Phase Separation of Polymerized Mixed Liposomes: Analysis of Release Behavior of Entrapped Molecules with Skeletonization

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ABSTRACT: The mixed liposomes, composed of a polymerizable lipid, 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC), and nonpolymerizable membrane constituents, DPPC, cholesterol, and sodium didodecyl phosphate, were prepared by an extrusion method. After polymerization, nonpolymerizable constituents were removed to obtain the polymerized framework of the liposome (the skeletonized liposome). The release of small molecules from the skeletonized liposomes through resulting holes was analyzed. A 5(6)-carboxyfluorescein (CF) and saccharides with various molecular weights were applied as release molecules. The molecular weight of dextran, whose retention ratio in the skeletonized liposomes is 50%, relates to the apparent size of the holes, i.e., the apparent domain size of the phase separation of a polymerized mixed liposome. The size of the holes increases with an increase in the mole fraction of nonpolymerizable lipids. This also depends on the polymerization temperature and the structure of nonpolymerizable lipids.

Introduction

Liposomes have been studied mainly as microcapsules for drug carriers or functional particles as well as models for a biomembrane.[1] However, the stability of the liposome is still a serious problem. In the last decade, many methods have been proposed for stabilizing liposomes.[2] One possible approach is the polymerization of membrane components as liposomes. Various polymerizable lipids have been synthesized and polymerized as liposomes to investigate their stability.[3-12] We have mentioned the importance of the selection of proper polymerization methods in order to obtain stable liposomes.[13,14] Since polymerized liposomes with phase transition like nonpolymerized liposomes are difficult to make, the polymerization of mixed liposomes containing both polymerizable and functional membrane constituents has been proposed.[15,16]

In polymerized mixed liposome systems, polymerized regions are phase separated from monomeric regions because polymerization itself induces phase separation by the direct binding of polymerizable lipids through covalent bonds regardless of their affinities. There are several techniques to analyze the phase separation such as the observation of transmission electron micrographs with a freeze-fractured method,[17] DSC,[18] and fluorescence measurements.[19] In addition, the analyses of phase separation in monolayer systems were recently possible with fluorescence microscopy[20] or phase-contrast electron microscopy.[21] We succeeded in visualizing the phase separation of polymerized mixed liposomes.[15] The monomeric regions of the mixed liposome were removed by washing with chloroform after drying or by adding surfactants to the liposome suspension. This method was named *skeletonization*.[15] Porous (skeletonized) liposomes were directly observed with scanning electron microscopy (SEM). The pores are known to reflect monomeric regions of polymerized mixed liposome. On the other hand, we clarified that the polymerized mixed liposomes possessed both the stability of polymerized liposomes and the functions of monomeric ones because of the complete phase separation between monomeric and polymerized regions. The release of fluorescent molecules (CF) from an internal phase of liposome should be controlled by the stability of monomeric regions.[16]

In this paper, we quantitatively analyzed the release behavior of encapsulated molecules with various molecular weights from skeletonized liposomes.

Experimental Section

Materials. 1,2-Bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) and 1,2-dipalmitoyl-3-phosphatidylcholine (DPPC) were purchased from Nippon Oil & Fats Co., Ltd. The purity of these lipids was confirmed by thin-layer chromatography (Merck, silica gel plates) as a single spot on the plate with chloroform/methanol/water (65:35:5 by volume) as the eluent. Cholesterol was purchased from Kanto Chem. Co., Ltd., and purified by recrystallization twice from methanol. Sodium didodecyl phosphate ($(C_{12}H_{25})_2PO_4^-Na^+$) was synthesized from phosphoryl chloride and dodecyl alcohol and purified by recrystallization twice from acetone.[22] Azobis(isobutyronitrile) (AIBN) and azobis(2-amidinopropane) dihydrochloride (AAPD) were purchased from Tokyo Kasei Co., Ltd., and Wako Pure Chemical Industries, Ltd., respectively. AIBN and AAPD were purified by recrystallization twice from methanol and pure water, respectively. Sodium cholate was purchased from Kanto Chem. Co., Ltd., and used without further purification. Fluorescence-grade 5(6)-carboxyfluorescein (CF), purchased from the Kodak Co., was used without further purification. Dextran-4000, -9400, -40 200, and -72 200 ($\bar{M}_w$ = 4000, 9400, 40 200, and 72 200, respectively) and maltopentaose ($\bar{M}_w$ = 878) were purchased from Sigma and Seikagaku Kogyo Co., Ltd., respectively.

Methods. (1) Preparation of Liposomes. An amount of the lipid mixture (0.15 g) was dissolved in dehydrated chloroform and slowly evaporated in a sample tube to prepare a thin film on an inner surface of the tube. A 2.5 wt % aqueous solution (5.0 mL) of glucose, saccharose, maltopentaose, or dextran was added to the tube and vortexed with glass beads at 50 °C. The resulting multilamellar liposomes were extruded through polycarbonate filters (pore sizes: 1.0, 0.4, 0.2, and 0.1 μm in sequence) to convert them into small unilamellar liposomes. The average diameter of resulting liposomes was measured to be 96.5 ± 24 nm with a Coulter submicron particle analyzer (Model N4 SD).

(2) Radical Polymerization of DODPC in Mixed Liposome.[14] A THF solution of AIBN (5.0×10^{-2} mol/L) was added to a liposome suspension (5.0 mol % to DODPC). In order to incorporate water-insoluble AIBN into the hydrophobic regions of the liposome membrane, THF was completely removed by N_2 bubbling at 30 °C. Water-soluble AAPD (5 mol % to DODPC) was then added to the suspension. Liposomes were polymerized at 60 °C under a nitrogen atmosphere. Polymerization conversion

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was successively analyzed from a decrease in the maximum absorbance at about 255 nm, attributed to the diene groups of DODPC. The polymerization conversion of diene groups reached about 90% after 12 h. The liposomes were estimated to be completely polymerized because the polymerization of 1-acyl chains and that of 2-acyl chains occurred independently.

(3) Photosensitized Polymerization of DODPC in Mixed Liposome.[23] A liposome suspension containing both AIBN and AAPD was prepared with the same method as mentioned above. The UV light around 360 nm was irradiated from a UV light source (a high-pressure mercury lamp; Riko UVL-100P) to the suspension in a Pyrex tube (1.5 cm diameter). Temperature was controlled to 30 °C by a thermostated bath. AIBN and AAPD radicals generated by UV irradiation initiate the polymerization of liposomes (photosensitized polymerization). A filter (Hoya L-38), which cut off the UV light less than 355 nm, was used to avoid the direct UV-induced polymerization of DODPC.

(4) Measurement of CF Release from Skeletonized Liposomes.[24] CF was encapsulated in an internal aqueous phase of polymerized mixed liposome (SUV, prepared by sonication) by the incubation of the liposomes in a CF solution (0.1 mol/L) for 100 h at 60 °C.[24] CF in an exogenous aqueous phase was removed by gel permeation chromatography (Sepharose CL-4B column, 20 mm diameter, 150 mm height). At this time, the entrapped CF was self-quenched. The amount of the released CF was measured with a fluorescence spectrometer (Jasco FP-550) as an increase in fluorescence intensity at 520 nm with excitation at 330 nm. Skeletonization was carried out by adding sodium cholate to the solution up to its concentration of 0.45 wt %. The complete release of CF was achieved by the destruction of the liposomes with a sonicator (Tomy Seiko UR-200P, at 60 W for 5 min) in the presence of Triton X-100.

(5) Measurement of the Retention Ratios of Encapsulated Molecules in Skeletonized Liposomes. A liposome solution containing saccharides or CF was subjected to gel permeation chromatography to expel them in an exogenous aqueous phase and fractionated (2 mL each in sample tubes). Liposomes were detected from turbidities at 600 nm. In this case, the CF in an internal aqueous phase of liposome was not self-quenched because of its low concentration. The amount of CF retained in the liposomes was determined from fluorescence intensity. On the other hand, the amount of saccharides retained in the liposomes was determined with a phenol–sulfuric acid method as follows.[25,26] Concentrated sulfuric acid (5 mL) was quickly added to a mixture of a liposome suspension (1 mL) containing saccharides and a phenol aqueous solution (5 wt %, 1 mL). The absorption intensity at 485 nm was measured with a UV spectrophotometer (Shimadzu MPS-2000).

The skeletonization of polymerized mixed liposomes containing saccharides was performed by adding 0.2 mL of sodium cholate solution (22 wt %) to 2 mL of liposome suspension. After the incubation for 1 h at 30 °C, the suspension was subjected to gel permeation chromatography. The amount of encapsulated molecules in the skeletonized liposomes was quantiatively analyzed as mentioned above.

The retention was defined as the ratio of the amount of encapsulated molecules retained in skeletonized liposomes (A_s) to that in the liposomes before skeletonization (A).

$$\text{retention}\ (\%) = A_s/A \times 100 \qquad (1)$$

In the case of saccharides, A_s and A were detected as the absorption intensity at 485 nm after the phenol–sulfuric acid treatment.

Results and Discussion

Skeletonization of Polymerized Mixed Liposomes. The release of CF molecules from liposomes is induced by skeletonization as shown in Figure 1. Monomeric DODPC or DPPC liposomes show rapid release after the addition of sodium cholate, whereas a little CF release occurs from polymerized DODPC (poly-DODPC) liposomes. In the case of poly-DODPC/DPPC liposomes, CF is released faster from the liposomes that have a larger mole fraction of DPPC. On the other hand, the turbidities of poly-

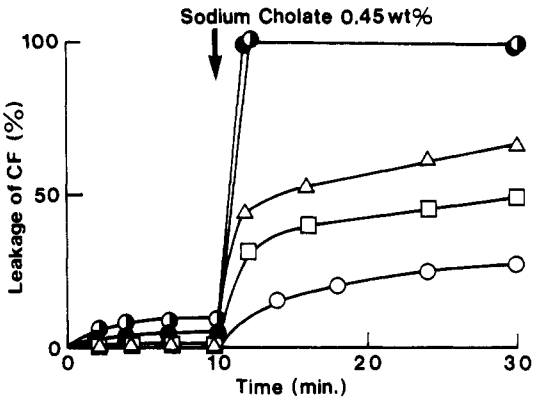

Figure 1. Skeletonization of poly-DODPC/DPPC (10:1) (□) and poly-DODPC/DPPC (5:1) (△) liposomes by adding sodium cholate at 30 °C observed from CF release behavior. Release behavior of poly-DODPC (○), DPPC (●), and DODPC (◑) liposomes is also depicted as reference.

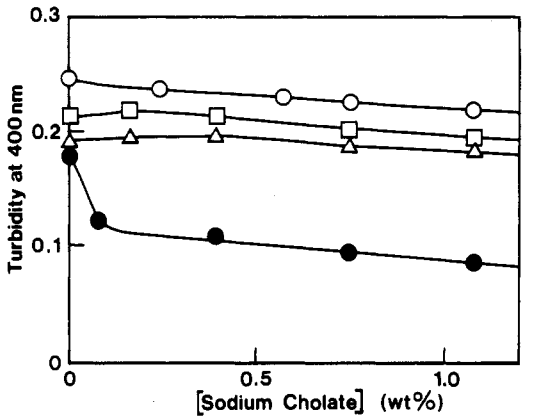

Figure 2. Stability of poly-DODPC (○), poly-DODPC/DPPC (10:1) (△), poly-DODPC/DPPC (5:1) (□), and DPPC (●) liposomes against sodium cholate.

DODPC and poly-DODPC/DPPC liposome suspensions do not change after the addition of sodium cholate, whereas that of the monomeric liposome suspension decreases significantly as shown in Figure 2. It shows that the liposome structure is kept in poly-DODPC systems after skeletonization. From both release and turbidity measurements, DPPC molecules are removed from the poly-DODPC/DPPC liposome by the addition of sodium cholate,[27] and CF molecules are released through the resulting holes. The CF release was confirmed to deeply depend on the fraction of DPPC. The resulting porous liposomes (skeletonized liposomes) were actually observed by SEM.[15] However, the liposome structure was destroyed by skeletonization when polymerized regions were not large enough to maintain a whole liposome structure (data not shown).

Polymerized DODPC liposomes containing CF in both exogenous and internal aqueous phases were skeletonized and subjected to gel permeation chromatography as shown in Figure 3a. The poly-DODPC liposomes were fractionated at the fraction number from 6 to 11. The detection of CF at the fractions of liposomes means the retention of CF in the internal aqueous phase of the liposomes. The CF in the exogenous aqueous phase was detected in the fraction number of more than 15. In the case of poly-DODPC/cholesterol (9:1 by mole) liposomes, no CF was detected in the fractions of liposomes after skeletonization (Figure 3b). It shows that poly-DODPC liposomes are stable against sodium cholate, whereas the polymerized mixed liposomes having phase-separated cholesterol regions are skeletonized by sodium cholate and the released

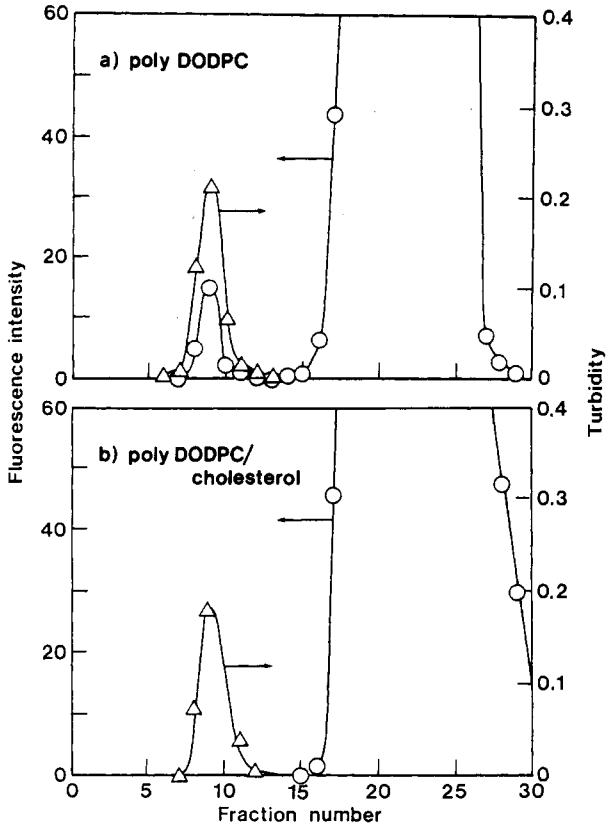

Figure 3. Elution profiles of polymerized liposomes (△) and CF (○), which were detected by turbidity at 600 nm and fluorescence intensity at 520 nm, respectively.

CF molecules are completely separated from the liposomes during gel chromatography.

Analysis of the Domain Sizes of the Phase Separation in Polymerized Mixed Liposomes. The quantitative analysis of released or encapsulated molecules after skeletonization with gel permeation chromatography has made it possible to calculate the retention ratios of encapsulated molecules. As mentioned above, the phase-separated monomeric regions in polymerized mixed liposomes are removed by skeletonization. If encapsulated molecules were hydrodynamically smaller than the sizes of holes, they should be released, while, if they were larger than the holes, they should be retained completely in the liposomes. Actually, since both encapsulated molecules and holes have certain size distributions, the retention ratios are between 0 and 100%. We chose mono-, oligo-, and polysaccharides (from glucose to dextran-72 200) as encapsulated molecules because they had no electrostatic charge to interact with the bilayer.

Figure 4 shows the relationship between the mole fraction of DPPC in poly-DODPC/DPPC liposomes and the retention ratio of each saccharide after skeletonization. In the case of poly-DODPC liposomes containing no DPPC, both glucose ($\bar{M}_w$ = 180) and saccharose ($\bar{M}_w$ = 342) were completely released after skeletonization, but 50% of the maltopentaose ($\bar{M}_w$ = 878) was retained. The dextrans with average molecular weights of more than 4000 were retained completely. This indicates that molecular packing in the poly-DODPC membrane is disturbed by sodium cholate and the apparent size of the resulting cracks would be similar to the hydrodynamical size of maltopentaose. Interestingly, CF ($\bar{M}_w$ = 376), which had about the same molecular weight of saccharose, was considerably retained in poly-DODPC liposomes as shown in Figure 3. It may support the previous result about the difference in

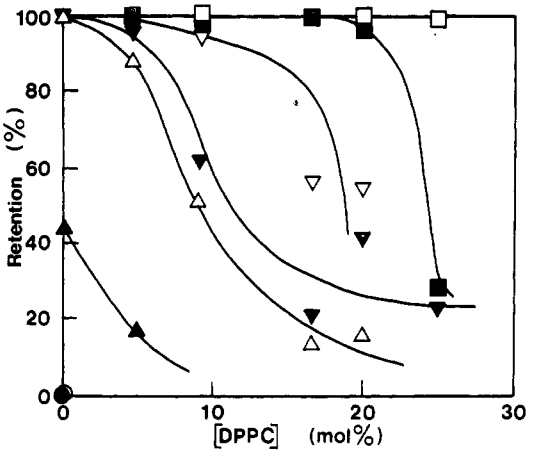

Figure 4. Retention ratios of encapsulated molecules in skeletonized liposomes as a function of the mole fraction of DPPC: (●) glucose, (○) saccharose, (▲) maltopentaose, (△) dextran-4000, (▼) -9400, (▽) -19 500, (■) -40 200, (□) -72 200.

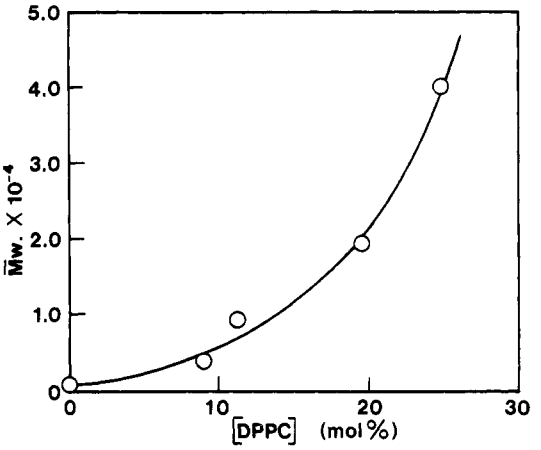

Figure 5. Analysis of the domain sizes of DPPC regions of poly-DODPC/DPPC liposomes from the 50% retention of polysaccharides in skeletonized liposomes.

the permeation properties between ionic and nonionic molecules,[28] though the difference in the structure of those molecules should be taken into consideration. When the concentration of DPPC was 20 mol %, the retention ratios of dextran-19 500 and dextran-40 200 were 50% and almost 100%, respectively. This indicates that the skeletonized liposomes have holes that permit the release of dextran-19 500 but no dextran-40 200.

Figure 5 shows the relationship between the mole fraction of DPPC in poly-DODPC/DPPC liposomes and the molecular weight of saccharides that was released 50% from the liposomes after skeletonization. This figure means that larger molecules are released from the mixed liposomes that have a larger mole fraction of DPPC. In other words, the phase separation becomes larger as the mole fraction of DPPC increases.

log–log plots of the data in Figure 5 were depicted as a linear relationship (Figure 6). The broken line represents the molecular weight of maltopentaose, which was released 50% from the poly-DODPC membrane by the addition of sodium cholate. The relationship intercepts the line at the concentration of DPPC of about 4.0 mol %. This means that the cracks on the poly-DODPC membrane after skeletonization are about the same size as the holes on the skeletonized poly-DODPC/DPPC membrane with 4.0 mol % of DPPC. However, taking the mole fraction into consideration, the estimated holes seems to be fairly small. This can be explained as follows. Polymerizable

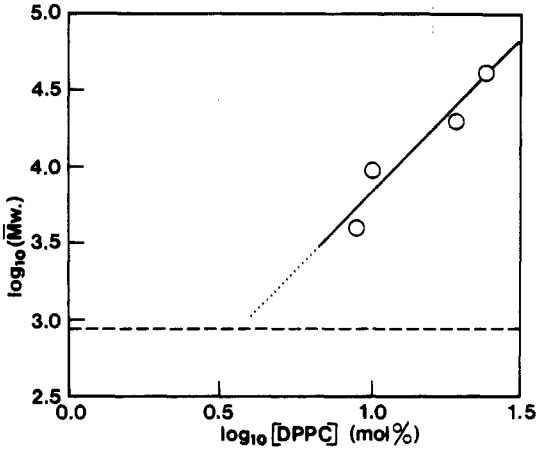

Figure 6. log–log plots between mole percent of DPPC and the $\bar{M}_w$ of polysaccharide with 50% retention. The broken line means the log $\bar{M}_w$ of maltopentaose, which is retained 50% in poly-DODPC liposomes after adding sodium cholate.

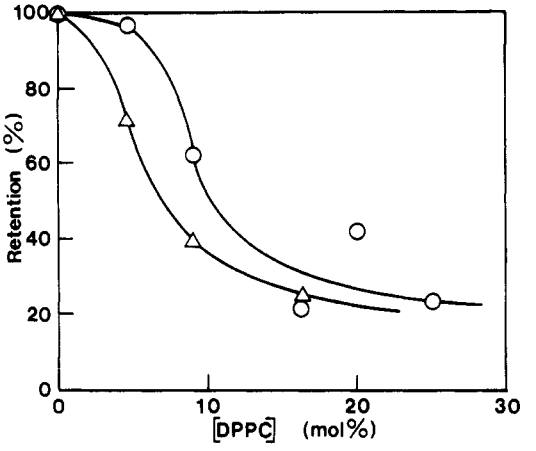

Figure 7. Retention profiles of dextran-9400 in poly-DODPC/DPPC liposomes polymerized at 30 °C (△) and 60 °C (○) after skeletonization.

diene groups of DODPC locate at the interface between the hydrophilic and the hydrophobic regions of the bilayer membrane.[12,14,23] After the polymerization of these mixed liposomes, phase separation occurs independently in the outer and the inner half of the bilayer. The size of the holes of the skeletonized liposomes should be related not only to the size and the number of phase-separated monomeric regions in each layer but also to the portions of monomeric regions overlapped between the outer and the inner half of the bilayer. The average size of the overlapped holes should be considerably small, especially when the mole fractions of DPPC are small.

de Kruijff and co-workers reported the average size of the defects in the DMPC bilayer to be 1.5–1.8 nm from the release measurement of radioactive molecules such as polyethylene glycols and dextrans.[29] They calculated the radii of the molecules by using the cubic root of molecular weights under the assumption that these molecules were spherical. If the radii of encapsulated molecules are calculated by their method, the apparent sizes of the holes in the skeletonized liposomes can be estimated from Figure 6. Namely, the diameters of maltopentaose and dextran-4000, -9400, -19 500, -40 200, and -72 200 are calculated to be 1.5, 2.3, 3.0, 4.0, 5.0, and 6.0 nm, respectively. For example, when the concentration of DPPC is 10 mol %, the 50% of retention can be confirmed for dextran-9400. The apparent size of the holes of the skeletonized liposomes is estimated to be about 3.0 nm. In the same manner, when the mole fraction of DPPC is 20%, the estimated size of the holes is 4.0 nm from the 50% retention of dextran-19 500. These values seem to be small in comparison with the size of the liposomes (about 100 nm in diameter). This should be attributed to the low probability of the overlap of the two phase-separated DPPC regions between the outer and the inner half of the bilayer.

We have already observed the clear holes of the skeletonized liposomes made of polymerized 1-[9(*p*-vinylbenzoyl)nonanoyl]-2-*O*-octadecyl-*rac*-glycero-3-phosphorylcholine (VNOP) and cholesterol by SEM.[15] Since VNOP has a styryl group at the end of the 1-acyl chain, it locates at the interface between the inner and the outer half of the bilayer.[30] Polymerization of VNOP occurs both in the inter- and intralayers of the bilayer. Accordingly, the resulting phase-separated regions should always overlap between both layers. This was confirmed as large penetrated holes after skeletonization.[15] Ringsdorf et al. also reported the "uncorked liposomes", which had many holes on the surface of liposomes, and studied the release behavior of encapsulated molecules.[2] Monomeric regions of the mixed polymerized liposome were removed by chemical cleavage of the disulfide group of a monomeric lipid. When they used fluorocarbon lipids or membrane-spanning hydrocarbon lipids as monomeric lipids, rapid release of encapsulated molecules from the polymerized mixed liposomes was observed after the removal of non-polymerized regions. On the other hand, slow release was observed for polymerized mixed liposomes containing monomeric hydrocarbon lipids. In the case of their fluorocarbon or membrane-spanning hydrocarbon lipids, since phase separation should occur simultaneously in both layers, rapid release was observed. In a hydrocarbon lipid system, the phase-separated monomeric regions in the outer layer should not completely overlap with those in the inner layer. Their experimental results support out conclusion.

Influence of Polymerization Conditions on the Domain Sizes of Phase Separation. For the poly-DODPC/DPPC liposomes polymerized at 30 or 60 °C, the retention of dextran-9400 after skeletonization was measured and the data were shown in Figure 7. The retention ratios in the liposomes polymerized at 30 °C were lower than those polymerized at 60 °C. Polymerization at 60 °C was carried out with both AIBN and AAPD, while polymerization at 30 °C was done with both AIBN and AAPD by irradiating UV light at 360 nm.[23] Different ways to generate radicals are considered to influence the polymerization, especially the rate of initiation. From the rate of polymerization and the molecular weight distribution of lipid polymers, the initiation of polymerization could be almost the same if conditions such as distance from a light source were adjusted. The results in Figure 7 certainly show that the phase separation of liposomes polymerized at 30 °C is larger than that of liposomes polymerized at 60 °C. A phase diagram of DODPC/DPPC mixed liposomes (data not shown) indicates that DODPC/DPPC (1:1) liposomes show a gel state below 21 °C, a mixture of gel and liquid-crystalline states between 21 and 35 °C, and a liquid-crystalline state above 35 °C. The two lipids are completely miscible at temperatures above 35 °C. In this case, phase separation should be induced by polymerization. On the other hand, at 30 °C, the phase separation has already occurred before polymerization. This phase separation is considered to be larger than that induced by polymerization.

We reported the temperature dependence of the rate of CF release from the polymerized mixed liposomes.[16] CF was rapidly released from DPPC liposomes at a gel-to-

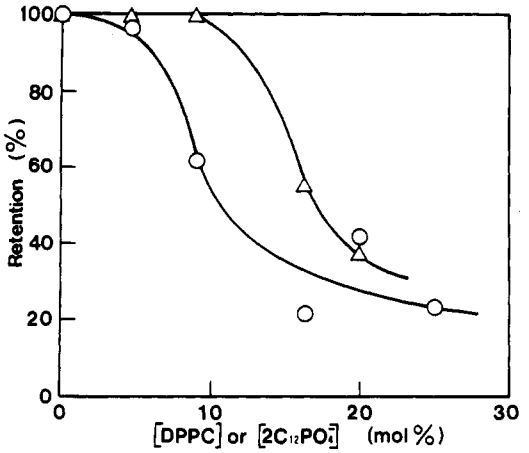

Figure 8. Retention profiles of dextran-9400 of poly-DODPC/DPPC (○) and poly-DODPC/$(C_{12}H_{25})_2PO_4^-Na^+$ (△) liposomes after skeletonization.

liquid-crystalline phase-transition temperature (T_c = 37 °C for small unilamellar liposomes) attributed to the disordered lipid packing at phase transition. In poly-DODPC liposomes, no significant release was observed, showing no distinct phase transition of polymerized liposomes. In the case of poly-DODPC/DPPC (1:1 by mole) liposomes polymerized at 60 °C, CF release should occur at the phase transition of DPPC because of the complete separation of DPPC regions. However, the sensitivity of the CF release response to phase transition was low in comparison with that of pure DPPC liposomes. CF was released gradually below the T_c because the disordered packing at the interface between DPPC and polymerized DODPC regions would be responsible for this low sensitivity, while the same liposomes polymerized in turn at 30 °C showed the higher sensitivity of CF release. It suggests that the phase separation of the mixed liposomes polymerized at 30 °C is larger than that polymerized at 60 °C. The results in Figure 7 qualitatively support this.

To clarify the influence of the kinds of nonpolymerizable lipids on the phase separation of polymerized mixed liposomes, DPPC was replaced with $(C_{12}H_{25})_2PO_4^-Na^+$. Dextran-9400 was used as encapsulated molecules. As shown in Figure 8, when the mole fraction of DPPC was 0.1, the retention was 60%. On the other hand, despite the same mole fraction, the retention in a $(C_{12}H_{25})_2PO_4^-Na^+$ system was still 100%. This indicates that domain sizes of phase separation in the $(C_{12}H_{25})_2PO_4^-Na^+$ system are smaller than those in the zwitterionic DPPC system. This should be due to the electrostatic repulsion among $(C_{12}H_{25})_2PO_4^-$ molecules in the mixed liposomes. It seems that the size of the phase separation can also be controlled by the use of different nonpolymerizable amphiphiles, which have different assembling characteristics in the bilayer membrane.

Conclusions

The phase separation of polymerized mixed liposomes was analyzed indirectly and quantitatively from the molecular weights of molecules released from skeletonized liposomes. This method is useful to investigate factors that influence the phase separation of the polymerized mixed liposomes. The domain size of phase separation can be controlled by changing the composition of lipid components in the mixed liposomes, the polymerization temperature, and the structure of polymerizable and nonpolymerizable components. Furthermore, the skeletonized liposomes thus prepared have a possibility as intelligent carriers that deliver and release various sizes of entrapped materials.

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