Notes

Bilayer Properties of the Multiple-Chain Ion Pair Amphiphiles

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Ion pair amphiphile (IPA), composed of two separate chains with a positive and a negative charge on each head group, is reported to form vesicular aggregate.[1] Each single chain is supposed to form micellar aggregate, but the formation of cylindrical shape by combination of two single chains is presumed to be the driving force of bilayer formation like the double-chained phospholipid. However, the stability of double chain IPA is not good enough to encapsulate biomolecules (enzymes, DNA etc.) or markers for a period, which deters further development toward widespread application in microencapsulation. In order to overcome the shortcoming, polymerizable IPA containing polymerizable vinyl group at the head group of one chain was introduced, resulting in a considerable enhancement in the stability of double chain IPA.[2] Meanwhile, synthetic quaternary ammonium surfactants have been known to form vesicular aggregate like the phospholipids long ago.[3] But due to the loose packing and biocompatibility problem ammonium type surfactant was not desirable for encapsulation and controlled release of small molecules. If permeation properties of ammonium type surfactant can be modified to a desired level, it would be highly valuable in the point of replacing the pricy phospholipid analogues and developing a new type of carrier.

In this paper, we would like to report the enhanced membrane tightness of multiple-chain ion pair amphiphile (MIPA) with 14, 16, and 18 carbon-long alkyl chain, prepared by inserting a surfactant with malonate moiety into two quaternary ammonium surfactants, *viz*, (1) bis(*N*,*N*-dimethyl-*N*,*N*-ditetradecylammonium) tetradecylmalonate, (2) bis(*N*,*N*-dimethyl-*N*,*N*-dihexadecylammonium) hexadecylmalonate, (3) bis(*N*,*N*-dimethyl-*N*,*N*-dioctadecylammonium) octadecylmalonate.

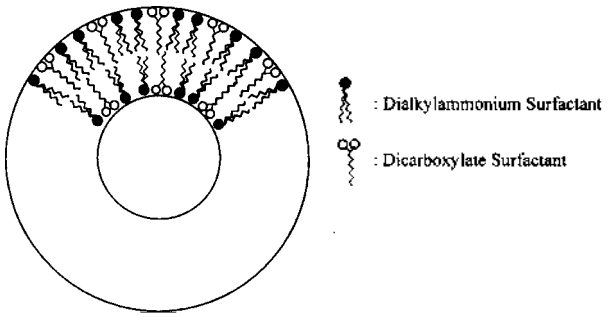

Figure 1. Schematic drawing of the liposome composed of MIPA.

Results and Discussion

In Figure 1 for the schematic drawing of bilayer formation, MIPA is composed of two positively charged surfactants and one dual negatively charged one, designed to fill up the gap between two quaternary ammonium surfactants. Tighter membrane can be formed by the electrostatic interaction between carboxylate and ammonium head groups in addition to hydrophobic interaction among alkyl chains. Some other modifications may be tried to enhance the tightness of membrane. For instance, other ionic head groups such as phosphate or sulfate is substituted for the carboxylate, chain length and number of chains are varied, and the mismatched MIPA which has different chain length within MIPA is tested. Leaving a room for other derivations, we narrow our focus on three MIPA candidates and their bilayer properties are compared in this paper.

As in Figure 2 for synthesis, diethylmalonate deprotonated with sodium ethoxide was coupled with *n*-alkyl bromide (chain length=14, 16, 18) by refluxing in ethanol, and the coupled product was hydrolyzed by aqueous NaOH solution

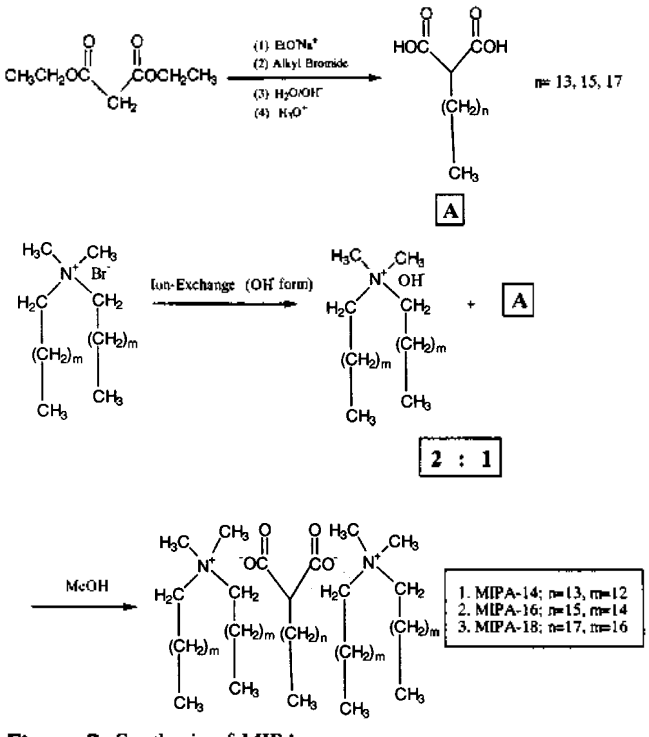

Figure 2. Synthesis of MIPAs.

to remove ester protecting group.[4] On the other hand, the quaternary ammonium surfactant was ion-exchanged to hydroxide form and, subsequently, paired with the dicarboxylate compound (A) at 2 : 1 molar ratio in methanol, followed by recrystallization from methanol/chloroform. Completeness of the pairing was confirmed by TLC in which R_f ($CHCl_3$/MeOH, 9/1) dropped from 0.6 of dicarboxylate compound to the bottom after ion pairing. Additionally, IR peak at 1700 cm^{-1} for carboxyl group shifted to 1570-1590 cm^{-1}. As for the proton NMR spectra, chemical shift of the protons at around the ammonium group moved from 3.5-3.6 ppm for free ammonium form to 3.2-3.4 ppm for MIPA and the chemical shift of the proton at the α-position of dicarboxylate compound (A) moved from 3.2 ppm to 3.0 ppm after ion pairing.

Formation of vesicular structure was confirmed by transmission electron microscopy (TEM) and the bilayer structure was observed for the sonicated vesicle of MIPA-16 stained with 2% uranyl acetate (Figure 3). The TEM picture was magnified by 100,000 times with the 50 nm bar at the bottom and the diameter of vesicle was about 500 Å (50 nm) or less, which was quite resonable for a sonicated vesicle. With the three MIPAs in hand, encapsulation of 4(5)-carboxyfluorescein (CF) was checked and efflux rates were measured by dialysis method.[5] Typically, 3 mg of MIPA in chloroform was dried to a thin uniform film, dispersed in 1 mL of 1 mM CF solution in deionized water, and the milky mixture was extruded through 0.1 μm Nuclepore polycarbonate membrane under argon pressure to get large unilamellar vesicle (LUV).[6] After removing unencapsulated CF by gel-filtration, encapsulated CF within MIPA vesicle was allowed to release slowly from dialysis bag at 45 °C, and the fluorescence intensity of retained CF was periodically measured to calculate permeation rate as presented in Table 1.

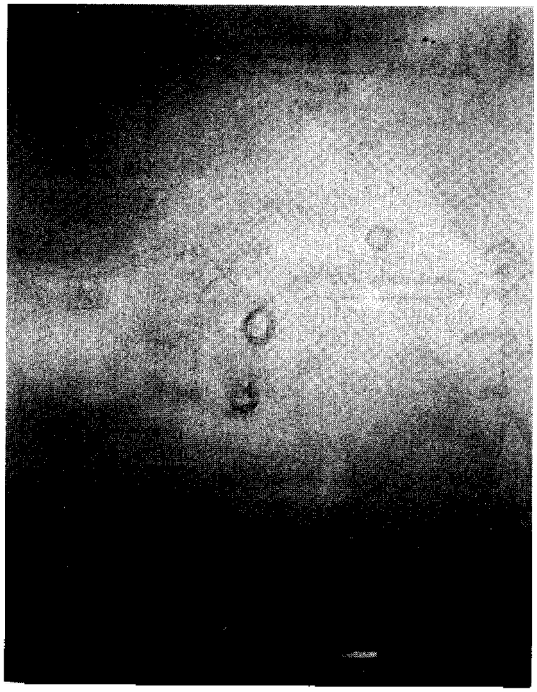

Figure 3. Transmission electron micrograph of the sonicated vesicle of MIPA-16 stained with 2% uranyl acetate.

Among the three MIPAs, MIPA-14 and MIPA-18 showed excellent encapsulation and stability during dialysis, while MIPA-16 had so low percentage of encapsulation after gel-filtration that permeation data were not available. Permeation result from the quaternary ammonium surfactant, *viz*, *N,N*-dimethyl-*N,N*-tetradecyllammonium bromide, *N,N*-dimethyl-*N,N*-hexadecylammonium bromide, *N,N*-dimethyl-*N,N*-dioctadecylammonium bromide, was not attainable due to the very low encapsulation efficiency of them, attributing to the loose packing of quaternary ammonium surfactant vesicle.

Meanwhile, LUV from MIPA-14 and MIPA-18 kept the markers so well that the amount of CF released during dialysis experiment at 45 °C was not noticeable. To induce further leakage of CF from the MIPA vesicle, small mole % of surfactant such as sodium dodecylsulfate (SDS) or Triton X-100 was added into the vesicular solution.[5] As shown in Figure 4 for the release profile of MIPA-14, percentage of encapsulated CF slowly decreased in the presence of surfactant and the permeation rate constant as calculated by literature method increased with further addition of surfactant.[7] SDS was quite effective in disrupting the tight bilayer of both MIPA-14 and MIPA-18, while Triton X-100 was not comparable to SDS in increasing the permeation rate proportionally with the addition of more surfactant. Reasonably, MIPA-18 showed slower permeation rate than MIPA-14, resulting from the longer chain length and tighter membrane packing. Initial fast decrease observed for all of the vesicle/surfactant solution was probably caused by the small amount of adsorbed CF (*ca.* 10%) on vesicular surface, so that permeation rate constant was calculated, based on the region after that. Although not included in this paper, turbidity data for LUV measured at 300 nm with the addition of SDS or Triton X-100 also showed that SDS was more effective than Triton X-100 in transforming the turbid solution into a clear one.

Membrane fluidity could be detected from the change in polarization intensity of the probe, 1,6-diphenyl-1,3,5-hexa-

Table 1. Permeability of CF from Vesicle of Multiple-chain Ion Pair Amphiphiles[a]

MIPA[b]	Surfactants	% of Surfactant (w/v)	10^8 *P* (cm/hr)[c]
14	None	-	NR[d]
14	SDS	0.5	3.8
14	Triton X-100	0.5	2.2
14	SDS	1.0	2.9
14	Triton X-100	1.0	1.8
14	SDS	1.5	5.8
14	Triton X-100	1.5	1.7
18	None	-	NR
18	SDS	0.5	1.9
18	Triton X-100	0.5	1.3
18	SDS	1.0	3.0
18	Triton X-100	1.0	1.0
18	SDS	1.5	5.3
18	Triton X-100	1.5	1.1

[a] All of the measurements were carried out at 45 °C. In each case, the extent of release was followed for more than 100 hrs and the permeation coefficient is an average of two independent experiments. [b] 14; MIPA-14, 18; MIPA-18. [c] See ref. 7 for the calculation of permeation coefficient. [d] NR; No release.

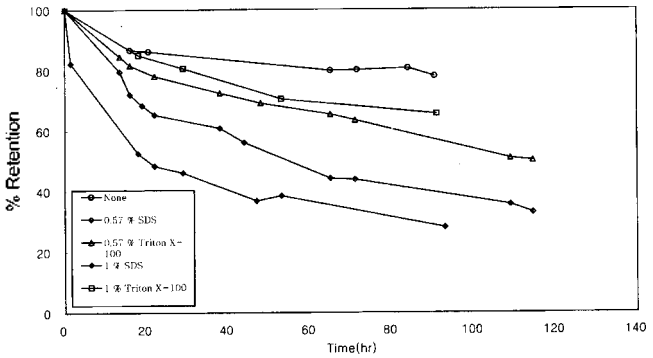

Figure 4. CF release from MIPA-14 with various surfactants at 45 °C.

triene (DPH), and the temperature range was set at every 5 °C between 15 °C and 70 °C.[8] Phase transition along the temperature range was not observed for all of the MIPAs, judging from that polarization intensity did not show any abrupt change as in phospholipid vesicle. As we were not clear about the polarization data, differential scanning calorimetry (DSC) was also applied for detecting thermal transition of all the MIPAs. The DSC thermogram for multilamellar vesicle (MLV) of three MIPAs did not present any conspicuous transition from 15 °C to 80 °C after three heating and cooling cycles. Both polarization and DSC data suggest that the phase transition of the MIPAs is very complicated to result in broad transition peaks because the MIPAs are composed of five alkyl chains which may not cooperate in transition as in double chain analogues. It is also suspected that we can not detect the broad transitions by the above methods.

Overall, we propose that MIPA-14 and MIPA-18 demonstrate excellent properties in sealing the leaky ammonium type membrane, and the release of encapsulated molecule within the MIPA vesicle can be induced by the addition of surfactant. If the above MIPA can be polymerizable, vesicular membrane, thus formed, would be comparable to the polymerized phospholipid vesicle in long-term stability and tightness of membrane. We are now preparing a set of MIPAs which differ in the number of chain, and head group. As for MIPA-16, we are very curious about the result of low encapsulation, because a second batch was prepared by the same method, resulting in the same low efficiency of encapsulation. Further structural modification of MIPA-16 remains to be established and permeability control through variation in chain length and combination of chains with opposite charge may offer a new horizon in the arena of microencapsulation.

Experimental Section

General Methods. DSC thermogram was scanned by MAC science DSC 3100 at Ajou university, Suwon, Korea. Fluorescence and polarization intensity was measured by Perkin-Elmer LS-50B Luminescence Spectrometer equipped with polarizer and temperature control. Elemental analysis was carried out at Seoul Branch Analytical Lab of Korea Basic Science Institute. TEM picture was taken at Inter-University Center for Natural Science Research Facilities at Seoul National University. The extruder was purchased from Lipex Biomembrane Co (B.C., Canada), and operated by Ar pressure. All reagents were obtained from Aldrich and Sigma Chemical Co. except diethylmalonate from Lancaster Synthesis, UK.

Hexadecylmalonic Acid. 560 mg (24.3 mmole) of sodium was dissolved in 20 mL of dry ethanol (dried over CaH_2) and the sodium ethoxide solution was slowly added into a solution of 3.00 g (18.7 mmole) of diethylmalonate in 10 mL of ethanol, followed by stirring for 1 hr under nitrogen. Into the solution was slowly added 6.85 g of *n*-hexadecyl bromide (22.4 mmol) in 20 mL of ethanol for 1 hr, and the whole mixture was refluxed under nitrogen overnight. After complete removal of ethanol, 30 mL of 0.3 M KOH was added and the mixture was refluxed for 30 hrs. The reaction mixture was neutralized with conc. HCl to get white precipitate and extracted with ethylacetate (100 mL × 3). The organic layer was washed with water, brine and dried over $MgSO_4$. After evaporation of solvent, the crude product was recrystallized 3 times from ethylacetate/hexane to get 5.09 g (15.5 mmol, 83%) of white solid: R_f=0.7 ($CHCl_3$/MeOH, 20/1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.9 (t, 3H, C$\underline{H}_3$), δ 1.1-1.3 (s, 28H, C$\underline{H}_2$), δ 1.7 (m, 2H, C$\underline{H}_2$CH(CO_2)$_2$), δ 3.2 (t, 1H, C$\underline{H}$(CO_2)$_2$); IR (KBr) 1700 cm^{-1} (C=O), 1472 cm^{-1} (C-H).

Tetradecylmalonic Acid. 2.07 g (7.50 mmole) of *n*-tetradecylbromide was substituted for the hexadecylbromide in the above procedure and other reagents were used according to the above scale. 1.15 g (3.83 mmol, 62%) of tetradecylmalonic acid was obtained as a white solid: R_f=0.7 ($CHCl_3$/MeOH, 20/1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.9 (t, 3H, C$\underline{H}_3$), δ 1.1-1.3 (s, 24H, C$\underline{H}_2$), δ 1.7 (m, 2H, C$\underline{H}_2$CH(CO_2)$_2$), δ 3.2 (t, 2H, C$\underline{H}$(CO_2)$_2$); IR (KBr) 1700 cm^{-1} (C=O), 1472 cm^{-1} (C-H).

Octadecylmalonic Acid. 2.50 g (7.50 mmole) of *n*-octadecylbromide was substituted for the hexadecylbromide in the above procedure and other reagents were used according to the above scale. 1.15 g of octadecylmalonic acid (3.22 mmol, 52%) was obtained as a white solid: R_f=0.7 ($CHCl_3$/MeOH, 20/1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.9 (t, 3H, C$\underline{H}_3$), δ 1.1-1.3 (s, 32H, C$\underline{H}_2$), δ 1.7 (m, 2H, C$\underline{H}_2$CH(CO_2)$_2$), δ 3.2 (t, 2H, C$\underline{H}$(CO_2)$_2$); IR (KBr) 1700 cm^{-1} (C=O), 1472 cm^{-1} (C-H).

Bis(*N*,*N*-dimethyl-*N*,*N*-dihexadecylammonium) Hexadecylmalonate. The ion-exchange resin (Bio-Rad AG-1X8, hydroxide form, 3.2 meq/g) was washed with methanol for 8 hrs by Soxhlet apparatus, and packed into a column (1 × 10 cm) prior to use. 350 mg (0.608 μmol) of *N*,*N*-dimethyl-*N*,*N*-dihexadecylammonium bromide in 10 mL of methanol was converted into hydroxide form by passing through the column (× 10), and mixed with 100 mg (0.3 μmol) of *n*-hexadecylmalonic acid. After stirring overnight, the solvent was evaporated, and the residue was recrystallized 3 times from methanol/chloroform to get 350 mg of colorless solid (87%): mp 87 °C; R_f=0.6 ($CHCl_3$/MeOH, 1/1), R_f=0 ($CHCl_3$/MeOH, 9/1); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.90 (t, 15H, C$\underline{H}_3$), δ 1.0-1.5 (s, 130H, C$\underline{H}_2$), δ 1.65 (m, 8H, C$\underline{H}_2$C$\underline{H}_2$N$^+$), δ 1.9 (m, 2H, CH$_2$C$\underline{H}_2$CH(CO_2)$_2$), δ 3.0 (t, 1H, C$\underline{H}$(CO_2)$_2$), δ (3.2-3.4, 20H, C$\underline{H}_3$N$^+$C$\underline{H}_2$); IR (KBr) 1573 cm^{-1} (C=O), 1468 cm^{-1} (C-H), 719 cm^{-1} (N-CH$_3$). Anal Calcd for $C_{87}H_{178}N_2O_4$·5.5H_2O: C, 73.82; N, 1.97. Found: C, 74.01; N, 1.97.

Bis(*N,N*-dimethyl-*N,N*-ditetradecylammonium) Tetradecylmalonate. 345 mg (0.665 μmol) of *N,N*-dimethyl-*N,N*-ditetradecylammonium bromide was used and the same procedure was followed to get a 320 mg of colorless solid (82%): mp 79-80 °C, R_f=0.6 ($CHCl_3$/MeOH, 1/1), R_f=0 ($CHCl_3$/MeOH, 9/1); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.90 (t, 15H, C$\underline{H}_3$), δ 1.0-1.5 (s, 110H, C$\underline{H}_2$), δ 1.65 (m, 8H, C$\underline{H}_2CH_2N^+$), δ 1.9 (m, 2H, C$\underline{H}_2CH_2CH(CO_2)_2$), δ 2.3 (m, 2H, C$\underline{H}_2CH(CO_2)_2$, δ 3.0 (t, 1H, C$\underline{H}(CO_2)_2$), δ (3.2-3.4, 20H, C$\underline{H}_3N^+C\underline{H}_2$); IR (KBr) 1592 cm^{-1} (C=O), 1468 cm^{-1} (C-H), 720 cm^{-1} (N-CH_3). Anal Calcd for $C_{77}H_{158}N_2O_4 \cdot 4.5H_2O$: C, 73.56; N, 2.23. Found: C, 73.73; N, 2.14.

Bis(*N,N*-dimethyl-*N,N*-dioctadecylammonium) Octadecylmalonate. 353 mg (0.560 μmol) of *N,N*-dimethyl-*N,N*-dioctadecylammonium bromide was used and the above procedure was followed to get a 300 mg of colorless solid (73%): mp 47 °C, R_f=0.6 ($CHCl_3$/MeOH, 1/1), R_f=0 ($CHCl_3$/MeOH, 9/1); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.90 (t, 15H, C$\underline{H}_3$), δ 1.0-1.5 (s, 150H, C$\underline{H}_2$), δ 1.65 (m, 8H, C$\underline{H}_2CH_2N^+$), δ 2.1 (m, 4H, C$\underline{H}_2C\underline{H}_2CH(CO_2)_2$), δ 3.0 (t, 1H, C$\underline{H}(CO_2)_2$), δ (3.2-3.4, 20H, $(C\underline{H}3)_2N^+(C\underline{H}_2)_2$); IR (KBr) 1592 cm^{-1} (C=O), 1468 cm^{-1} (C-H), 720 cm^{-1} (N-CH_3). Anal Calcd for $C_{97}H_{198}N_2O_4 \cdot 9H_2O$: C, 71.97; N, 1.73. Found: C, 71.76; N, 2.26.

Preparation of Liposome and the Measurement. MIPA was stored in chloroform at 4 °C and an aliquot (3 mg) of MIPA in chloroform was evaporated by blowing with nitrogen and dried under high vacuum to get a thin lipid film. After the addition of 1 mL of 1 mM CF solution in deionized water, vortex mixing, brief sonication, and freeze-thawing (×5), the solution was used as MLV or extruded through 0.4, 0.1 μm Nuclepore polycarbonate membrane (×5) to prepare LUV solution. Unencapsulated CF was removed by Sephadex G-50 gel-filtration column (1.5×40 cm), eluting with deionized water. Fractions containing encapsulated CF were collected and 2 mL out of the collected vesicle solution was put into a dialysis bag (Spectrapore No. 2 cellulose tubing, 1.5 cm×5.0 cm). Appropriate percent of surfactant solution (w/v) was added into the vesicle solution to see the effect of surfactant as needed. The bag was kept in 500 mL of deionized water at 45 °C and 100 μL of sample was taken periodically from the inside of the bag, followed by mixing with 2 mL of water for fluorescence measurement (E_x: 480 nm, E_m: 518 nm). Permeation rate constant was calculated according to the literature method.[5] LUV solution was prepared for the turbidity experiment, in which 100 μL of 2.5% (w/v) surfactant solution was added sequentially into 2 mL of vesicular solution, and turbidity measured at 300 nm after each addition was corrected for the added volume of surfactant.

Polarization and DSC. For the measurement of polarization intensity, 35 μL of 4×10^{-3} M DPH in THF was added into 2 mL of MLV solution (3 mg of MIPA). Polarization intensity was measured at every 5 °C from 15 °C to 70 °C (E_x: 350 nm, E_m: 450 nm), and calculated automatically by the fluorescence data management (FLDM) operating system in the luminescence spectrometer.

For DSC, 50 μL of MLV solution was loaded into an aluminum pan and sealed with a cap under pressure. As for the reference, same amount of deionized water was filled. Thermal transition was monitored 3 times between 15 °C and 70 °C, and the third scan was recorded for comparison.

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