Synthesis and Assembly of Poly(ethylene glycol)−Lipids with Mono-, Di-, and Tetraacyl Chains and a Poly(ethylene glycol) Chain of Various Molecular Weights

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Abstract: We synthesized a series of amphiphiles with poly(ethylene glycol) [MW 2000 (PEG20), 5000 (PEG50), 12 500 (PEG125)] as a headgroup and one, two, or four palmitoyl chains (1C16, 2C16, or 4C16), using a lysine monodendron as a connector. The relationship between the hydrophilic−hydrophobic balance of the multiacyl PEG-lipids and the physicochemical characteristics in self- or co-assembly with phospholipids were studied. The PEG-lipids were easily synthesized by combination of a general liquid-phase peptide synthesis and the acylation of an amino acid. The PEG part of the PEG−lipid films was crystallized to show a typical spherulite pattern. The thermal properties and microscopic observation revealed the phase separation of PEG and acyl chain parts. The critical micelle concentrations (cmcs) mainly depend on the number of acyl chains rather than the molecular weight of the PEG chain, although the area per molecule is dependent on the molecular weight of the PEG chain rather than the number of the acyl chains. The gel-to-liquid crystalline phase transition temperature was increased with the increasing number of acyl chains and the decreasing molecular weight of the PEG chain. The PEG−lipids in the aqueous dispersions assemble to take fibrous structures with bimolecular thickness because of the intermolecular hydrogen bonding. The PEG−lipids were immobilized onto the surface of the phospholipid vesicles by simply adding their aqueous dispersions to the vesicle dispersion; however, they dissociated from the vesicles on dilution of the mixed dispersion because they were incorporated into the vesicles in an equilibrium state. To prevent the dissociation of the PEG−lipids, at least two and four acyl chains were required for PEG with M_w 5000 and 12 500, respectively. The aggregation of the vesicles by the addition of water-soluble polymers was significantly inhibited with the increasing molecular weight of the PEG chain. For the tight immobilization of the PEG−lipids with the long PEG chain onto the vesicular surface, an increased number of acyl chains is necessary, and the surface modification with the long PEG chains significantly increases the dispersion stability of the vesicles.

Introduction

The hydrophilic−hydrophobic balance of amphiphiles is the most important factor in characterizing their physicochemical properties.[1] In biological systems, phospholipids with a small hydrophilic headgroup, such as a glycerophosphocholine group, assemble to form a bilayer membrane by hydrophobic interaction among the diacyl chains with a carbon number of 16 or 18. The critical micelle concentrations (cmcs) of the phospholipids are so small ($<10^{-10}$ M) that the concentrations of free monomers are negligible. However, in the case of the amphiphiles such as glycoproteins with a large hydrophilic headgroup, their large headgroup can be immobilized in the bilayer membrane with a large hydrophobic polypeptide as a membrane-spanning anchor.[2] The poly(ethylene glycol) (PEG)-conjugated phosphatidylethanolamines (PEG−phospholipids) have been widely used for the modification of the vesicular surface to prolong the blood circulation time or to stabilize their dispersion states.[3−6] However, their cmcs are relatively high ($>10^{-5}$ M) because of the large headgroup, and the interbilayer transfer rates of the PEG−phospholipids are also very fast compared with those of phospholipids.[7] In general, the molecular weights of the PEG chain are between 2000 and 5000, and the hydrophobic parts are usually taken from two choices: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE) or 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE). Though there are many papers on the molecular weight of PEG, the hydrophobic part has not been paid much attention. We clarified that the PEG−phospholipids were incorporated into the bilayer membrane at an equilibrium state and removable from the membrane by simple dilution.[8] This result indicates that the hydrophobic group of the PEG−phospholipids is not large enough to anchor the PEG chain to the membrane. Therefore, we designed macromolecular amphiphiles to immobilize water-soluble polymers or proteins on the membrane as illustrated in Figure 1. Our goals in designing such macromolecular amphiphiles are (1) simple syntheses, (2) chemically

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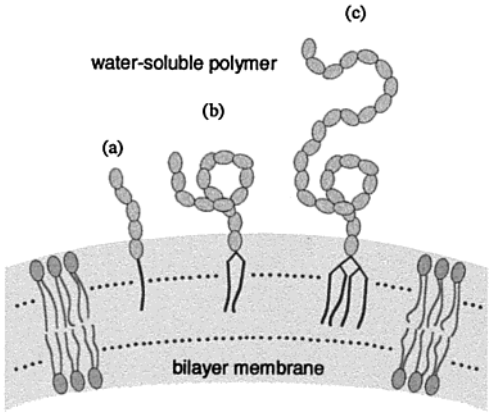

Figure 1. The concept to immobilize the large water-soluble polymers on the bilayer vesicles. The PEG−lipids are spontaneously incorporated into the bilayer membrane. The large number of acyl chains would be necessary to immobilize the PEG chain with large molecular weight. (a) mono-, (b) di-, and (c) tetraacyl PEG−lipids.

distinct structures, (3) an appropriate hydrophilic−hydrophobic balance for immobilization, (4) a parallel orientation of the acyl chains not to disturb the orientation of phospholipids in bilayer membrane, and (5) biocompatibility and biodegradability.

Dendrons, which constitute one part of a dendrimer and have functional groups such as amino, hydroxy, or carboxylic acid groups at the ends, are potent candidates to bind plural molecules, and the number of the functional groups is controllable by changing the generation of the dendrons.[9−14] A peptide dendrimer based on lysine has been used to bind the saccharides[15] or peptides[16,17] and is considered to be suitable for biomaterials because it should be biocompatiable and biodegradable. An amphiphilic lysine dendrimer,[18] a PEG−dendrimer,[19−21] and others have been reported, and the properties at the air−water interface or aqueous phase were studied.[22−25] We use the branched amino end groups and the carboxylic acid group of lysine monodendrons to bind palmitoyl chains and a α-methyl-ω-aminomethylpoly(oxyethylene) chain, respectively, to synthesize PEG−lipids.

In this paper, we report on the syntheses and properties of a series of the PEG−lipids (PEGm−nCk, m•10^2 is the molecular weight of PEG, n is the number of acyl chains, k is the carbon number of the acyl chains) in solid and liquid states. Furthermore, the co-assembling properties of the PEG−lipids in a mixed state with phospholipid vesicles were clarified in relation to the structure of the PEG−lipids.

Experimental Section

Materials. L-Lysine, *p*-toluenesulfonic acid, benzyl alcohol (Bzl-OH), and palmitic acid were purchased from Kanto Chemical Co. α-Methyl-ω-aminomethylpoly(oxyethylene)s [M_n; 2050, 5050, 12 500, M_w/M_n <1.03 gel permeation chromatography (GPC)] were purchased from NOF Co. The mixed lipid powders of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG) at 5:5:1 molar ratio (Presome PPG-I) were purchased from Nippon Fine Chemical Co.

General Methods and Procedures. Melting points were determined with a Seiko 120 DSC thermal analysis unit. The infrared (IR) spectra were measured for the films and aqueous dispersions. The film was prepared by casting the $CHCl_3$ solution of the PEG−lipid on the KBr plate. For the aqueous dispersion, the PEG−lipids were dispersed into pure water at 60 °C. The sample dispersions were sandwiched between the CaF_2 windows, and the IR spectra were recorded on a JASCO FT-IR-410. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of solutions in $CDCl_3$ or d_6-DMSO were recorded on a JEOL JNM-LA500 (500 MHz) spectrometer. Fast-atom bombardment mass spectroscopy (FAB-MS) spectra were obtained with a JEOL JMS−SX102A spectrometer. Analytical thin-layer chromatography (TLC) was performed on commercial plates coated with silica gel (TLC MERK, silica gel 60 F_{254}). The silica for flash chromatography was Merck Kieselgel 60 (230−400 mesh). The differential scanning calorimetry (DSC) analysis was performed with a Seiko 120 DSC from 10 to 90 °C at a scan rate of 2 °C min^{-1}. The aqueous dispersion of the sample ([PEG−lipid] = 15 mM, 60 μL) was sealed in a silver pan. The compounds dissolved in chloroform ([PEG−lipid] = 10^{-3} kg dm^{-3}) were cast on a glass plate and observed with a polarized optical microscope.

Syntheses. The PEG−lipids were synthesized by a liquid-phase peptide synthetic method.[18,26]

PEG50−1C16. Palmitic acid (51 mg, 0.2 mmol) and *N*, *N*′-dicyclohexylcarbodiimide (DCC; 41 mg, 0.2 mmol) were dissolved in chloroform and stirred for 1 h at 4 °C. The solution was dropped into a chloroform solution of PEG50-NH_2 (0.5 g, 0.1 mmol) and (dimethylamino)pyridine (DMAP) (24 mg, 0.2 mmol). The reaction was examined by TLC (silica gel plate, chloroform/methanol = 4/1, v/v), and the PEG50-NH_2 spot had completely disappeared after 2 h. The product reprecipitated from ether was further purified by column chromatography (silica gel, chloroform/methanol = 6/1, v/v). The PEG50−1C16 was obtained as a white powder (0.45 g, yield 86%). TLC (silica gel) chloroform/methanol (4/1): R_f 0.70. IR (film; cm^{-1}): 3375 (νN−H, amide), 1671 (νC═O, amide), 1540 (δN−H, amide); ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 3H, $-CH_3$), 1.25 (s, 24H, $-CH_2-$), 1.68 (m, 2H, $-CH_2-C-NH-$), 1.84 (t, 2H, $-CO-C-CH_2$), 2.46 (t, 2H, $-CO-CH_2-$), 2.46 (t, 2H, $-CO-CH_2-$), 3.38 (s, 3H, $-OCH_3$), 3.46 (t, 2H, $-N-CH_2$), 3.64 (PEG). ^{13}C NMR ($CDCl_3$, 500 MHz, δ ppm): 14.13, 22.69, 29.36, 29.66, 31.93, 59.03, 70.58, 71.82. Anal. Calcd for $C_{246}H_{493}N_1O_{115}$: C 55.52, H 9.34, N 0.26; found: C 55.51, H 9.61, N 0.53.

Bis-palmitoyl-L-Lys. The L-lysine (Lys; 5.1 g, 35.2 mmol) and *p*-toluenesulfonic acid (monohydrate, 14.7 g, 77.3 mmol) were dissolved into benzyl alcohol (Bzl-OH; 14.0 g, 124.1 mmol) and benzene (30 mL).[27] The mixture was refluxed for 6 h at 100 °C with trapping generated water with a Dean−Stark receiver. The reaction mixture was allowed to cool to room temperature and reprecipitated three times with

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ether (200 mL) at 4 °C. After recrystallization from methanol/ether at 4 °C, L-Lys-*O*-Bzl $(TsOH)_2$ was obtained as a white solid (18.0 g, yield 88%).

The palmitic acid (3.2 g, 12.4 mmol) and DCC (2.6 g, 12.4 mmol) were dissolved in chloroform and stirred for 1 h at 4 °C. The L-Lys-*O*-Bzl$(TsOH)_2$ (3.0 g, 5.12 mmol) and DMAP (1.4 g, 11.4 mmol) were added to the mixture and stirred at 4 °C for 14 h and then at 25 °C for 2 h. The crude product was washed with a Na_2CO_3 saturated aq. solution. After recrystallization from methanol at 4 °C, bis-palmitoyl-L-Lys-*O*-Bzl was obtained as a white solid (2.9 g, yield 79%).

Bis-palmitoyl-L-Lys-OBzl (1.52 g, 2.13 mmol) was dissolved in a mixed solvent (43 mL, chloroform/methanol = 10/7, v/v), and a 1 N NaOH solution (3.4 mL) was added and stirred for 4 h at 25 °C. The 1 N HCl solution was added to the solution until a pH of 3.0 was attained. The product was washed with water and then methanol. Bis-palmitoyl-L-Lys (1.30 g, yield 98%) was obtained as a white solid. TLC (silica gel) chloroform/methanol (4/1): R_f 0.45. IR (film; cm^{-1}): 3305 (υN−H, amide), 1721 (υC═O, carbonyl), 1638 (υC═O, amide), 1553 (δN−H, amide); ^{1}H NMR (DMSO-d_6, 500 MHz, δ ppm): 0.84 (t, 6H, $-CH_3$), 1.24 (s, 50H, $-CH_2-CH_2-$, Lys γ-CH_2), 1.36 (m, 2H, Lys δ-CH_2), 1.47 (m, 4H, $-N-CO-C-CH_2-$), 1.55, 1.67 (m, 2H, Lys β-CH_2), 2.02, 2.09 (t, 4H, $-N-CO-CH_2-$), 2.99 (m, 2H, Lys ϵ-CH_2), 4.14 (m, 1H, Lys α-CH), 7.55 (br, 1H, −NH−CO−), 7.78 (d, 1H, −NH−CO−), 12.23 (br, 1H, −COOH). MS (FAB): calcd for $C_{38}H_{74}N_2O_4$: 623.0; found: 623.5 $(M^+H)^+$. Anal. Calcd for $C_{38}H_{74}N_2O_4$: C 73.26, H 11.97, N 4.50; found C 72.39, H 12.43, N 4.74.

PEG-L-Lys. The L-Lys (1.5 g, 10.3 mmol) and *t*-buthoxyoxycarbonyl anhydride (6.3 g, 29.0 mmol) were dissolved into a mixed solvent of dioxan (10 mL), water (10 mL), and 1 N NaOH (10 mL) and stirred for 6 h at pH 7.3, and 25 °C. The reaction mixture was concentrated up to 10 mL, and pH was adjusted to 2.4 by adding a $KHSO_4$ aq. solution. The product was extracted with ethyl acetate and reprecipitated from hexane. The L-Lys-$(Boc)_2$ was obtained as a white solid (2.64 g, yield 74%).

The L-Lys-$(Boc)_2$ (83 mg, 0.24 mmol) and DCC (50 mg, 0.24 mmol) were dissolved in chloroform and stirred for 1 h at 4 °C. The reaction mixture was added to a PEG50-NH_2 (1.0 g, 0.2 mmol) and DMAP (24 mg, 0.2 mmol) chloroform solution and stirred for 6 h at 4 °C. The crude product was purified by reprecipitation from ether. The PEG-L-Lys-$(Boc)_2$ (0.97 g, yield 90%) was thus obtained as a white solid.

The PEG50−L-Lys-$(Boc)_2$ (0.5 g, 0.09 mmol) was dissolved in trifluoroacetic acid (5 mL) and stirred for 1 h at 4 °C. The product was purified by reprecipitation from ether. The PEG50−L-Lys (0.44 g, yield 91%) was obtained as a white solid. TLC (silica gel) chloroform/methanol (4/1): R_f 0.32. ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 1.60 (m, 2H, Lys γ-CH_2), 1.80−1.90 (m, 4H, $-CH_2-C-N-$, Lys δ-CH_2), 2.05 (m, 2H, Lys β-CH_2), 3.06 (t, 2H, $-CH_2-N-CO-$), 3.25−3.40 (m, 2H, Lys ϵ-CH_2), 3.38 (s, 3H, $-O-CH_3$), 3.65 (PEG), 4.15 (m, 1H, Lys α-CH), 7.64, 7.83, 8.41 (br, 7H, −NH−CO−, $-NH_3^+$).

PEG50−2C16. Bis-palmitoyl-L-Lys (125 mg, 0.2 mmol) and DCC (41 mg, 0.2 mmol) were dissolved in chloroform and stirred for 30 min at 25 °C. PEG50-NH_2 (0.5 g, 0.1 mmol) and DMAP (24 mg, 0.2 mmol) were added to the mixture and stirred at 25 °C for 6 h. The product reprecipitated from ether was further purified by column chromatography (silica gel, chloroform/methanol = 6/1, v/v). The PEG50−2C16 was obtained as a white powder (500 mg, yield; 88%). TLC (silica gel) chloroform/methanol (4/1): R_f 0.73. IR (film; cm^{-1}): 3294 (υN−H, amide), 1634 (υC═O, amide), 1553 (δN−H, amide); ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 6H, $-CH_3$), 1.25 (s, 50H, $-CH_2-CH_2-$, Lys γ-CH_2), 1.32 (m, 2H, Lys δ-CH_2), 1.63−1.80 (br, 8H, $-CH_2-C-N-$, $-N-CO-C-CH_2-$, Lys β-CH_2), 2.27, 2.38 (t, 4H, $-N-CO-CH_2-$), 3.29 (m, 2H, Lys ϵ-CH_2), 3.38 (s, 3H, $-O-CH_3$), 3.43 (br, 2H, $-CH_2-NH-$), 3.66 (PEG), 4.39 (m, 1H, Lys α-CH). ^{13}C NMR ($CDCl_3$, 500 MHz, δ ppm): 14.12, 22.68, 25.74, 28.75, 29.34, 29.53, 29.65, 31.92, 36.35, 38.13, 59.02, 70.44, 71.95. Anal. Calcd for $C_{268}H_{535}N_3O_{117}$: C 56.59, H 9.48, N 0.74; found C 56.88, H 9.87, N 1.07.

PEG20−2C16s. Yield 60%. TLC (silica gel) chloroform/methanol (4/1): R_f 0.75. IR (film; cm^{-1}): 3311 (υN−H, amide), 1637 (υC═O, amide), 1556 (δN−H, amide). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 6H, $-CH_3$), 1.25 (s, 50H, $-CH_2-CH_2-$, Lys γ-CH_2), 1.32 (m, 2H, Lys δ-CH_2), 1.63−1.80 (br, 8H, $-CH_2-C-N-$, $-N-CO-C-CH_2-$, Lys β-CH_2), 2.27, 2.38 (t, 4H, $-N-CO-CH_2-$), 3.29 (m, 2H, Lys ϵ-CH_2), 3.38 (s, 3H, $-O-CH_3$), 3.43 (br, 2H, $-CH_2-NH-$), 3.66 (PEG), 4.39 (m, 1H, Lys α-CH). ^{13}C NMR ($CDCl_3$, 500 MHz, δ ppm): 14.12, 22.68, 25.74, 28.75, 29.34, 29.53, 29.65, 31.92, 36.35, 38.13, 59.02, 70.44, 71.95.

PEG125−2C16. Yield 61%. TLC (silica gel) chloroform/methanol (4/1): R_f 0.63. IR (film; cm^{-1}): 3305 (υN−H, amide), 1638 (υC═O, amide), 1556 (δN−H, amide). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 6H, $-CH_3$), 1.25 (s, 50H, $-CH_2-CH_2-$, Lys γ-CH_2), 1.32 (m, 2H, Lys δ-CH_2), 1.63−1.80 (br, 8H, $-CH_2-C-N-$, $-N-CO-C-CH_2-$, Lys β-CH_2), 2.27, 2.38 (t, 4H, $-N-CO-CH_2-$), 3.29 (m, 2H, Lys ϵ-CH_2), 3.38 (s, 3H, $-O-CH_3$), 3.43 (br, 2H, $-CH_2-NH-$), 3.66 (PEG), 4.39 (m, 1H, Lys α-CH). ^{13}C NMR ($CDCl_3$, 500 MHz, δ ppm): 14.12, 22.68, 25.74, 28.75, 29.34, 29.53, 29.65, 31.92, 36.35, 38.13, 59.02, 70.44, 71.95.

PEG50−4C16. Bis-palmitoyl-L-Lys (100 mg, 0.15 mmol) and DCC (30 mg, 0.15 mmol) were dissolved in chloroform and stirred for 1 h at 4 °C. The reaction mixture was added to a PEG50−L-Lys (200 mg, 0.038 mmol) and DMAP (4.5 mg, 0.037 mmol) chloroform solution and stirred for 6 h at 25 °C. The reaction mixture was filtered with a glass filter, and the filtrate was reprecipitated from ether. The crude product was further purified by column chromatography (silica gel plate, solvent: chloroform/methanol = 6/1, v/v). The PEG50−4C16 was obtained as a white powder (180 mg, yield 78%). TLC (silica gel) chloroform/methanol (4/1): R_f 0.78. IR (film; cm^{-1}): 3305 (υN−H, amide), 1637 (υC═O, amide), 1560 (δN−H, amide). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 12H, $-CH_3$), 1.25 (s, 110H, $-CH_2-CH_2-$, Lys γ-CH_2), 1.35−1.90 (br, 22H, Lys δ-CH_2, $-CH_2-C-N-$, $-N-CO-C-CH_2-$, Lys β-CH_2), 2.00−2.45 (br, 8H, $-N-CO-CH_2-$), 3.30 (br, 6H, Lys ϵ-CH_2), 3.38 (s, 3H, $-O-CH_3$), 3.43 (br, 2H, $-CH_2-NH-$), 3.66 (PEG), 4.48 (br, 3H, Lys α-CH_2). ^{13}C NMR ($CDCl_3$, 500 MHz, δ ppm): 14.12, 22.68, 25.79, 26.15, 29.36, 29.42, 29.59, 29.72, 31.93, 59.03, 70.58, 71.95. Anal. Calcd for $C_{312}H_{619}N_7O_{121}$: C 58.51, H 9.74, N 1.53; found C 58.20, H 10.04, N 1.7.

The PEG125−4C16 was obtained as a white powder (yield: 77%) by the same method. TLC (silica gel) chloroform/methanol (4/1): R_f 0.68. IR (film; cm^{-1}): 3292 (υN−H, amide), 1635 (υC═O, amide), 1552 (δN−H, amide). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 12H, $-CH_3$), 1.25 (s, 110H, $-CH_2-CH_2-$, Lys γ-CH_2), 1.35−1.90 (br, 22H, Lys δ-CH_2, $-CH_2-C-N-$, $-N-CO-C-CH_2-$, Lys β-CH_2), 2.00−2.45 (br, 8H, $-N-CO-CH_2-$), 3.30 (br, 6H, Lys ϵ-CH_2), 3.38 (s, 3H, $-O-CH_3$), 3.43 (br, 2H, $-CH_2-NH-$), 3.66 (PEG), 4.48 (br, 3H, Lys α-CH_2). ^{13}C NMR ($CDCl_3$, 500 MHz, δ ppm): 14.12, 22.68, 25.79, 26.15, 29.36, 29.42, 29.59, 29.72, 31.93, 59.03, 70.58, 71.95.

Critical Micelle Concentration (cmc). The cmc was measured by an iodine solubilization method.[28] The PEG−lipids with different amounts were dispersed into an iodine aqueous solution ($[I_2]$ = 30 mg L^{-1}). The transmittance at the maximum absorption wavelength of 360 nm was measured with a UV−vis spectrophotometer (MPS-2000, Shimadzu Co.). The relationship between the concentration of the PEG−lipids and the logarithm of the transmittance was used to determine the cmc from the inflection point of the relation.

Langmuir−Blodgett Experiment. A monolayer of the PEG−lipids was spread from a portion (7 or 20 μL) of the chloroform solutions (0.5 mM) of the PEG−lipids on the subphase of pure water at 25 °C. The surface area was varied by moving a Teflon-coated barrier automatically at a rate of 28 cm^2 min^{-1} in a thermostated Langmuir trough (HBM-type balancemeter; Kyowa Interface Science Co.). The surface pressure was measured by a Wilhelmy-plate method.[29] The surface pressure and total surface area were recorded with an *X-Y* recorder.

Transmission Electron Microscopy (TEM). To prepare samples for TEM, the PEG−lipids were dispersed in pure water at 60 °C. One

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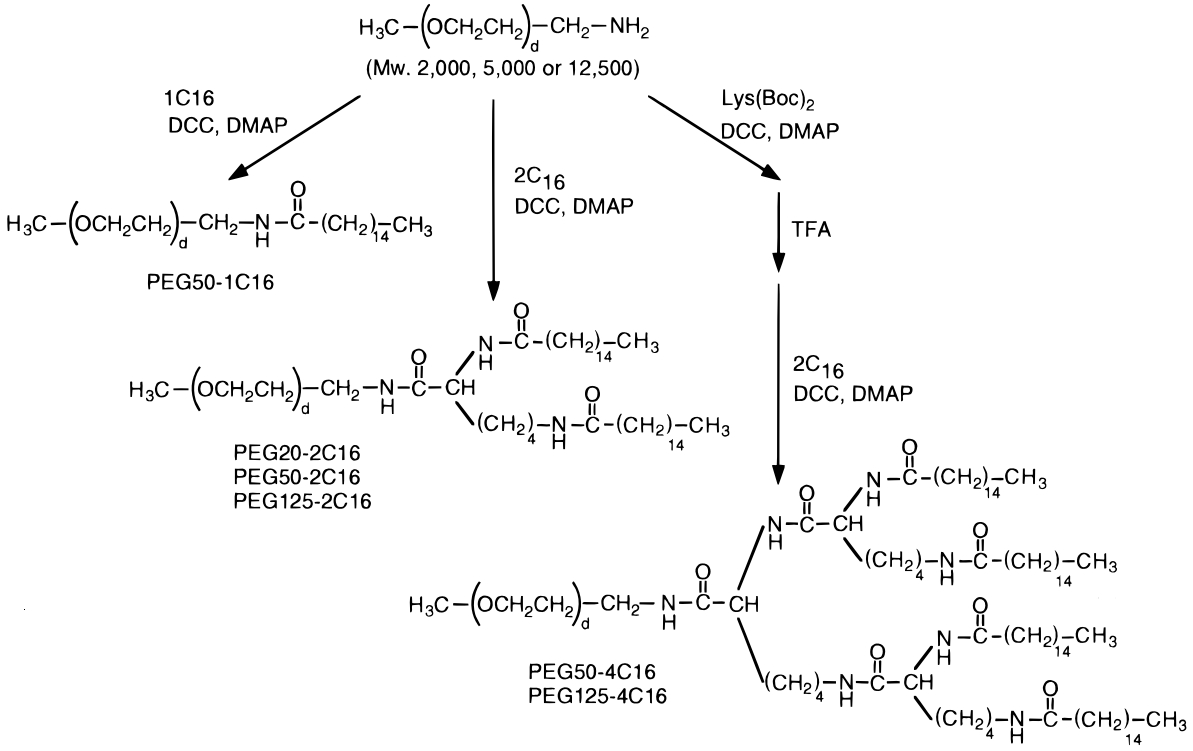
Scheme 1. Synthesis of the PEG−Lipids



drop of the dispersion was dropped onto a carbon-coated copper grid, and then the excess solution was immediately removed with the help of a filter paper. One drop of an aqueous solution of sodium phosphotungstate (Kanto Chemical Co.; 0.01 kg dm^{-3}) was then dropped on the grid and treated as just described. The grid, kept in a desiccator for 12 h, was observed with a TEM (JEM-1010, JEOL Co.) using an acceleration voltage of 100 kV.

Fluorescence Depolarization Method. For the fluorescence depolarization method, a 1,6-diphenyl-1,3,5-hexatriene (DPH) solution [5 μL, [DPH] = 0.5 mM, in tetrahydrofuran (THF)] was added to a dispersion of the PEG−lipids (2.5 mL, [PEG−lipids] = 1 mM). A THF solution in the absence of DPH was added to the dispersion of the PEG−lipids as a blank. These samples were incubated at 37 °C for 2 h. Fluorescence spectroscopy was carried out with a JASCO FP-770 spectrofluorometer equipped with polarizers. DPH was excited at 357 nm and then detected at 430 nm.

Preparation of PEG-Incorporated Vesicles. Mixed lipids were dissolved in chloroform and dried in vacuo to form a film on the wall of a flask. After the film was hydrated and dispersed into a Tris-HCl buffer solution ([Tris] = 20 mM, [NaCl] = 140 mM, pH 7.4) with glass beads, it was extruded through polycarbonate membrane filters (the final pore size of the filter was 0.2 μm). The diameter of the resulting vesicles was determined to be 250 ± 40 nm with a COULTER submicron particle analyzer (Coulter, Hialeah, FL). The total surface area of the vesicles in the dispersion was measured with 6-*p*-toluidino-2-naphthalenesulfonic acid (Tns),[30] and the average number of bilayer membranes of the vesicle was calculated to be 2.1.

A dispersion of the PEG−lipids (19 mL, [PEG−lipids] = 17.5 μM) was added to the vesicle dispersion (25 mL, [lipids] = 17.3 mM) and stirred at 37 °C. The incorporation of the PEG−lipids into the surface of the vesicles was continuously monitored as a thermal change with an isothermal titration microcalorimeter (MCS ITC, Microcal, Inc.). For the measurement of the incorporation ratio, the sample was centrifuged (100 000 g, 60 min) at 37 °C to remove the unincorporated PEG−lipids as a supernatant. The precipitate was freeze-dried and dissolved in $CDCl_3$. The peak area ratio (R_1) of the choline methyl proton of DPPC (3.39 ppm) to the methylene proton of PEG (3.63 ppm) was measured by ^{1}H NMR spectroscopy (JEOL JNM-LA500) to determine the incorporated ratio of the PEG−lipids.[8] The same measurement was carried out for the control samples, where unincorporated PEG−lipids were not removed, to determine the peak area ratio (R_0) of the choline methyl proton of DPPC to the methylene proton of PEG for the total amount of the PEG−lipids. The incorporation ratio was calculated from a percentage of R_1 to R_0.

Critical Molecular Weight (M_c). To determine a critical molecular weight (M_c) of water-soluble polymers for the aggregation of vesicles, we used plain PEG with various molecular weights. We added a solution of the PEGs (500 μL, 0.15 kg dm^{-3}) to the vesicle dispersion (2 mL, 1.39 mM) in a cuvette (l = 1 cm) at 37 °C. The turbidity change (ΔOD) at 600 nm was monitored 15 min after the addition of PEGs with a UV−vis spectrophotometer (MPS-2000, Shimadzu Co.).[31] The M_c was determined as the minimum molecular weight of the PEGs for the initiation of turbidity increase.

Results and Discussion

Syntheses of PEG−Lipids. We synthesized a series of PEGm−nCk compounds with PEG [MW 2000 (PEG20), 5000 (PEG50), 12 500 (PEG125)] as a headgroup and one, two, or four palmitoyl chains (1C16, 2C16, or 4C16) as shown in Scheme 1. The PEGm−1Ck and PEGm−2Ck could be quite easily synthesized by coupling the PEGm with 1Ck and 2Ck, respectively. The PEGm−4Ck was also easily synthesized, even if a reaction step to couple the lysine protected by *t*-butoxyoxycarbonyl groups was added. The yields of those compounds were high (~80%).

Our synthetic route can be generalized as follows. A water-soluble polymer (**P-B**) with a reactive functional group (**B**) at one end is coupled with the functional group **A** of a monomer [**A-2**(**BC**)], which also has two functional groups **B** protected by **C**. After removing **C**, the monodendron structure can be built at the end of the **P** by repeating the coupling reaction of **B** with **A** of the other monomers. Finally, the di-acyl derivatives [**A-2**(**BD**)] are coupled with the end groups **B**. The number of

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Table 1. Melting Points of the PEGm−nCk Amphiphiles

compound	T_m (°C)	ΔH (kcal mol^{-1})
PEG50−1C16	59.5	211.0
PEG20−2C16	47.0, 95.7	42.4, 18.1
PEG50−2C16	59.3, 86.4	199.8, 21.7
PEG125−2C16	62.4, 77.6	448.7, 13.2
PEG50−4C16	56.2, 154.7	164.8, 12.7
PEG125−4C16	61.5, 145.9	415.2, 6.9
2C16	119.5	24.4
	125.4	
4C16	121.1	9.3
	159.4	12.5
PEG20	54.1	78.9
PEG50	63.0	229.8
PEG125	63.5	514.8

acyl chains is calculated to be 2^n, where n is the generation number of the monodendron. In the case of PEG−4C16, the repeating number of the coupling reaction was 2 and n was 2.

Solid State Properties. The melting points are summarized in Table 1. PEG50−1C16 has a single melting point at 59.5 °C. Interestingly, the other samples have two distinct melting points; one melting point is near 60 °C, which can be the melting point of the PEG chain [the melting point of pure PEG with a high molecular weight (M_w > 5000) is 63 °C]. The melting enthalpy increases with the molecular weight of PEG, and each enthalpy corresponds to that of the plain PEG with the same molecular weight. The other higher melting point increases with the increasing number of acyl chains. When the number of acyl chains is constant, the increasing molecular weight of the PEG part results in a drop in the latter melting point. Therefore, the latter melting point should be identified as the melting point of the acyl chains. The number of acyl chains in one PEG−lipid would increase the packing of the acyl chains, leading to the enhancement of the intramolecular interaction of hydrogen bonding, whereas the steric hindrance of the PEG part would disturb the intermolecular packing of the acyl chains. The two melting points indicate the phase-separation between the PEG part and the acyl chain part. The melting behavior depends on the ratio of the hydrophilic and hydrophobic parts of the PEG−lipids, such as the block copolymers reported elsewhere.[19,20]

The spherulite structure, typical of crystallized PEG, was observed with a polarized optical microscope, indicating that the phase-separated PEG phase characterizes the morphology of the PEG−lipid crystal. However, the diameter of the spherulites of the PEG−lipids tends to be small compared with that of pure PEG. Especially, a unique morphology band-like texture was observed for PEG50−4C16, suggesting a different orientation due to the influence of the bulky hydrophobic region. This structure is assumed to be a lyotropic liquid crystal typically observed for amphiphiles.[32,33]

Critical Micelle Concentration (cmc). PEG50−1C16 is easily dispersed into pure water to become a transparent solution, whereas PEG50−2C16 and PEG50−4C16 are dispersed with difficulty even by heating above 45 and 60 °C, respectively. Table 2 summarizes the cmcs of the synthesized PEG−lipids. The cmc decreases with the increasing number of acyl chains (i.e., with the enlargement of the hydrophobic part). There is little difference in the cmcs between PEG50−2C16 (59 μM) and PEG125−2C16 (63 μM); however, PEG20−2C16 has a somewhat lower cmc (34 μM). From these results, the molecular weight of PEGs with molecular weight >5000 would not affect the cmc of the PEG−lipids because of the polymer effect. There are many papers on the properties of monoalkyl-PEGs, including the relationship between the molecular weight of the PEG part and the cmcs of those surfactants.[34−36] The increment rate of the cmc with the increasing degree of polymerization gradually decreases when the degree of polymerization becomes high. This phenomenon is the polymer effect; namely, a smaller effect of the PEG molecular weight on the cmc when the molecular weight becomes higher. The cmcs of PEG50−4C16 and PEG125−4C16 were 26 and 25 μM, respectively. The cmc was decreased with the increasing number of the acyl chains.

The cmcs of the PEG−lipids were generally quite high in comparison with those of phospholipids. For instance the cmc of DPPC is $\sim 10^{-10}$ M,[37] and it jumps tremendously due to the conjugation with hydrophilic PEG. For example, the cmc of PEG50−DPPE is 70 μM (Tris buffer, pH 7.4, 37 °C),[8] that of PEG50−DSPE is 9 μM (Tris buffer, pH 7.4, 37 °C),[8] and that of PEG20−DSPE is 5.8 μM (saline).[38] Of course, direct comparison among these data is impossible because the experimental conditions are not the same. The cmc roughly depends on the length of the acyl chains. It should be noted that based on the cmc, the PEG−lipids still have strong hydrophilicity even if they have four acyl chains.

Monolayer Study. Figure 2 is the surface pressure (π)−surface area (A) isothermal curves of monolayers of the PEG−lipids and the lipids without PEG parts (1C16, 2C16, 4C16). The areas per molecule are summarized in Table 2. As shown in Figure 2(a), the area per molecule of the PEG−free lipids clearly depends on the number of acyl chains. The areas per molecule of 1C16, 2C16, and 4C16, calculated from the extrapolation of the slope to zero surface pressure, were 0.21, 0.52, and 1.10 nm^2, respectively. The limiting areas per molecule were 0.20, 0.40, and 0.92 nm^2 for 1C16, 2C16, and 4C16, respectively. If the acyl chains of the PEG−lipid would order in parallel with each other, the occupied areas of the acyl chains for 1C16, 2C16, and 4C16 would be calculated from Corey−Pauling−Koltun (CPK) models to be 0.2, 0.5, and 1.2 nm^2, respectively. These values show good agreement with the observed ones. The di- or triacyl amphiphiles with two headgroups (so-called "gemini surfactants") were studied from the $\pi-A$ isotherms.[39] The area per molecule of a triacyl amphiphile was also larger than that of a diacyl one.

Interestingly, the isotherms of the 2C16 and 4C16 reached a limiting area without showing a clear collapsing point. This result indicates that some cohesive force between those molecules would exist in the monolayer membrane to stabilize the membrane. This force would be hydrogen bonding among the amide groups of the lipids.

The monoacyl PEG−lipid (PEG50−1C16) shows no steep increase in the surface pressure during compression, indicating the solubilization of the PEG−lipid into the subphase, as shown in Figure 2(b). The other PEG−lipids have an expanded liquid membrane phase, which shows the gradual increase of the surface pressure over the wide surface area. This phase should be a process to compress the entangled PEG chain. Therefore,

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Table 2. Physicochemical Parameters of PEGm−nCk Amphiphiles in Aqueous Solution

compound	HLB	cmc (μM)[a]	area per molecule (nm^2)		T_c (°C)[b]	ΔH (kcal mol^{-1})	frequency of amide I (cm^{-1})
PEG50−1C16	23.1	147	soluble		non (L)	non	1651
PEG20−2C16	15.2	34	2.6[c]		62	2.8	1641
PEG50−2C16	19.8	59	5.4[c]		54	2.0	1641
PEG125−2C16	24.3	63	11.6[c]		non (L)	non	1645
PEG50−4C16	16.6	26	8.2[c]		non (G)	non	1637
PEG125−4C16	20.9	25	11.6[c]		non (G)	non	1642
1C16	−	−	0.21[d]	0.20[e]	−	−	−
2C16	−	−	0.52[d]	0.40[e]	−	−	−
4C16	−	−	1.10[d]	0.92[e]	−	−	−

[a] In pure water at 37 °C. [b] L, liquid crystalline phase; G, gel phase. [c] At 30 mN/m in pure water at 25 °C. [d] Area per molecule calculated from extrapolation of slope to 0 mN/m. [e] Limited area per molecule.

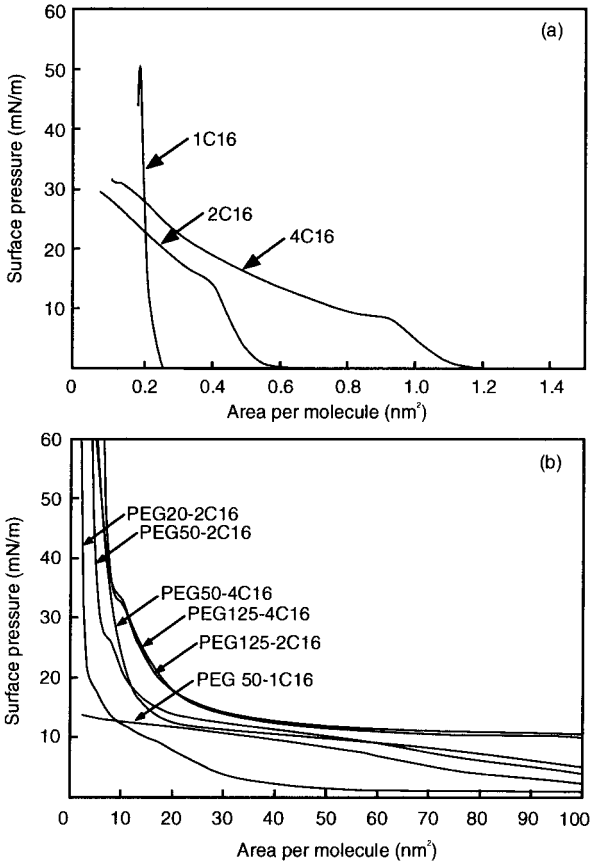

Figure 2. Surface pressure (π)−surface area (A) isotherms of the (a) nC16 and (b) PEGm−nC16 monolayer membranes at the air−water interface at 25 °C.

the PEG chain with a higher molecular weight shows a larger surface pressure for the same molecular area.

A steep increase in the surface pressure was observed from 10 nm^2 for PEG20, from 15 nm^2 for PEG50, and from 20 nm^2 for PEG125, independent of the number of acyl chains. This increase represents the phase transition from the expanded liquid membrane to the compressed solid one. However, the molecular areas obtained by extrapolation from the steep increasing part of the curves to the X-axis in Figure 2(b) were considerably larger than the corresponding surface areas already calculated and depicted in Table 2. This difference indicates that the molecular area would be determined from the maximum cross-sectional area of the entangled PEG chain of the PEG−lipids as a typical characteristic of the amphiphiles with a flexible linear chain as a headgroup. The expansion of the entangled polymer chain in a good solvent can be calculated from the Flory's empirical equation as $R_F = aN^{3/5}$, where R_F is the inertial diameter of the polymer entanglement.[40] The R_F of the PEG−lipids with molecular weights of PEG20, 50, and 125 were calculated to be 3.5, 6.0, and 10.4 nm, respectively, and thus the molecular areas of those lipids were estimated to be 9.6, 28.3, and 84.9 nm^2, respectively. Therefore, the PEG chains would not be freely entangled but be compressed with each other to form a condensed structure.

Assembling Structures. Fibrous assembling structures were observed by TEM for PEG20−2C16, PEG50−4C16, and PEG125−4C16 with almost uniform diameters of 6, 5, and 5 nm, respectively. The standard deviation of the diameters was within 1 nm. Considering the CPK models, the lengths of the hydrophobic part of 2C16 and 4C16, including the dendron part, were calculated to be 2.3 and 2.8 nm, respectively. The lengths of the bilayer were thus estimated to be 4.6 and 5.6 nm, respectively. Those values roughly agree with the negatively stained parts of the objects obtained from the TEM photographs (Figure 3). This result suggests that the base of the assembling structure would be constructed from a bimolecular unit. In addition, many fibers take parallel orientation, leaving spaces of 4, 6, and 11 nm for PEG20, PEG50, and PEG125, respectively, which are in good agreement with the R_F values of the PEG chains. This result indicates the interdigital arrangement of the PEG entanglement between neighboring fibers. Therefore, the stained space between the fibers in Figure 3 is considered to be filled with PEG chains, which would prevent further access to each other by steric hindrance. The staining reagent, sodium phosphotungstate, should penetrate into the PEG chain domain because PEG could solubilize this salt by strong ion−dipole interaction.

The assembling structure of the PEG50−1C16 and PEG50−2C16 seems to be a ribbonlike structure. The ribbons of the PEG50−1C16 were frequently twisted, and the observed width varied from 5 to 22 nm. This variation means that the ribbons of PEG50−1C16 have a bilayer thickness because the minimum width (5 nm) corresponds to the bimolecular layer of 1C16 (4.2 nm). On the other hand, the ribbons of PEG50−2C16 are rather flat. The width varied from 14 to 36 nm. PEG125−2C16 has a globular structure of 20 ± 5 nm diameter.

In general, amphiphilic molecules, of which the headgroup is considerably large compared with the acyl chains, tend to form globular micelles.[1] For instance, PEG19−DSPE and PEG50−DSPE are known to form micelles.[4] In contrast, our PEG−lipids tend to form fibrous structures. If the amphiphilic molecule would have asymmetric carbon atoms or intermolecular hydrogen bonding in addition to hydrophobic interaction in assemblies, the amphiphile would orient to form fibers.[41]

Fourier Transform-Infrared (FT-IR) Measurement. The band shift of the FT-IR spectra provides useful information about

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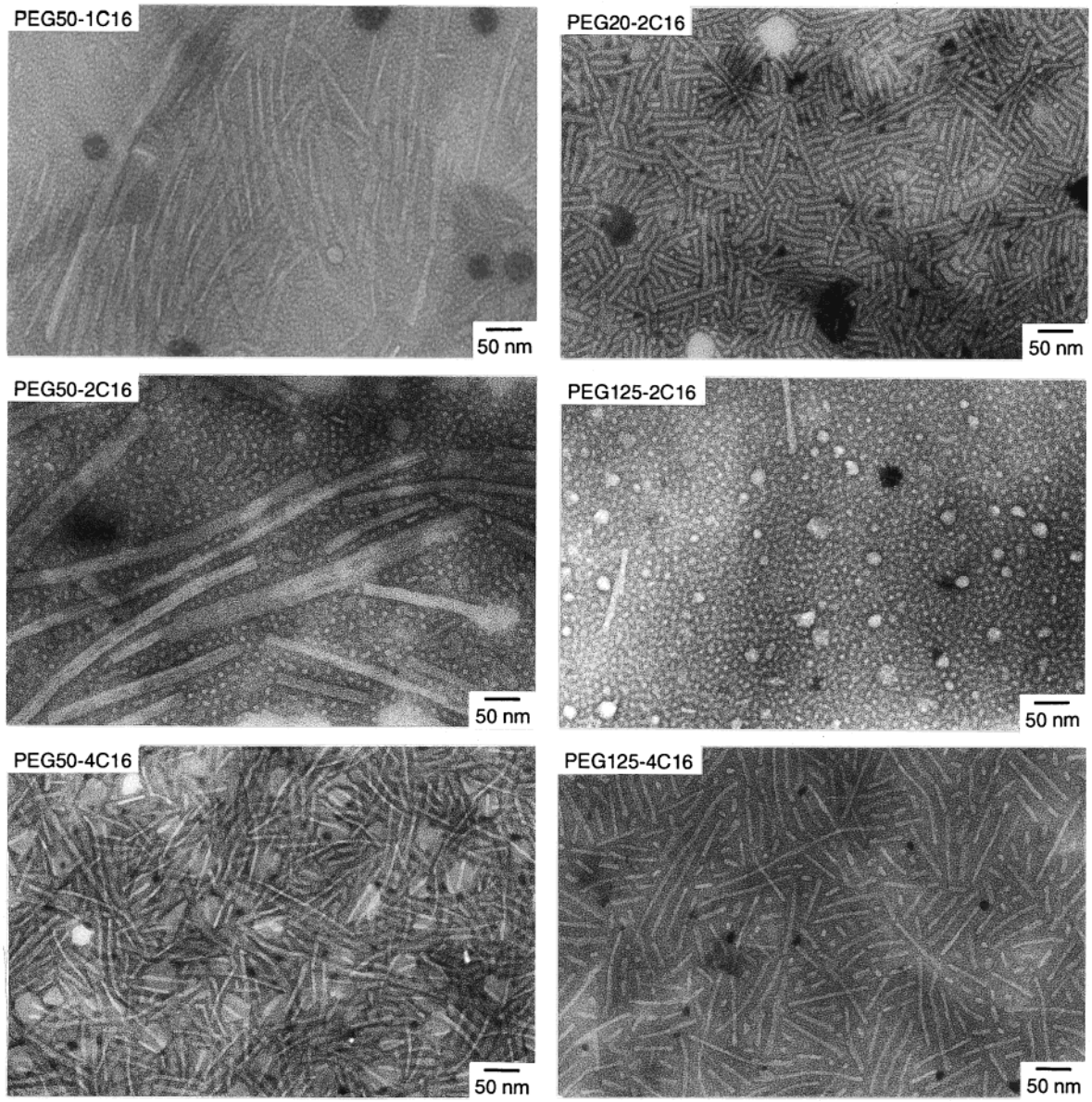

Figure 3. Transmission electron micrographs of the self-assemblies of the PEGm−nC16 amphiphiles. The PEGm−nC16 amphiphiles were dispersed in pure water at 60 °C.

hydrogen bonding.[42,43] The amide I band of the free amide bond is located at ∼1680 cm^{-1} and shifts to the lower wavenumber depending on the strength of the hydrogen bonding. The amide I band of the aqueous dispersions of the PEG−lipids appears at 1651, 1641, 1641, 1645, 1637, and 1642 cm^{-1} for PEG50−1C16, PEG20−2C16, PEG50−2C16, PEG125−2C16, PEG50−4C16, and PEG125−4C16, respectively, as shown in Table 2. The larger degree of deviation from the wavenumber of the free amide bond indicates the stronger hydrogen bonding of the PEG−lipids in assemblies. Therefore, PEG50−1C16 and PEG125−2C16 have relatively weak hydrogen bonding, whereas PEG50−4C16 has the strongest bonding. The strength of the hydrogen bonding tends to increase with the increasing number of the acyl chains and the lowering molecular weight of the PEG chains. Therefore, our PEG−lipids, which have strong hydrogen bonding at the lysine dendron part, would assume fibrous structures rather than micelles.

Thermal Properties of the PEG−Lipids in Assembling States. An endothermic peak at the gel-to-liquid crystalline phase transition was observed by DSC at 64 °C (ΔH = 2.8 kcal mol^{-1}) for PEG20−2C16 and at 54 °C (ΔH = 2 kcal mol^{-1}) for PEG50−2C16. The other PEG−lipids did not show any peaks in a temperature region from 5 to 90 °C. As a reference, DPPC with the same chain length as the PEG−lipids just mentioned, showed a phase transition temperature of 41 °C. Therefore, the lysine dendron structure would account for the higher phase transition temperatures of the PEG−lipids because it would contribute to the intermolecular interaction by hydrogen bonding.

DPH was added to the PEG−lipid assemblies as a hydrophobic fluorescence probe. The emission of fluorescence indicates the existence of a hydrophobic region in the PEG−lipid assemblies. The packing state in the PEG50−nC16 assemblies estimated from DPH fluorescence anisotropy shows a clear dependence of the number of acyl chains, as shown in Figure 4. In general, the anisotropy of the bilayer membrane in a gel state is between 0.2 and 0.3, and it decreases at a gel-to-liquid crystalline phase transition temperature (T_c), indicating the low packing state of the acyl chains in a liquid crystalline

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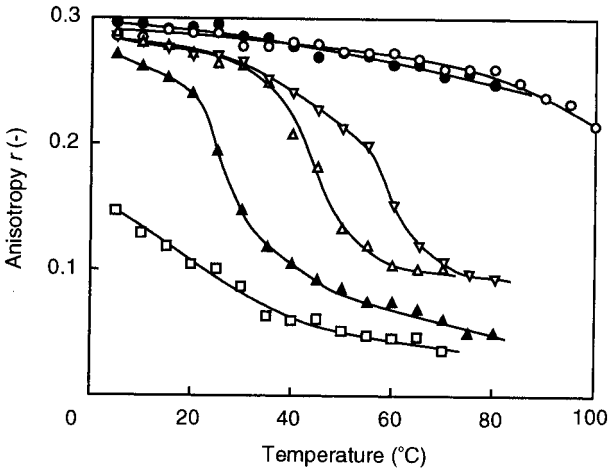

Figure 4. Temperature dependence of the fluorescence anisotropy of DPH in self-assemblies of the PEGm−nC16 amphiphiles in pure water. Key: (○) PEG50−4C16; (●) PEG125−4C16; (▽) PEG20−2C16; (△) PEG50−2C16; (▲) PEG125−2C16; (□) PEG50−1C16.

phase. As shown in Figure 4, PEG50−1C16 has low anisotropy, even at 5 °C, and decreases gradually with temperature. PEG50−1C16 assumes a ribbonlike structure of an amorphous liquidlike phase.

On the other hand, the PEG−lipids with two acyl chains show a steep decrease in anisotropy around the temperature considered T_c. These values nearly correspond to the T_c measured by DSC. Interestingly, the molecular weight of the PEG chain of the PEG−lipids influences the T_c; that is PEG20−2C16, PEG50−2C16, and PEG125−2C16 have T_c values of 60, 45, and 23 °C, respectively. The lowering of T_c by increasing the molecular weight of the PEG part would be attributed to the lower packing of the acyl chains by steric hindrance of the PEG headgroup. The PEG−lipid with 4C16 shows high anisotropy, at least from 10 to 90 °C, suggesting a gel state in the observed temperature region that was independent of the molecular weight of the PEG. Therefore, the PEG−lipid with four acyl chains has the highest packing density. This conclusion is supported by DSC and $\pi-A$ curve measurements as already described (that is, the highest melting point and a solid membrane with the highest collapsing pressure). This conclusion can also be supported by the property of a natural phospholipid, such as cardiolipin, with four acyl chains.[44]

Synthetic amphiphiles with tetraacyl chains were reported by Kunitake et al.[45] They also used amino acids such as glutamic acid or lysine as the spacers of the tetraacyl lipids and speculated that the amphiphiles spaced with lysine were superior to glutamic acid in orienting the acyl chains. For the design of the hydrophobic part with several acyl chains, the orientation of each acyl chain is the most important factor as an anchor part so as to fit the bilayer membrane. Using a lysine dendron structure, the orientation of all acyl chains to one side would be possible because the lysine has enough flexibility to adjust the direction of acyl chains[24] and intramolecular hydrogen bonding to fix those acyl chains appropriately.[46]

Incorporation of the PEG−Lipids into Vesicles. When the dispersion of the PEG−lipids was mixed with a vesicle dispersion, the PEG−lipid molecules were spontaneously incorporated into the surface of the vesicles. This event was detected as exothermic behavior by isothermal titration microcalorimetry. The exothermic rate, which would roughly represent the incorporation rate, reached zero within 3 min, indicating that the incorporation was in an equilibrium state. The initial incorporation rate decreased with the increasing number of the acyl chains of the PEG−lipids. Although the hydrophobicity of the PEG−lipids increased with the increasing number of the acyl chains, the probability for thermodynamically generating enough space in the bilayer membrane to accept the PEG−lipids should become low, which would be a determinant to the incorporation rate. The incorporation ratios were 62, 70, and 53% for PEG50−1C16, PEG50−2C16, and PEG50−4C16, respectively. These ratios are reflected by equilibrium constants, which are determined by the incorporation and dissociation rate constants. The lower incorporation ratios of the PEG50−1C16 and PEG50−4C16 than that of PEG50−2C16 would be caused by a larger dissociation rate constant and a smaller incorporation rate constant, respectively.

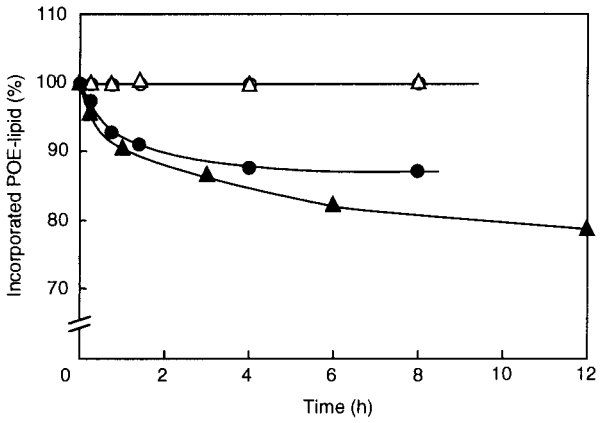

Figure 5. The dissociation of the PEGm−nC16 from the vesicles by dilution at 37 °C. Key: (○) PEG125−4C16; (△) PEG50−2C16; (●) PEG125−2C16; (▲) PEG50-DPPE.

Dissociation of the PEG−Lipids from Vesicles. Because the PEG−lipids are incorporated into the surface of the phospholipid vesicles in an equilibrium state, they dissociate after diluting the vesicle dispersion. In general, PEG50 was used for PEG−phospholipids. We have studied the dissociation behavior of PEG50−DPPE and PEG50−DSPE from the palmitoyl-based vesicle (DPPC/cholesterol/DPPG at 5:5:1 molar ratio) or stearoyl-based vesicle [1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC)/cholesterol/1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol (DSPG) at 5:5:1 molar ratio] and have confirmed the dissociation of PEG50−DPPE from both membranes and the relatively slow dissociation of the PEG50−DSPE.[8]

We prepared vesicles, the surfaces of which were modified with 0.2 mol % PEG chains, as a sample because the equilibrium constant of the PEG−lipid incorporation was stable below this incorporation ratio. After 6-fold dilution of the vesicles with a buffer solution, the change in the incorporation ratio was monitored as shown in Figure 5. Interestingly, PEG50−2C16 and PEG125−4C16 did not dissociate from the palmitoyl-based vesicle but PEG125−2C16 did. Therefore, the PEG chains with molecular weights of 5000 and 12 500 could be tethered to the vesicular surface with two and four acyl chains, respectively. When the PEG−lipid is compared with the PEG−phospholipid, the former shows a higher affinity for the phospholipid bilayer membrane. The major reason for high affinity should be the hydrogen bonding between the ester bond of the phospholipid and the amide bond of the PEG−lipid. Furthermore, the PEG−phospholipid has a negative charge on the headgroup of the

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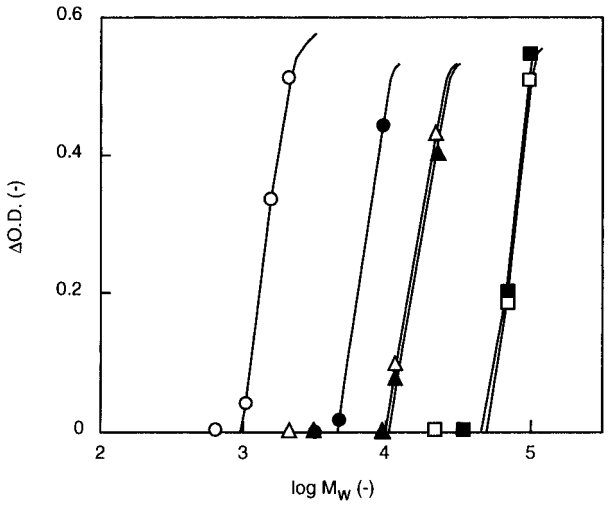

Figure 6. The critical molecular weight (M_c) of the water-soluble polymer for the aggregation of the vesicles modified with PEGm−nC16 at 37 °C. Key: (○) plain vesicles; (●) PEG20−2C16; (△) PEG50−DSPE; (▲) PEG50−2C16; (□) PEG125−4C16; (■) PEG120−DSPE.

phospholipid, which should lead to the low affinity of the PEG−phospholipid for the phospholipid membrane because the surface charge of the vesicles is negative.

Critical Molecular Weight. The solutions of PEG with different molecular weights were added to the dispersion of the phospholipid vesicles with 0.2 mol % PEG−lipids on the surface for the study of the M_c for the aggregation of the vesicles. The larger M_c indicates the higher dispersion stability of the vesicles because of the restriction of the interaction between the vesicular surface and the added polymers.[31] The M_c of the phospholipid vesicles (DPPC/cholesterol/DPPG = 5/5/1 m/m) without PEG modification is 1000, and that with PEG50−DSPE has an M_c of 12 000. In the cases of PEG20−2C16, PEG50−2C16, and PEG125−4C16, the M_c values are 4900, 11 700, and 49 500, respectively (Figure 6). These results clearly indicate that the longer PEG chain has a larger effect on preventing the aggregation of the vesicles because the thickness of the PEG layer covering the surface of the vesicle increases with the PEG molecular weight. This effect would result in the suppression of the access of macromolecules or vesicles to the surface of the vesicle.

Conclusions

We succeeded in controlling the number of acyl chains of a PEG−lipid by using the lysine dendron structure. The increased number of the acyl chains resulted in the enlargement of the hydrophobic region with a high intramolecular orientation of the acyl chains. However, the molecular weight of the PEG chain influenced the intermolecular packing because of steric hindrance. The PEG−lipid with diacyl chains anchored the PEG chain on the surface of the vesicles up to a molecular weight of 5000, whereas the PEG−lipid with tetraacyl chains anchored the PEG chain up to a molecular weight of 12 500. Therefore, if one would like to incorporate a longer PEG chain, one should enlarge the hydrophobic part by increasing the generation number of the dendron to increase the number of acyl chains. The dispersion stability of the vesicles significantly increased with the increasing molecular weight of the PEG chain. This effect would lead to the prolongation of the intravenous circulation time of the vesicles. These dendron-type hydrophobic groups are also available to fix hydrophilic macromolecules, such as PEG derivatives, polysaccharides, and proteins, etc, for drug delivery systems.

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