Destruction and Reconstitution of Polyphospholipid Vesicles with Size Memory

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ABSTRACT: 1,2-Bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine (DODPC), constructing the bilayer membrane of a vesicle, was polymerized by UV irradiation. The freeze-dried vesicles were completely dissolved in chloroform to obtain cross-linked phospholipid polymer (polyDODPC). The polyDODPC consisted of high molecular weight components and oligomers. The polyDODPC was dispersed by sonication, and two size distributions of the reconstituted vesicles were confirmed, larger vesicles from high molecular weight polyDODPC and smaller vesicles from oligomers. When the polyDODPC vesicles of various sizes and unimodal size distribution were reconstituted by an extrusion method, the vesicles of the same size as the vesicles at polymerization showed excellent stability compared with the other sizes. Interestingly, the original size of the polyDODPC vesicles could be completely restored by a cholate dialysis method. It was also clarified that vesicles larger than the original size were reconstituted from the higher molecular weight component, and the smaller vesicles were from the oligomers. Furthermore, a linear phospholipid polymer obtained from 1-palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine could not restore the original size. Those results indicate that the original size of the vesicles can be restored completely by all polyDODPCs having the original composition of large and small molecular weights and cross-linked structure.

Introduction

There are various kinds of molecular assemblies such as micelles, vesicles, lamella, tubes, and fibrils. Molecular assemblies with complicated but regulated structure can be constructed by interaction between constituent molecules and their structural conformability because the degree of freedom in molecular packing should be closely restricted.[1] Such molecular assemblies can generally be seen in biological systems, e.g., viruses and biomembranes, due to the conformability of biopolymers such as membrane proteins and proteins associated with the membrane.

Focusing on phospholipid vesicles, constituent molecules orientate with relatively high regularity and form a bilayer structure. However, the vesicles generally have various numbers of bilayer membranes and various sizes, depending on the preparation methods or conditions as well as the structure and composition of constituent molecules, because varied curvature of the bilayer membrane is possible by changing the molecular number of the outer and the inner leaflets of the bilayer membrane. Only the minimum size of vesicles with one bilayer membrane, which are usually prepared by sonication, can be determined by membrane constituents.[2] Fuhrhop and Fritch prepared vesicles using bipolar amphiphiles which have two different polar head groups at both ends of the long chain.[3] The resulting vesicle has a unimodal size and one monolayer. This indicates that the conforming structure of the amphiphiles can determine the curvature of the monolayer.

We synthesized phospholipid polymers by polymerization of the vesicles from 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine (DODPC)[4] or 1-palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine (POPC).[5] When the DODPC vesicles were polymerized by UV light, the freeze-dried vesicles were soluble in an organic solvent such as chloroform and dissociated to phospholipid polymers (polyDODPC), while if they were polymerized with radical initiators,[6-8] they were not soluble in any solvent. We clarified that this was due to the difference in their molecular weight of polymerization and cross-linked structure.[9] On the other hand, POPC vesicles freeze-dried after polymerization were soluble in organic solvent independent of the polymerization method because of the linear structure of the resulting polymer (polyPOPC).[10] We studied the possibility of restoration of the original vesicular structure for reconstituted polyPOPC vesicles by a polymerization technique.[10] However, the size of the reconstituted vesicles strongly depends on the reconstitution methods, and there was no restoration of the original size of the vesicles.

In this paper, polyDODPC prepared by UV irradiation as vesicles was used to prove whether the cross-linked polyDODPC restored the original size of the vesicles after reconstitution because the polyDODPC has a cross-linked structure but is soluble in organic solvent. We studied the molecular weight of polyDODPC and separated the fractions by molecular weight to determine the contribution of each fraction constructing the original size of the vesicles.

Experimental Section

Materials. 1,2-Bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine (DODPC) was purchased from Nippon Oil & Fats Co., Ltd. 1-Palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine (POPC) was a gift from Toyo Soda Co., Ltd. The purity of the lipid was confirmed by thin-layer chromatography (Merck, silica gel plates) with chloroform/methanol/water (65/25/4 by volume). Sodium cholate was purchased from Kanto Chem. Co., Inc. and used without further purification.

Methods. (a) Preparation of Phospholipid Polymer by Polymerization as Vesicles. A thin film of DODPC (500 mg) prepared by evaporating the chloroform solution (12 mM) was dispersed with a vortex mixer in pure water (10 mL). The obtained turbid suspension was placed in an Extruder (Lipex Biomembranes Inc.) and forced to penetrate through the pores of polycarbonate membrane filters (Nuclepore, Coster Ltd.) with a stepwise reduction of the pore size of the filters from 1 to 0.2 μm. The vesicle suspension was sealed in a quartz tube

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Table 1. Average Molecular Weights of Samples and polyODA Obtained from the Samples[a]

sample	yield[b] (%)	VPO of sample, $M_n \times 10^3$	VPO of polyODA, $M_n \times 10^3$	SEC of polyODA $M_n \times 10^2$	SEC of polyODA $M_w \times 10^2$	SEC of polyODA M_w/M_n
polyDODPC		7.5 (9.6)	1.0(3.7)	4.9(1.7)	26 (9.2)	5.5
fraction 1	15	1.6 (2.0)	0.5 (1.8)	3.6 (1.3)	7.4 (2.6)	2.0
fraction 2	35	2.6 (3.3)	0.8 (2.8)	4.3 (1.5)	8.1 (2.9)	1.9
fraction 3	50	12 (15.8)	1.2 (4.2)	5.7 (2.0)	41 (14.5)	7.2
polyPOPC[c]		5.5 (7.0)		4.9 (1.7)	11 (3.9)	2.2

[a] Value in parentheses is the corresponding degree of polymerization. [b] These values were obtained from the weights of each fraction based on the used polyDODPC. [c] SEC data were calculated after ignoring the peak of palmitic acid.

under a N_2 atmosphere. The UV light was irradiated from two UV light sources (low-pressure mercury lamp, Riko UVL-32LP) onto the quartz tube rotated at 100 rpm. The temperature was controlled to 30 °C. The minimum distance from the light sources to the surface of the tube was 5 mm, and the entire apparatus was completely covered with reflecting metal sheets. The UV absorption at 254 nm, attributed to the diene groups ($\epsilon_{max} = 3.4 \times 10^{-4}$ m^2/mol), was analyzed with a UV spectrometer (Shimadzu, MPS-2000) to determine the polymerization conversion of DODPC. Monomeric phospholipid, DODPC, was completely polymerized in a bilayer membrane by 7 h of UV irradiation. The phospholipid polymer (polyDODPC) was obtained by completely solublizing the freeze-dried powder of the vesicles in chloroform and freeze-drying the solution again after replacement with benzene.

(b) Fractionation of PolyDODPC with Different Molecular Weights. Because polyDODPC with a higher molecular weight becomes more difficult to dissolve in ethanol, the polyDODPC in an ethanol solution can be precipitated by adding a smaller amount of water as a poor solvent. Using this property, the lowest molecular weight component (1) was first extracted from the freeze-dried powder (100 mg) with a mixed solvent of water/ethanol = 1/4 (v/v) (10 mL). A mixed solvent of water/ethanol = 1/19 (v/v) (10 mL) was then added to the residue to extract fraction 2, and the resulting residue was fraction 3; the highest molecular weight.

(c) Molecular Weight Measurement of PolyDODPC. Preparation of polyODA. PolyDODPC was dispersed in 1 N NaOH aqueous solution and refluxed (12 h) for hydrolysis to separate the 1- and 2-acyl chains from the hydrophilic head group, glycerophosphocholine. A 1 N HCl aqueous solution was added to the dispersion until the pH changed to 3.0, and then poly(2,4-octadecadienoic acid) (polyODA) precipitated. The polyODA was extracted with chloroform, washed twice with water and dried *in vacuo* (yield: 95%).

(1) VPO Method. The number-average molecular weight of polyDODPC or polyODA was determined by a vapor pressure osmometry (VPO) method (Corona, vapor pressure osmometer No. 117). The measurement was carried out at 37 °C with chloroform solutions of polyDODPC or polyODA. The calibration constant was obtained with 1,2-bisstearoyl-*sn*-glycero-3-phosphocholine and stearic acid.

(2) SEC Method. The molecular weight distribution of polyODA was measured by size exclusion chromatography (SEC) (Shimadzu, GPC-80M, 804 column, and a refractive index detector (RID-6A)) after dissolution in THF.

(d) Reconstitution of Vesicles from PolyDODPC. (1) Sonication Method. PolyDODPC (40 mg) was dispersed in pure water (4 mL) by vortex mixing. The dispersion was sonicated with a probe-type sonicator (Tomy Seiko UR-200P, 80W, 10 min × 2, 10 °C) under a N_2 atmosphere.

(2) Extrusion Method. PolyDODPC (100 mg) was dispersed into pure water (10 mL) by vortex mixing. The resulting dispersion was extruded through the pores of membrane filters. The vesicular size was controlled by selecting the final pore size (0.1, 0.2, or 0.4 μm) of the membrane filter.

(3) Cholate Dialysis Method. PolyDODPC (100 mg) was dispersed into 5 mM Tris-HCl buffer (10 mL, pH 7.0) by vortex mixing. Sodium cholate was added to the suspension until the molar ratio of DODPC to cholate became 2−3. The solution was stirred with a magnetic stirrer until the solution became clear and was then dialyzed using a cellulose tube (Viskase Sales Co., UC8-32-25) for 36 h against the same solution described above (3 L).

(e) Turbidity Measurement. The reconstituted vesicle suspension (1 g/dL) prepared by extrusion was put into a cuvette (light-pass length 1 cm), and then the absorbance was measured at 800 nm with a UV spectrometer to evaluate the stability in the dispersion state.

(f) Size Measurements of Vesicles. (1) Transmission Electron Microscopy. An aqueous solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (0.3 mL, 11 g/dL) was added to 1 mL of the vesicle suspension (0.1 g/dL). This was incubated for 6 h at room temperature to stain the inner aqueous phase of the vesicles. A drop (6 μL) of the suspension was then placed on a carbon-coated copper grid and blotted with filter paper. Finally, the sample was observed with a JEM-100CX (JEOL) electron microscope operated at 100 kV.

(2) Dynamic Light Scattering. All dynamic light scattering (DLS) measurements were performed on a Coulter submicrometer particle analyzer (Model N4SD). The vesicle suspension (0.1 g/dL; 4 mL) was placed in a chamber set at 25 °C, and data (SDP weight results) were collected automatically to give an "amount vs size" histogram.

Results and Discussion

Characterization and Fractionation of PolyDODPC. Scanning electron microscopy (SEM) was used to confirm the complete dissociation of polyDODPC from the vesicular structure. The sphere structure was clearly observed for freeze-dried polyDODPC vesicles by SEM (data not shown). When the powder was dissolved in chloroform and freeze-dried again after replacement with benzene, no sphere structure or other regular assembling structures were observed. This indicates the complete dissociation of the polyDODPC in the organic solvents.

The number-average molecular weight of the soluble polyDODPC without fractionation was measured by vapor pressure osmometry and compared to that of polyODA, which was obtained by the hydrolysis of the ester linkage of polyDODPC. The number-average molecular weights (the degrees of polymerization) of polyDODPC and polyODA were 7.5 × 10^3 (9.6) and 1.0 × 10^3 (3.7), respectively, as listed in Table 1. This shows the cross-linked structure of the polyDODPC of which the glycerol groups act as cross-linking points of polyODA, as schematically shown in Figure 1.

Because polyDODPC does not completely dissolve in THF as a solvent for SEC, polyODA, which is soluble in THF, was subjected to SEC analysis to obtain more detailed information about polyDODPC. As shown in Figure 2, the molecular weight distribution of polyODA measured by SEC includes high and low molecular weight components. Four peaks were observed at elution times of 18.8, 21.3, 22.0, and 22.5 min. Though a comb-type polymer such as polyODA cannot be exactly calibrated with polystyrene standards, the number-average molecular weight of the first peak is 5.8 × 10^4, and the corresponding degree of polymerization (DPn)

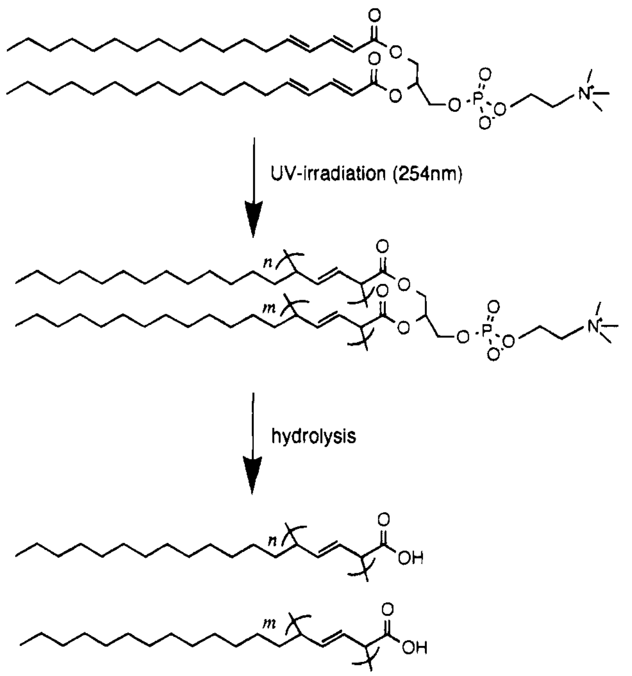

Figure 1. Preparation of polyDODPC and polyODA shown schematically. 1- and 2-acyl chains of DODPC are individually polymerized and then polyDODPC is formed. PolyODA for SEC analysis is obtained by hydrolysis of polyDODPC.

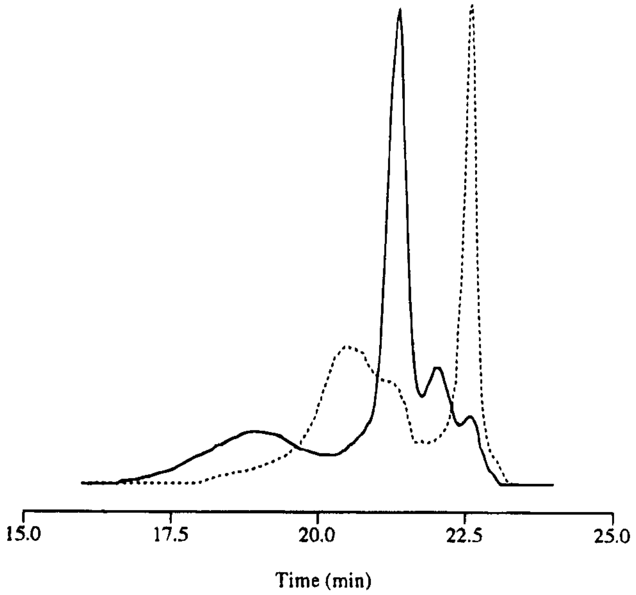

Figure 2. SEC analysis of polyODAs, which were obtained from vesicles of polyDODPC (—) and polyPOPC (- - -).

is ~20. The second peak includes the pentamer, tetramer, and trimer of polyODA (average, tetramer). The third and fourth peaks are the dimer and monomer, respectively. The average molecular weights of the polyODA are listed in Table 1. There are clear differences between the number-average molecular weights of polyODA from VPO and SEC. SEC data did not calibrate accurately.

The polyDODPC obtained from polymerized vesicles of 0.2-μm diameter were separated into three fractions by extraction with mixed solvents, as described in the Experimental Section. Freeze-dried fractions 1 and 2 were soft powders, but fraction 3 consisted of hard particles. Table 1 also summarizes the yield, the number-average molecular weights of polyDODPC measured by VPO, and the SEC data of the corresponding polyODA in each fraction. The order of M_n is fractions 3 > 2 > 1. The VPO data indicate that fractions 1 and 2 are oligomers, whereas fraction 3 is a relatively high molecular weight material. Figure 3 shows the SEC curves of polyODA obtained from the polyDODPC of fractions 1–3. Fraction 1 is composed of the second, third, and fourth peaks of the polyODA from polyDODPC before fractionation as shown in Figure 2. Namely, fraction 1 is the mixture from monomer to tetramer or pentamer of polyODA. Fraction 2 has a distribution similar to fraction 1, but the ratios of the monomer and dimer are lower than those in fraction 1. Fraction 3 shows a different feature; it has the first peak, besides the other three peaks, and the third and fourth peaks become smaller than those in fraction 2. Therefore, it was clarified that polyDODPC consists of the oligomer and polymer of polyODA, and the solubilities of the polymers and oligomers in aqueous ethanol solution drastically change at a mixing ratio of 1/19 (v/v). Moreover, considering the yield of each fraction, the ratio of the low and high molecular weights of polyDODPC was 1/1 under this polymerization condition.

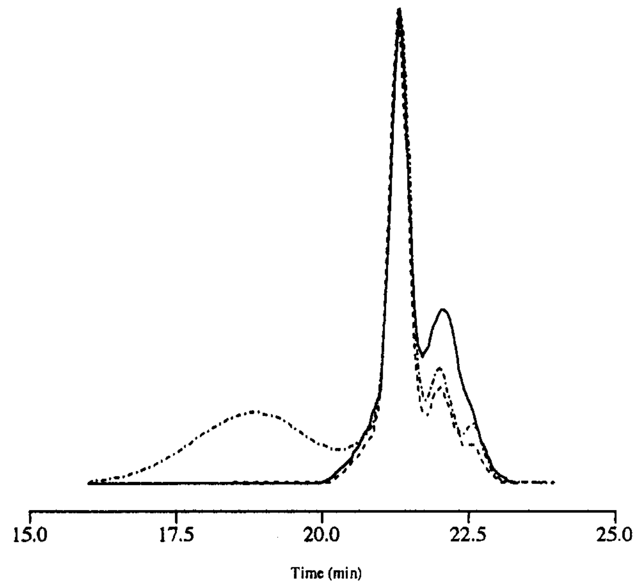

Figure 3. SEC analysis of polyODAs, which were obtained from three fractions by ethanol/water extraction. Only fraction 3 included a broad peak in 16–20 min, and there are three peaks (>20 min) in all fractions: (—) 1, (- - -) 2, and (– • –) 3.

Because DODPC has 2,4-diene groups in both acyl chains, polyDODPC polymerized as vesicles should be a structure different from polyDODPC polymerized in a solution state. Namely, the former can be estimated as a fragment of the bilayer membrane because of the cross-linking property of DODPC.[10] The cross-linking in polymerization can be explained in terms of the orientation of DODPC in the bilayer membrane. Because the glycerol backbone of phospholipids is oriented vertically to the membrane plane,[11,12] the 1-acyl chain also extends vertically. Although the 2-acyl chain first extends along the membrane plane, it then folds at the 6-carbon atom and parallels the 1-acyl chain. Consequently, the position of the diene group in the 1-acyl chain is different from that of the 2-acyl chain in the bilayer membrane.[10] It is therefore obvious that the polymerization of the 1- and 2-acyl chains proceeds individually in the bilayer membrane, and the glycerol groups of DODPC act as cross-linking points of polyDODPC.

Such a highly cross-linked polymer is generally considered not to dissolve in organic solvents. In fact, polyDODPC obtained from vesicles polymerized by radical initiators such as azobis(isobutyronitrile) and azobis(2-amidinopropane) dihydrochloride[4-8] or γ-ray irradiation[13] cannot be dissolved in any organic solvent. Such insoluble polyDODPC becomes soluble in THF after methanolysis of the ester linkage of the DODPC unit.[9] The resulting poly(methyl octadecadienoate) showed main components with high molecular weights in the SEC data.[14] On the other hand, polyDODPC obtained by UV irradiation is soluble in organic solvents such as chloroform or ethanol because polyDODPC is a mixture of polymers and oligomers, and oligomers are the main components.[14]

However, the molecular weight distribution of polyODA does not indicate the real distribution of the molecular weight of polyDODPC. There are three possibilities that explain the two components in the SEC data of polyODA. The first is that polyDODPC is a mixture of polymers and oligomers, the second is that polyDODPC is composed of polymers and oligomers of polyODA, and the last is a mixed system of the first and the second cases. In order to clarify these possibilities, polyDODPC was carefully divided into three fractions by making use of the different solubility of the polymers or oligomers in a mixed solvent of ethanol and water. Oligomeric fractions (1 and 2) of polyDODPC were completely separated from the high molecular weight fraction (3). PolyODA from fraction 3 showed two distributions in SEC as shown in Figure 3, indicating that the high molecular weight fraction of polyDODPC is composed of high molecular weight polyODA and oligomers, while polyODA from fractions 1 and 2 showed oligomers. Therefore, it is concluded that polyDODPC is a mixture of cross-linked polymers and oligomers, and the cross-linked polymers consist of high molecular weight polyODA and oligoODA.

The POPC vesicles were polymerized by UV irradiation in the same way as the polyDODPC vesicles. The degree of polymerization of polyPOPC measured with VPO was ~7. As shown in Figure 2, the profile of the SEC of polyODA from polyPOPC was quite different from that from polyDODPC. Because the 1-acyl chains of polyPOPC cannot be polymerized, the peak at an elution time of 22.5 min is confirmed to be palmitic acid. PolyODA from polyPOPC shows a broad peak, of which the number-average and weight-average degrees of polymerization are 1.6 and 5.4, respectively. The molecular weight of polyPOPC is lower than the high molecular weight fraction of polyDODPC.

From the SEC data obtained from polyDODPC and polyODA, the cross-linked structure of polyDODPC would provide high molecular weight polymers and oligomers to compensate for the loose molecular packing of the cross-linking polymers by oligomers in the bilayer membrane. It is still unclear why the average molecular weight of polyODA from polyPOPC is smaller than that of the polymer part of polyODA from polyDODPC.

Reconstitution of Vesicles. (1) Sonication Method. The vesicles (1 g/dL) reconstituted from polyDODPC by sonication show two distributions: small (~0.05 μm) and large vesicles (~1 μm), measured with a Coulter N4SD. The size of the small vesicles was almost the same as that of the vesicles prepared by sonication of monomeric DODPC (0.03 μm). The large vesicles were separated from the small ones by centrifugation (1900*g*, 20 min). Figure 4 shows the size distribution of the small vesicles, superimposed on another size distribution of separated large vesicles. It is recognized that the large vesicles were completely separated from the small ones. Furthermore, when the large vesicles were sonicated again, few small vesicles were prepared, suggesting that the polyDODPC constructing the large vesicles should be different from that constructing the original polyDODPC vesicles.

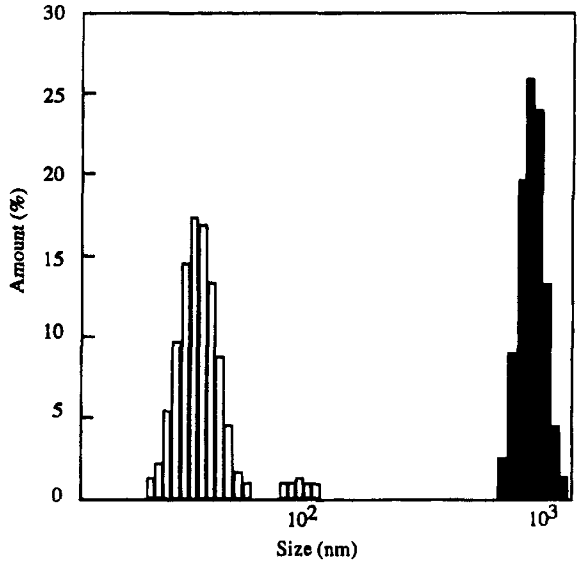

Figure 4. Size distributions by light-scattering results of small vesicles (□) and large vesicles (■) reconstituted by sonication method.

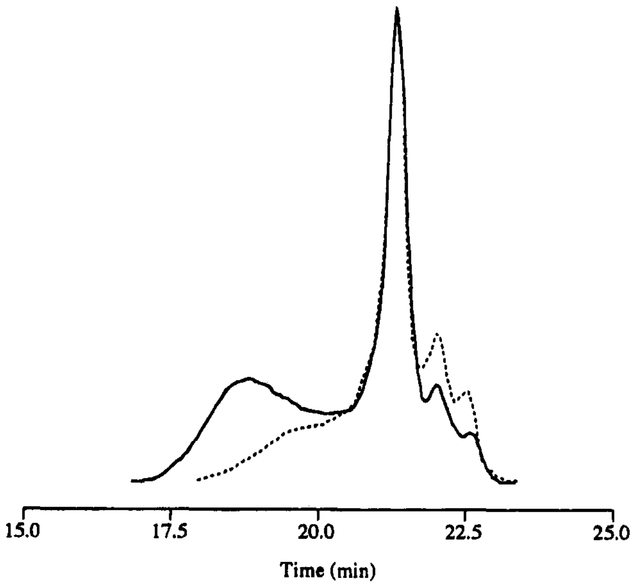

Figure 5. SEC analysis of polyODAs obtained from vesicles reconstituted by sonication method. There is high molecular weight distribution of polyODA from small vesicles (- - -). On the other hand, high molecular weight distribution increases in the case of polyODA from large vesicles (—).

The freeze-dried powders of large and small vesicles, separated as mentioned above, were hydrolyzed, and the molecular weight distribution of the polyODA constructing each size of vesicles was measured by SEC. As shown in Figure 5, the polyODA from small vesicles was clarified to be oligomers, and no peak was observed on the high molecular weight side, as was observed for the original vesicles as shown in Figure 2. In the case of polyODA from the large vesicles, however, a peak on the high molecular weight side was confirmed in addition to the low molecular weight peak, and the height of the peak on the high molecular weight side was larger than that of the original polyODA.

Table 2. Vesicle Sizes and Turbidities (at 800 nm) of Vesicle Suspensions Prepared by Extrusion

pore size, μm	original		reconstituted		
			size, nm		
	size, nm	turbidity	DLS	TEM (n = 50)	turbidity
0.4	389 ± 140	0.38	398 ± 180	420 ± 110	
0.2	203 ± 42	0.21	290 ± 130	270 ± 82	0.40
0.1	162 ± 28	0.19	210 ± 60	198 ± 23	0.21
0.05	138 ± 53	0.18			

Considering that molecular motion is reinforced by sonication, the mobility of polyDODPC decreases with increasing molecular weight, and molecules with a similar mobility tend to assemble. Therefore, the separation between polyDODPC with high and low molecular weights is considered to occur on sonication, and also polyDODPC with a cross-linked structure could not undergo extensive curvature, leading to the separation of vesicles into large and small vesicles. Consequently, the dimers or trimers of DODPC are effectively dispersed in water to form small vesicles, as the vesicles prepared from monomeric DODPC, and polyDODPC molecules with high molecular weight were forced to form larger vesicles. Therefore, this method was not adequate to restore the original size of the vesicles from polyDODPC.

(2) Extrusion Method. PolyDODPC powder was dispersed in water, and the dispersion (1 g/dL) was extruded through membrane filters by applying N_2 gas pressure. In the case of reconstitution of polyDODPC vesicles by extrusion, the relationship between the pore size of the filters and the size of the resulting vesicles is shown in Table 2. Vesicles penetrated repeatedly (~10 times) until 5 mL of the suspension had penetrated within 1 min at 10 kg/cm^2 gas pressure. The lipid concentration after extrusion was almost the same as that before treatment. As a reference, the same method was performed for monomeric DODPC vesicles. The size of the vesicles almost coincided with the penetrated pore size of the membrane filters when the pore size was larger than 0.2 μm, while the size of the vesicles was larger than the penetrated pore size when the pore size was smaller than 0.1 μm. It would be more difficult for smaller vesicles to penetrate through smaller size pores because smaller vesicles are more stable than larger ones. In this case, the vesicles cannot penetrate through pores by destroying their structure but by deforming in size. In the case of polyDODPC vesicles reconstituted by extrusion, it is interesting that the vesicular sizes were always larger than the pore sizes and had a unimodal size distribution as shown in Table 2. Vesicles reconstituted from polyDODPC were of 0.4-μm diameter after passing through a 0.4-μm pore size filter and 0.3- and 0.2-μm diameter after passing through filters of which the pore sizes were 0.2 and 0.1 μm, respectively. The vesicles could not pass through the 0.05-μm pore size filter.

Polycarbonate membrane filters for extrusion have a unimodal size of penetrated pores. When phospholipid vesicles larger than the pore size are forced to pass through the pores, three phenomena are considered to be possible: (1) The vesicles cannot pass through the pores due to plugging. (2) The vesicles can pass through the pores by deforming their structure. Therefore, the resulting vesicles are larger than the pore size. (3) The vesicles can pass by being cleaved to form vesicles smaller than the pore size.

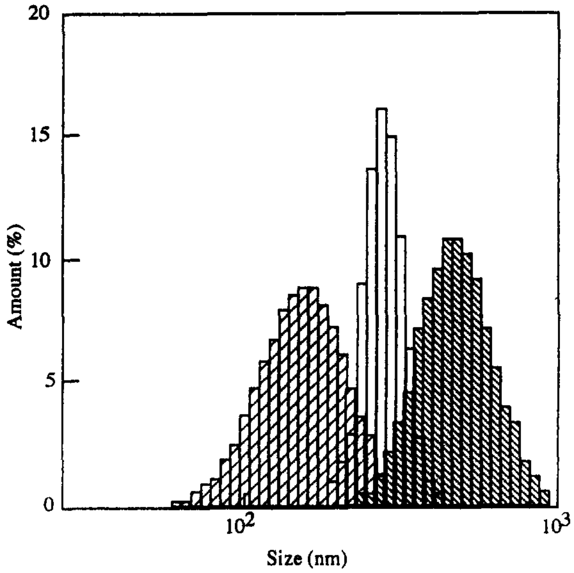

Figure 6. Size distributions by light-scattering results of vesicles reconstituted from polyDODPC (open bar), fraction 1 (right hatch), and fractions 3 (left hatch) with cholate dialysis method.

Monomeric phospholipid vesicles pass through the smaller pores by cleavage due to the relatively weak shear stress. However, stronger shear stress should be necessary with decreasing vesicular size due to the higher stability of the vesicles, and those vesicles pass through the smaller pores by deforming their size. Extrusion of vesicles with polyDODPC is more difficult than that with monomeric DODPC because of the higher molecular weight of polyDODPC used in the extrusion.

Interesting phenomena were observed for the turbidity measurements of the vesicles reconstituted from polyDODPC. The data are also summarized in Table 2. It could be easily understood that vesicles reconstituted by extrusion using a filter pore size larger than 0.2 μm were larger than the original size of the polyDODPC vesicles (0.2 μm). However, the turbidity was much greater than that of monomeric vesicles of the same size and concentration. This clearly indicates the aggregation of the reconstituted vesicles. For example, the reconstituted vesicles of 0.4-μm diameter show more than twice the turbidity of monomeric vesicles of the same diameter. On the other hand, reconstituted vesicles of which the size was just 0.2 μm were prepared by extruding polyDODPC vesicles through a 0.1-μm pore size filter. The reconstituted vesicles of 0.2-μm diameter showed a turbidity similar to that of monomeric vesicles of 0.2-μm diameter, indicating no aggregation of the resulting vesicles.

Because the size of the original vesicles at polymerization was 0.2-μm, polyDODPC obtained from the polymerized vesicles memorized the packing states of the polyDODPC in the bilayer membrane having the curvature of the 0.2-μm vesicles. The polyDODPC vesicles were demonstrated to have the most stable molecular packing when the vesicles assumed the original size. If the reconstituted vesicles were larger than the original size, the vesicles should tend to aggregate because of the loose packing of polyDODPC. Because the reconstruction of the vesicles smaller than the original ones was impossible, only the vesicles of 0.2-μm diameter were stable.

(3) Cholate Dialysis Method.[15,16] When a transparent solution of mixed micelles of polyDODPC and

Table 3. Sizes of Vesicles Reconstructed by the Cholate Dialysis Method

	size, nm		
		after	
sample	before by DLS	by DLS	by TEM ($n = 100$)
DODPC	210 ± 41	76 ± 21	
polyDODPC	138 ± 52	124 ± 36	
polyDODPC	208 ± 48 (219 ± 28)[a]	202 ± 52	213 ± 14
fraction 1		180 ± 50	190 ± 30
fraction 2		325 ± 58	
fraction 3		452 ± 110	410 ± 89
polyPOPC	212 ± 43	282 ± 100	

[a] This size was measured in TEM photograph.

sodium cholate was dialyzed against 5 mM Tris-HCl buffer (pH 7.0)[17,18] to remove the cholate, the solution gradually became turbid, and the size of the particles measured with a Coulter submicrometer particle analyzer increased from ~20 nm to a constant value of ~200 nm after 36-h dialysis. The concentration of obtained vesicles was 0.3−0.6 g/dL. The mixed micelles were gradually converted to the bilayer phospholipid vesicles by the gradual removal of sodium cholate during dialysis. Therefore, the constant size can be estimated to be the most stable size of the vesicles reconstituted spontaneously from the polyDODPC.

Table 3 shows the size of vesicles reconstituted from polyDODPC (or DODPC). Figure 7 is the transmission electron micrograph of the reconstituted vesicles. The same results were obtained by this method as listed in Table 3. In the case of monomeric DODPC, the size of the reconstituted vesicles was 76 ± 21 nm, which is far smaller in comparison with the original DODPC vesicles (210 ± 41 nm). On the other hand, in the case of polyDODPC from the original vesicles of 208 ± 48 nm diameter, the reconstituted vesicles were the same size (202 ± 52 nm) as the original ones. Moreover, when the same operation was performed for polyDODPC obtained from the smaller vesicles (138 ± 52 nm), the reconstituted vesicles showed the same size (124 ± 36 nm) as the original ones. Based on the results mentioned above, polyDODPC vesicles were determined to be capable of memorizing their assembled states and restoring their original size. The good correspondence of the diameter of the reconstituted polyDODPC vesicles with that of the vesicles before reconstitution showed that the diameter of the polyDODPC vesicles was restored after the reconstitution.

On the other hand, in order to study the importance of the two-dimensional structure of polyDODPC, polymerized POPC, having a linear structure, was used for reconstitution. As shown in Table 3, when the reconstitution by cholate dialysis was carried out with polyPOPC, the size of the reconstituted vesicles was larger than the original size of the vesicles (282 ± 100 nm). Therefore, the polyPOPC cannot duplicate the original structure.

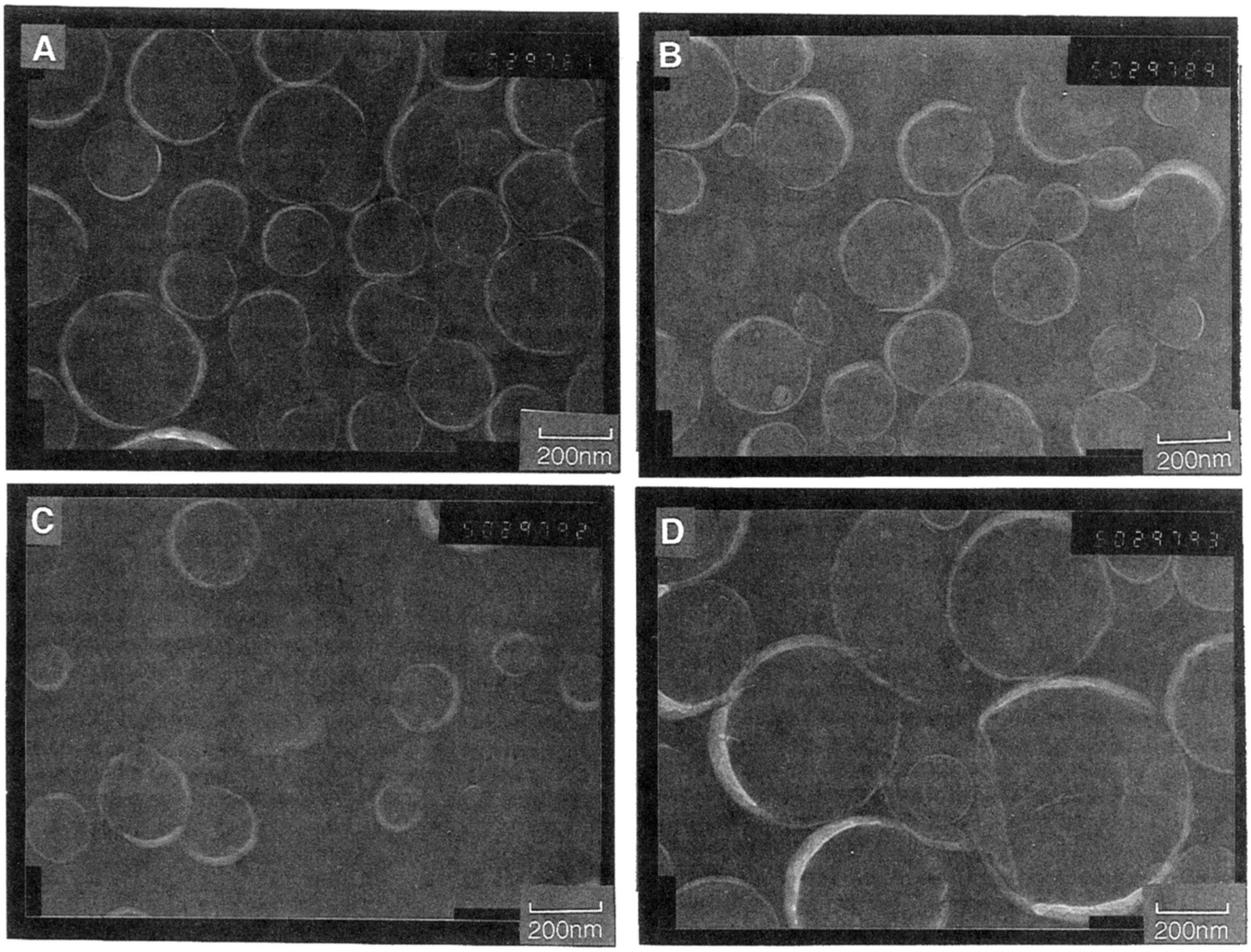

Figure 7. TEM photographs (×50000) of (A) original vesicles, and vesicles reconstituted from (B) polyDODPC, (C) fraction 1, and (D) fraction 3.

(4) Molecular Weight of PolyDODPC and Size of Reconstituted Vesicles. In order to study the mechanism of the restoration of the original polyDODPC vesicles by cholate dialysis, the three fractions of polyDODPC, which were fractionated by molecular weight, were used for reconstitution. The size distributions of vesicles reconstituted from fractions 1–3 are summarized in one figure (Figure 6), and the TEM micrographs are shown in Figure 7.

The solubility of polyDODPC in water by the addition of sodium cholate decreased with increasing molecular weight. After being stirred for ~10 min, all solutions became transparent. Interestingly, the size of the resulting reconstituted vesicles depends on the molecular weight of polyDODPC. When vesicles were reconstituted from fraction 3, which had the highest molecular weight of the three fractions, the size of the reconstituted vesicles was 452 ± 110 nm, larger than the original size (208 ± 48 nm). On the other hand, the vesicles reconstituted from fraction 1, having the smallest molecular weight, were 180 ± 50 nm, smaller than the original size. The same results could also be confirmed by transmission electron micrography as shown in Figure 7. Therefore, the size of polyDODPC vesicles can be controlled by the molecular weight of the polyDODPC. A similar result was also confirmed in the extrusion method of each fraction. The vesicles from fraction 3 can penetrate only through a 0.4-μm pore size filter, but those from fractions 1 and 2 can penetrate through a 0.2- and 0.1-μm filter, respectively. This also indicates that the lower the molecular weight, the smaller the reconstituted vesicles.

As described above, polyDODPC was composed of cross-linked high molecular weight polyDODPC and low molecular weight oligoDODPC. It can be easily understood that the high molecular weight components have a role in forming a membrane with small curvature, and the oligomeric component tends to form a membrane with high curvature. The curvature of the vesicles was determined by the number and the packing state of phospholipid molecules in the bilayer membrane. In the case of polyPOPC vesicles, the distance among the repeating units of linear polyPOPC is estimated to be constant in the direction of the main chain, although the units had freedom in the two-dimensional direction. Therefore, the molecular packing can be adjusted in two dimensions. This would explain the single distribution in molecular weight of polyPOPC as shown in Figure 2. After the polyPOPC vesicle powder was dissolved in an organic solvent, the memory of the two-dimensional state packing was completely lost. PolyPOPC formed vesicles with the most stable molecular packing state and the original structure was not restored after reconstitution.

On the other hand, with cross-linked polyDODPC, two-dimensional packing was maintained even after dissolution in chloroform. The packing state among DODPC units in two-dimensional cross-linked polyDODPC is considered to be constant. Therefore, the curvature of the polyDODPC vesicles would be adjusted roughly by the number of polyDODPC and finely by the packing state among two-dimensional polyDODPC. When the polyDODPC fraction with high molecular weight was dispersed in water, vesicles of larger size (~450 nm) were formed. If the vesicles were forced to become smaller than this, cracking would occur among the polyDODPC fragments, which leads to the destabilization of the vesicles. Therefore, it is impossible for such high molecular weight cross-linked polyDODPC to form a high curvature. On the other hand, monomeric DODPC assembled to form the smallest vesicles of 70-nm diameter. This indicates that the monomeric lipid can form the high curvature because of the good packing of the lipid. Reconstituted polyDODPC vesicles took the original curvature when they were reconstituted only from all components of the original vesicles. Those results lead to the explanation that the molecular packing state even under high curvature becomes stable if the cracks in cross-linked polyDODPC are filled with oligoDODPC. Therefore, the original curvature is restored by the molecular weight and molecular weight distribution of the polyDODPC, which are determined from the original size of the vesicles at polymerization. This also means that the size of the polyDODPC vesicles can be adjusted by changing the molecular weight distribution, by changing the mixing ratio of the cross-linked polyDODPC and oligomers.

Conclusions

DODPC vesicles were polymerized by UV irradiation to form polyDODPC. The polyDODPC was composed of cross-linked polymers and oligomers. The size of the vesicles reconstituted by a cholate dialysis method was restored from all components of the polyDODPC, but polymeric and oligomeric DODPC afforded larger and smaller vesicles, respectively. The linear polymers did not restore the size of the original vesicles. Therefore, all components of polyDODPC constituting polymerized vesicles should form the most stable assembling structure, the size of which was the same as the vesicles at polymerization.

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