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Gemini peptide lipids with ditopic ion-recognition site. Preparation and functions as an inducer for assembling of liposomal membranes

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Abstract—Gemini amphiphiles having two peptide lipid units and a spacer group connected at the polar heads were synthesized. A gemini peptide lipid bearing L-histidyl residues, hydrophobic double-chain segments, and a tri(oxyethylene) spacer performed as an inducer of the reversible assembling of liposomal membranes through ditopic ion recognition toward transition metal ions and alkali metal ions. The vesicular assembling behavior induced by the gemini peptide lipids was sensitive to the structural difference in the amino acid residue and the spacer group of the lipids.

1. Introduction

Liposomal membranes formed with phospholipids or bilayer-forming synthetic lipids have been widely employed as biomembrane models, drug carriers, nano-reactors, and scaffolds for supramolecular devices.[1–9] On functionalization of the liposomal membranes, the lipid assemblies have been usually handled as the uni-vesicular state, but not as the multi-vesicular assembly. In the light of the superiority of the multi-cellular organisms in biological systems as compared with the corresponding unicellular states, however, establishment of the methodology to construct the multi-vesicular assemblies as artificial tissues is of great significance. On these grounds, many kinds of approaches for assembling of the liposomal membranes have been proposed up to the present time.[10–18] In this article, we are to report a novel strategy to create reversible assembling system of the liposomal membranes by using an ion-recognizable gemini peptide lipid (**1**) as an inducer (Fig. 1).

Our molecular design of the lipid **1** was inspired by a naturally occurring gemini lipid, cardiolipin, having unique dimeric lipid structure and interesting biological functions.[19,20] Especially, we paid attention to the specific ion binding behavior of the cardiolipins toward divalent ions such as Ca^{2+} and Mg^{2+} to influence the aggregate morphology of the lipid membranes. Up to the present time, a considerable number of investigations have been reported on synthetic gemini surfactants consist of two surfactant molecules via a spacer group.[21–23] The gemini surfactants provide novel and interesting opportunities to investigate various supramolecular morphologies depending on the structural characteristics following to attractive

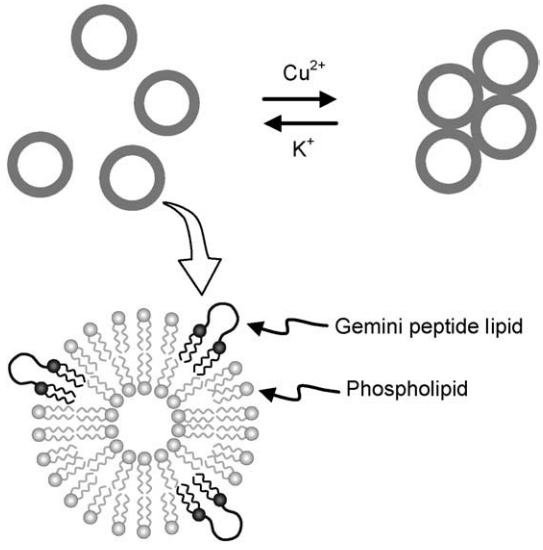

Figure 1. Schematic representation of the assembling of liposomes containing the gemini peptide lipid as triggered by specific ion recognition.

Keywords: Gemini peptide lipid; Ditopic ion-recognition; Vesicular assembling; Pseudo-crown ether; Supramolecular chemistry.
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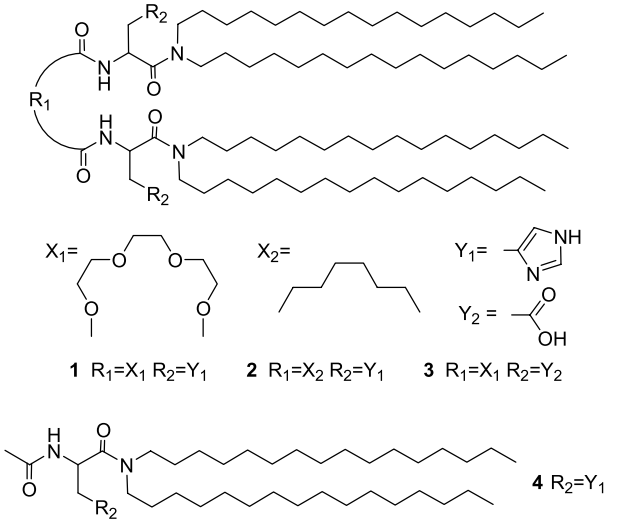
applications. Although much attention has been focused on physicochemical properties of the synthetic gemini surfactants, there are few reports on the synthetic gemini lipids composed of two double-chain segments like the cardiolipin. Thus, we prepared the gemini lipids having two double-chain units and two kinds of ion recognition sites, by extending our previous studies on the bilayer-forming synthetic peptide lipids having an amino acid moiety interposed between the polar head moiety and a hydrophobic double-chain segment.[24]



2. Results and discussion

2.1. Synthesis of the gemini peptide lipids

In order to enhance the ion-recognition ability of the synthetic gemini lipid in the liposomal membranes, we introduced two L-histidyl residues as the polar heads and a tri(oxyethylene) group as a spacer between the head moieties, expecting that the former and the latter groups act as effective binding sites for transition metal ions and alkali metal ions, respectively. The gemini peptide lipid (**1**) was synthesized through condensation of the peptide lipids with the corresponding bis(acyl chloride). As the reference lipids of **1**, we also prepared a gemini peptide lipid having a hydrocarbon spacer (**2**), a gemini peptide lipid bearing L-aspartate residues (**3**), and a non-gemini peptide lipid (**4**). The syntheses of these lipids were basically referred to the synthetic procedures of the peptide lipids.[24]

2.2. Ion recognition behavior of the gemini peptide lipids and its consequence in assembling of the liposomal membranes

The hybrid bilayer vesicles were prepared by sonication of an aqueous dispersion of dimyristoylphosphatidylcholine (DMPC) and a gemini peptide lipid in a 10:1 molar ratio with a cup-type sonicator at 30 W for 6 min. The hydrodynamic diameter (D_{hy}) of the hybrid vesicle of **1** was evaluated to be 160 nm by means of dynamic light scattering (DLS) measurements. Upon addition of Cu^{2+} ions to the hybrid vesicles at pH 9 and 30 °C, the D_{hy} value was significantly increased, suggesting the assembling of the vesicles or the followed fusion (Fig. 2). The similar behavior was also observed for the hybrid vesicles of **2**, but not for the hybrid vesicles containing the gemini lipid **3** or the non-gemini lipid **4** and the liposomes formed with DMPC alone under the comparable concentration conditions.

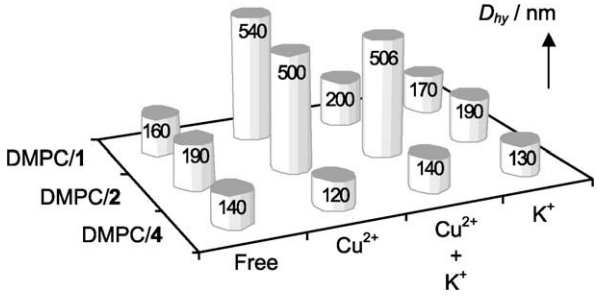


Figure 2. Effects of Cu^{2+} and K^+ ions for the vesicular assembling as evaluated by DLS at pH 9 and 30 °C. Concentrations in mmol dm^{-3}: [DMPC], 0.5; [gemini lipid], 0.05; [**4**], 0.1; [Cu^{2+}], 0.5; [K^+], 5.0.

The present hybrid vesicle of **1** showed a phase transition from gel to liquid-crystalline state with the peak maximum (T_m) at 24.3 °C and its enthalpy change (ΔH) of 4.3 kJ mol^{-1} per the matrix lipid, as evaluated by differential scanning calorimetry (DSC). Upon addition of Cu^{2+} ions to the vesicular solution, the phase transition behavior was scarcely affected, reflecting that the metal ions did not give perturbation in the packing shape of the lipid molecules in the membrane so as to change the aggregate morphology; the T_m and ΔH values were 24.3 °C and 4.0 kJ mol^{-1}, respectively, in the presence of Cu^{2+} ions in an equimolar amount to the phospholipid. In general, remarkable changes in the phase transition can be detected by DSC for the morphological transformation from small vesicles to the corresponding larger ones.[25,26] In addition, the increased D_{hy} value was immediately recovered to the original value in the metal-free system upon addition of an excess amount of EDTA. The results clearly indicate that the assembling of the hybrid vesicles formed with DMPC and **1** was triggered by Cu^{2+} ions without accompanying fusion of the vesicles as illustrated in Figure 1.

The ion binding to the surface of the lipid bilayer vesicle was qualitatively examined by ζ-potential measurements.[27] pH-Dependences of the ζ-potential for the DMPC vesicles containing the gemini peptide lipid **1** in the presence and absence of Cu^{2+} ions were shown in Figure 3. In the metal-free system, deprotonation of the imidazolyl groups in **1** caused the decrease in the ζ-potential from +40 to −20 mV. On the other hand, significant increase in the ζ-potential was observed in a pH region over 7 in the presence of Cu^{2+} ions. The results strongly suggest that Cu^{2+} ions bind to the histidyl residues of the gemini peptide lipids to induce the vesicular assembling.

Specificity of the ion recognition by the gemini peptide lipid to induce the vesicular assembling was evaluated from the extent of the increased D_{hy} values upon addition of various transition metal ions (Table 1). The ion selectivity for the hybrid vesicles composed of DMPC with **1** or **2** was well correlated to the general stability sequence of divalent

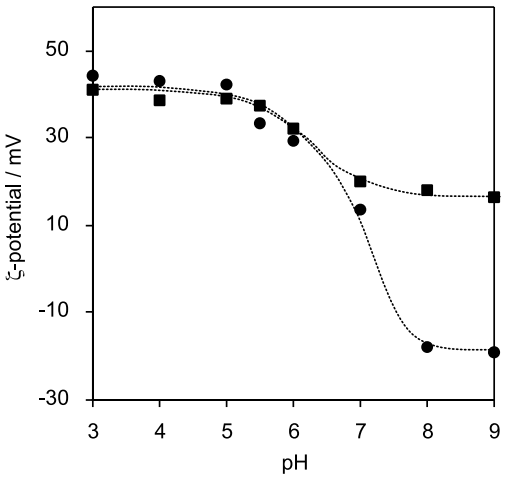


Figure 3. pH-Dependences of ζ-potential for the DMPC vesicle containing the gemini peptide lipid **1** at 30 °C in the presence (square) and absence (circle) of Cu^{2+} ions (0.5 mmol dm^{-3}). Concentrations in mmol dm^{-3}: [DMPC], 0.5; [**1**], 0.05; [Na^{+}] 0.5.

Table 1. Hydrodynamic diameters for the DMPC vesicles containing the gemini peptide lipid at pH 9.0 and 30 °C[a]

Metal ions	Hydrodynamic diameter/nm[b]	
	Lipid **1**	Lipid **2**
None	150 (20)	190 (20)
Mn^{2+}	140 (10)	190 (20)
Fe^{2+}	150 (10)	210 (20)
Co^{2+}	270 (20)	230 (30)
Ni^{2+}	320 (40)	240 (20)
Cu^{2+}	540 (20)	500 (30)
Zn^{2+}	350 (50)	360 (20)

[a] Hydrodynamic diameters were determined by DLS. Concentrations in mmol dm^{-3}: [DMPC], 0.5; [**1**] or [**2**], 0.05, [metal ions], 0.5.
[b] Standard deviation of three independent measurements is in parenthesis.

transition metal complexes, the Irving–Williams series; $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$.[28,29] Under the similar concentration conditions, alkali and alkaline-earth metal ions did not act as the trigger for assembling of the hybrid vesicles.

In order to elucidate the stoichiometry and the binding constant for the copper complexes formed in the hybrid vesicles, circular dichroism (CD) spectra were taken in aqueous solution by monitoring the conformational changes in the chiral histidyl moieties of the lipid upon complexation.[30,31] The CD spectral changes of the gemini peptide lipid **1** embedded in the DMPC vesicle upon addition of Cu^{2+} ions are shown in Figure 4(A). The titration isotherm shown in Figure 4(B) was obtained by monitoring the CD intensity change at 214 nm ($\Delta\Delta\varepsilon_{214}$), which was applied to determine the binding constant of Cu^{2+} ions with **1**. The Job's plot analyses in the vesicular system revealed that Cu^{2+} ions bind to the imidazolyl groups in a ratio of 1:4. The binding constants for the other lipids, **2** and **4**, were also determined in the similar manner. Thus the binding constants for the 1:2 complexes of Cu^{2+} ions with the gemini lipids **1** and **2** in the DMPC vesicle were 1.6×10^{11} and 1.3×10^{11} dm^{6} mol^{-2}, respectively. These values are also represented to be 2.6×10^{22} and 1.7×10^{22} dm^{12} mol^{-4} for the lipid **1** and **2**, respectively, as apparent binding

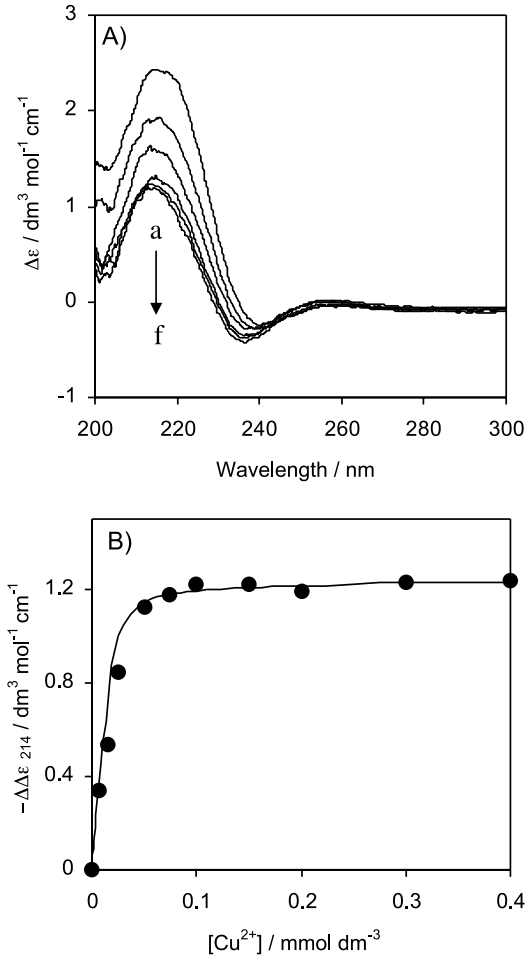


Figure 4. (A) CD spectral changes for the DMPC (0.5 mmol dm^{-3}) vesicle containing the gemini peptide lipid **1** (0.05 mmol dm^{-3}) upon addition of Cu^{2+} ions. The concentrations of Cu^{2+} ions in mmol dm^{-3}: a, 0; b, 0.015; c, 0025; d, 0.05; e, 0.2; f; 0.5. (B) Change in the molar CD at 214 nm ($\Delta\Delta\varepsilon_{214}$) upon titration of the gemini peptide lipid **1** with Cu^{2+} ions.

constants for the 4:1 complex of the imidazolyl groups in the lipid and Cu^{2+} ions. Whereas the binding constant of Cu^{2+} ions toward the non-gemini lipid **4** with one imidazolyl group per one lipid molecule was 1.5×10^{17} dm^{12} mol^{-4} for the 1:4 complex, being much weaker than those of the corresponding gemini lipids. Under the conditions in Figure 2, for example, the imidazolyl groups of the gemini lipid **1** completely form the copper complex, whereas only 75% of the histidyl residue of **4** binds Cu^{2+} ions and 25% of the imidazolyl group is free from the metal coordination.

Thus, we can image the mechanism for the Cu^{2+}-triggered assembling of the liposomal membranes containing the gemini peptide lipid as follows. In order to keep two lipid bilayer surfaces at separation less than about 2 nm in aqueous media, some attractive force which overcomes the repulsive hydration force observed on the surface of the hydrated lipid membranes[32,33] would be necessary. Thus, our observation that the DMPC liposomes containing the non-gemini lipid **4** did not form the vesicular assembly in the presence of Cu^{2+} ions indicates that the intra-vesicular 1:4 complex of the metal ions with the imidazolyl groups is

thermodynamically more stable than the corresponding inter-vesicular complex under the present conditions, presumably owing to the repulsive hydration force. On the other hand, the gemini peptide lipids with two imidazolyl groups can stabilize the inter-vesicular complex reinforced with the linkage of the spacer unit, as shown in Figure 5. Although the corresponding intra-vesicular complex may be also formed in the hybrid liposome of the gemini peptide lipid, the inter-vesicular complex would play pivotal role as the membrane junction to induce the vesicular assembling. We also observed that the assembling of the hybrid vesicles was triggered by Cu^{2+} ions in the liquid crystalline state above the T_m of the lipid membrane, but not below the T_m at which the inter-vesicular complex would be hardly formed in the rigid gel matrix with highly restricted molecular motion. This observation strongly support that the inter-vesicular complex shown in Figure 5 is a key species for the vesicular assembling in this study.

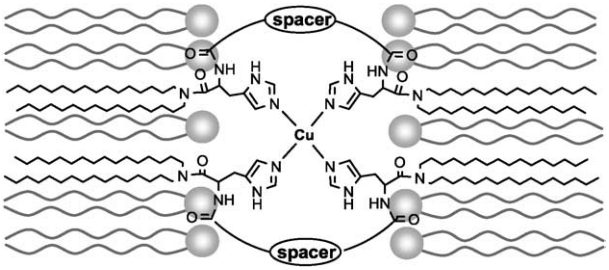


Figure 5. Plausible key species of the copper complex of the gemini peptide lipid having L-histidyl residues which stabilizes the vesicular assembly by forming the membrane junction.

2.3. Heteroditopic ion-recognition by the gemini peptide lipid and its consequence in disassembling of the liposomal membranes

The gemini peptide lipid **1** has a pseudo-crown ether moiety at the spacer unit, and is expected to behave as a ditopic ion receptor. As shown in Figure 2, the hydrodynamic diameter of the assembled vesicles in the presence of Cu^{2+} ions was decreased from 540 to 200 nm upon further addition of K^+ ions (5.0 mmol dm^{-3}). On the other hand, there was no significant change of the vesicular size upon addition of K^+ ions in the absence of Cu^{2+} ions. The results clearly indicate that the assembling of the liposomal membranes is reversibly controlled by ditopic ion recognition of **1** with Cu^{2+} and K^+ ions. Since the disassembling of the vesicles was not observed for the hybrid vesicles of DMPC and the gemini peptide lipid lacking the pseudo-crown ether moiety **2** upon addition of K^+ ions after the Cu^{2+}-triggered vesicular assembling. Thus the disassembling behavior of the hybrid vesicles of DMPC and **1** was interpreted by replacement of the Cu^{2+}-binding to the imidazolyl groups with the K^+-binding to the pseudo-crown ether moiety to breakdown the inter-vesicular copper complex.

In addition, marked selectivity for alkali metal ions was observed in the vesicular disassembling behavior (Fig. 6). Tendency of the disassembling effect seems to be correlated with the binding affinity of alkali metal ions to the pseudo-crown ether moiety;[34,35] $K^+ > Na^+ > Li^+ > Rb^+ \approx Cs^+$. Our present results would bring up possibilities to extend the interesting ditopic ion-recognition systems in solutions[36–38] to those in the lipid membrane systems as the biomembrane models.

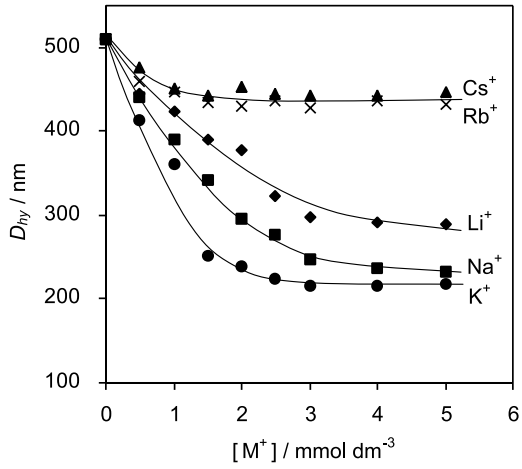


Figure 6. Effect of alkali metal ions (M^+) on hydrodynamic diameter (D_{hy}) of the DMPC vesicles containing gemini peptide lipid (**1**) in the presence of Cu^{2+} ion at 30 °C and pH 9. Concentrations in mmol dm^{-3}: [DMPC], 0.5; [**1**], 0.05; [Cu^{2+}], 0.5.

3. Conclusion

We have shown here that the transformation between the assembling and disassembling of the liposomal membranes can be reversibly controlled as a response of heteroditopic ion recognition by the gemini peptide lipid embedded in the vesicles. We believe our results revealed a new function of the gemini surfactants and propose a guidepost to design supramolecular assemblies exhibiting ditopic molecular recognition and its response.

4. Experimental

4.1. General

Organic solvents were purified, dried by standard procedures and kept over a drying agent before use. Unless otherwise mentioned, reagents were commercially available as a guaranteed grade and used without further purification. Thin layer chromatography (TLC) was performed on a silica gel 70 FM plate-Wako (Wako Pure Chemical Industries) with fluorescence detection under UV light. Liquid chromatography was performed using a silica gel column (Wako-gel C-300) and/or high performance liquid chromatography (HPLC) using a JAI Model instrument (Japan Analytical Instruments) equipped with JAIGEL−1H and -2H columns. ^{1}H NMR spectra were taken on a JEOL JNM-LA400 spectrometer. HR-MS (FAB+) was recorded with a JEOL JMS-700 MStation mass spectrometer.

4.2. Synthesis

4.2.1. *N*,*N*-Dihexadecyl-N^2-*t*-butoxycarbonyl-N^τ-tosyl-L-histidinamide [Boc-His(Tos)2C_{16}]. N^2-*t*-Butoxycarbonyl-N^τ-tosyl-L-histidine (3.00 g, 7.33 mmol) was added to a solution of dihexadecylamine (2.85 g, 6.11 mmol) and

triethylamine (0.74 g, 7.33 mmol) in dry dichloromethane (20 dm^{-3}) at 0 °C with stirring for 15 min. Diethylcyano phosphate (1.20 g, 7.33 mmol) was added to the solution and stirred for 30 min at 0 °C and then for 1 h at 25 °C. The solution was evaporated in vacuo to give a solid. The crude product was purified by liquid chromatography on a column of silica gel (Wako-gel C-300) with chloroform–methanol (99.5:0.5 v/v) as eluent giving a white solid, yield 5.18 g (99%); mp 52.6–53.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 0.88 (6H, t, J=6.7 Hz, $(CH_2)_{13}CH_3$), 1.24 (52H, m, $NCH_2(CH_2)_{13}CH_3$), 1.35 (9H, s, $(CH_3)_3CO$), 1.43 (4H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 2.42 (3H, s, $C_6H_4CH_3$), 2.80 (2H, m, $CHCH_2Im$), 2.99–3.42 (4H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 4.81 (1H, m, $CHCH_2Im$), 5.27 (1H, d, J=8.3 Hz, CONH), 7.09 (1H, s, Im-5H), 7.33 (2H, d, J=8.4 Hz, o-C_6H_4), 7.79 (2H, d, J=8.4 Hz, m-C_6H_4), 7.89 (1H, s, Im-2H). Anal. calcd for $C_{50}H_{88}N_4O_5S$: C, 70.05; H, 10.35; N 6.54%. Found: C, 70.21; H, 10.62; N, 6.55%.

4.2.2. N^2,$N^{2'}$-2,5,8,11-Tetraoxadodecanedioyl-bis(*N*,*N*-dihexadecyl-N^{τ}-tosyl-L-histidinamide) [Teo-Bis(His(Tos)2C_{16})].

Trifluoroacetic acid (8.90 g, 78.0 mmol) was added to a solution of Boc-His(Tos)2C_{16} (3.34 g, 3.90 mmol) in dry dichloromethane (10 dm^{-3}) at 25 °C with stirring for 2 h. The solvent was evaporated in vacuo, and the residue and triethylamine (3.95 g, 39.0 mmol) was dissolved in dry dichloromethane at 0 °C. 2,5,8,11-Tetraoxadodecanedioyl dichloride (0.536 g, 1.95 mmol) dissolved in dichloromethane (10 dm^{-3}) was added to the solution for 20 min. The mixture was stirred for 1 h at 25 °C and washed with brine. The solvent was evaporated in vacuo, and the crude product was purified by liquid chromatography on a column of silica gel (Wako-gel C-300) with chloroform–methanol (99:1 v/v) as eluent and then HPLC with chloroform as eluent to give a colorless oil, yield 0.75 g (22%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 0.88 (12H, t, J=6.7 Hz, $(CH_2)_{13}CH_3$), 1.26 (104H, m, $(CH_2)_{13}CH_3$), 1.41 (8H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 2.42 (6H, s, $C_6H_4CH_3$), 2.84 (4H, m, $CHCH_2Im$), 2.93–3.42 (8H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 3.62 (4H, s, $CH_2CH_2O(CH_2)_2OCH_2CH_2$), 3.65 (4H, m, $CH_2CH_2OCH_2CH_2OCH_2CH_2$), 4.12–4.32 (4H, m, $CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2$), 4.81 (2H, m, $CHCH_2Im$), 5.73 (2H, d, J=8.3 Hz, CONH), 7.09 (2H, s, Im-5H), 7.33 (4H, d, J=8.4 Hz, o-C_6H_4), 7.79 (4H, d, J=8.4 Hz, m-C_6H_4), 7.89 (2H, s, Im-2H). Anal. calcd for $C_{98}H_{170}N_8O_{12}S_2\cdot 5H_2O$: C, 65.15; H, 10.04; N, 6.20%. Found: C, 65.15; H, 9.67; N, 6.12%.

4.2.3. N^2,$N^{2'}$-2,5,8,11-Tetraoxadodecanedioyl-bis(*N*,*N*-dihexadecyl-L-histidinamide) (1).

Aqueous NaOH (1.0 mol dm^{-3}, 10 dm^{-3}) was added to Teo-Bis(His(Tos)2C_{16}) (0.43 g, 0.28 mmol) in methanol (100 dm^{-3}) with stirring for 1 h at 0 °C. The solvent was removed in vacuo, and the residue was purified by liquid chromatography on a column of silica gel (Wako-gel C-300) with chloroform–methanol (97:3 v/v) to give a colorless oil, yield 0.145 g (41%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 0.88 (12H, t, J=6.7 Hz, $(CH_2)_{13}CH_3$), 1.26 (104H, m, $(CH_2)_{13}CH_3$), 1.47 (8H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 3.02 (4H, m, $CHCH_2Im$), 3.22–3.39 (8H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 3.64 (4H, s, $CH_2CH_2OCH_2CH_2OCH_2CH_2$), 3.67 (4H, m, $CH_2CH_2OCH_2CH_2OCH_2CH_2$), 4.12–4.32 (4H, m, $CH_2CH_2OCH_2CH_2OCH_2CH_2$), 4.81 (2H, m, $CHCH_2Im$), 6.41 (2H, br, CONH), 6.88 (2H, s, Im-5H), 7.51 (2H, s, Im-2H). Anal. calcd for $C_{84}H_{158}N_8O_8$: C, 71.64; H, 11.31; N, 7.96%. Found: C, 71.30; H, 11.53; N, 7.68%. HR-MS (FAB$^+$): exact mass calcd for $C_{84}H_{159}N_8O_8$ $[M+H]^+$ 1408.2281, found 1408.2275.

4.2.4. *N*,*N*-Dihexadecyl-N^2-*t*-butoxycarbonyl-N^{τ}-benzyloxymethyl-L-histidinamide [Boc-His(Bom)2C_{16}].

N^2-*t*-Butoxycarbonyl-N^{τ}-benzyloxymethyl-L-histidine (6.76 g, 18 mmol) was added to a solution of 1-hydroxybenzotiazole (2.43 g, 18.0 mmol) in dry dichloromethane (20 dm^{-3}) at 0 °C with stirring for 5 min. Then *N*,*N*′-dicyclohexylcarbodiimide (2.97 g, 14.4 mmol) was added to the solution and stirred at 0 °C. After 15 min, dihexadecylamine (5.74 g, 12.0 mmol) was added to the solution and stirred for 3 h at 0 °C and then for 42 h at room temperature. The precipitation was removed by filtration and the solvent was removed in vacuo. The residue dissolved in ethyl acetate (300 dm^{-3}) was washed sequentially with 10% aqueous citric acid, 5% aqueous sodium hydrogen carbonate and saturated aqueous sodium chloride. After being dried, the solution was evaporated in vacuo to give a yellow oil. The crude product was purified by liquid chromatography on a column of silica gel (Wako-gel C-300) with chloroform–methanol (99.5:0.5 v/v) as eluent giving a white solid, yield 3.02 g (31%); mp 49.8–50.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 0.88 (6H, t, J=6.7 Hz, $(CH_2)_{13}CH_3$), 1.26 (52H, m, $(CH_2)_{13}CH_3$), 1.39 (9H, s, $(CH_3)_3CO$), 1.47 (4H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 2.92 (2H, m, $CHCH_2Im$), 3.00–3.44 (4H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 4.45 (1H, d, J=11.9 Hz, $ImCH_2OCHHC_6H_5$), 4.51 (1H, d, J=11.9 Hz, $ImCH_2OCHHC_6H_5$), 4.79 (1H, m, $CHCH_2Im$), 5.29 (1H, d, J=11.0 Hz, $ImCHHOCH_2C_6H_5$), 5.32 (1H, d, J=8.5 Hz, CONH), 5.41 (1H, J=11.0 Hz, $ImCHHOCH_2C_6H_5$), 6.89 (1H, s, Im-5H), 7.32 (5H, m, $OCH_2C_6H_5$), 7.46 (1H, s, Im-2H). Anal. calcd for $C_{51}H_{90}N_4O_4$: C, 74.40; H, 11.02; N 6.81%. Found: C, 74.27; H, 10.91; N, 6.87%.

4.2.5. N^2,$N^{2'}$-Octanedioyl-bis(*N*,*N*-dihexadecyl-N^{τ}-benzyloxymethyl-L-histidinamide) [C_6-Bis(His(Bom)2C_{16})].

Trifluoroacetic acid (8.16 g, 71.6 mmol) was added to a solution of Boc-His(Bom)2C_{16} (2.95 g, 3.58 mmol) in dry dichloromethane (40 dm^{-3}) at room temperature with stirring for 2 h. The solvent was evaporated in vacuo, and the residue and triethylamine (2.95 g, 28.6 mmol) was dissolved in dry dichloromethane (40 dm^{-3}) at 0 °C. Octanedioyl dichloride (0.61 g, 2.86 mmol) dissolved in dichloromethane (7 dm^{-3}) was added to the solution for 1 h. The mixture was stirred for 4 h at 25 °C and the solvent was removed in vacuo. The residue dissolved in dichloromethane (200 dm^{-3}) was washed sequentially with 10% aqueous citric acid, 5% aqueous sodium hydrogen carbonate and saturated aqueous sodium chloride. After being dried, the solution was evaporated in vacuo to give a yellow oil. The crude product was purified by liquid chromatography on a column of silica gel (Wako-gel C-300) with chloroform–methanol (99:1 v/v) as eluent giving a colorless oil, yield 2.42 g (85%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 0.88 (12H, t, J=6.7 Hz, $(CH_2)_{13}CH_3$), 1.26 (108H, m, $(CH_2)_{13}CH_3$, $CO(CH_2)_2(CH_2)_2(CH_2)_2CO$), 1.47 (8H, m, $NCH_2CH_2(CH_2)_{13}CH_3$), 1.58 (4H, m, $COCH_2CH_2(CH_2)_2CH_2CH_2CO$), 2.12 (4H, m, $COCH_2(CH_2)_4CH_2CO$), 3.02 (4H, m, $CHCH_2Im$), 3.11–3.43 (8H, m, $NCH_2CH_2(CH_2)_{13}CH_3$),

4.42 (2H, d, $J=11.9$ Hz, ImCH$_2$OC*H*HC$_6$H$_5$), 4.54 (2H, d, $J=11.9$ Hz, ImCH$_2$OCH*H*C$_6$H$_5$), 5.17 (2H, m, C*H*CH$_2$Im), 5.31 (2H, d, $J=11.0$ Hz, ImC*H*HOCH$_2$C$_6$H$_5$), 5.41 (2H, $J=11.0$ Hz, ImCH*H*OCH$_2$C$_6$H$_5$), 6.89 (2H, s, Im-5H), 7.22 (2H, d, $J=8.5$ Hz, CON*H*), 7.32 (10H, m, OCH$_2$C$_6$*H*$_5$), 7.51 (2H, s, Im-2H). Anal. calcd for $C_{100}H_{174}N_8O_7\cdot 1H_2O$: C, 74.95; H, 11.07; N, 6.99%. Found: C, 74.87; H, 10.90; N, 6.92%.

4.2.6. N^2,$N^{2'}$-Octanedioyl-bis(*N*,*N*-dihexadecyl-L-histidinamide) (2). Pd-C 10% (0.36 g) was added to C_6-Bis(His(Bom)2C_{16}) (1.13 g, 0.69 mmol) dissolved in 80% aqueous acetic acid (20 dm^{-3}). The mixture was placed under hydrogen at atomospheric pressure and room temperature for 15 h with stirring. The mixture was filtered on celite and the filtrate was concentrated under reduced pressure. The crude product was purified by liquid chromatography on a column of silica gel (Wako-gel C-300) with chloroform–methanol (97:3 v/v) as eluent giving a white solid, yield 72.1 mg (17%); mp 95.4–96.1 °C. ^{1}H NMR (400 MHz, CDCl$_3$, TMS): δ 0.88 (12H, t, $J=6.7$ Hz, (CH$_2$)$_{13}$C*H*$_3$), 1.26 (108H, m, (C*H*$_2$)$_{13}$CH$_3$, CO(CH$_2$)$_2$(C*H*$_2$)$_2$(CH$_2$)$_2$CO), 1.58 (4H, m, COCH$_2$C*H*$_2$(CH$_2$)$_2$C*H*$_2$CH$_2$CO), 2.12 (4H, m, COC*H*$_2$(CH$_2$)$_4$C*H*$_2$CO), 3.02 (4H, m, CHC*H*$_2$Im), 3.13–3.39 (8H, m, NC*H*$_2$CH$_2$(CH$_2$)$_{13}$CH$_3$), 5.17 (2H, m, C*H*CH$_2$Im), 6.83 (2H, s, Im-5H), 7.22 (2H, d, $J=8.5$ Hz, CON*H*), 7.63 (s, 2H, Im-2H). Anal. calcd for $C_{84}H_{158}N_8O_4\cdot 2CH_3CO_2H$: C, 72.18; H, 11.43; N, 7.65%. Found: C, 72.45; H, 11.47; N, 7.72%. HR-MS (FAB$^+$): exact mass calcd for $C_{84}H_{159}N_8O_4$ [M+H]$^+$ 1344.2484, found 1344.2483.

4.2.7. N^2-Acetyl-*N*,*N*-dihexadecyl-N^τ-benzyloxymethyl-L-histidinamide [Ac-His(Bom)2C_{16}]. Trifluoroacetic acid (7.04 g, 61.8 mmol) was added to a solution of Boc-His(Bom)2C_{16} (2.54 g, 3.08 mmol) in dry dichloromethane (10 dm^{-3}) at room temperature with stirring for 2 h. The solvent was evaporated in vacuo, and the residue and triethylamine (3.13 g, 30.1 mmol) was dissolved in dry dichloromethane (10 dm^{-3}) at 0 °C. Acetic anhydride (0.921 g, 9.02 mmol) was added to the solution for 1 h. The mixture was stirred for 8 h at room temperature and the solvent was removed in vacuo. The residue dissolved in dichloromethane (100 dm^{-3}) was washed sequentially with 10% aqueous citric acid, 5% aqueous sodium hydrogen carbonate and saturated aqueous sodium chloride. After being dried, the solution was evaporated in vacuo to give a yellow oil. The crude product was purified by liquid chromatography on a column of silica gel (Wako-gel C-300) with chloroform–methanol (99:1 v/v) as eluent giving a colorless oil, yield 1.13 g (49%). ^{1}H NMR (400 MHz, CDCl$_3$, TMS): δ 0.88 (6H, t, $J=6.7$ Hz, (CH$_2$)$_{13}$C*H*$_3$), 1.26 (52H, m, (C*H*$_2$)$_{13}$CH$_3$), 1.47 (4H, m, NCH$_2$C*H*$_2$(CH$_2$)$_{13}$CH$_3$), 2.00 (3H, s, C*H*$_3$CO), 2.98 (2H, m, CHC*H*$_2$Im), 3.01–3.50 (4H, m, NC*H*$_2$CH$_2$(CH$_2$)$_{13}$CH$_3$), 4.46 (1H, d, $J=11.7$ Hz, ImCH$_2$OC*H*HC$_6$H$_5$), 4.58 (1H, d, $J=11.7$ Hz, ImCH$_2$OCH*H*C$_6$H$_5$), 5.11 (1H, m, C*H*CH$_2$Im), 5.31 (1H, d, $J=11.0$ Hz, ImC*H*HOCH$_2$C$_6$H$_5$), 5.41 (1H, $J=11.0$ Hz, ImCH*H*OCH$_2$C$_6$H$_5$), 6.36 (1H, d, $J=8.3$ Hz, CON*H*), 6.83 (1H, s, Im-5H), 7.32 (5H, m, OCH$_2$C$_6$*H*$_5$), 7.46 (1H, s, Im-2H). Anal. calcd for $C_{48}H_{84}N_4O_3$: C, 75.34; H, 11.06; N, 7.32%. Found: C, 74.97; H, 11.18; N, 7.27%.

4.2.8. N^2-Acetyl-*N*,*N*-dihexadecyl-L-histidinamide (4). Pd-C 10% (0.223 g) was added to Ac-His(Bom)2C_{16} (0.893 g, 1.16 mmol) dissolved in 80% aqueous acetic acid (20 dm^{-3}). The mixture was placed under hydrogen at atomospheric pressure and room temperature for 24 h with stirring. The mixture was filtered on celite and the filtrate was concentrated under reduced pressure. The crude product was purified by liquid chromatography on a column of silica gel (Wako-gel C-300) with chloroform–methanol (97:3 v/v) as eluent giving a white solid, yield 0.254 g (33%); mp 60.7–61.5 °C. ^{1}H NMR (400 MHz, CDCl$_3$, TMS): δ 0.88 (6H, t, $J=6.7$ Hz, (CH$_2$)$_{13}$C*H*$_3$), 1.26 (52H, m, (C*H*$_2$)$_{13}$CH$_3$), 1.47 (4H, m, NCH$_2$C*H*$_2$(CH$_2$)$_{13}$CH$_3$), 2.00 (3H, s, C*H*$_3$CO), 2.98 (2H, m, CHC*H*$_2$Im), 3.01–3.50 (4H, m, NC*H*$_2$CH$_2$(CH$_2$)$_{13}$CH$_3$), 5.03 (1H, m, C*H*CH$_2$Im), 6.57 (1H, d, $J=8.5$ Hz, CON*H*), 6.83 (1H, s, Im-5H), 7.57 (1H, s, Im-2H). Anal. calcd for $C_{40}H_{76}N_4O_2\cdot 0.5H_2O$: C, 73.45; H, 11.87; N, 8.57%. Found: C, 73.35; H, 11.83; N, 8.39%. HR-MS (FAB$^+$): exact mass calcd for $C_{40}H_{77}N_4O_2$ [M+H]$^+$ 645.6047, found 645.6041.

4.2.9. N^2,$N^{2'}$-2,5,8,11-Tetraoxadodecanedioyl-bis(*N*,*N*-dihexadecyl-L-aspartic 1-amide) (3). The gemini peptide lipid having aspartate residues (**3**) was prepared in a manner analogous to that for the synthesis of **1** by using N^2-*t*-butoxycarbonyl-O^4-benzyl-L-aspartic acid instead of N^2-*t*-butoxycarbonyl-N^τ-tosyl-L-histidine. In the final step, the benzyl groups were removed by the Pd-C catalyzed hydrogenation. A colorless oil. ^{1}H NMR (400 MHz, CDCl$_3$, TMS): δ 0.88 (12H, t, $J=6.7$ Hz, (CH$_2$)$_{13}$C*H*$_3$), 1.26 (104H, m, (C*H*$_2$)$_{13}$CH$_3$), 1.58 (8H, m, NCH$_2$C*H*$_2$(CH$_2$)$_{13}$CH$_3$), 2.65 (2H, m, CHC*H*$_2$COOH), 3.21–3.43 (8H, m, NC*H*$_2$CH$_2$(CH$_2$)$_{13}$CH$_3$), 3.67 (4H, s, CH$_2$CH$_2$OC*H*$_2$C*H*$_2$OCH$_2$CH$_2$), 3.69 (4H, m, CH$_2$C*H*$_2$OCH$_2$CH$_2$OC*H*$_2$CH$_2$), 4.14–4.33 (4H, m, C*H*$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$C*H*$_2$), 4.97 (2H, m, C*H*CH$_2$COOH), 6.89 (2H, br, CONH). Anal. calcd for $C_{80}H_{154}N_4O_{12}\cdot 0.5H_2O$: C, C, 69.98; H, 11.38; N, 4.08%. Found: C, 69.98; H, 11.50; N, 4.16%. HR-MS (FAB$^+$): exact mass calcd for $C_{80}H_{155}N_4O_{12}$ [M+H]$^+$ 1364.1642, found 1364.1649.

4.3. Preparation of hybrid vesicles

Hybrid liposomes employed in this study were generally prepared according to established protocol[1] as follows. Appropriate amounts of synthetic lipid and dimyristoylphosphatidylcholine (DMPC) were dissolved in chloroform. The solvent was evaporated under nitrogen gas flow and the residual trace solvent was completely removed in vacuo. Hydration of the thin film thus obtained on the wall of a vial was performed at 40 °C with an appropriate amount of water. Multi-walled bilayer vesicles were formed upon vortex mixing of the aqueous dispersion. The corresponding single-walled vesicles were prepared by sonication of the dispersion sample with a cup-type sonicator above the phase transition temperature for 5 min.

4.4. Measurements

4.4.1. Differential scanning calorimetry (DSC). The phase transition behavior of the lipid bilayer vesicles was measured with a differential scanning calorimeter (VP-DSC, MicroCal). The measurements were performed

between 10 and 50 °C with a 0.5 °C/min heating rate. Phase transition temperature (T_m), its enthalpy change (ΔH) was evaluated.

4.4.2. Dynamic light scattering (DLS) measurement. Hydrodynamic diameter of the lipid bilayer vesicles was measured by a dynamic light scattering spectrometer equipped with 633 nm He–Ne laser (DLS-6000, Otsuka electronics) at 30 °C. Time course of the light scattering from the sample was analyzed by the Cumulant method at an angle of 90° from the incident light.

4.4.3. ζ-Potential measurement. Binding of metal ions to the vesicular surface was evaluated from change in the ζ-potential value by using an electrophoretic light scattering apparatus by a laser doppler system (ELS-6000, Otsuka Electronics) at 30 °C. Latex beads of polystyrene with a 200 nm diameter were employed as a standard. The vesicular solution was mixed with NaCl as an electroryte and its pH was adjusted by aqueous HCl or NaOH.

4.4.4. Circular dichroism spectroscopy. In order to evaluate the Cu^{2+}-binding to the gemini peptide lipid, circular dichroism (CD) measurements were carried out by a spectropolarimeter (J-820, JASCO). The CD spectra of the peptide lipid embedded in the DMPC liposome were measured at pH 9.0 and 30 °C by changing the Cu^{2+} concentrations. Titration isotherms were obtained from the changes in CD intensity, and the data were fitted to an appropriate binding model. An iterative curve-fitting method yielded the binding constant and the maximum change in CD intensity. The binding constants mentioned in the text are the average of three independent measurements. Job's method was used to determine the stoichiometry of the complex of the lipid with Cu^{2+} ions.

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