Synthesis and Spectroscopic Analysis of Chromophoric Lipids Inducing pH-Dependent Liposome Fusion

Takenori Tomohiro,*,†,‡ Yoshikatsu Ogawa,† Hiroaki Okuno,†,§ and Masato Kodaka*,†

Contribution from the Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan, and Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

Received August 7, 2003; E-mail: ttomo@ms.toyama-mpu.ac.jp; m.kodaka@aist.go.jp.

Abstract: We design novel chromophoric amphiphiles **6a**−**c**, which lead to pH-dependent membrane fusion of egg phosphatidylcholine (eggPC) liposome containing them. Lipids **6a**−**c** comprise double alkyl chains, a single chain with a 2-nitrophenol group as a pH trigger, and dipeptide (Asp-Asp) between them. The pKa values of 2-nitrophenol groups of **6a**−**c** in liposome are larger than that of hydrophilic compound **9** in an aqueous solution. Absorption spectra indicate that the fields around 2-nitrophenol of **6a**−**c** situated in liposome membranes are more hydrophobic than that of **9** in an aqueous solution, whereas the environments around deprotonated 2-nitrophenolate of **6b** and **6c** are not so hydrophobic as that of **6a**. This means that protonated 2-nitrophenol groups of **6a**−**c** are embedded in bilayer membranes. Deprotonated 2-nitrophenol groups of **6b** and **6c** must be located in less hydrophobic circumstances, while that of **6a** is still embedded in bilayer membranes because of its larger hydrophobicity. Absorption spectra and ¹H NMR spectra respectively suggest that protonated 2-nitrophenol groups of **6a** and those of **6c** might take face-to-face associations in bilayer membranes.

Introduction

Compared with a viral vector, liposomes are good candidates as efficient carriers of oligonucleotides or drugs into a variety of cells in the fields of gene therapy[1] or chemotherapy.[2] One of problems is, however, the stability of liposomes in vivo due to their rapid uptake into the mononuclear phagocyte system. Various types of lipid derivatives and thus liposomes have been designed to solve this problem and furthermore to improve the targeting efficiency toward tissues.[3] Some challenges are focused on the artificial mimetics of the biological uptake systems through endocytosis or phagocytosis pathways, e.g., the DNA introduction of influenza virus into a cell via membrane fusion.[4] The fusion mechanism is not clear yet, whereas it is known that conformational change of influenza hemagglutinin at low

pH induces membrane interaction by insertion of the fusion peptide. There are some observations that pores are formed in membranes after the perturbation is caused by insertion of the fusion peptide, which leads to complete fusion.[5] A similar phenomenon was recently reported for protein Bax that promoted apoptosis in neurons.[6] One of the attractive methods is to use pH-sensitive liposomes.[7] Therefore, interaction between these liposomes and cells has been tested in vitro, which showed possibilities for the application of the liposomes to gene or drug delivery.[8] Their applications are nevertheless limited, since most of these liposomes consist of only natural lipids or simply modified lipids. To achieve biomimetic pH-dependent liposomes, we designed new artificial lipids with a carboxyl group[9] or a 2-nitrophenol group,[10] which act as adhesion triggers toward cell membranes. In the lipids bearing a carboxylate trigger, the efficiency of liposome fusion in an acidic solution is clearly

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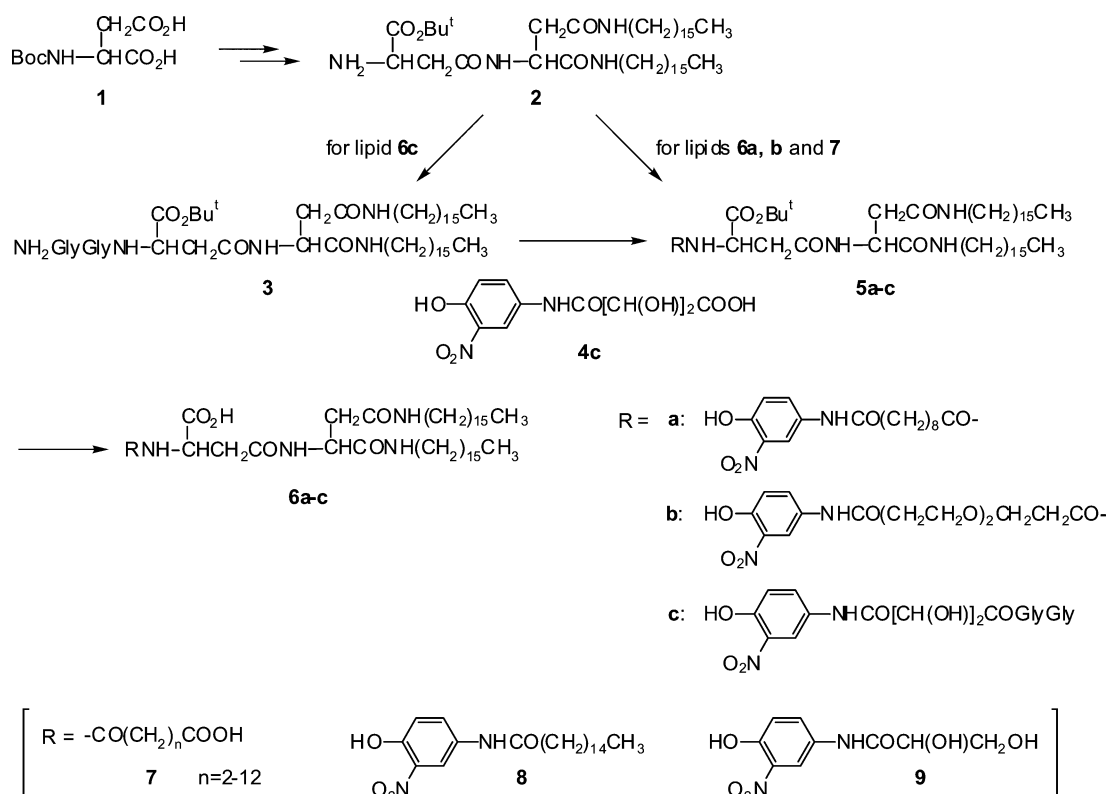* To whom inquiries about the paper should be addressed.
† National Institute of Advanced Industrial Science and Technology.
‡ Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University.
§ Present address: School of Pharmaceutical Sciences, Toho University.

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Scheme 1

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dependent on the length of aliphatic trigger chains; that is, the balance of hydrophobicity and hydrophilicity of the trigger part is important for efficient liposome fusion.[9] We also used a 2-nitrophenol group, because it normally has pKa at neutral pH and therefore we can expect direct transfer of liposome contents into cells at the physiological pH, in analogy with a vector in vitro. Actually the lipid (**6a**) with a 2-nitrophenol trigger can induce liposome fusion at pH lower than 8.[10]

In the present study, a series of lipids bearing a 2-nitrophenol group, **6a** and its analogues (**6b** and **6c**), is synthesized and analyzed in detail by spectroscopic methods to evaluate their microscopic environments in bilayer membranes.

Results

Preparation of Lipids and Liposomes. Synthetic routes of lipids bearing a 2-nitrophenol group (**6a**−**c**) derived from **1** are shown in Scheme 1. Compound **2** composed of two aspartic acids and double long alkyl chains was prepared and characterized as previously described.[9]

The synthetic lipids consist of two aspartic acids, double long alkyl chains, and the additional third chain with a 2-nitrophenol group at the end. These fragments connect by amide bonds that are stable under ordinary acidic and basic conditions. This property is important for further combination between the lipids and peptides, and we have directly synthesized lipopeptides by a peptide synthesizer on a solid phase after preparing peptides.[11,12] The hydrophilic carboxyl group of the aspartic residue has a role to maintain its position on the surface of the liposome membrane. The 2-nitrophenol group is employed as a pH trigger,

since its pKa value is normally around 7 and thus liposome fusion is possible at physiological pH. In addition, this is a good chromophore for spectroscopic analyses of its microscopic environments, since the absorption wavelength is sensitive to surrounding polarity, molecular interaction, and so on. Three kinds of linkers, *n*-alkyl, polyethylene-type, and protic chains between aspartic acid and 2-nitrophenol were designed to evaluate the effect of hydrophobicity of trigger chains. In the previous study using lipids bearing a carboxylate trigger, efficiency of lipid mixing was largely dependent on the length of trigger alkyl chains.[9] Namely, when the pH of the solution was changed from the neutral region toward the acidic side, hydrophobicity of the trigger chain was increased by protonation of the carboxylate anion. The trigger chain should be inserted into the bilayer membrane of another liposome, which resulted in membrane perturbation.[13] These results indicated that the balance of hydrophilic and hydrophobic character should be important for membrane fusion. To reveal the effect of hydrophobicity of the trigger chains on the microscopic structures of lipids, two other types of analogous lipids (**6b,c**) with less hydrophobicity were designed and synthesized as well as **6a**.

The liposome solution was prepared by sonication using a probe-type sonicator. The liposomes were composed of eggPC and the synthetic lipids (5−20 mol % of the whole lipids). Purification of the liposomes was achieved by gel filtration on Sephadex G75 (eluant: 10 mM Taps buffer, 0.1 M NaCl, pH 9.0) with monitoring the absorbance at 415 nm due to 2-nitrophenolate moiety. There was one peak with the same retention time as the peak of liposomes detected by light scattering at 420 nm (irradiated at 350 nm) as shown in Figure 1. This
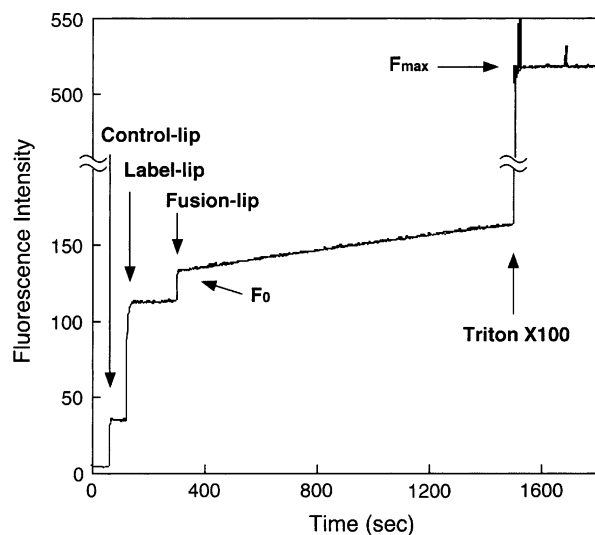
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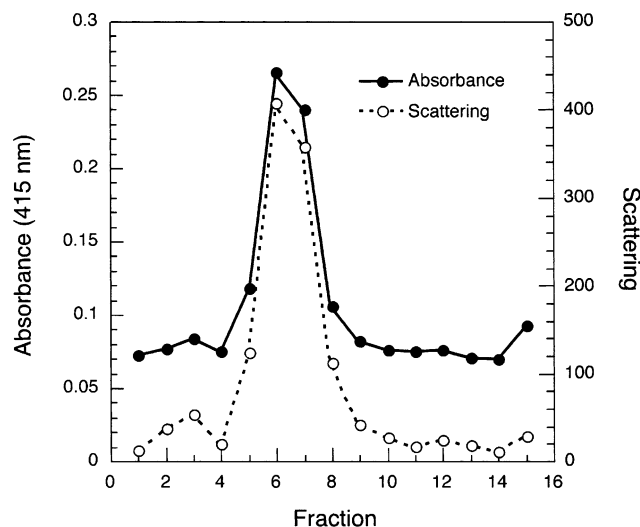
Figure 1. Gel filtration of liposome using a Sephadex G75 column eluted with 10 mM Taps buffer (pH 9.0, 0.1 M NaCl). Left: absorbance at 415 nm (solid line). Right: light scattering (detection 420 nm/irradiation 350 nm, dotted line).

Figure 2. Time course of lipid mixing (%) of Control-lip and S10% Fusion-lip including **6c** (pH 7.4, 37 °C).

indicates that the synthetic lipids bearing 2-nitrophenol groups are surely incorporated into eggPC liposomes.

pKa of the 2-Nitrophenol Group. There are some methods to estimate pKa values of compounds embedded in membranes.[14] Here we investigated the properties of synthetic lipids in the liposome membrane by measuring pKa of the 2-nitrophenol group. Compound **9** was derived from glyceric acid as a water-soluble standard derivative. In addition, lipid **8** was synthesized by coupling with palmitic acid as a hydrophobic 2-nitrophenolate probe with a single alkyl chain to compare the hydrophobic strengths of organic solvents and lipid bilayer membranes. The pKa value of the 2-nitrophenol group of water-soluble derivative **9** was determined by a pH-metric titration in an aqueous solution as 6.78. In synthetic lipids **6a−c** and **8**, the pH-metric titration was performed by addition of citric acid into the liposome solution containing 10 mol % of each synthetic lipid (S10% Fusion-lip). The pH-titration was monitored by the absorbance of the 2-nitrophenolate moiety at 20°C (e.g., see

Table 1. pKa Values of Compounds **6a−c**, **8**, and **9**

compound	condition[a]	pKa
6a	S10% EggPC liposome	>9
6b	S10% EggPC liposome	8.97
6c	S10% EggPC liposome	8.55
8	S10% EggPC liposome	8.73
9	H$_2$O	6.78

[a] The pKa values were measured in Fusion-lip solution at 20 °C for compound **6** and **8** and in an aqueous solution for compound **9**.

the supporting information), where the absorbance was measured after the pH of the solution became steady. As summarized in Table 1, all the pKa values of synthetic lipids **6a−c** and **8** in liposome were larger than 8.5, which were quite different from the pKa of **9** (6.78) in an aqueous solution. In **6a**, nitrophenolic protons were not completely dissociated even at pH 11.5 (see the supporting information).

Electronic Absorption Spectra. Some spectroscopic methods are known to be useful to study hydrophobic environments in membranes.[15] In the present study, the microscopic environment around the 2-nitrophenol trigger parts were analyzed by absorption spectra of the protonated and deprotonated species in aqueous solutions, organic solvents, and lipid bilayer membranes.

Absorption maxima of **9** were observed at 367 nm (λp) due to the protonated form (pH 3.1) and at 427 nm (λd) due to the deprotonated one (pH 11.3) in an aqueous solution. These absorption bands showed a red shift (379 and 430 nm, respectively) in MeOH, where the protonated 2-nitrophenol form gave a particularly large red shift. This suggests that the change (viz., red shift) in the absorption maxima (λp and λd) is a good measure of the hydrophobic environment around 2-nitrophenol group. The absorption maxima of **8** in MeOH−CHCl$_3$ appeared at 383 nm (λp) for the protonated form and at 433 nm (λd) for the deprotonated one, which were close to those of **9** in MeOH (379 and 430 nm, respectively). In CHCl$_3$, λp and λd of **8** showed a further red shift up to 390 and 446 nm, respectively. In the liposomes, **8** gave λp at 384 nm and λd at 449 nm, which were very close to those in CHCl$_3$. This obviously indicates that **8** should be located in a lipid bilayer membrane whose hydrophobic strength is comparable to that of CHCl$_3$. Since there seems to be a good relation between the red shift and the hydrophobic strengths, we measured absorption spectra of the synthetic lipids **6a−c** in a similar manner (Table 2). Though **6a−c** in liposomes all exhibited appreciable red shift as compared to **9** in an aqueous solution, the behavior of **6a** is clearly different from those of **6b** and **6c**. In fact, **6a** shows a small red shift (374 nm) in λp and a large red shift (448 nm) in λd, while **6b** and **6c** show large red shifts (389 and 396 nm) in λp and small red shifts (431 and 436 nm) in λd.

NMR Spectra of Liposome. The liposome solutions containing synthetic lipids were prepared in deuterium oxide (D$_2$O), and then the pD was adjusted to around 10. The chemical shifts of aromatic protons (H$_a$, H$_b$, H$_c$) in **6c** were compared under both basic and acidic conditions, namely, for deprotonated and protonated states of the nitrophenol group (Table 3). Whereas aromatic protons of **6a**, **6b**, **6c**, **8**, and **9** in protonated form showed their signals at comparable positions in 10% CD$_3$OD−CDCl$_3$, the corresponding signals of lipid **6c** obviously moved

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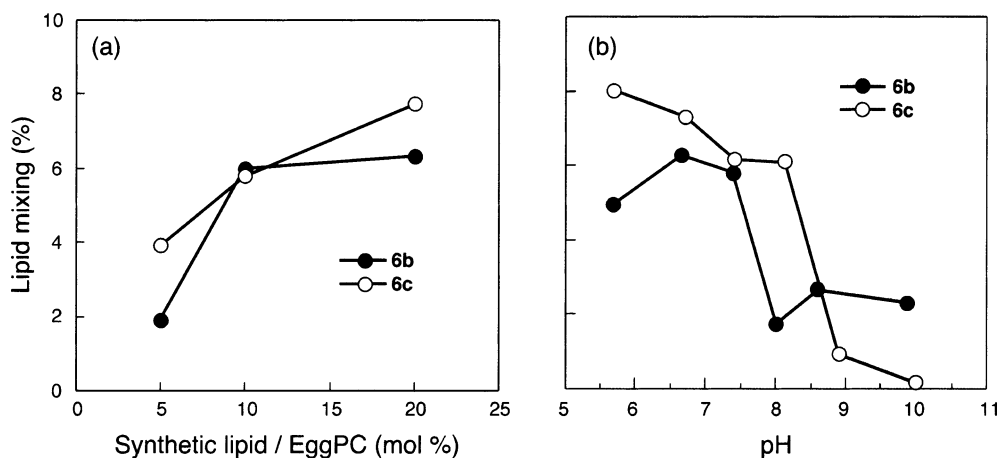
Table 2. Electronic Absorption Maxima of Lipids **6a**−**c**, **8**, and **9**

compound	condition	absorption maxima λp, nm[a]	absorption maxima λd, nm[b]
6a	50% MeOH−CHCl$_3$	384	433
	S10% liposome	374	448
6b	S10% liposome	389	432
6c	S10% liposome	396	438
8	10% MeOH−CHCl$_3$	383	433
	CHCl$_3$	390	446
	S10% liposome	384	449
9	H$_2$O	367	427
	MeOH	379	430
	10 mM Hepes	368	428

[a] The absorption maxima of protonated species in each condition at 20 °C. [b] The values of deprotonated species.

Table 3. Chemical Shifts (ppm) of Aromatic Protons in Compounds **6a**−**c**, **8**, and **9**

solvent compound	CDCl$_3$-CD$_3$OD 6a	6b	6c	8	9	CDCl$_3$ 8	D$_2$O 9
temp, °C	50	50	50	rt	rt	rt	rt
Ha	8.32	8.45	8.43	8.41	8.52	8.29	8.18
Hb	7.84	7.87	7.81	7.79	7.81	7.77	7.60
Hc	7.10	7.14	7.11	7.10	7.15	7.13	7.11

6c	liposome[a] protonated	liposome[a] deprotonated	CDCl$_3$−CD$_3$OD protonated	DMF-d$_7$ protonated	DMF-d$_7$ deprotonated
Ha	8.16	7.85	8.43	8.73	7.75
Hb	7.59	7.21	7.81	8.00	7.60
Hc	7.08	6.66	7.11	7.20	7.30

[a] S10% Fusion-lip was prepared in deuterated 10 mM Hepes buffer followed by pD adjustment to around 10 with NaOD, and the solution was acidified with deuterated citric acid.

to higher magnetic field in liposome. These signals of **6c** are also situated at higher magnetic field compared with that in DMSO-d_6. In lipid **9**, on the other hand, the chemical shifts moved to higher magnetic field when the solvent was changed from 10% CD$_3$OD−CDCl$_3$ to D$_2$O. These two tendencies are not consistent, since the hydrophobicity of the liposome membrane should be as large as that of CHCl$_3$ in view of the results of the absorption spectra in Table 2. The NMR shift to higher magnetic field on deprotonation of **6c** in liposome and in DMF-d_7 is reasonably understood, because electron density on the hydrogen atoms of 2-nitrophenolate should be increased by deprotonation. Contrary to **6c**, **6a**, and **6b** did not give NMR spectra in liposome that are suitable for quantitative analysis of chemical shift.

Lipid Mixing. The lipid mixing assay was performed by monitoring FRET (fluorescence resonance energy transfer) between two fluorescent labeled lipids, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl-*sn*-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl-*sn*-phosphatidylethanolamine (Rh-PE), using a method of Struck et al.[16,17] Fluorescent labeled liposome (Label-lip)

Figure 3. Time-resolved electronic absorption spectra of lipid mixing solution of Control-lip and S10% Fusion-lip including **6b** (pH 4.0, 20 °C).

contained 2 mol % each of fluorescent lipids. To a Hepes buffer with appropriately adjusted pH at 37 °C in a quartz cell was added an eggPC liposome solution (Control-lip) followed by addition of Label-lip, then the lipid mixing was initiated by the addition of Fusion-lip (eggPC liposomes containing certain amount of synthetic lipids). Figure 2 showed the lipid mixing of Control-lip and S10% Fusion-lip including **6c** at pH 7.4. After Control-lip and Label-lip were mixed, fluorescent intensity at 530 nm was not changed during 5 min before Fusion-lip was added. All runs were done in duplicate or more. It was confirmed in a control experiment, where Control-lip was added instead of Fusion-lip, that the lipid mixing did not occur during the whole measurement.

Time-Resolved Electronic Absorption Spectra. The initial fusion process was detected by a multichannel spectrophotometer. Absorbance at 432 nm due to 2-nitrophenolate anion was immediately decreased within 100 ms after the addition of citric acid to a stirring Fusion-lip (10 mol % of **6b**) solution and an absorption band due to the protonated species emerges at 390 nm (Figure 3). After several minutes, the absorption was gradually increased throughout the range from 350 to 600 nm, and the solution became turbid. This suggests that the fusion or the phase transition of lipid membranes takes place after the rapid protonation of 2-nitrophenolate anion. Kinetic analysis of the initial decrease of absorbance at 432 nm gave the apparent first-order rate constant of protonation to be 5.2 s^{-1}.

Discussion

Perturbation of membranes is known to be induced by Ca^{2+},[18] fusion peptides, and proteins such as hemagglutinin and Bax, as well as by cooperation of these proteins and some lipid such as cardiolipin.[19] In the previous studies, we found that the balance of hydrophilic and hydrophobic character of trigger chains is important for liposome membrane fusion.[9] To initiate lipid mixing, the trigger chain should penetrate into the membrane or disturb the surface. However, there is a hydrophobic barrier of the phospholipid bilayer membrane.[20] Actually,

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Figure 4. Dependence of lipid mixing (%) on content of synthetic lipids (a) and on pH (b).
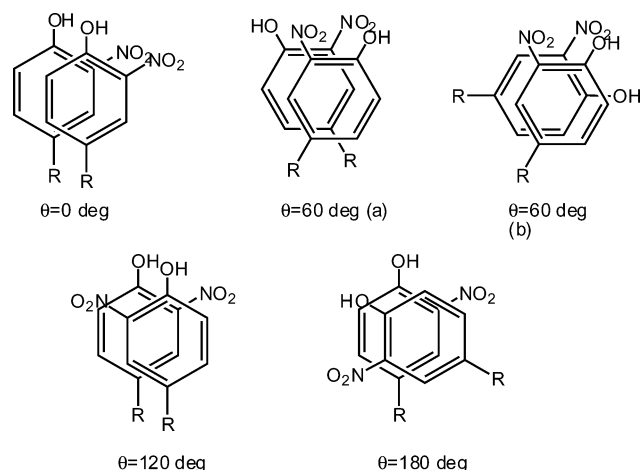

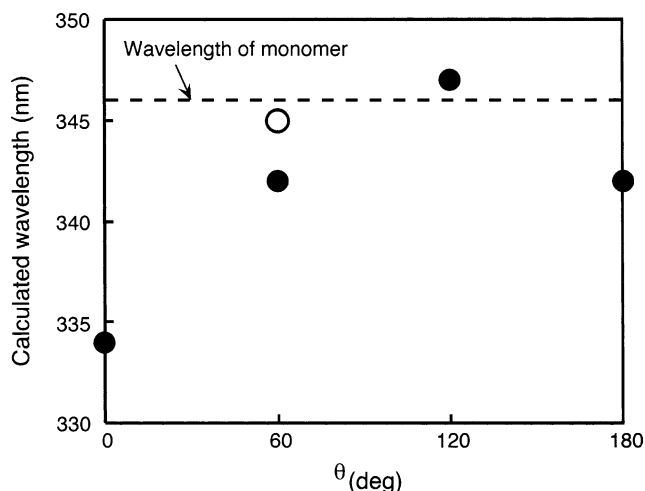**Figure 5.** Typical parallel arrangements between two 2-nitrophenol derivative molecules.

Figure 6. Dependence of calculated wavelength on angle θ.

efficiency of lipid mixing largely depends on the length of the trigger chain, *n*-alkylcarboxylate (**7**, $2 \leq n \leq 12$, as described in Scheme 1); that is, the short trigger ($n = 2, 4$) chain has only a little effect owing to the poor hydrophobicity. In this report, 2-nitrophenolate was chosen as a pH-sensitive and chromophoric group that is more hydrophobic than carboxylate.

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The efficiency of lipid mixing in this series (**6a**−**c**) was lower compared with that using fusion peptide or proteins[10] but apparently increased with the amount of the synthetic lipids from 5 to 20 mol % (Figure 4a). The data of lipid mixing used here were the values 20 min after the addition of Fusion-lip in the lipid mixing experiment. There was no large difference in the efficiency between lipids **6b** and **6c**; viz., the lipid mixing was around 5−8%, which is rather small in comparison with those of **6a** (ca. 10%)[10] and **7** (9−13% on using S10% Fusion-lip including **7**).[9] The efficiency of lipid mixing also depends on the solution pH; viz., the efficiency is increased on protonation of a 2-nitrophenol group (Figure 4b). Molecular shape of these synthetic lipids might affect the stability of the liposome membrane. The result that the shape of the synthetic lipids in minimum-energy states calculated by MM3 (CAChe system) was not very different from that of eggPC suggests that on protonation the hydrophobic trigger chain induces membrane perturbation leading to lipid mixing between liposomes.

Absorption spectra give useful information on the microscopic environments around the deprotonated and protonated 2-nitrophenol groups. Table 2 shows the relation between λd and λp, in which the both wavelengths on the whole show red shift as the environments around the 2-nitrophenol become more hydrophobic. It should be noted, however, that the red shift of λp for **8**, **9**, **6b**, and **6c** situated in MeOH, MeOH−CHCl$_3$ or liposome is as a whole larger ($\Delta\lambda = 12$−29 nm) than that of $\Delta\lambda$ ($\Delta\lambda = 3$−22 nm). Here $\Delta\lambda$ is defined as the increase in the wavelength (red shift) from that of **9** in an aqueous solution. Red shift is also observed when the individual lipid exists in different solvents. For example, the red shift of λd in **8** is 13 nm between CHCl$_3$ (446 nm) and MeOH (433 nm) and 16 nm between liposome in Hepes buffer (449 nm) and MeOH (433 nm). These comparable values clearly demonstrate that the hydrophobic fields surrounding 2-nitrophenolate anions of **8** resemble each other in liposome and CHCl$_3$. Another example is that **9** exhibits the red shift of λp by 12 nm when the solvent is changed from H$_2$O (367 nm) to MeOH (379 nm). In **6a**, a similar phenomenon was observed in two lipid conditions; that is, λd is 448 nm for liposome, which is close to λd of **8** in liposome (449 nm), and 433 nm for MeOH−CHCl$_3$. This suggests that the trigger of **6a** exists in a liposome membrane even when the 2-nitrophenol headgroup takes an anionic form. In contrast to **6a**, **6b** and **6c** in liposomes gave λd (432 and 438

nm) comparable to **9** in an aqueous solution (427 nm), possibly because the triggers of **6b** and **6c** exist out of the liposome membrane. The fact that the p*K*a values of lipid **6a**−**c** and **8** (>8.55) in liposome solutions were much larger than that of **9** (6.78) definitely proves that the former lipids in the protonated state are located in a more hydrophobic field than the latter, because electrostatic attraction is stronger and thus deprotonation is more depressed in higher hydrophobic surroundings. The anionic chromophore can probably form hydrogen bonds with MeOH in **6a** (50% MeOH−CHCl$_3$) and **8** (10% MeOH−CHCl$_3$), which makes λd smaller than expected ($\Delta\lambda = 6$ nm). Actually, in CHCl$_3$ **8** gives large red shifts of λd ($\Delta\lambda = 19$ nm) and λp ($\Delta\lambda = 23$ nm). Also, in liposome the 2-nitrophenol group of **8** is probably buried inside the bilayer membrane and thus hydrogen bonding with water is thought to be prohibited. Contrary to all these compounds, **6a** in liposome shows obviously unusual behavior in that the red shift of λp ($\Delta\lambda = 7$ nm) is too small compared with that of λd ($\Delta\lambda = 21$ nm).

Though the large red shift of λd ($\Delta\lambda = 21$ nm) in **6a** indicates that the anionic chromophore is situated in the hydrophobic region, the small red shift of λp ($\Delta\lambda = 7$ nm) in **6a** cannot be explained by small hydrophobicity of the fields, since the protonated form should be more stable in hydrophobic environments than the deprotonated form. Therefore, one has to find another explanation for the above unusual result. Since there is a possibility that **6a** forms aggregates in bilayer membranes, we evaluated the effect of association on the shift of λp with a molecular orbital calculation method (INDO/1 in ZINDO of CAChe system). We calculated λp for typical face-to-face associations between two 2-nitro-4-acetamidophenol molecules (Figure 5). The angle (θ) between the two nitro groups is changed from 0 deg to 180 deg by 60 deg, where there are two arrangements for $\theta = 60$ deg. The distance between the two benzene rings is fixed to 3.7 Å. Dependence of the calculated λp is illustrated in Figure 6, which proves that λp shows blue shift from the wavelength of the monomer when the aggregate is formed and that the blue shift is especially large when θ is near zero. These results might account for the observation that **6a** in liposome gives λp smaller than **6b** and **6c** in liposome.

It is known that interaction between aromatic lipids such as azobenzene- or pyren-modified lipids in ordinal aliphatic lipid membrane results in phase separation.[21] However, since electrostatic repulsion is stronger than the aromatic attractive interaction, aromatic lipids are separated from each other when they have charge with the same sign.[21a] There is a possibility that the present synthetic lipids bearing 2-nitrophenol groups should show a similar phenomenon. Actually, the absorption spectra and the ^{1}H NMR spectra, respectively, showed that **6a** and **6c** in the protonated state might associate in the membrane through the aromatic π−π interaction. This association can change their electronic states, resulting in the blue shift of the absorption spectra and the NMR shift toward higher magnetic field due to diamagnetic shielding of each aromatic ring. The reason **6c** does not have so small a λp as **6a** is not clear. One of the possible reasons might be that two **6c** molecules take an arrangement near $\theta = 120$ deg as shown Figures 5 and 6, while **6a** has a possibility to take an arrangement near $\theta = 0$ deg.

The 2-nitrophenolate trigger chain of **6c** should penetrate into a membrane, and thus **6c** becomes a so-called tridentate-type lipid, which can interact with each other to generate a microdomain. This might destabilize membranes and induce membrane fusion via lipidic particle formation (inverted micelles) in a joining part of liposomes, such as the cases of phosphatidylserine (PS)-PC[22] or cardiolipin with a calcium metal ion.[23]

Conclusions

Liposome membrane fusion was investigated using the chromophoric synthetic lipids having 2-nitrophenol derivatives as trigger chains. The lipid mixing assay applying FRET showed that the liposome fusion proceeded in the pH region lower than the p*K*a of 2-nitrophenol groups. The p*K*a values of the chromophoric lipids in a lipid bilayer membrane were larger than 8.5, which were quite different from that of **9** (6.78) in an aqueous solution. The protonation of 2-nitrophenolate anions in membranes was not rapid, and membrane fusion gradually took place after protonation. Electronic absorption of these synthetic lipids indicated different environments around the chromophoric groups, depending on the hydrophobicity of the trigger chains. The deprotonated hydrophilic trigger chains could be out of the membrane and then penetrate into the membrane of another liposome after protonation. The higher hydrophobic trigger chains, however, could not remain outside the membrane even in the deprotonated form. The synthetic lipids having three chains (**6a**−**c**) led to membrane fusion, but the one having a single chain (**8**) did not induce membrane fusion. In addition, membrane fusion was not clearly observed for the Fusion-lip including **6a** in the deprotonated form. These data suggested that the synthetic lipid might form a tripodal lipid and they might interact each other after penetration of the protonated trigger chains into the membrane.

Experimental Section

Materials. Egg phosphatidylcholine (PC) was obtained from Kawahara Petrochemical Co., Ltd. *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl-*sn*-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl-*sn*-phosphatidylethanolamine (Rh−PE) were commercially available from Molecular Probes (Eugene, OR). *N*-*tert*-Butyloxycarbonyl-L-aspartic acid (Boc-Asp), *N*-9-fluorenylmethoxycarbonyl-L-aspartic acid α-*tert*-butyl ester (Fmoc-Asp-(αBu^t)), glycylglycine, 1-hydroxybenzotriazole hydrate (HOBt·H$_2$O), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). 1-Hexadecylamine, 4-amino-2-nitrophenol, and 3,6-dioxaoctanedioic acid were obtained from Aldrich Chemical Co., Inc. (St. Louis, MO). 4-Dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide monohydrochloride (WSCI), sebacic acid, and DL-tartaric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Instruments. Fluorescent studies were performed by a JASCO FP-777 spectrofluorometer. Stationary electronic absorption spectra and time-resolved electronic absorption spectra were, respectively, measured by JASCO V-550 UV/ Vis and OTSUKA ELECTRONICS MCPD-1000 spectrophotometers. IR spectra were measured on a JASCO FT/IR-7300 spectrometer, and NMR spectra were recorded at 270 MHz on a JEOL JNM EX-270 FT-NMR spectrometer.

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Synthesis of Lipids. The synthetic route of the lipids bearing a 2-nitrophenol group derived from *N*-(*tert*-butoxycarbonyl)-L-aspartic acid (**1**) via 2-amino-*N*-(1,2-bis-hexadecylcarbamoylethyl)succinamic acid *tert*-butyl ester (**2**) is shown in Scheme 1.

2-[2-(2-Aminoacetylamino)acetylamino]-*N*-(1,2-bishexadecylcarbamoylethyl)succinamic acid butyl ester (3). FmocGlyGly (57 mg, 0.16 mmol) was dissolved in 1 mL of DMF followed by addition of 20 mL CHCl₃ solution of **2** (100 mg, 0.13 mmol) and HOBt·H₂O (25 mg, 0.16 mmol) at room temperature. A solution of TBTU in 5 mL of DMF was added to the reaction mixture at room temperature and was stirred overnight under nitrogen. The product was purified by column chromatography on silica gel eluted by 10% MeOH−CHCl₃. Yield: 42%. ¹H NMR (CDCl₃+ CD₃OD) δ 7.77 (d, 2 H, J = 7.6 Hz), 7.63 (d, 2 H, J = 7.9 Hz), 7.38 (t, 2 H, J = 7.9 Hz), 7.31 (t, 2 H, J = 7.6 Hz), 4.6−4.7 (m, 2 H, CH), 4.43 (d, 2 H, J = 6.6 Hz), 4.23 (t, 1 H, J = 7.9 Hz), 3.93 (s, 2 H), 3.87 (s, 2 H), 3.1−3.3 (m, 4 H), 2.4−2.8 (m, 4 H), 1.47 (s br, 4 H), 1.44 (s, 9 H), 1.26 (s br, 52 H), 0.88 (t, 6 H, J = 6.6 Hz). The product (25 mg) was treated with 5% piperidine−CHCl₃ (5 mL) at room temperature overnight, and the solvent was removed. The residue was washed with EtOAc, and **3** was obtained in 65% yield. ¹H NMR (CDCl₃+ CD₃OD) δ 10.41 (s, 1 H), 8.97 (s, 1 H), 8.42 (d, 1 H, J = 2.6 Hz), 7.96 (dd, 1 H, J = 2.6, 9.2 Hz), 7.85 (d, 1 H, J = 8.2 Hz), 7.35−7.45 (m, 2 H), 7.12 (d, 1 H, J = 9.2 Hz), 6.00 (t, 1 H, J = 5.6 Hz), 4.81 (ddd, 1 H, J = 5.3, 5.6, 8.2 Hz), 4.59 (ddd, 1 H, J = 2.6, 7.3, 7.3 Hz), 4.22 (d, 1 H, J = 15.7 Hz), 4.21 (d, 1 H, J = 15.7 Hz), 4.09 (d, 1 H, J = 16.0 Hz), 4.06 (d, 1 H, J = 16.0 Hz), 3.81 (s, 4 H), 3.1−3.3 (m, 4 H), 2.7−2.9 (m, 3 H), 2.38 (dd, 1 H, J = 7.3, 15.3 Hz), 1.47 (s br, 4 H), 1.40 (s, 9 H), 1.25 (s br, 52 H), 0.88 (t, 6 H, J = 6.6 Hz).

9-(4-Hydroxy-3-nitrophenylcarbamoyl)nonanoic Acid (4a). To a solution of sebacic acid (1.0 g, 5.0 mmol in 3 mL DMF) and 4-amino-2-nitrophenol (0.765 g, 5.0 mmol in 87 mL CHCl₃) was added WSC (1.0 g, 5.0 mmol in 10 mL of CHCl₃), and the mixture was stirred at room temperature overnight. The reaction mixture was washed with 50 mL of dilute HCl twice and brine and was then dried over MgSO₄. Purification of the product was performed by column chromatography on silica gel eluted with 50% EtOAc−hexane, followed by recrystallization from the same solvent. Yield: 27%. IR (KBr) 3305, 1697, 1667, 1542 cm⁻¹. ¹H NMR (CDCl₃) δ 8.34 (d, 1 H, J = 2.3 Hz), 7.82 (dd, 1 H, J = 2.6, 8.9 Hz), 7.11 (d, 1 H, J = 9.2 Hz), 2.37−2.26 (m, 4 H), 1.70 (t, 2 H, J = 6.9 Hz), 1.61 (t, 2 H, J = 6.9 Hz), 1.33 (br s, 8 H). Anal. Calcd for C₁₆H₂₂N₂O₆: C, 56.80; H, 6.55; N, 8.28. Found: C, 57.31; H, 6.22; N, 8.08.

3-{2-[2-(4-Hydroxy-3-nitrophenylcarbamoyl)ethoxy]ethoxy}-propionic Acid (4b). WSC (0.90 g, 5.0 mmol) was added to a solution of 3,6-dioxaoctanedioic acid (0.99 g, 5.0 mmol) and HOBt·H₂O (0.77 g, 5.0 mmol) in 50 mL of DMF followed by addition of 4-amino-2-nitrophenol (0.77 g, 5.0 mmol), and the reaction mixture was stirred overnight at room temperature under nitrogen. After removal of the solvent, EtOAc was added to the residue. The products were extracted with 5% aqueous NaOH solution, which was washed with EtOAc twice. The aqueous phase was acidified with concentrated HCl and extracted with EtOAc 3 times. The organic phase was washed with water and brine and was dried over MgSO₄. The crude product was purified by short ODS column chromatography eluted with 20% MeOH−H₂O after 1% HCl. Yield: 0.50 g (32%). ¹H NMR (CDCl₃) δ 10.41 (s, 1 H), 8.89 (S, 1 H), 8.32 (d, 1 H, J = 2.6 Hz), 7.96 (dd, 1 H, J = 2.6, 9.2 Hz), 7.13 (d, 1 H, J = 9.2 Hz), 4.26 (s, 2 H), 4.15 (s, 2 H), 3.82 (s, 4 H).

2,3-Dihydroxy-*N*-(4-hydroxy-3-nitrophenyl)succinamic Acid (4c). DL-Tartaric acid (2.25 g, 15.0 mmol) and 4-amino-2-nitrophenol (0.77 g, 5.0 mmol) were dissolved in 50 mL of DMF. HOBt·H₂O (0.77 g, 5.0 mmol) was added to the solution at 0 °C under nitrogen followed by addition of WSC (0.90 g, 5.0 mmol). The reaction mixture was stirred at room temperature under nitrogen overnight. After removal of the solvent, the residue was purified by ODS column chromatography

eluted with 2−5% CH₃CN−H₂O. The yellow solid was washed with 20% THF−CH₂Cl₂ and dried under vacuum. Yield: 55%. ¹H NMR (DMSO-d_6) δ 9.88 (s, 1 H), 8.48 (d, 1 H, J = 2.6 Hz), 8.34 (dd, 1 H, J = 2.6, 8.9 Hz), 7.09 (d, 1 H, J = 8.9 Hz), 4.26 (s, 1 H), 4.15 (s, 1 H), 3.82 (s, 2 H).

5a. Compounds **2** (0.25 g, 0.33 mmol) and **4a** (0.11 g, 0.32 mmol) were dissolved in 25 mL of CHCl₃. HOBt·H₂O (0.05 g, 0.33 mmol in 0.9 mL of DMF and 9.1 mL of CHCl₃) and DMAP (0.04 g, 0.33 mmol in 5 mL of CHCl₃) were added to the reaction mixture followed by addition of TBTU (0.11 g, 0.33 mmol in 0.9 mL of DMF and 9.1 mL of CHCl₃). The reaction mixture was stirred overnight at room temperature. After washing with 1 N HCl 3 times and brine, the solution was dried over Na₂SO₄ and evaporated in vacuo. Purification was performed by column chromatography on silica gel eluted with 3% MeOH−CHCl₃. Yield: 77%. IR (KBr): 3285, 1740, 1648, 1537 cm⁻¹. ¹H NMR (CDCl₃) δ 10.4 (s, 1 H), 8.38 (s, 1 H), 8.32 (d, 1 H, J = 2.3 Hz), 7.96 (dd, 1 H, J = 2.6 and 2.6 Hz), 7.51 (d, 1 H, J = 7.3 Hz), 7.42 (t, 1 H, J = 5.8 Hz), 7.11 (d, 1 H, J = 9.2 Hz), 6.59 (d, 1 H, J = 8.3 Hz), 5.99 (t, 1 H, J = 5.7 Hz), 4.79 (ddd, 1 H, J = 6.3, 6.6, 6.6 Hz), 4.66 (ddd, 1 H, J = 3.6, 7.2 Hz), 3.21 (m, 4 H), 1.46 (s, 9 H), 1.25 (br s, 60 H), 0.87 (t, 6 H, J = 6.6 Hz). Anal. Calcd for C₆₀H₁₀₆N₆O₁₀: C, 67.25; H, 9.97; N, 7.84. Found: C, 67.36; H, 10.20; N, 7.63.

5b. A solution of TBTU (128 mg, 0.40 mmol) in 25 mL of DMF was added to the mixture of **2** (0.2 g, 0.27 mmol), **4b** (84 mg, 0.27 mmol), and HOBt·H₂O (61 mg, 0.40 mmol) in 50 mL of CHCl₃ at room temperature, and the reaction mixture was stirred overnight under nitrogen. After removal of the solvent, the residue was dissolved in hot EtOAc and CHCl₃ and washed with 3 N HCl and brine and then dried over MgSO₄. The solvent was evaporated in vacuo, and yellow solid was filtrated and washed with MeOH, EtOAc, and ether. Yield: 0.159 g (57%). ¹H NMR (CDCl₃) δ 10.41 (s, 1 H, COOH), 8.97 (s, 1 H), 8.42 (d, 1 H, J = 2.6 Hz), 7.96 (dd, 1 H, J = 2.6, 9.2 Hz), 7.85 (d, 1 H, J = 8.2 Hz), 7.35−7.45 (m, 2 H), 7.12 (d, 1 H, J = 9.2 Hz), 6.00 (t, 1 H, J = 5.6 Hz), 4.81 (ddd, 1 H, J = 5.3, 5.6, 8.2 Hz), 4.59 (ddd, 1 H, J = 2.6, 7.3, 7.3 Hz), 4.22 (d, 1 H, J = 15.7 Hz), 4.21 (d, 1 H, J = 15.7 Hz), 4.09 (d, 1 H, J = 16.0 Hz), 4.06 (d, 1 H, J = 16.0 Hz), 3.81 (s, 4 H), 3.1−3.3 (m, 4 H), 2.7−2.9 (m, 3 H), 2.38 (dd, 1 H, J = 7.3, 15.3 Hz), 1.47 (s br, 4 H), 1.40 (s, 9 H), 1.25 (s br, 52 H), 0.88 (t, 6 H, J = 6.6 Hz). Anal. Calcd for C₅₈H₁₀₂N₆O₁₂: C, 64.77; H, 9.56; N, 7.81. Found: C, 64.58; H, 9.77; N, 7.69.

5c. Compounds **3** (100 mg, 0.12 mmol), **4c** (33 mg, 0.12 mmol), and HOBt·H₂O (21 mg, 0.14 mmol) were dissolved in 15 mL of DMF and 5 mL of CHCl₃. TBTU (45 mg, 0.14 mmol) was added to the solution followed by stirring at room temperature under nitrogen overnight. The solvent was removed in vacuo, and the yellow precipitate was washed with MeOH, EtOAc, CH₂Cl₂, and 10% MeOH−CH₂Cl₂ to give 58 mg (44%) of the product. ¹H NMR (CDCl₃) δ 10.38 (s, 1 H), 8.41 (s, 1 H), 8.32 (d, 1 H, J = 2.6 Hz), 7.99 (dd, 1 H, J = 2.6, 9.0 Hz), 7.52 (d, 1 H, 7.3 Hz), 7.44 (t, 1 H, 5.6 Hz), 7.12 (d, 1 H, J = 9.0 Hz), 6.55 (d, 1 H, J = 8.1 Hz), 5.88 (s, 1 H), 4.82 (ddd, 1 H, J = 4.7, 6.8, 8.1 Hz), 4.68 (ddd, 1 H, J = 3.4, 6.4, 7.3 Hz), 3.13−3.30 (m, 4 H), 2.88 (dd, 1 H, J = 3.4, 15.2 Hz), 2.80 (dd, 1 H, J = 4.7, 14.8 Hz), 2.66 (dd, 1 H, J = 6.8, 14.8 Hz), 2.45 (dd, 1 H, J = 6.4, 15.2 Hz), 2.29−2.37 (m, 2 H), 2.15−2.29 (m, 2 H), 1.68−1.75 (m, 2 H), 1.54−1.67 (m, 4 H), 1.44−1.54 (m, 13 H), 1.25 (s br, 52 H), 0.88 (t, 6 H, J = 7.1 Hz).

6a. Compound **5a** (74 mg, 0.069 mmol) was dissolved in 8 mL of 30% TFA−CHCl₃ and stirred overnight at room temperature. After the solvent was evaporated, the residue was suspended in MeOH and the precipitate was collected by filtration and washed with MeOH, dried under vacuum for overnight. Yield: 87%. IR (KBr): 3284 (OH), 1717, 1647, 1542 cm⁻¹. ¹H NMR (CDCl₃ + CD₃OD at 50 °C) δ 8.32 (d, 1 H, J = 2.5 Hz), 7.84 (dd, 1 H, J = 9.0, 2.5 Hz), 7.10 (d, 1 H, J = 9.0 Hz), 4.76 (t, 1 H, J = 5.6 Hz, NHC*H*CH₂CO), 4.63 (dd, 1 H, J = 5.6, 6.7 Hz), 3.17−3.22 (m, 4 H), 2.78 (dd, 1 H, J = 5.6, 15.1 Hz), 2.77

(dd, 1 H, J = 5.6, 15.1 Hz), 2.67 (dd, 1 H, J = 5.1, 14.9 Hz), 2.49 (dd, 1 H, J = 6.7, 14.9 Hz), 2.34 (t, 2 H, J = 7.6 Hz), 2.22 (t, 2 H, J = 7.6 Hz), 1.40−1.54 (m, 4 H), 1.26 (s br, 60 H), 0.88 ppm (t, 6 H, J = 6.6 Hz). Anal. Calcd for $C_{56}H_{98}N_6O_{10}$: C, 66.24; H, 9.73; N, 8.28. Found: C, 66.34; H, 9.72; N, 8.10.

6b. Compound **5b** (50 mg, 0.048 mmol) was dissolved in 20 mL of 30% TFA−CHCl₃, and the reaction mixture was stirred overnight at room temperature. After the solvent was evaporated, the residue was suspended in MeOH, and the precipitate was collected by filtration, washed with MeOH and CHCl₃, and dried under vacuum for overnight. Yield: 71%. ¹H NMR (CDCl₃ + CD₃OD at 50 °C) δ 8.45 (d, 1 H, J = 2.6 Hz), 7.87 (dd, 1 H, J = 2.6, 9.2 Hz), 7.14 (d, 1 H, J = 9.2 Hz), 4.82 (dd, 1 H, J = 5.0, 5.3 Hz), 4.60 (dd, 1 H, J = 5.6, 6.6 Hz), 4.18 (s, 2 H), 4.08 (s, 2 H), 3.75−3.85 (m, 4 H), 3.05−3.20 (m, 4 H), 2.85 (dd, 1 H, J = 5.0, 15.6 Hz), 2.83 (dd, 1 H, J = 5.3, 15.6 Hz), 2.61 (dd, 1 H, J = 5.6, 14.9 Hz), 2.48 (dd, 1 H, J = 6.6, 14.9 Hz), 1.46 (s br, 4 H), 1.27 (s br, 52 H), 0.89 (t, 6 H, J = 6.6 Hz).

6c. Compound **5c** (20 mg, 18 mmol) was dissolved in 6 mL of CHCl₃ and 2 mL of TFA, and the reaction mixture was stirred at room temperature overnight. The solvent and TFA were evaporated. To the residue was added MeOH, and the yellow precipitate was filtrated and washed with MeOH, EtOAc, CHCl₃, and 10% MeOH−CHCl₃ to obtain 18 mg (95%) of the yellow solid. ¹H NMR (CDCl₃ + CD₃OD) δ 8.43 (d, 1 H, J = 2.6 Hz), 7.81 (dd, 1 H, J = 2.6, 9.0 Hz), 7.11 (d, 1 H, J = 9.0 Hz), 4.77 (t, 1 H, J = 6.0 Hz), 4.63 (dd, 1 H, J = 6.0, 6.2 Hz), 3.09−3.20 (m, 4 H), 2.78 (dd, 1 H, J = 6.0, 15.4 Hz), 2.76 (dd, 1 H, J = 6.0, 15.4 Hz), 2.64 (dd, 1 H, J = 6.0, 15.0 Hz), 2.51 (dd, 1 H, J = 6.2, 15.0 Hz), 2.34 (t, 2 H, 7.7 Hz), 2.20−2.26 (m, 2 H), 1.66−1.73 (m, 2 H), 1.58−1.65 (m, 2 H), 1.47 (s br, 4 H), 1.26 (s br, 52 H), 0.88 (t, 6 H, J = 6.8 Hz).

Hexadecanoic Acid 4-Hydroxy-3-nitrophenylamide (8). Palmitic acid (0.256 mg, 1 mmol), 4-amino-2-nitrophenol (0.154 mg, 1 mmol), and DMAP (0.134 mg, 1 mmol) were dissolved in 10 mL of CHCl₃. HOBt (0.184 mg, 1.2 mmol) was dissolved in 10 mL of DMF and CHCl₃ (1:9), and the solution was added dropwise to the above reaction mixture followed by addition of WSC (0.212 mg, 1.1 mmol) dissolved in 5 mL of CHCl₃. The reaction mixture was stirred overnight. The solvent was evaporated and the residue was dissolved in EtOAc. The solution was then washed with 1N HCl 3 times and brine, and dried over Na₂SO₄. Purification by preparative thin-layer chromatography with 30% EtOAc−hexane gave the product in 87% yield. IR (KBr): 3310, 1669, 1542, 1314 cm⁻¹. ¹H NMR (CDCl₃) δ 10.40 (s, 1 H), 8.29 (d, 1 H, J = 2.6 Hz), 7.77 (dd, 1 H, J = 2.6, 8.9 Hz), 7.13 (d, 1 H, J = 8.9 Hz), 2.36 (t, 2 H, J = 7.3 Hz), 1.73 (t, 2 H, J = 7.3 Hz), 1.26 (s, 24 H), 0.87 (t, 6 H, J = 6.6 Hz). Anal. Calcd for $C_{22}H_{36}N_2O_4$: C, 67.32; H, 9.24; N, 7.14. Found: C, 67.64; H, 9.12; N, 6.93.

2,3-Dihydroxy-*N*-(4-hydroxy-3-nitrophenyl)propionamide (9). DL-Glyceric acid (0.577 mg, 3.76 mmol), 4-amino-2-nitrophenol (0.400 mg, 3.76 mmol), DMAP(0.460 mg, 3.76 mmol), HOBt (0.577 mg, 3.76 mmol), and WSC (0.751 mg, 3.77 mmol) were dissolved in 30 mL of DMF, and the mixture was stirred overnight. The solvent was evaporated in vacuo, and the residue was dissolved in CHCl₃ followed by washing with dilute HCl and was extracted with 1N NaOH and water. After reducing the solution volume, the product was purified by ODS column eluted with 5% MeCN−H₂O. IR (KBr) 3305, 1666, 1550, 1330 cm⁻¹. ¹H NMR (CD₃CN) δ 10.14 (s, 1 H), 8.96 (br s, 1 H), 8.60 (d, 1 H, J = 2.6 Hz), 7.78 (dd, 1 H, J = 2.6, 8.9 Hz), 7.14 (d, 1 H, J = 8.9 Hz), 4.16 (t, 1 H, J = 4.2 Hz), 3.76 (d, 2 H, J = 6.9, 4.2 Hz).

Preparation of Liposomes. The lipids were dissolved in MeOH−CHCl₃ at the desired molar ratio. Small amount of DMF or DMSO was used to dissolve **6b** and **6c**. The solvent was evaporated by nitrogen gas stream and completely dried under vacuum pressure to deposit the lipid on the wall of glass test tube as a thin film. One milliliter of Hepes buffer (10 mM Hepes, 0.1 M NaCl, pH 9) was then added and vortexed. The resulting suspension of multilameller vesicles (MLVs) was sonicated for 5−10 min with a probe-type sonicator (Branson Sonifier 250) until the solution became clear with luster. The liposome solutions were directly used for the experiments. The liposome solutions containing the synthetic lipids (Fusion-lip) were freshly prepared before use and kept at 0 °C, whose pH was adjusted to around 10 with aqueous NaOH.

pH-Metric Titration of Chromophoric Lipids. After preparing S10% Fusion-lip, 2 mL of the Hepes buffer was added and the pH of the solution was adjusted to around 11 with aqueous NaOH in a quartz cell at 20 °C. To the stirred liposome solution, 0.5 M HCl was added stepwise with a syringe, and the pH and electronic absorption due to each nitrophenolate anion were measured at 20 °C. In the case of **8**, it was directly dissolved in water and the solution was acidified with concentrated HCl, and 1N NaOH was then added into the stirring solution followed by measurement of pH at 20 °C.

Measurement of NMR Spectra. A dried lipid film of eggPC and synthetic lipid was suspended in a Hepes buffer prepared using deuterium oxide. After sonication of the solution to prepare the liposome, the solution was directly used for the NMR measurement. The solution was adjusted at pD around 10 with an aqueous NaOD solution and transferred to a NMR tube. The measurement was performed under basic and acidic conditions at 20 °C.

Lipid Mixing Assay. Label-lip containing both NBD-PE and Rh-PE was freshly prepared before use. Hepes buffer (1.89 mL, 10 mM) containing 0.1 M NaCl was adjusted at appropriate pH with aqueous NaOH or citric acid in a quartz cell at 37 °C. To the solution was added 90 μL of Control-lip solution followed by addition of 10 μL of Label-lip solution; then lipid mixing was initiated by the addition of 10 μL of Fusion-lip solution containing certain amount of synthetic lipids. The time course of lipid mixing was measured on a fluorescent spectrometer (λ_{ex} = 470 nm, λ_{em} = 530 nm). After 20 min, the liposomes were completely collapsed by adding Triton X-100 to give maximum fluorescence intensity, F_{max}. The percentage of lipid mixing at a given time was calculated by $[(F_t - F_0)/(F_{max} - F_0)]100$, where F_0, F_t, and F_{max} are the fluorescent intensity at time 0, t, and after collapsing the liposomes. The assay was carried out at various pHs using S10% Fusion-lip and was also studied using Fusion-lip containing 5, 10, and 20 mol % of the corresponding synthetic lipid at pH 7.4.

Time-Resolved Electronic Absorption Spectra. S10% Fusion-lip containing **6b** was transferred into a quartz cell at 4 °C under nitrogen and the pH was adjusted to around 10. The solution was mixed with a Control-lip at a molar ratio of 9:1 (Control-lip/Fusion-lip). To start membrane fusion, the pH of the solution was acidified to pH 4 at 20 °C by addition of 2 M citric acid. Absorption spectra were measured every 100 ms for several minutes by a multichannel spectrophotometer.

Supporting Information Available: Additional experimental details, pH-metric titration for **6b** and **6a** in the liposomes. This material is available free of charge via the Internet at http://pubs.acs.org.

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