Calorimetry and Cryo-Transmission Electron Microscopic Studies of PEG2000-Grafted Liposomes

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The phase behavior of poly(ethylene glycol) grafted liposomes (PEG-liposomes) was investigated by differential scanning calorimetry (DSC), dynamic light scattering (DLS) and cryo-transmission electron microscopy (cryo-TEM). PEG-liposomes were prepared from mixtures of dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylethanolamine with a covalently attached PEG molecular weight of 2000 (DSPE-PEG2000). From the results of DLS measurements, the coexistence of PEG-liposomes and small molecular assemblies were confirmed at mole fractions of DSPE-PEG2000 above about 0.1. Moreover, it was confirmed that small molecular assemblies were disk micelles by cryo-TEM. However, the phase transition enthalpies of PEG-liposomes were hardly changed according to the DSC measurement, though the mole fraction of the PEG lipid increased. From these results, it was suggested that the phase transition enthalpies hardly changed despite mixed micelles being formed because the bilayer structure of the disk micelle maintains high cooperativity between the DPPC molecules.

Key words liposome; disk micelle; cryo-transmission electron microscopy; differential scanning calorimetry; dynamic light scattering

Many successful experimental achievements have been reported on the medical application of liposomes, consisting of the lipid bilayer membrane, as a drug carrier for the purpose of the reduction of drug toxicity or the targeting of drugs to specific cells.[1)] Especially, the development competition of liposomes to aim at the extension of the half-life in the blood circulation is remarkable. Allen *et al.*[2,3)] and Gabizon *et al.*[4)] reported that the use of ganglioside G_{M1} as a lipid component of the liposomes allowed them to avoid or delay uptake by the reticuloendothelial system (RES). More recently, many researchers have reported that liposomes (PEG-liposomes) conjugated with amphipathic polyethylene glycol (PEG) significantly increase the blood circulation liposome half-life compared to those without PEG.[5—8)] Many studies, however, have been carried out from the viewpoint of biochemistry. It is necessary to clarify the physicochemical properties of PEG-liposomes. We have reported the particle size, zeta potential, microviscosity and permeability in the previous papers and discussed the optimum amount of PEG-lipid in liposomes and the optimum PEG chain length of PEG-lipid.[9—12)] In this study, the effects of PEG-lipid on the phase behavior and aggregate structure of liposomes were investigated using differential scanning calorimetry (DSC), dynamic light scattering (DLS) and cryo-transmission electron microscopy (cryo-TEM).

Experimental

Materials L-α-Dipalmitoylphosphatidylcholine (DPPC, 99.6% pure) and distearoyl-*N*-monomethoxy poly(ethylene glycol)-succinyl-phosphatidylethanolamine (DSPE-PEG) were acquired from NOF Co., Ltd. (Tokyo, Japan). The weight-average molecular weight of poly(ethylene glycol) was 2000. Dulbecco's phosphate-buffered saline (PBS) powder, composed of NaCl, KCl, Na_2HPO_4 and KH_2PO_4 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PBS powder was dissolved in water for injection (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), and an isotonic solution of pH 7.4 was used. All other chemicals were commercial products of reagent grade.

Preparation of PEG-Liposomes PEG-liposomes were prepared using DPPC and DSPE-PEG2000. DPPC and DSPE-PEG2000 were first dissolved in chloroform in a test tube and the solvent was removed by blowing nitrogen gas into the test tube, and the residual solvent was then further dried overnight at room temperature in a desiccator under vacuum. PBS was added to the lipid film and warmed (55—60 °C) above the phase transition temperature of DPPC (41 °C) for 30 min. The test tube was then vigorously shaken in a vortex mixer, and multilamellar vesicles (MLV) were obtained. Large unilamellar vesicles (LUV) were obtained from MLV suspensions extruded five times through a double-stacked polycarbonate membrane filter (pore size: 200 nm) using an Extruder (Lipex Biomembranes Inc., British Columbia, Canada) at 55 °C.

Differential Scanning Calorimetry The phase transition enthalpy of liposomal bilayer membranes was measured with a high-sensitivity differential scanning calorimeter (DSC 8230, Rigaku Co., Tokyo, Japan) equipped with a thermal controller (F-25, Julabo Labortechnik GmbH, Seelbach, Germany). The liposome solution (20 μl) was put in a sampling vessel, made of stainless steel (resistance pressure: 50 atm, vessel size: 3×5 (ϕ) mm), and then the vessel was sealed. The reference was PBS with the same volume as that of the sample. When the cycle heating was carried out, samples were heated to 50 °C and cooled to 20 °C, and then heated again. The measurement conditions were 1 °C min^{-1} for the scanning rate.

Dynamic Light Scattering The particle size of liposomes was determined using a dynamic light scattering method. The particle size distribution of liposomes was measured at 37 °C with a particle size analyzer (NICOMP 380ZLS, Particle Sizing Systems Inc., CA, U.S.A.). The light source was a diode pump solid state laser (DPSS laser) with a wavelength of 535 nm and the scattering angle was 90°.

Cryo-Transmission Electron Microscopy A small amount (3—5 μl) of liposome solution for cryo-TEM was placed on the surface of a TEM copper grid covered by a porous carbon film, which was held by a pair of self-locking tweezers mounted on a spring-loaded shaft with a cryo preparation system (Leica EM CPC, Leica Microsystems AG, Wetzlar, Germany). The sample drop was blotted with a filter paper to form a thin liquid film on the grid (<300 nm), and immediately plunged into liquid ethane cooled by liquid nitrogen (−175 °C). The grid was transferred onto the tip of a cryospecimen holder (CT3500, Oxford Instruments plc., Oxon, U.K.) under liquid nitrogen. The specimens were kept at about −173 °C and imaged in a transmission electron microscope (H-7650, Hitachi High-Technologies Co., Tokyo, Japan) at an accelerating voltage of 120 kV under a low electron dose.

Results and Discussion

Figure 1 shows the DSC curves for DPPC liposomes con-

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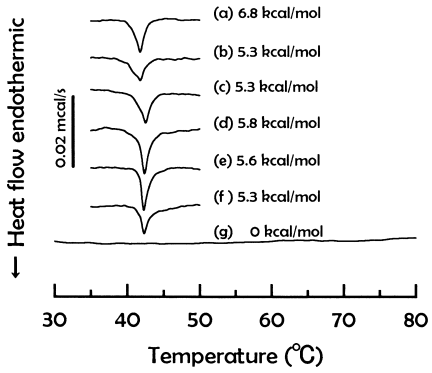

Fig. 1. DSC Curves of Liposomes with Various Amounts of DSPE-PEG2000

Mole fraction of DSPE-PEG2000: (a), 0; (b), 0.030; (c), 0.060; (d), 0.090; (e), 0.117; (f), 0.145; (g), 1.

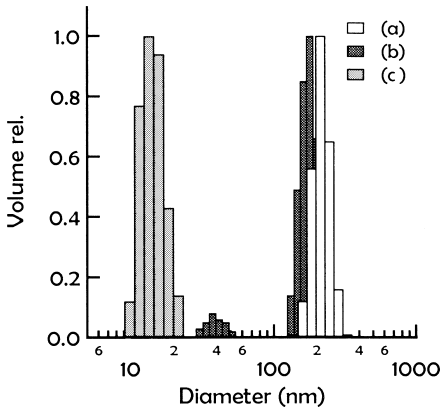

Fig. 2. Particle Size Distribution of DPPC (a), DPPC/DSPE-PEG ($X_{DSPE\text{-}PEG2000}$=0.145) (b), DSPE-PEG2000 (c)

taining DSPE-PEG2000. The DSC curves showed an endothermic peak at about 41 °C (Figs. 1a—f). On the other hand, the peak was not present in the DSC curve of the single PEG-lipid system (Fig. 1g). In our previous study,[12] it was clarified that the encapsulation efficiency of PEG-liposomes that contained DSPE-PEG2000 above 0.1 mole fraction decreased by about 50% compared with liposomes without the PEG-lipid. Furthermore, Sriwongsitanont *et al.*[13] examined the effects of PEG-lipid concentration on liposomal structure using a gel exclusion chromatography technique. They reported that liposomes coexisted with mixed micelles when the concentration of DSPE-PEG2000 was greater than 10 mol%. This is in good agreement with our previous results. It is well known that the spherical micelle, having a lower aggregation number and a larger curvature does not show the phase transition peak in DSC thermograms because the cooperativity required to show the phase transition can not be kept. Therefore, it is guessed that as mixed micelles are generated, the phase transition enthalpies of PEG-liposomes decrease. However, the mole fraction of the PEG-lipid was hardly changed into the phase transition enthalpies of liposomes up to 0.145. Furthermore, this reason was examined in detail using DLS and cryo-TEM.

Figure 2 shows the particle size distribution of DPPC/DSPE-PEG2000 systems. A single peak was shown at a mean size of about 200 nm in DPPC-liposome. The PEG-liposomes containing DSPE-PEG2000 with a 0.145 mole fraction showed the peak in two places (170 nm, 35 nm). The single PEG-lipid system showed a single peak around 15 nm. Figure 3 shows the cryo-TEM images of DPPC/DSPE-PEG2000 systems. Only liposomes were observed in Fig. 3a. As for Figs. 3b and c, the coexistence of liposomes and other molecular assemblies was confirmed. Moreover, a spherical micelle with a mean size of about 15 nm was observed in Fig. 3d. Israelachvili defined the critical packing parameter (*CPP*) describing the shape and volume ratio of the molecules, which allows prediction of the structure of the aggregates being formed.[14,15] The *CPP* value was calculated according to the following equation:

$$CPP = V/a_0 l_c \quad (1)$$

where V is the volume of the hydrophobic portion, a_0 is the effective area per hydrophilic group, and l_c is the length of the hydrophobic group. It was clarified that the preferred mo-

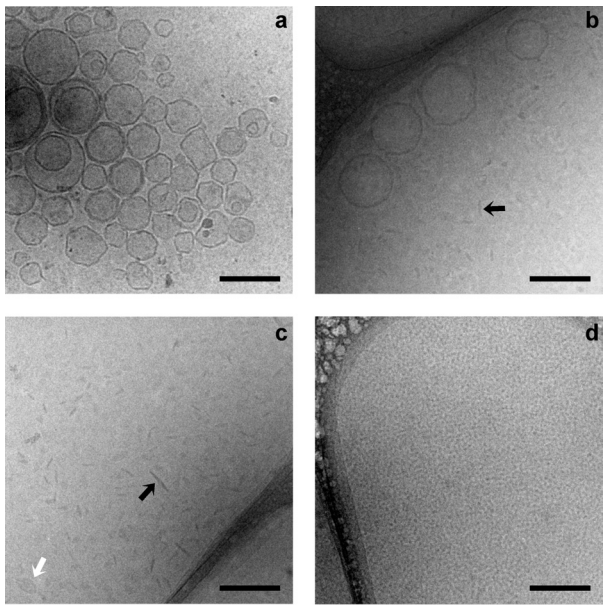

Fig. 3. Cryo-TEM Images of DPPC (a), DPPC/DSPE-PEG ($X_{DSPE\text{-}PEG2000}$ =0.145) (b) and (c), DSPE-PEG2000 (d)

The bar length is 200 nm.

lecular assembly depends on the *CPP* value. For instance, $CPP<1/3$ corresponds to a spherical micelle, $1/3<CPP<1/2$ corresponds to a rod-like micelle, $1/2<CPP<1$ corresponds to a vesicle (bilayer), $CPP\sim1$ corresponds to a planar bilayer and $CPP>1$ corresponds to an inverted micelle. The volume of a saturated carbon chain and the maximum length of a fully extended hydrocarbon chain are calculated using Tanford's equations[16]:

$$V = 0.027(n_c + n_{Me}) \quad (2)$$

$$l_c = 0.15 + 0.127 n_c \quad (3)$$

where n_c is the total number of carbon atoms per chain and n_{Me} is the number of methyl groups. Then, the molecular assemblies of the lipids used in this research were considered. The *CPP* value of the DPPC molecule is 0.65 from Eq. 1 (V=0.92 nm^3 (from Eq. 2), a_0=0.65 nm^2,[17] l_c=2.18 nm (from Eq. 3)), and it is understood that DPPC molecules form vesicles to satisfy the condition of $1/2<CPP<1$. In the case of DSPE-PEG2000, V and l_c values were estimated to be

1.03 nm^3 and 2.44 nm, respectively. In addition, when PEG has dissolved in water in the random coil form, the size of the PEG chain that is the hydrophilic group of the PEG-lipid can be shown as the Flory dimension (R_F).[18] R_F is calculated according to the following equation:

$$R_F = aN^{3/5} \quad (4)$$

where a is the monomer size ($a = 0.35$ nm for PEG2000) and N is the degree of polymerization ($N = 45$ for PEG2000). According to Eq. 4, R_F for PEG2000 is 3.44 nm, and the effective area per hydrophilic group of DSPE-PEG2000 is estimated as $a_0 = \pi(R_F/2)^2 = 9.4\ nm^2$. Therefore, the *CPP* value of DSPE-PEG2000 is 0.045 from Eq. 1, and it is understood that DSPE-PEG2000 molecules form spherical micelles to satisfy the condition of $CPP < 1/3$. This prediction agrees with the result of Fig. 3d. On the other hand, the existence of the disk micelle has also been reported by Edwards *et al.*,[19,20] who proposed the model of the disk micelle. That is, it is composed of a bilayer portion that consists mainly of DPPC and the edge portion that consists mainly of the PEG-lipid. The model of this disk micelle is appropriate from the *CPP* value of DPPC and DSPE-PEG2000. From Fig. 3c, it was understood that the cylinder shape assembly (black arrow) and circular shape assembly (white arrow) denoted the disk micelles observed edge-on and face-on, respectively. Furthermore, the reason why PEG-lipids localize to the edge portion of the disk micelle was considered. In our previous paper,[11] we confirmed that the addition of PEG-lipid to the DPPC liposomal bilayer membrane caused lateral phase separation, as seen by freeze-fracture electron microscopic observation. Bedu-Addo *et al.*[21] suggested that the occurrence of the phase separation in the PEG-liposomes due to the property of the PEG chain of the PEG-lipid, that is, the phase separation of the PEG-liposome, is generated by the PEG chain-chain entanglement due to the van der Waals forces, and also inter- and intra-chain hydrogen bonds that act in the PEG chains. Therefore, it was assumed that the localization of PEG-lipid on the edge of the disk micelle originated from the phase separation by the tangling of the PEG-chains. From these results, it was suggested that the phase transition enthalpies hardly changed, though the mixed micelles were formed because the bilayer structure of the disk micelle maintained the high cooperativity between DPPC molecules.

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