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Polymerization of Liposomes Evaluated from Molecular Weight Distribution of Diene-Type Phospholipid Polymers

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ABSTRACT: 1,2-Bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) or 1-palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (POPC) was dispersed in water by sonication to form liposomes. DODPC liposomes were polymerized by UV-irradiation or with radical initiator such as azobis(2-amidinopropane)dihydrochloride (AAPD) at 60°C and methanolyzed to obtain THF-soluble polymers. The molecular weight distribution obtained from a gel permeation chromatography reveals that the UV-irradiation provides low-molecular-weight products and a small amount of polymers, while free radical polymerization gives polymers having relatively high-molecular-weight. The molecular weight of the phospholipid polymers is also influenced by the assembling state. The constrained lipid packing of small unilamellar POPC liposomes provides relatively low-molecular-weight polymers in comparison with those of larger liposomes. In addition, POPC liposomes polymerized at the temperature above the gel-to-liquid crystalline phase transition temperature (T_c) show the higher molecular weight of polymers than those polymerized below the T_c.

KEY WORDS Diene-Containing Phospholipid / Liposomes / Molecular Weight Distribution / Polymerization / UV-Irradiation / Radical Initiator / Lipid Packing / Phase Transition Temperature /

Liposomes have been studied as microcapsules for drugs or functional particles as well as models for biomembranes.[1-5] The drawback of this system is the stability of their structures. Polymerization of liposomes is one of the potent techniques to obtain stable liposomes.[5] 1,2-Bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC), which has diene groups in 2,4-position of 1- and 2-acyl chains, is completely polymerized by UV-light.[6] On the other hand, when DODPC is polymerized with water-insoluble azobis-(isobutyronitrile) (AIBN) and water-soluble azobis(2-amidinopropane)dihydrochloride (AAPD), the final polymerization conversion reaches 49% and 56%, respectively.[6] In addition, the simultaneous use of both radical initiators provides a 93% polymerization conversion. These phenomena are explained by different packing of diene-groups of 1- and 2-acyl chains in the bilayer membrane. Polymerization of diene-groups in 1-acyl chains is selectively initiated by AIBN because they are in a hydrophobic region while the polymerization of 2-acyl chain is achieved by AAPD because those in 2-acyl chains face the outer aqueous phase.[6,7] This selectivity is supported by the result that 1-palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (POPC) which has a diene-group in a 2-acyl chain is

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completely polymerized with AAPD but not with AIBN.[8] The selectivity of the polymerization is of course influenced by the lipid packing, the phase state, and so on.[9,10] The selectively polymerized phospholipids give linear polymers in which diene groups exist in the other acyl chains. Reconstruction of polymeric liposomes is realized with excellent solubility of these polymers in organic solvents. Furthermore, more stable liposomes can be obtained by the postpolymerization (crosslinking) of remaining diene groups of reconstructed liposomes.[11] Besides the selectivity of the polymerization, the stability of polymerized liposomes also depends on the polymerization methods. Namely, the liposomes polymerized with radical initiators are more stable than those obtained by UV-irradiated polymerization.[6] Therefore, analysis of molecular weights of resulting polymers should be useful to evaluate the stability of polymerized liposomes as well as release measurements of encapsulated molecules.[12-15]

In this paper, the results of average molecular weight of DODPC or POPC polymers are used to clarify the differences of polymerization methods, between UV-irradiated polymerization and AAPD-initiated polymerization, as well as to investigate the effects of factors such as average radius, temperature, and composition.

EXPERIMENTAL

Materials

1,2-Bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) was purchased from Nippon Oil & Fats Co., Ltd. 1-Palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (POPC) was a gift from Toyo Soda Co., Ltd. The purity of these lipids was confirmed by thin-layer chromatography (Merck, silica gel plates) with chloroform–methanol–water (65:35:5 by vol.) as the solvent. Samples showing a single spot with an *Rf* value of around 0.3 were used for the experiments.[6] Azobis(2-amidinopropane)dihydrochloride (AAPD) was purchased from Wako Pure Chemical Industries, Ltd. and purified by recrystallization twice from distilled water.

METHODS

Preparation of Liposomes with Various Sizes

A total of 0.20 g of the polymerizable lipid was dissolved in dehydrated chloroform and slowly evaporated in a rotating sample tube to prepare a thin film on the inner surface of the tube. Then, 20 ml of distilled water were added. Small unilamellar liposomes with an average radius of about 14 nm were prepared by sonication (Tip type, Tomy Seiko UR-200P) at 60 W for 20 min under a nitrogen atmosphere.[16] Freshly prepared liposomes were stored at 25°C (above the gel-to-liquid crystalline phase transition temperature (T_c) of DODPC) to prevent liposomal fusion.[10] On the other hand, large unilamellar liposomes with an average radius of 43 nm were prepared with an Extruder. Multilamellar liposomes prepared by mechanical mixing of the thin film with glass beads were passed five times through polycarbonate Nuclepore filters (pore size: 0.4 and then 0.2 μm) with Extruder.[9] The average radius of the obtained liposomes was confirmed by dynamic light scattering particle size analyzer (Coulter N4SD).

Polymerization with Water-Soluble Radical Initiator (AAPD)

AAPD (5.0 mol% to the polymerizable lipids) was added directly to a liposome suspension to avoid decomposition by sonication. To introduce AAPD molecules into the internal aqueous phase, the suspension was slightly sonicated.[11] Polymerization was carried out at 60°C under a nitrogen atmosphere. Polymerization conversion was successively analyzed by monitoring the decrease in absorption intensity at 255 nm attributed to the diene groups of the polymerizable lipids.[16]

UV-Irradiated Polymerization

Liposome suspensions (1.0 wt%) containing no radical initiator were put into quartz tubes (1 cmϕ) and sealed under a nitrogen atmosphere. UV-irradiated polymerization was carried out with a low-pressure mercury lamp (Riko UVL-32W) in a thermostated bath with a distance of 3 cm from the UV-light source.[16]

Photosensitized Polymerization with AAPD

Liposome suspensions containing AAPD (5.0 mol% to the polymerizable lipids) were put into Pyrex tubes (5 mmϕ) under a nitrogen atmosphere and fixed in a thermostated bath with a distance of 5 cm from the UV-light source (high-pressure mercury lamp: Riko UVL-100P). The UV-light with wavelength shorter than 360 nm was cut with an UV-filter (Hoya L-38) to avoid UV-irradiated polymerization of POPC.[11]

Methanolysis of Phospholipid Polymers[12]

Polymerized and lyophilized liposomes (200 mg) were dissolved in absolute methanol (50 ml). Acetyl chloride (5 ml) was added to the solution. The solution was refluxed for 3 days in an oil bath at 100°C. The precipitation was dissolved in chloroform and washed with pure water. At this stage, a part of the solution, which contained oligomers and/or monomers other than polymers was sampled for gel permeation chromatography (GPC). The other solution was pored into cold methanol to isolate the polymer component by precipitation. The precipitate was washed with cold methanol and subjected to gel permeation chromatography.

Molecular Weight Distribution of Methanolyzed Lipid Polymers

Methanolyzed lipid polymers were dissolved in tetrahydrofuran. Gel permeation chromatography (Water Associates Co., Ltd.) using a differential refractive index detector was conducted with a combination of 10^4, 10^3, 500 and 100 A μ-Styragel connected in series. Molecular weight of polystyrene standards (Toyo Soda Co. Ltd.), 1.3×10^6, 4.3×10^5, 1.9×10^5, 4.2×10^4, 1.0×10^4, 4.0×10^3, 9.6×10^2, and 5.4×10^2 were used.

Leakage of Fluorescent Probes

Since 5(6)-carboxyfluorescein* (CF) interfered with the polymerization of lipid liposomes, CF molecules were introduced into an internal aqueous phase of liposomes after polymerization.[17] CF (0.1 mol l^{-1}) was mixed with the polymerized liposomes and incubated for 100 h at 60°C.[17] A CF-containing liposome suspension was subjected to gel permeation chromatography (Sepharose CL-4B column, 30 mmϕ, 600 mm h) to expel CF molecules dissolved in an exogenous aqueous phase. At this concentration, CF was self-quenched in the internal aqueous phase of the liposomes. The leaked CF was detected as an increase in fluorescence intensity at 520 nm by fluorescence spectrometer (JASCO FP-550) with an excitation at 330 nm. Liposomes were disrupted to release 100% of CF by sonication in the presence of Triton X-100.

RESULTS AND DISCUSSION

Effect of Polymerization Method on Molecular Weight Distribution

1,2-Bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) was polymerized by two different methods, UV-irradiated polymerization and polymerization with radical initiators. Since DODPC has polymerizable groups in both 1- and 2-acyl chains, the simultaneous polymerization of both acyl chains by UV-irradiation provides crosslinking of lipid polymers.[6,9] Against this, lyophilized poly-DODPC liposomes are easily soluble in methanol because polymerized 1- and 2-acyl chains are separated each other by methanoly-

* 5-Carboxyfluorescein containing 6-carboxy isomer.

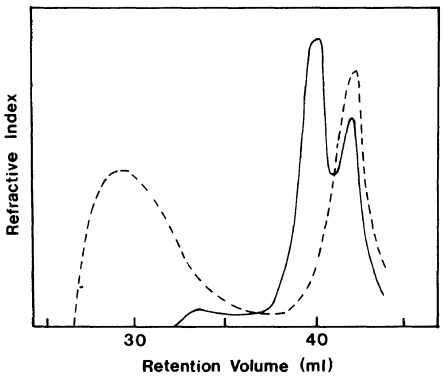

Figure 1. Effects of polymerization methods on the molecular weight distribution of methanolyzed DODPC polymers. DODPC molecules were polymerized as liposomes with UV-irradiation (—) or with AAPD (---), and then methanolyzed.

sis, and there is no crosslinkage.[12] After the complete replacement with THF, the molecular weight distribution of the solute shows three typical peaks as shown in Figure 1. The peak seen in the largest retention volume is the same as that in the methanolyzed monomeric DODPC solution. The middle and last peaks are observed in the case of a polymerized system. They are attributed to oligomers and polymers, respectively. The last peak is very small in comparison with the other two peaks. This shows that UV-irradiated polymerization is not an effective method to give high-molecular weight polymers in spite of complete disappearance of diene groups. The weight-average molecular weight of poly-DODPC obtained by UV-irradiated polymerization was analyzed with VPO method and calculated to be 4.5×10^3.[6] It should correspond mainly to the molecular weight of oligomers (degree of polymerization is about 6). On the other hand, in the case of radical polymerization by AAPD, the methanolyzed DODPC polymers show two large fractions. One can be attributed to monomeric component. Since AAPD radical fragments attack diene-groups only in the 2-acyl chains of DODPC,[6] the diene-groups in the 1-acyl chains should remain unreacted. Therefore, the unpolymerized 1-acyl chains

Table I. Average molecular weight of methanolyzed poly-DODPC polymerized as liposomes

Polymn. method	$\bar{M}_n$	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$
UV-irradiation	5.4×10^3	6.9×10^3	1.2
AAPD	1.7×10^4	3.9×10^4	2.3

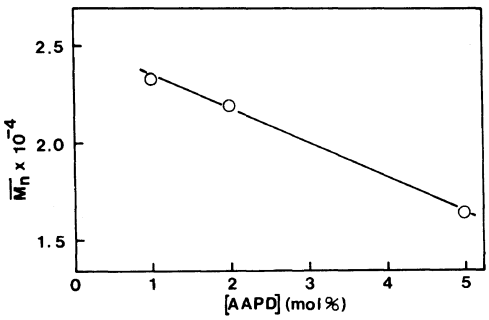

Figure 2. Effects of AAPD concentration on number-average molecular weight of methanolyzed poly-PODPC.

result in the monomer fraction after methanolysis. The other peak represents phospholipid polymers derived from polymerized 2-acyl chains. The average molecular weight of polymer fraction prepared by UV-light or by AAPD is calculated from calibration curve of polystyrene standard samples (Table I), though application of this method to such comb-like polymers is not suitable. The weight-average molecular weight ($\bar{M}_w$) of polymers obtained from UV-irradiated polymerization is 6.9×10^3, considerably smaller than that from radical initiated polymerization ($\bar{M}_w = 3.9 \times 10^4$). This supports our previous results that the UV-irradiated polymerization could not provide enough stability, while polymerization with radical initiators could.

The molecular weight of polymers is generally regulated by the amount of radical initiators. Figure 2 indicates that the decrease in molar ratio of radical initiators to DODPC certainly gives polymers with larger moelcular weight. However, it influences more strictly the rate of polymerization. For example, the number-average molecular weight of polymers obtained

with 1 mol% of AAPD is 1.4 times that with 5 mol% of AAPD, while it takes 3 times longer to reach 50% polymerization conversion (not shown). It is concluded for the polymerization of lipods that lowering the concentration of radical initiators is not a convenient method to get higher molcular weight of polymers.

In the case of UV-irradiated polymerization, polymerization is initiated directly by the activation of diene-groups of the lipids. There is no effect of monomer concentration on the molecular weight of phospholipid polymers because polymerization is carried out on the bilayer membrane where monomer concentration is always constant at a constant liposomal size. However, at high concentration of liposomes, excess scattering (turbidity) of the solution actually prevents UV-irradiated polymerization. UV-irradiation generates locally concentrated lipid radicals.[18] These radicals would take several fates other than being lipid polymers.[13] Namely, some lipid radicals would be converted to photoproducts having no diene groups but showing similar molecular weights to monomer lipids as seen in Figure 1. Some would contribute to the formation of oligomers.

The concentration of lipid radicals can be controlled by change of the distance from UV-light source to quartz vessel. Molecular weights of lipid polymers should increase with decreasing radical concentration, *i.e.*, increasing the distance from the vessel to the light source. There is no difference in the retention volume of three molecular weight distribution peaks, but the intensity ratio of the polymeric to the ligomeric peak increases with distance as shown in Figure 3. Therefore, the average molecular weight surely depends on radical concentration, namely it increases with distance. However, the existence of two distinct peaks, *i.e.*, for polymers and oligomers, and no change in retention volume with changing radical concentration still remain unclear.

Fendler *et al.* used a laser pulse technique for UV-irradiated polymerization to get longer

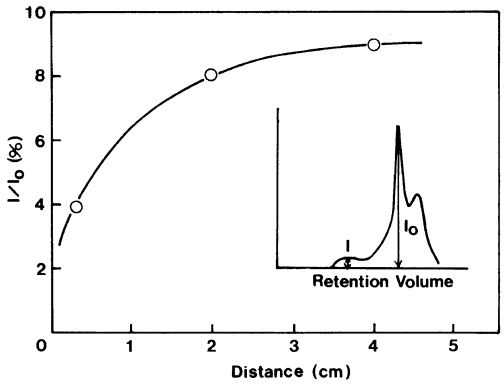

Figure 3. Relationship between I/I_0 and distance from UV-light source. I and I_0 were indicated in the insertion.

lipid polymers.[19] This technique is effective to give a high degree of polymerization because the interval between pulses for generating radicals is much longer than the period for complete transfer reaction of radicals. The number of radicals by one pulse would be controlled by changing the intensity or width of a laser pulse. Under a suitable condition, polymerization by UV-irradiation should give longer molecular weight of lipid polymers.

The degree of polymerization is also a function of the kind of polymerizable lipids. We found that polymerized liposomes with high molecular weight could be obtained even by UV-irradiated polymerization in the case of a polymerizable lipid that had a styryl group at the end of an acyl chain.[12] Prolonged irradiation, however, led to degradation of the lipod polymers. In the polymerization of this lipid with water-insoluble AIBN, a high degree of polymerization was also obtained without any degradation of polymers (data not shown). Bolikal and Regen pointed out that methacryloyl type poly-lipids obtained by UV-irradiation showed lower molecular weights than those from AIBN-induced polymerization, due to the photodegradation of polymers by UV-light.[17]

Effect of Lipid Packing

The molecular weight distribution of lipid

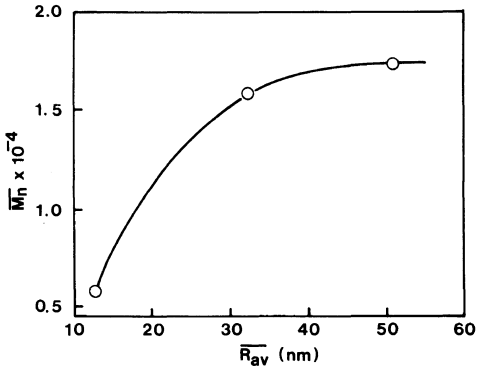

Figure 4. Effects of average radius ($\bar{R}_{av}$) of liposomes on the number-average molecular weight of methanolyzed poly-POPC. POPC liposomes were polymerized with AAPD at 60°C.

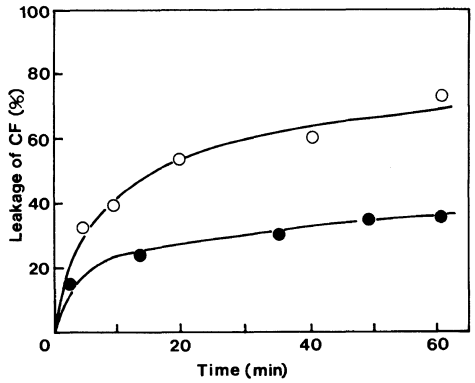

Figure 5. Stability of polymerized liposomes whose average radius was 14 nm (○) or 43 nm (●). Liposomes were polymerized with AIBN at 60°C.

polymers should also depend on the lipid packing of liposomes. Lipid packing can be easily controlled by changing the size of liposomes. We prepared liposomes with various size distributions through fusion of small unilamellar liposomes by treating them below the gel-to-liquid crystalline phase transition temperature.[10] DODPC is not suitable for this experiment because small DODPC liposomes allow unselective polymerization of diene groups in 1- and 2-acyl chains due to the disordered lipid packings.[10] It results in crosslinking which makes the lipid polymers insoluble in methanol. Therefore, POPC, which has a diene-group in only 2-acyl chain, was used for this experiment. Figure 4 shows the dependence of liposomal size on the number-average molecular weight of polymers prepared with AAPD at 60°C. The average molecular weight increases when the average radius of liposomes increases from 14 nm to 30 nm. There is no size dependence on the molecular weight of the polymers for liposomes whose average radius is more than 30 nm.[10] Small unilamellar vesicles (SUV) are well-known to show size dependence.[20-22] For example, with increasing size, the gel-to-liquid crystalline phase transition temperature (T_c) shift to higher temperature and cooperativity of phase transition is larger.[20] These are explained by the improvement of lipid packing with increasing size. In the case of large unilamellar liposomes (LUV), since the change of size does not cause the change of molecular packing, the phase transition temperature shows no size dependence.[20] The size dependence of the molecular weight distribution of methanolyzed POPC polymers, therefore, corresponds well with that of the lipid packing of liposomes. Since molecular packings in an inner- and an outer-layer of small unilamellar liposomes are differently constrained because of their large curvature, cracks and defects should therefore be formed in accordance with progress of polymerization to maintain their high curvature. Then polymerization should terminate at these defects, followed by radical transfer to such as water molecules. Figure 5 shows the leakage of CF from large or small DODPC liposomes polymerized with AAPD. The release rate from large unilamellar liposomes is restricted by polymerization. However, in small unilamellar liposomes, significant release occurs because of more disordered packing of shorter lipid polymers in small unilamellar liposomes.

Effect of Polymerization Temperature

Since photosensitized polymerization with AAPD could be initiated regardless of

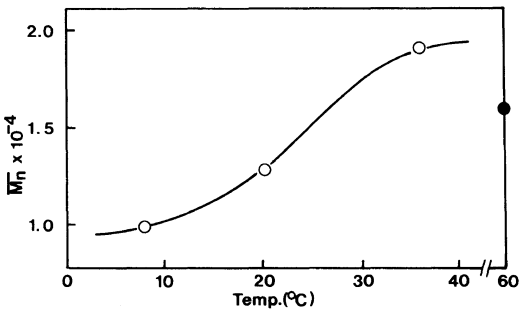

Figure 6. Effects of polymerization temperature on number-average molecular weight of methanolyzed poly-POPC. POPC liposomes were polymerized with photosensitized AAPD radicals.

temperature, the polymerization of POPC liposomes was carried out at temperatures below or above the gel-to-liquid crystalline phase transition temperature ($T_c = 28°C$).[11,23] Figure 6 shows the temperature dependence of number-average molecular weight of the POPC polymers. Since AAPD starts to decompose thermally above 40°C, it is quite difficult to evaluate the temperature dependence of polymerization at higher the temperature at the same radical concentration. The number-average molecular weight of polymers, polymerized with AAPD at 60°C without UV-irradiation, is shown as a closed circle in Figure 6. When the temperature was elevated above T_c, the molecular weight of the obtained lipid polymers was about twice that obtained below the T_c. Two reasons can be presented for the higher molecular weight of polymers obtained above T_c. One relates to conformational change at polymerization. Polymerization of 2,4-diene containing lipids should propagate *via* coupling at 2- and 5-position of carbon between diene groups on neighboring acyl chains at the same position.[24] Coupling at 2- and 5-C between 1- and 2-acyl chains in the same lipid (intra-polymerization) is, however, impossible because of structural mismatching attributed to the location of glycerol groups. The propagation of polymers should be achieved followed by significant conformational change of the acyl chains. The liquid crystalline state is favorable for this type of polymerization because the high segmental motion increases the possibility for diene groups or radicals to take suitable conformation for polymerization. The other reason is related to the lipid packing of liposomes. Since th packing of acyl chains in the lipid polymer is different from that of acyl chains of monomeric lipid, polymerization simultaneously would produce cracks at the interface between polymers and polymers or between polymers and monomers. These cracks become large as polymerization proceeds, causing the suppression of further propagation of polymers. In the liquid crysalline state, higher lateral diffusion of monomeric lipids and polymers would provide more chances to meet monomeric lipids with propagating chain ends. Polymerization of diene-containing lipid liposomes accompanies conformational and/or packing change. Polymerization at a temprature above the T_c is therefore favorable to obtain higher-molecular-weight polymers.

CONCLUSIONS

Polymerization of phospholipids which contain 2,4-diene groups is influenced by polymerization methods. High-molecular-weight of polymers is obtained with radical initiators but not with UV-light. This would be due to the difference of initiation mechanism, namely difference in the amount of radicals in the initial stage. Furthermore, the molecular weight is influenced by lipid packing or mobility. Larger molecular weight is obtained when polymerization is performed either for larger liposomes or at temperatures above the gel-to-liquid crystalline phase transition temperature. The molecular weight of polymerization is concluded to depend on the assembling state of lipid molecules besides general polymerization conditions such as concentrations of the initiated radicals.

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