liposomes enter cells and the pH of the lumen of endocytic vesicles is mildly acidic (6), pH-sensitive liposomes have been extensively studied as vehicles for cytoplasmic delivery of drugs, enzymes, and nucleic acids (7–9). It is believed that these liposomes would be destabilized in the endocytic lumen and fuse eventually with the endosome membranes, thereby releasing the entrapped contents into the cytoplasm before they are destroyed in lysosomes (10).

In previous studies, we have shown that sulfatide helps to stabilize 1,2-dioleoyl-3-*sn*-phosphatidylethanolamine (DOPE) bilayers, and the hydration and partial dehydration of its headgroup with changing pH play an essential role in determining the pH sensitivity of DOPE/sulfatide vesicles (11). Sulfatide is also known to be able to prolong liposome circulation half-lives (12), a clear indication that clearance by the reticuloendothelial system is reduced. Furthermore, this glycosphingolipid appears to be able to assist liposomes to pass through the blood–brain barrier, implying that the sulfatide-containing liposomes may be useful for treatment of various kinds of brain diseases (13–18). It is therefore interesting to examine the effect of sulfatide on Ca^{2+}-induced fusion of DOPE/sulfatide vesicles and see whether the fusion process is modulated by pH. The results obtained from such studies should aid in the design of sulfatide-containing liposomes for drug delivery and may also help to shed some light on the general features of liposome-cell membrane interactions.

It is generally accepted that membrane fusion can be separated into two distinct stages that are coupled kinetically (19, 20). The first step is dehydration of the surfaces of apposed bilayers resulting mainly from charge neutralization. The next step is destabilization of the bilayer structure by formation of nonbilayer lipid intermediates, leading to mixing of membrane lipids and aqueous con-

Abbreviations: DOPE, 1,2-dioleoyl-3-*sn*-phosphatidylethanolamine; SUVs, small unilamellar vesicles; FRET, fluorescence resonance energy transfer; DPA, dipicolinate; GM_1, galactosyl-*N*-acetylgalactosaminyl (*N*-acetylneuraminyl) galactosylglucosylceramide; PS, bovine brain phosphatidylserine; PE, phosphatidylethanolamine; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; PC, phosphatidylcholine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; DNS-Lys, N^{ε}-dansyl-l-Lys; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; H_{11}, hexagonal phase.

[1] To whom correspondence should be addressed.

tents. In this study, the rate of membrane fusion, as measured by lipid mixing, was monitored by a fluorescence resonance energy transfer (FRET) assay based on probe dilution (21). This method allows continuous measurement of the decrease in energy transfer efficiency, which under suitable experimental conditions is directly proportional to the extent of lipid mixing (21). Because content mixing is a necessary and sufficient indicator of fusion, the Tb/DPA assay was also briefly used (22). In addition, to model the fusion process experienced by sulfatide-containing liposomes during endocytosis, synaptosomes were used as the target biological membrane at varying pH levels. Our results show that sulfatide, at all concentrations tested, inhibits the fusion of DOPE/sulfatide vesicles. However, the pH sensitivity of these liposomes is suppressed only at higher sulfatide concentrations.

MATERIALS AND METHODS

Materials

Bovine brain sulfatide, GM_1, chloroform stock solutions of DOPE, 1,2-dioleoyl-3-*sn*-phosphatidylcholine (DOPC), and bovine brain PS were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The percentage of nonhydroxy and α-hydroxy fatty acyl chains of the sulfatide is approximately 70% and 30%, respectively, as determined by thin-layer chromatography using the solvent system of chloroform–methanol–acetone–acetic acid–water 8:2:4:2:1 (v/v). N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE), N^{ε}-dansyl-l-Lys (DNS-Lys), $TbCl_3$, and DPA were obtained from Molecular Probes (Eugene, OR). Other reagents were of analytical reagent grade and deionized water was used for all experiments.

Liposome preparations

DOPE/sulfatide small unilamellar vesicles (SUVs) were prepared by sonication of an aqueous suspension of the lipids as described by Thulborn and Sawyer (23). Briefly, chloroform solutions of the lipids were placed in Pyrex glass tubes, and the solvent was evaporated under a stream of nitrogen. Residues of the solvent were removed in a vacuum container overnight at 4°C. The thin lipid film formed on the walls of the glass tubes was hydrated at room temperature with Tris buffers (10 mm Tris-HCl, 150 mm NaCl, pH 6.0, 7.4, or 9.0). For Tb/DPA content mixing assay, SUVs were prepared in either of the following aqueous media: *a*) 15 mm $TbCl_3$, 10 mm TES, and 150 mm sodium citrate (pH 7.4); *b*) 150 mm sodium dipicolinate and 10 mm TES (pH 7.4). Sonication was carried out under nitrogen for 10 min (30 s on and 30 s off for each cycle) with a Heat Systems Sonicator XL at the maximum power output. Temperature was controlled with an ice-water bath and no lipid degradation was found as examined by thin-layer chromatography. Unencapsulated solutes (i.e., $TbCl_3$ and DPA) were removed by passing the vesicles down a Sephadex G-50 column equilibrated in and washed by an osmotically balanced TES buffer (10 mm TES, 1 mm EDTA, 100 mm NaCl) at pH 7.4. 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 phospholipids and the results were comparable with those determined according to Bartlett (24). The concentration of sulfatide was determined by the method of Kean (25).

Fusion assays by lipid mixing

Vesicle fusion, as assayed by lipid mixing, was followed by decrease in the efficiency of FRET resulted from the dilution of nonexchangeable fluorophores (21). In this method, 0.8 mol% of NBD-PE, the energy donor, and Rh-PE, the energy acceptor, were included in one population of vesicles. These labeled SUVs and a 3-fold excess of unlabeled vesicles of similar lipid composition were then mixed in a fluorescence cuvette at 25°C. The solution was stirred continuously with a small magnet and $CaCl_2$ was added at the desired final concentration from a stock solution (2 m). The NBD fluorescence emission at 530 nm was measured with a Perkin-Elmer LS-50B spectrofluorometer with the excitation at 470 nm. The total lipid concentration used in these experiments was 0.2 mm.

Fusion assays by content mixing

Mixing of the aqueous contents of the vesicles was followed using the Tb/DPA assay as described by Wilschut et al. (26, 27). Tb-containing vesicles and DPA-containing vesicles were mixed in a TES buffer (10 mm TES, 0.1 mm EDTA, and 100 mm NaCl, pH 7.4). $CaCl_2$ was added to the desired final concentration from a stock solution. With the excitation at 276 nm, fusion of vesicles was monitored by increase in the fluorescence intensity at 492 nm, arising from the formation of a highly fluorescent Tb/DPA complex.

Fluorescence emission of DNS-Lys

DOPC vesicles containing 0–40 mol% sulfatide were prepared in the presence of 2 mol% DNS-Lys. The excitation wavelength was 330 nm and the emission spectra were recorded at 25°C from 450 to 600 nm with a scan rate of 60 nm/min. The excitation and emission slit widths were 5 nm, respectively.

Preparation of synaptosomes

Synaptosomes were prepared from the whole brain of male Wistar rat (200–250 g) by the method of Abdel-Latif (28) with slight modifications. All procedures were carried out at 0–4°C. Briefly, about 3.2 g of the rat brain tissue in 10 volumes of sucrose buffer (0.25 m sucrose, 10 mm Tris-Cl, pH 7.4) were homogenized by 10 up and down strokes in a motor-driven glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1,000 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged again at 12,000 *g* for 15 min. The pellet obtained was washed twice with the sucrose buffer and then resuspended in it. Further purification was carried out by centrifugation (90,000 *g* for 1 h) on a four-step discontinuous Ficoll density gradient (2%:8%:12%:20% in 0.25 m sucrose medium). The synaptosomal fraction at the interface between 8% and 12% Ficoll concentrations was harvested, diluted with 2 volumes of the sucrose buffer, and then spun down at 120,000 *g* for 20 min. The resulting pellet was resuspended in the sucrose buffer. Protein concentration of the synaptosomes obtained was determined according to the method of Lowry et al. (29). The synaptosomes were then diluted to 2 mg protein/ml, stored at −20°C and used within 3 days of preparation.

Trypsinization of synaptosomal membranes was performed at 4°C for 1 h at a trypsin concentration of 10 μg/ml. After the incubation, a 4-fold concentration of soybean trypsin inhibitor was added to the reaction mixture. The synaptosomes were then washed twice with the sucrose buffer.

Fusion of sulfatide-containing liposomes to synaptosomes

Synaptosomes (60 μl) from the stock solution were added into 1.94 ml Tris buffer at pH 6.0, 7.4, or 9.0 and incubated in a cuvette at 37°C for 5 min. Fusion was initiated by rapid injection of 0.5 ml NBD/Rh-labeled liposomes into the synaptosome solu-

tion. The increase in NBD fluorescence intensity at 530 nm was followed by the Perkin-Elmer LS-50B spectrofluorometer for 30 min with an excitation wavelength of 470 nm.

Fusion kinetics

In order to assess the fusion rate obtained at different pHs for vesicles of different compositions, the kinetic data of NBD fluorescence intensity (F(t)) were numerically fitted by weighted nonlinear least-squares regression to the following equation:

$$F(t) = F(0) + \Sigma F(i)\exp(-t/\tau_i)$$

where F(0) is the fluorescence intensity before the addition of Ca^{2+}, F(i) is the pre-exponential factor, t is the time, and the characteristic lipid mixing half-time is given by $t_{1/2,i} = \tau_i \ln 2$. The data fitting was carried out with the TableCurve™ curve-fitting software version 3.0 (Jandel Scientific, CA) based on the Levenburg-Marquardt algorithm. Goodness-of-fit was determined by evaluating the degree-of-freedom adjusted coefficient of determination (r^2) (30).

RESULTS

The fusion assay based on probe dilution is insensitive to aggregation of lipid vesicles as both fluorescent probes (i.e., NBD-PE and Rh-PE) are in the same lipid bilayer (31). There is also no significant probe transfer from the labeled vesicles to the unlabeled ones during vesicle aggregation (31). In the case where both probes are present at 0.8 mol%, the fluorescence emission of the energy donor, NBD-PE, is significantly quenched as the fluorophores are in close proximity. If the labeled vesicles fuse with the unlabeled ones, lipid mixing takes place, resulting in an increase in the proximity and release of the quenched fluorescence of NBD-PE. Typical time courses of fluorescence development at 10 mm Ca^{2+} added to mixtures of various sulfatide-containing SUVs and their labeled counterparts of similar lipid compositions are shown in **Fig. 1**. Although the millimolar Ca^{2+} concentration used may have little physiological relevance, it caused the vesicles to fuse at measurable rates. At 20 mol% sulfatide, an initial rapid NBD fluorescence enhancement was observed for DOPE/sulfatide SUVs (Fig. 1, curve d), suggesting that lipid mixing occurred probably as a result of membrane fusion. The NBD fluorescence intensity reached its maximum 5 min after the addition of Ca^{2+}. When the sulfatide content was increased to 30 mol%, where stable DOPE/sulfatide vesicles were formed at the physiological pH and in the presence of human plasma (11), the increase in NBD fluorescence was much slower but continued for the entire duration of the assay (Fig. 1, curve c). This implies that the higher concentration of sulfatide was inhibitory to fusion of DOPE/sulfatide vesicles. In the extreme case where the particles consisted only of sulfatide, there was no detectable increase in NBD fluorescence in the presence of 10 mm Ca^{2+} (Fig. 1, curve a). As anticipated, DOPC/sulfatide vesicles did not fuse under the experimental conditions (Fig. 1, curve b), indicating that the ability of DOPE to readily adopt nonbilayer structures played a critical role in membrane fusion.

In order to establish that the lipid mixing assay reflected the mixing of internal aqueous contents, the fluorescence development upon injection of a 1:1 (v/v) mixture of Tb- and DPA-containing DOPE/sulfatide SUVs into a medium containing 10 mm Ca^{2+} was studied (**Fig. 2**). An initial rapid mixing of internal aqueous contents, as shown by the marked increase in Tb/DPA fluorescence emission, was observed for vesicles containing both 20 and 30 mol% sulfatide. There was no detectable difference between the rates of fluorescence enhancement for these

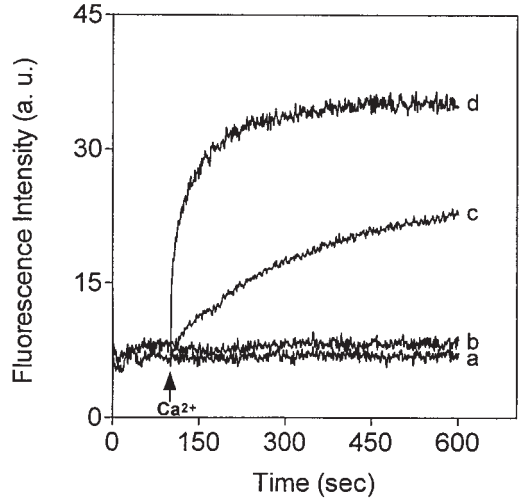

Fig. 1. Time courses of lipid mixing monitored by enhancement of NBD fluorescence intensity for sulfatide-containing vesicles in the presence of 10 mm Ca^{2+}. NBD/Rh-labeled and unlabeled vesicles were mixed in the fluorescence cuvette equipped with an automatic stirrer. Arrow indicates the time at which Ca^{2+} was added. The lipid compositions of the vesicles are: (a) sulfatide; (b) DOPC/sulfatide (20 mol%); (c) DOPE/sulfatide (30 mol%); and (d) DOPE/sulfatide (20 mol%). The experiments were carried out at 25°C and pH 7.4.

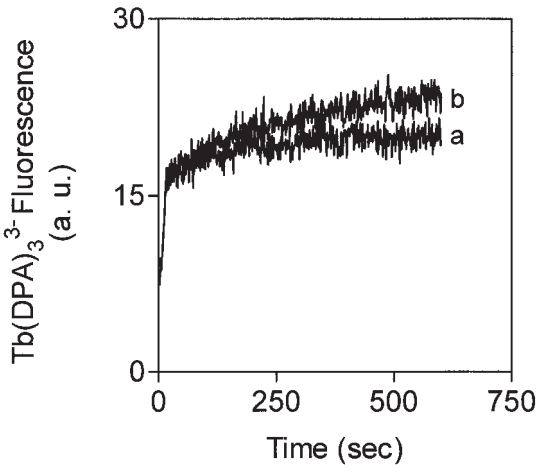

Fig. 2. Representative time courses of content-mixing experiments. Tb- and DPA-containing DOPE/sulfatide vesicles were mixed together before the addition of 10 mm Ca^{2+} at time zero. The lipid compositions of the vesicles are (a) DOPE/sulfatide (30 mol%), and (b) DOPE/sulfatide (20 mol%). The experiments were carried out at 25°C and pH 7.4.

vesicles at this stage. However, as the fusion proceeded, the increase in Tb/DPA fluorescence intensity in vesicles containing 30 mol% sulfatide (Fig. 2, curve a) was slower than that in vesicles containing 20 mol% sulfatide (Fig. 2, curve b). At the end of the 10-min observation, the relative fluorescence increase in the former reached only 75% of that in the latter. Apparently, vesicle fusion observed by dilution of fluorescent lipid probes was confirmed by the assay of Tb/DPA content mixing. We have therefore carried out all of the remaining fusion experiments by using the lipid mixing assay for its simplicity. The Tb/DPA assay was conducted regularly for purpose of confirmation of full membrane fusion.

In order to get a deeper insight into the kinetics of Ca^{2+}-induced membrane fusion, we have fitted the NBD fluorescence data numerically to a sum of exponential terms (see Materials and Methods). The equations were sorted by the coefficient of determination, r^2, which indicated how strong the relationship really was. In our case, all NBD fluorescence data obtained from the calcium-induced vesicle–vesicle fusion studies gave a good fit to a single-exponential model ($r^2 = 0.95–0.99$). Fitting to the sum of a higher order of exponentials did not improve the goodness-of-fit, implying a lack of statistical support for additional floating parameters.

We first applied the above treatment to study the effect of sulfatide content in DOPE vesicles on the kinetics of Ca^{2+}-induced lipid mixing. The half-times of lipid mixing are plotted in **Fig. 3** as a function of sulfatide concentration. As can be seen, the half-time increased with increasing molar concentration of sulfatide in an approximately linear fashion, reaching an inflection point at 30 mol% sulfatide. At >30 mol% sulfatide, the half-time increased at a rate about 2-fold higher than that at <30 mol% sulfatide, implying a change in the mechanism of sulfatide-induced inhibitory effect on fusion of the DOPE/sulfatide vesicles.

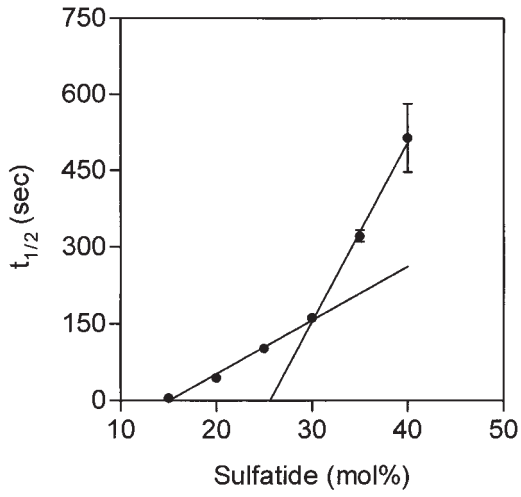

Fig. 3. Effects of sulfatide concentration in DOPE/sulfatide vesicles on the half-times of Ca^{2+} (10 mm)-induced lipid mixing. The experiments were carried out at 25°C and pH 7.4. Each point represents the mean ± SD of three independent experiments under identical conditions.

It is interesting to note that the sulfatide concentration at the above inflection point is close to the mol% of sulfatide needed to stabilize the DOPE/sulfatide vesicles (11).

It is generally agreed that membrane apposition, destabilization, and mixing of lipids and aqueous contents take place during fusion of liposomes (32, 33). In unsaturated PE-containing vesicles, membrane destabilization can be triggered by conditions such as a reduction in pH, where the PE component reverts from lamellar to nonbilayer inverted hexagonal (H_{II}) phase (reviewed in refs. 1 and 34). It has been proposed that sulfatide acts by increasing the hydration at the lipid–water interface, thereby stabilizing the lamellar phase (11). To confirm that sulfatide did increase the level of hydration at the lipid–water interface, we examined the characteristics of DNS-Lys fluorescence emission in DOPC vesicles at increasing sulfatide concentrations (**Fig. 4**). DOPE is not suitable for this type of study as its lamellar phase is unstable at low sulfatide concentrations (11). Although the maximum hydration levels of PC and PE are different (35), it should be reasonable to deduce the effect of sulfatide on membrane hydration in DOPE vesicles from the results obtained in DOPC vesicles. Figure 4 shows that the fluorescence emission of DNS-Lys undergoes a marked reduction in intensity and a small red-shift (3–4 nm) in spectral shape at increasing content of sulfatide, indicating an increase in headgroup hydration at the lipid–water interface (36). The plot of DNS-Lys fluorescence intensity at 520 nm as a function of sulfatide concentration shows that at sulfatide concentrations below 20 mol%, surface hydration increased continuously with addition of the glycosphingolipid and then levelled out, approaching a limiting plateau beyond approximately 30 mol% sulfatide (Fig. 4, inset). This probably corresponds to the saturation of surface hydration under the experimental conditions used.

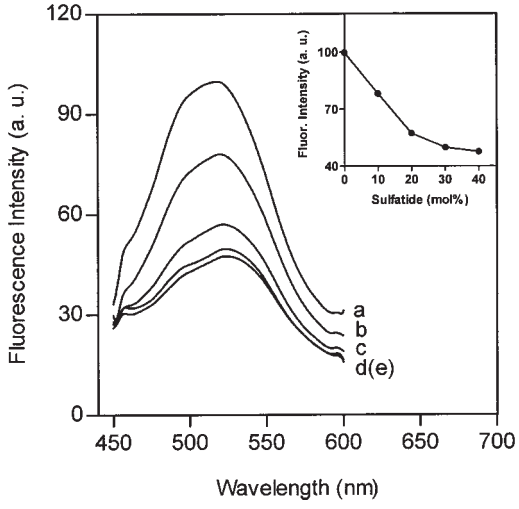

Fig. 4. Effect of sulfatide concentration on the fluorescence emission spectra of DNS-Lys in DOPC/sulfatide vesicles. The sulfatide concentrations are (a) 0%; (b) 10 mol%; (c) 20 mol%; (d) 30 mol%, and (e) 40 mol%, respectively. The inset shows the relationship between DNS-Lys fluorescence intensity and sulfatide concentration. The spectra were obtained at 25°C and pH 7.4.

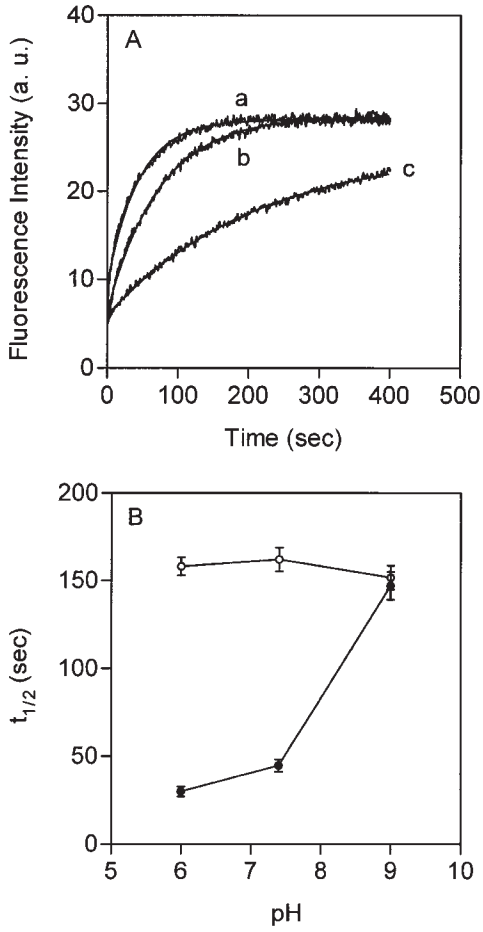

Fig. 5. Effects of pH on lipid mixing kinetics of DOPE/sulfatide vesicles (25°C). Panel A shows the time course of NBD fluorescence enhancement in DOPE/sulfatide (20 mol%) vesicles in the presence of 10 mm Ca^{2+} added at time zero. The experiments were carried out at (a) pH 6.0, (b) pH 7.4, and (c) pH 9.0, respectively. The solid lines represent the nonlinear least-squares fits of the experimental data to a single-exponential model. Panel B shows the effect of pH on the lipid mixing half-times in vesicles containing different sulfatide concentrations: (○) DOPE/sulfatide (30 mol%); (●) DOPE/sulfatide (20 mol%). Each point represents the mean ± SD of three independent experiments under identical conditions.

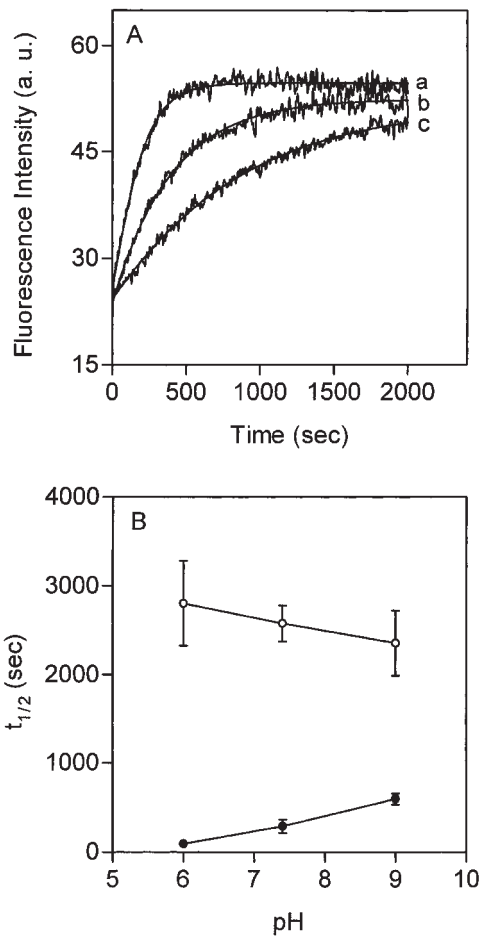

Fig. 6. Effects of pH on lipid mixing kinetics of DOPE/GM_1 vesicles (25°C). Panel A shows the time course of NBD fluorescence enhancement in DOPE/GM_1 (10 mol%) vesicles in the presence of 10 mm Ca^{2+} added at time zero. The experiments were carried out at (a) pH 6.0, (b) pH 7.4, and (c) pH 9.0, respectively. The solid lines represent the nonlinear least-squares fits of the data to a single-exponential model. Panel B shows the effect of pH on the lipid mixing half-times in vesicles containing different GM_1 concentrations: (○) DOPE/GM_1 (20 mol%); (●) DOPE/GM_1 (10 mol%). Each point represents the mean ± SD of three independent experiments under identical conditions.

In the following experiments, the rates of lipid mixing are examined at three pH levels to see whether the DOPE/sulfatide vesicles are pH-sensitive in terms of membrane fusion. This would be of great importance with respect to the development of fusogenic drug delivery vehicles. **Figure 5** shows the pH-dependent lipid mixing in the presence of 20 or 30 mol% sulfatide. A pH increase from 6.0 to 7.4 resulted in a 50% inhibition in the fusion rate at 20 mol% sulfatide; a further increase in pH to 9.0 resulted in a more remarkable inhibition (~2.3-fold). However, there was no significant difference among the fusion rates obtained for vesicles containing 30 mol% sulfatide at pH 6.0–9.0, indicating that the pH sensitivity of membrane fusion was virtually abolished at the higher sulfatide concentration.

Our results so far have revealed that the inhibitory effect of sulfatide on the rate and pH sensitivity of lipid mixing depends on the amount of glycosphingolipid present in the vesicle bilayer. As the initial steps of membrane fusion involve bilayer apposition and destabilization, the presence of bulky carbohydrate headgroups may play a central role in inhibiting these processes. If this is the case, the magnitude of the hindrance should increase with increasing size of the headgroup. We have therefore continued our investigation on the steric interference of lipid headgroup by using bilayer vesicles containing ganglioside GM_1. As shown in **Fig. 6**, the rate of Ca^{2+}-induced lipid mixing was pH-sensitive at 10 mol% GM_1, with a 5.4-fold inhibition to the half-time being observed at pH 9.0 compared to that at pH 6.0. At 20 mol% GM_1, however, the pH sensitivity of lipid mixing was completely abolished within the pH range examined (Fig. 6B). No evidence of lipid mixing was apparent at 30 mol% GM_1 and above (data not shown). These results show that the inhibitory effect of glycosphingolipids on the rate of Ca^{2+}-

induced lipid mixing increases dramatically with increasing size of their oligosaccharide headgroups, whilst more glycosphingolipids with smaller headgroups will be required to abolish the pH sensitivity of the fusion process.

Calcium-induced fusion of phosphatidylserine-containing lipid vesicles is probably the most widely studied model system for membrane fusion. As compared to the glycosphingolipids, the headgroup of this anionic phospholipid is relatively small and therefore lacks the steric barrier for close apposition of vesicles. The effects of PS concentration and pH on Ca^{2+}-induced lipid mixing of DOPE/PS vesicles are shown in **Fig. 7**. In these experiments, a lower calcium concentration (2.5 mm) was used as higher Ca^{2+} concentration would lead to very rapid fusion rates that were inconvenient to measure. Figure 7 shows that the fusion kinetics of liposomes containing 20 mol% PS was pH-sensitive, with the rate of lipid mixing being decreased by ~70% when pH was raised from 6.0 to 9.0. In contrast to the inhibitory effects of the glycosphingolipids at higher concentrations, increasing PS concentration to 30 mol% did not affect the pH sensitivity of the vesicles and the rate of lipid mixing decreased by about 2-fold when pH was raised from 6.0 to 9.0 (Fig. 7B). Furthermore, higher PS concentration resulted in higher rate of lipid mixing under otherwise identical experimental conditions.

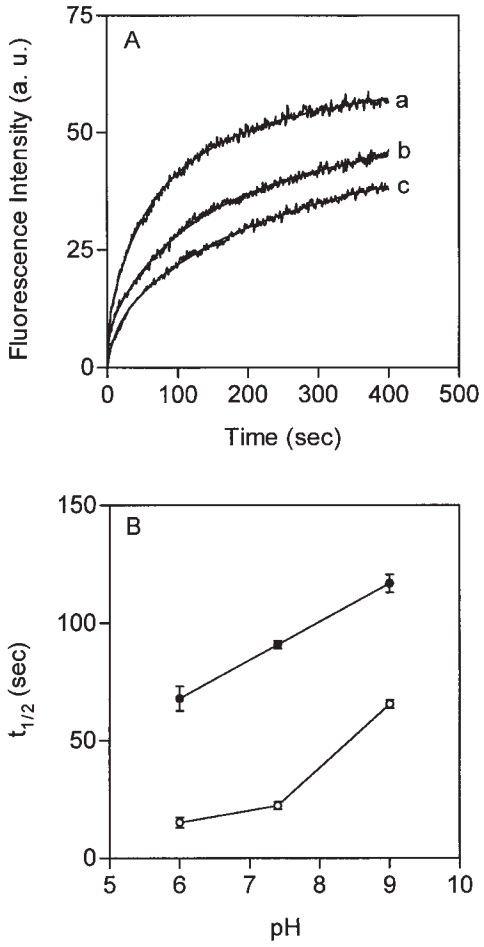

Fig. 7. Effects of pH on lipid mixing kinetics of DOPE/PS vesicles (25°C). Panel A shows the time courses of NBD fluorescence enhancement in DOPE/PS (20 mol%) vesicles in the presence of 2.5 mm Ca^{2+} added at time zero. The experiments were carried out at (a) pH 6.0, (b) pH 7.4, and (c) pH 9.0, respectively. The solid lines represent the nonlinear least-squares fits of the data to a single-exponential model. Panel B shows the effects of pH on the lipid mixing half-times in vesicles containing different PS concentrations: (○) DOPE/PS (30 mol%); (●) DOPE/PS (20 mol%). Each point represents the mean ± SD of three independent experiments under identical conditions.

Sulfatide-containing liposome-mediated delivery to the brain has been reported by several investigators (13–18, 37, 38). Synaptosomes appear to be a valid target membrane for studying fusion of the sulfatide-containing liposomes to the plasma membranes of neurons. The liposomes used in these experiments contain 20 mol% sulfatide and their rate of lipid mixing at 10 mm Ca^{2+} was previously found to be pH-dependent (Fig. 5). When these sulfatide-containing vesicles were mixed with synaptosomes, the NBD fluorescence appeared unfit for the exponential model used for lipid mixing between bilayer vesicles, suggesting that the fusion between liposomes and synaptosomes is more complex than that in pure liposome systems. However, the rate of lipid mixing, as judged by the net increase in NBD fluorescence intensity after incubation for 30 min at 37°C, appeared to be pH-sensitive (**Fig. 8**). At pH 6.0, the rate of lipid mixing was approximately 45% and 2-fold higher than those at pH 7.4 and 9.0, respectively.

To assess whether membrane protein(s) is(are) involved in the lipid mixing event, we treated the synaptosomes with trypsin, a proteolytic enzyme that catalyzes the hydrolysis of peptide bonds. Our results showed that treatment of synaptosomes with trypsin strongly inhibited lipid mixing between the liposomes and the synaptosomes (Fig. 8). Trypsinization for 60 min resulted in ~60% and ~70% inhibition in lipid mixing rate at pH 6.0 and pH 7.4, respectively, while at pH 9.0, the inhibition of lipid mixing

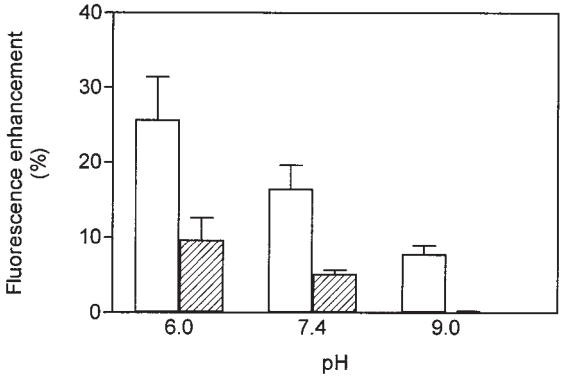

Fig. 8. Effects of pH on fusion of DOPE/sulfatide (20 mol%) vesicles to synaptosomes as measured by the enhancement of NBD fluorescence intensity. The synaptosomes (2 mg protein/ml) are either directly mixed with the DOPE/sulfatide vesicles (open bars) or pre-treated with trypsin (10 μg/ml) (hatched bars). The fluorescence enhancement was followed at 37°C for 30 min. Each bar represents the mean ± SD of two or three independent experiments under identical conditions.

was complete. These data have revealed the role of membrane protein(s) in mediating the fusion of pH-sensitive liposomes to cell membranes. In addition to the action of protein(s), nonspecific interactions may also be involved in the membrane fusion process, particularly under acidic conditions (Fig. 8). Our results are in general agreement with those obtained for fusion between large unilamellar PS liposomes and synaptosomes (39).

DISCUSSION

In our earlier studies (11), it was found that sulfatide is able to stabilize DOPE SUVs when its concentration is sufficiently high. Under the conditions used there, the vesicles were pH-sensitive in terms of membrane permeability to encapsulated solutes. The results reported here show that the DOPE/sulfatide SUVs may become pH-insensitive in terms of Ca^{2+}-induced membrane fusion if the concentration of sulfatide is too high (e.g., $\geqslant$30 mol% sulfatide at 10 mm Ca^{2+}). These data have important implications with respect to therapeutic delivery via the endosomal route which requires the liposomes not only to remain stable during storage and to have prolonged circulation time, but also to be pH-sensitive and able to fuse with the endosomal membranes under acidic conditions. These would ensure that the solutes carried by pH-sensitive liposomes be delivered into the cytoplasm. By carefully choosing the optimum lipid combinations (i.e., 20–30 mol% sulfatide), we have demonstrated that it should be feasible to generate stable sulfatide-containing DOPE vesicles whose pH-sensitivity towards membrane fusion is largely retained.

It has been proposed that the repulsive force for preventing the destabilization of DOPE/sulfatide vesicles is provided by the interfacial hydration and electrostatic interactions, brought about mainly by the negatively charged sulfate groups of sulfatide molecules (11, 40). This is probably particularly true when the sulfatide concentration is low. In this stage, Ca^{2+} acts on the vesicle surface either by neutralizing the charge repulsion and dehydrating the membrane surface (41, 42) or by triggering formation of non-bilayer fusion intermediates (43, 44). We have shown that this latter mechanism is essential to lipid mixing between the sulfatide-containing vesicles as replacement of DOPE with DOPC abolishes their ability of fusion (Fig. 1). On the other hand, H^+ functions in a similar way and is able to induce membrane destabilization and fusion (45, 46). It has been suggested that protonation of the sulfate headgroups, caused by a reduction in the pH, neutralizes the negative charge and the membrane surface becomes partially dehydrated (11). The PE component is thus allowed to revert to the H_{II} phase, resulting in destabilization and lipid mixing of the DOPE/sulfatide bilayer vesicles. Therefore, when sulfatide concentration is relatively low, e.g., $<$30 mol% of the total lipids under our conditions, lipid mixing of these sulfatide-containing vesicles is a pH-sensitive process (Fig. 5).

However, the Ca^{2+}-induced lipid mixing of sulfatide-containing vesicles seems to be affected not only by membrane dehydration and charge repulsion, but also by a steric barrier originated from the sulfated galactose headgroups. For Ca^{2+}-induced fusion of liposomes containing PS, the fusion rate increases with increasing PS concentration (Fig. 7). This can be conventionally explained by the enhanced dehydration and bilayer contact through the formation of more interbilayer Ca^{2+}–PS complexes (47). Presumably, higher sulfatide concentration should lead to higher fusion rate because more Ca^{2+}, which was in excess, would bind to the membrane surface and result in more dehydration, as in the case of PS system. However, the rate of lipid mixing in sulfatide-containing vesicles decreased with increasing sulfatide concentration (Fig. 3). This inhibitory effect on lipid mixing could be attributed to the structural difference between the headgroups of PS and sulfatide. In sulfatide, the negatively charged polar headgroup is more bulky than that of PS. The conical shape of sulfatide molecules may thus prevent the curvature distortion required for the formation of nonbilayer H_{II} phase from taking place.

On the other hand, at $\geqslant$30 mol% sulfatides, the Ca^{2+}-induced lipid mixing between sulfatide-containing vesicles is no longer pH-sensitive (Fig. 5). Moreover, the hydration at the bilayer surface has reached a maximum level (Fig. 4). Even the mode of the inhibitory effect of sulfatide on lipid mixing appears to be different from that for vesicles containing $<$30 mol% sulfatide (Fig. 3). Taken together, these results can be explained in terms of an emerging predominance of the steric interference of the sulfatide headgroups. This hypothesis is supported by following considerations. It has been reported that the surface area per molecule of sulfatide in a fluid state is 75 $Å^2$ (48, 49) and that per DOPE molecule is 38 $Å^2$ (50). This implies that in a DOPE bilayer vesicles containing 30 mol% sulfatide, about 45% of the surface area would be covered by the glycosphingolipid. If the cross-sectional shape of the sulfatide headgroup is assumed to be circular and a single layer of bound water molecules is 2 Å thick, the diameter of a hydrated sulfatide headgroup will be about 11.8 Å. With these data, a two-dimensional arrangement of the glycosphingolipid in a bilayer membrane can be depicted in **Fig. 9**. As can be seen (Fig. 9B), there are direct molecular contacts between the hydrated sulfatide headgroups at 30 mol% sulfatide. The restricted geometrical arrangement would therefore prevent phase separation and consequent destabilization of the bilayer vesicles from taking place easily. As a comparison, Fig. 9A shows that at 20 mol% sulfatide the space between sulfatide headgroups is large enough for pH-induced hydration–dehydration to play its role in membrane fusion.

If the pH sensitivity of membrane fusion can be inhibited by the steric hindrance of sulfatide headgroups, as has been suggested so far, a similar inhibitory effect should be observed in DOPE/GM_1 vesicles but at lower GM_1 molar concentrations, considering its larger carbohydrate headgroup. This is indeed the case (Fig. 6). At 20 mol% GM_1, the Ca^{2+}-induced fusion is already pH-insensitive. Because the surface area occupied by a GM_1 molecule is about 100

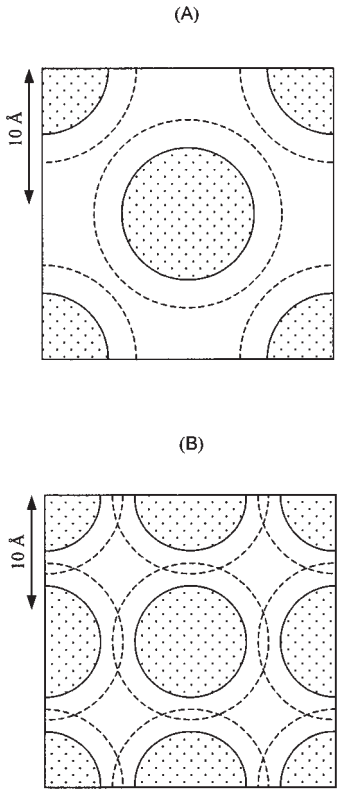

Fig. 9. Schematic diagrams showing the molecular organization of sulfatide in the surface of DOPE/sulfatide vesicles. A shaded circle denotes the average cross-section of a sulfatide headgroup and a circle of broken line represents the area occupied by a hydrated sulfatide headgroup. The relevant headgroup sizes were obtained from refs. 48–50 and at a surface pressure of 10 mN/m. (A) 20 mol% of sulfatide; (B) 30 mol% of sulfatide.

Å^2 (48, 49), the proportion of vesicle surface covered by 20 mol% GM_1 is about 40%, which is close to that occupied by 30 mol% sulfatide (see above). At this GM_1 concentration, direct molecular contacts between hydrated GM_1 are unavoidable, as revealed by models similar to Fig. 9.

It is noteworthy that the effect of sulfatide on the pH sensitivity of Ca^{2+}-induced fusion is not due to difference in the size of DOPE/sulfatide vesicles. In fact, difference in size between vesicles containing 20 and 30 mol% of sulfatide is negligible, as examined by elusion of the vesicles through a Sepharose CL-4B gel filtration column and electron microscopy (data not shown). On the other hand, our results did not rule out the possibility that GM_1 headgroup protrusion also played a role in inhibiting close approach and fusion of the bilayer vesicles (51, 52).

In recent years there has been an increasing interest in the possible roles played by membrane proteins in fusion of liposomes to biological membranes (39, 53–55). The pH sensitivity of our sulfatide-containing liposomes was retained when they were allowed to fuse with synaptosomes even in the absence of Ca^{2+} (Fig. 8). In fact, the rate of lipid mixing was not affected by increasing Ca^{2+} up to the physiological level. The implications of these results are unclear and too complex to be considered in detail in this work, but it is evident that membrane protein(s) played an important role in the fusion process as the latter was significantly inhibited by trypsin pretreatment of synaptosomes (Fig. 8). A close examination on the data found that the pH sensitivity of vesicle lipid mixing might largely be attributed to the nonspecific interactions, while membrane protein(s) enhanced the fusion rate almost equally at all pH values tested. It is interesting to note that proteins are able to induce formation of H_{II} phase in certain phospholipid bilayers (56–58). A protein-triggered phase separation and reversion of PE components to H_{II} phase may thus represent part of the mechanism for protein-mediated fusion between liposomes and synaptosomes. On the other hand, the nature of the nonspecific interactions should be associated to surface hydration as it is affected by pH of the aqueous medium (Fig. 8).

In conclusion, by carefully choosing the lipid composition of DOPE/sulfatide liposomes, it is possible to manipulate their pH-sensitive fusogenic capability. These liposomes should be suitable for intracellular drug delivery via the endocytosis pathway. Further experiments, aimed at elucidation of the contribution of sulfatide to liposome-cell uptake and fusion, are currently under way.

This work was funded by National University of Singapore Research Grant, RP920325. Xiaofeng Wu is the recipient of a National University of Singapore Research Scholarship.

Manuscript received 16 October 1998 and in revised form 9 March 1999.

REFERENCES

1. Litzinger, D. C., and L. Huang. 1992. Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. *Biochim. Biophys. Acta.* **1113:** 201–227.
2. Leventis, R., T. Diacovo, and J. R. Silvius. 1987. pH-dependent stability and fusion of liposomes combining protonatable double-chain amphiphiles with phosphatidylethanolamine. *Biochemistry.* **26:** 3267–3276.
3. Collins, D., D. C. Litzinger, and L. Huang. 1990. Structural and functional comparisons of pH-sensitive liposomes composed of phosphatidylethanolamine and three different diacylsuccinylglycerols. *Biochim. Biophys. Acta.* **1025:** 234–242.
4. Eastman, S. J., M. J. Hope, K. F. Wong, and P. R. Cullis. 1992. Influence of phospholipid asymmetry on fusion between large unilamellar vesicles. *Biochemistry.* **31:** 4262–4268.
5. Holland, J. W., C. Hui, P. R. Cullis, and T. D. Madden. 1996. Poly (ethylene glycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry.* **35:** 2618–2624.
6. Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* **55:** 663–700.
7. Connor, J., and L. Huang. 1986. pH-sensitive immunoliposomes as an efficient and target-specific carrier for antitumor drugs. *Cancer Res.* **46:** 3431–3435.
8. Ropert, C., C. Malvy, and P. Couvreur. 1993. Inhibition of the Friend retrovirus by antisense oligonucleotides encapsulated in liposomes: mechanism of action. *Pharm. Res.* **10:** 1427–1433.
9. Briscoe, P., I. Caniggia, A. Gravers, B. Benson, L. Huang, A. K. Tanswell, and B. A. Freeman. 1995. Delivery of superoxide dismutase to pulmonary epithelium via pH-sensitive liposomes. *Am. J. Physiol.* **268:** L374–380.
10. Straubinger, R. M. 1993. pH-sensitive liposomes for delivery of macromolecules into cytoplasm of cultured cells. *Methods Enzymol.* **221:** 361–376.
11. Wu, X., K. H. Lee, and Q-T. Li. 1996. Stability and pH sensitivity of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles. *Biochim. Biophys. Acta.* **1284:** 13–19.

12. Allen, T. M., C. Hansen, and J. Rutledge. 1989. Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. *Biochim. Biophys. Acta.* **981:** 27–35.
13. Naoi, M., and K. Yagi. 1980. Incorporation of enzyme through blood-brain barrier into the brain by means of liposomes. *Biochem. Int.* **1:** 591–596.
14. Yagi, K., M. Naoi, H. Sakai, H. Abe, H. Konishi, and S. Arichi. 1982. Incorporation enzyme into brain by means of liposomes of novel composition. *J. Appl. Biochem.* **4:** 121–125.
15. Yagi, K., and M. Naoi. 1986. Glycolipid insertion into liposomes for their targeting to specific organs. *In* Medical Application of Liposomes. K. Yagi, editor. Japan Scientific Societies Press, Tokyo. 91–97.
16. Chen, D., Q-T. Li, and K. H. Lee. 1993. Antinociceptive activity of liposome-entrapped calcitonin by systemic administration in mice. *Brain Res.* **603:** 139–142.
17. Chen, D., and K. H. Lee. 1993. Biodistribution of calcitonin encapsulated in liposomes in mice with particular reference to the central nervous system. *Biochim. Biophys. Acta.* **1158:** 244–250.
18. Sakamoto, A., and T. Ido. 1993. Liposome targeting to rat brain: effect of osmotic opening of the blood-brain barrier. *Brain Res.* **629:** 171–175.
19. Düzgünes, N. 1985. Membrane fusion. *In* Subcellular Biochemistry, Vol. 11. D. B. Roodyn, editor. Plenum, New York. 195–286.
20. Wilschut, J. 1991. Membrane fusion in lipid vesicle systems. *In* Membrane Fusion. J. Wilschut and D. Hoekstra, editors. Marcel Dekker, Inc., New York. 89–126.
21. Struck, D. K., D. Hoekstra, and R. E. Pagano. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry.* **20:** 4093–4099.
22. Wilschut, J., and D. Papahadjopoulos. 1979. Ca^{2+}-induced fusion of phospholipid vesicles monitored by mixing of aqueous contents. *Nature.* **281:** 690–692.
23. Thulborn, K. R., and W. H. Sawyer. 1978. Properties of locations of a set of fluorescent probes sensitive to the fluidity gradient of the lipid bilayer. *Biochim. Biophys. Acta.* **511:** 125–140.
24. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
25. Kean, D. L. 1968. Rapid, sensitive spectrophotometric method for quantitative determination of sulfatides. *J. Lipid Res.* **9:** 319–327.
26. Wilschut, J., N. Düzgünes, R. Fraley, and D. Papahadjopoulos. 1980. Studies on the mechanism of membrane fusion: kinetics of calcium ion induced fusion of phosphatidylserine vesicles followed by a new assay for mixing of aqueous vesicle contents. *Biochemistry.* **19:** 6011–6021.
27. Wilschut, J., S. Nir, J. Scholma, and D. Hoekstra. 1985. Kinetics of Ca^{2+}-induced fusion of cardiolipin-phosphatidylcholine vesicles: correlation between vesicle aggregation, bilayer destabilization, and fusion. *Biochemistry.* **24:** 4630–4636.
28. Abdel-Latif, A. A. 1966. A simple method for isolation of nerve-ending particles from rat brain. *Biochim. Biophys. Acta.* **121:** 403–406.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
30. Dawson-Saunders, B., and R. G. Trapp. 1990. Basic and Clinical Biostatistics. Appleton & Lange, Norwalk.
31. Düzgünes, N., T. M. Allen, J. Fedor, and D. Papahadjopoulos. 1987. Lipid mixing during membrane aggregation and fusion: why fusion assays disagree. *Biochemistry.* **26:** 8435–8442.
32. Bentz, J., N. Düzgünes, and S. Nir. 1983. Kinetics of divalent cation induced fusion of phosphatidylserine vesicles: correlation between fusogenic capacities and binding affinities. *Biochemistry.* **22:** 3320–3330.
33. Bentz, J., S. Nir, and D. G. Covell. 1988. Mass action kinetics of virus-cell aggregation and fusion. *Biophys. J.* **54:** 449–462.
34. Cullis, P. R., C. P. Tilcock, and M. J. Hope. 1991. Lipid polymorphism. *In* Membrane Fusion. J. Wilschut and D. Hoekstra, editors. Marcel Dekker, Inc., New York. 35–64.
35. Sen, A., and S-W. Hui. 1988. Direct measurement of headgroup hydration of polar lipids in inverted micelles. *Chem. Phys. Lipids.* **49:** 179–184.
36. Epand, R. M., and B. T-C. Leon. 1992. Hexagonal phase forming propensity detected in phospholipid bilayers with fluorescent probes. *Biochemistry.* **31:** 1550–1554.
37. Osanai, T., and Y. Nagai. 1984. Suppression of experimental allergic encephalomyelitis (EAE) with liposome-encapsulated protease inhibitor: therapy through the blood-brain barrier. *Neurochem. Res.* **9:** 1407–1416.
38. Laham, A., N. Claperon, J. J. Durussel, E. Fattal, J. Delatter, F. Puisieux, P. Couverur, and P. Rossignol. 1988. Liposomally entrapped adenosine triphosphate: improved efficiency against experimental brain ischaemia in the rat. *J. Chromatogr.* **440:** 455–458.
39. Almeida, M. T., J. Ramalho-Santos, C. R. Oliveira, and M. C. Pedroso de Lima. 1994. Parameters affecting fusion between liposomes and synaptosomes: role of proteins, lipid peroxidation, pH and temperature. *J. Membrane Biol.* **142:** 217–222.
40. Faure, C., J. F. Tranchant, and E. J. Dufourc. 1996. Comparative effects of cholesterol and cholesterol sulfate on hydration and ordering of dimyristoylphosphatidylcholine membranes. *Biophys. J.* **70:** 1380–1390.
41. Montich, G. G., M. M. Bustos, B. Maggio, and F. A. Cumar. 1985. Micropolarity of interfaces containing anionic and neutral glycosphingolipids as sensed by merocyanine 540. *Chem. Phys. Lipids.* **38:** 319–326.
42. Sundler, R., and J. Bondeson. 1991. Modulation of membrane fusion by glycolipids and lectin-mediated intermembrane contact. *In* Membrane Fusion. J. Wilschut and D. Hoekstra, editors. Marcel Dekker, Inc., New York. 255–271.
43. Hope, M. J., D. C. Walker, and P. R. Cullis. 1983. Ca^{2+} and pH induced fusion of small unilamellar vesicles consisting of phosphatidylethanolamine and negatively charged phospholipids: a freeze fracture study. *Biochem. Biophys. Res. Commun.* **110:** 15–22.
44. Allen, T. M., K. Hong, and D. Papahadjopoulos. 1990. Membrane contact, fusion, and hexagonal (H_{II}) transitions in phosphatidylethanolamine liposomes. *Biochemistry.* **29:** 2976–2985.
45. Ellens, H., J. Bentz, and F. C. Szoka. 1985. H^+- and Ca^{2+}-induced fusion and destabilization of liposomes. *Biochemistry.* **24:** 3099–3106.
46. Nir, S., N. Düzgünes, and J. Bentz. 1983. Binding of monovalent cations to phosphatidylserine and modulation of Ca^{2+}- and Mg^{2+}-induced vesicle fusion. *Biochim. Biophys. Acta.* **735:** 160–172.
47. Portis, A., C. Newton, W. Pangborn, and D. Papahadjopoulos. 1979. Studies on the mechanism of membrane fusion: evidence for an intermembrane Ca^{2+}-phospholipid complex, synergism with Mg^{2+}, and inhibition by spectrin. *Biochemistry.* **18:** 780–790.
48. Maggio, B., F. A. Cumar, and R. Caputto. 1978. Surface behaviour of gangliosides and related glycosphingolipids. *Biochem. J.* **171:** 559–565.
49. Maggio, B., J. M. Sturtevant, and R. K. Yu. 1987. Effect of calcium ions on the thermotropic behaviour of neutral and anionic glycosphingolipids. *Biochim. Biophys. Acta.* **901:** 173–182.
50. Hauser, H., I. Pascher, R. H. Pearson, and S. Sundell. 1981. Preferred conformation and molecular packing of phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta.* **650:** 21–51.
51. Hoekstra, D., and N. Düzgünes. 1986. Ricinus communis agglutinin-mediated agglutination and fusion of glycolipid-containing phospholipid vesicles: effect of carbohydrate head group size, calcium ions, and spermine. *Biochemistry.* **25:** 1321–1330.
52. Melikyan, G. B., N. S. Matinyan, and V. B. Arakelian. 1990. The influence of gangliosides on the hydrophilic pore edge line tension and monolayer fusion of lipid membranes. *Biochim. Biophys. Acta.* **1030:** 11–15.
53. Singh, D. M., X. Shan, J. H. Davis, D. H. Jones, and C. W. M. Grant. 1995. Oligosaccharide behavior of complex natural glycosphingolipids in multicomponent model membranes. *Biochemistry.* **34:** 451–463.
54. Vidal, M., and D. Hoekstra. 1995. In vitro fusion of reticulocyte endocytic vesicles with liposomes. *J. Biol. Chem.* **270:** 17823–17829.
55. Slepushkin, V. A., S. Simões, P. Dazin, M. S. Newman, L. S. Guo, M. C. Pedroso de Lima, and N. Düzgünes. 1997. Sterically stabilized pH-sensitive liposomes: intracellular delivery of aqueous contents and prolonged circulation in vivo. *J. Biol. Chem.* **272:** 2382–2388.
56. Batemburg, A. M., J. H. van Esch, and B. Dekruijff. 1988. Melittin-induced changes of the macroscopic structure of phosphatidylethanolamines. *Biochemistry.* **27:** 2324–2331.
57. Maggio, B., and R. K. Yu. 1992. Modulation by glycosphingolipids of membrane-membrane interactions induced by myelin basic protein and melittin. *Biochim. Biophys. Acta.* **1112:** 105–114.
58. Perillo, M. A., J. N. Scarsdale, R. K. Yu, and B. Maggio. 1994. Modulation by gangliosides of the lamellar-inverted micelle (hexagonal II) phase transition in mixtures containing phosphatidylethanolamine and dioleoylglycerol. *Proc. Natl. Acad. Sci. USA.* **91:** 10019–10023.