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Polymerization of Diene-Containing Lipids as Liposomes by Radical Initiators. 4.[1] Effect of Lipid Packing on the Polymerization Profile

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ABSTRACT: 1,2-Di(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) was polymerized as liposomes with a water-soluble radical initiator, azobis(2-amidinopropane) dihydrochloride (AAPD). DODPC liposomes were prepared with a tip-type sonicator and incubated at 4, 8, or 20 °C. When DODPC liposomes were incubated at lower temperatures than the gel-to-liquid crystalline phase transition temperature (16 °C for DODPC liposomes), these liposomes fused with each other to produce large unilamellar liposomes. An average radius was calculated from the ^{1}H NMR signal intensity ratio for the choline methyl protons split by Eu^{3+}. Generally AAPD initiated radical polymerization of diene groups on the 2-acyl chains of DODPC because of nonequivalent acyl chain packing in bilayer membranes. The polymerization conversion for larger DODPC liposomes reached about 50% by AAPD-initiated polymerization. An excess polymerization was initiated by AAPD when liposomes were not incubated unless the average radius was smaller than 30 nm. This is explained by the disordered lipid packing for small liposomes. This disordered lipid packing permits invasion of water molecules deeper into the hydrophobic region of the outer half of the bilayer membrane of the liposomes, attributed to a larger curvature. For small DODPC liposomes the AAPD radicals could therefore reach diene groups in even 1-acyl chains which were essentially not attacked by AAPD radicals from an aqueous phase as long as lipids were well oriented. The increase of polymerization conversion may be due to an entropically semistable lipid packing inevitable for smaller liposomes. It is concluded that the well-defined selective polymerization of diene groups in 2-acyl chains of DODPC lipids requires liposomes larger in size than with a 30-nm radius.

Introduction

Phospholipid bilayer liposomes are widely applied as microcapsules for drugs or functional molecules as well as models for biomembranes. These liposomes, however, are generally not stable and undergo aggregation and fusion. To stabilize these assembled structures, polymerizable amphiphiles have been incorporated as a major component to construct stable membrane structures by polymerization. In our previous papers, unilamellar liposomes composed of 1,2-di(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC), which contained diene groups in both acyl chains were polymerized by radical initiators.[2,3] Either water-insoluble azobis(isobutyronitrile) (AIBN) or water-soluble azobis(2-amidinopropane) dihydrochloride (AAPD) provided polymerization conversion of around 50% for this lipid. A simultaneous polymerization by these radical initiators resulted in complete polymerization, and the resulting polymerized DODPC liposomes showed excellent stability against physical or chemical stimuli such as sonication or detergent attack.[3] However, this monomeric lipid had polymerizable diene groups in the same position (2,4-diene) in two acyl chains, and it appeared very likely that these acyl chains showed nonequivalent reactivities against water-soluble or -insoluble radical initiators.[3] Polymerization of diene groups bound to the 1-acyl chains was initiated by the addition of AIBN. On the other hand, the diene groups on the 2-acyl chains were believed to face an aqueous phase and were polymerized by the addition of water-soluble radical initiators. This was confirmed by the same polymerization experiments with a monodiene-type polymerizable lipid, 1-palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine which possessed one diene group only in the 2-acyl chain. It was clearly demonstrated that the polymerization of diene groups in the 2-acyl chains was initiated only by water-soluble radical initiators.[4] All of the results strongly support the possibility of a selective polymerization of polymerizable amphiphiles in an assembled structure. This selective polymerization, however, was not so clear for very small DODPC liposomes. This suggested that the radical polymerization of these systems should be affected by the lipid packing in the liposomes. This study was therefore intended to elucidate the effect of lipid packing in liposomes on their polymerization profiles.

Experimental Section

Materials. 1,2-Di(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) was purchased form Nippon Oil & Fats Co., Ltd. This was characterized by thin-layer chromatography (Merck, silica gel plates) with chloroform/methanol/water (65/35/5, by vol) as eluant before use.[3] A polymerizable lipid which showed a single spot on the TLC plate was used without purification. Azobis(2-amidinopropane) dihydrochloride (AAPD) was purchased from Tokyo Kasei Co. Ltd. and was purified by recrystallization twice from water.

Methods. A total of 0.200 g of lipids was dissolved in dry chloroform and was slowly evaporated in a rotating sample tube to prepare a thin lipid film on the inner surface of the tube. Twenty milliliters of degassed distilled water was then added to the tube. The liposome suspension (1.0 wt %) was prepared with a tip-type sonicator (Tomy Seiko UR-200P) at 60 W for 10 min under a nitrogen atmosphere. Freshly prepared liposome suspensions were sealed and incubated at 4, 8, or 20 °C to induce liposomal fusion and to analyze the effect of molecular packing in liposomes upon polymerization. AAPD (3.5 mg; 5 mol % to

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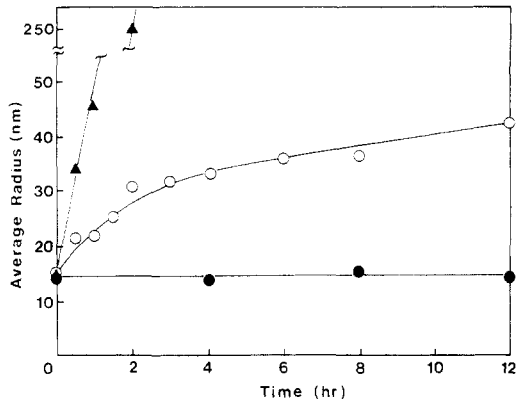

Figure 1. Effect of incubation time and temperature on the fusion of DODPC liposomes: (▲) 4 °C; (○) 8 °C; (●) 20 °C. Average radius was calculated from the intensity ratio of signal for outer- to that for inner-facing choline methyl protons split by the addition of Eu^{3+}.

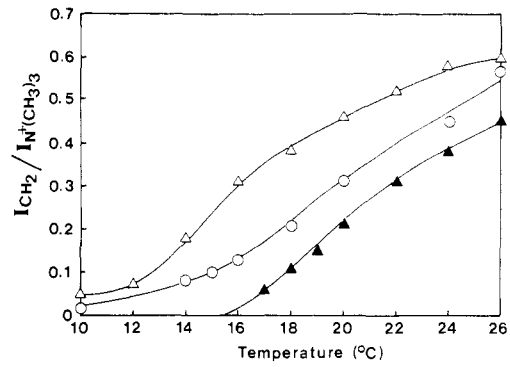

Figure 2. Temperature dependence of ^1H NMR intensity ratio of hydrophobic methylene signal to choline methyl signal for DODPC liposomes incubated under different condition: (△) freshly prepared by sonication (average radius = 14 nm); (○) incubated at 8 °C for 12 h (average radius = 42 nm); (▲) incubated at 4 °C for 12 h (average radius = 250 nm).

the monomeric lipids) was added to the liposome suspensions just before the polymerization. The DODPC liposomes were polymerized for 12 h at 60 °C. The liposome suspensions containing different amounts of AAPD were also prepared in the same manner to analyze the effect of concentration of AAPD on the polymerization conversion.

A liposome suspension was periodically pipetted from the sample tube during polymerization. A diluted sample solution was analyzed by UV spectrometry to quantitatively analyze the polymerization conversion.[2,3] The UV absorption at 255 nm, corresponding to the diene groups, was successively analyzed to determine the polymerization conversion for DODPC. Liposome suspensions were also prepared with D_2O instead of H_2O in the same manner as mentioned above and were put into 5-mm NMR tubes. FT-NMR spectrometry (JEOL FX-90Q) was carried out to characterize the effect of incubation on the phase transition behavior and segmental motion of hydrophobic acyl chains of DODPC lipids in the liposomes. $Eu(NO_3)_3 \cdot 6D_2O$ was added to the DODPC liposome suspension to a final concentration of 4.0 mM to shift the outerfacing choline methyl proton signals to the higher magnetic field.[5] The average radius of liposomes could be calculated by the signal intensity ratio of these choline methyl protons in the outer- and inner-facing lipid bilayer aqueous phase, employing a simple equation.[6]

Results and Discussion

To analyze the effect of liposome size on radical polymerization, it is necessary to prepare liposomes with different average radii. First, the general results of the fusion phenomenon of the DODPC liposomes were obtained by ^1H NMR spectrometry. Liposomes freshly prepared by sonication tend to fuse with each other because of their semistable state.[7] DODPC liposomes were prepared in D_2O and incubated at a different temperature. A shift reagent, Eu^{3+} was added to the suspension and the average radius of the liposomes was determined from the signal ratio for choline methyl protons facing toward the outer aqueous phase to those facing inward, according to a previously published calculation.[6] Figure 1 shows the increase of the average radius of DODPC liposomes as a result of liposomal fusion. The liposomal fusion was induced by incubation at temperatures under the gel-to-liquid crystalline phase transition temperature (T_c).[6,7] As the T_c of DODPC is 16 °C, incubation at 4 °C induced very fast liposomal fusion, as shown in Figure 1 (▲), and quite large liposomes with not only unilamellar but also multilamellar structure were obtained. This liposomal fusion has already been reported by Thompson et al.[7,8] A simple radius calculation from the ^1H NMR intensity ratio was no longer valid for these multilamellar systems because this calculation is effective only for unilamellar liposomes. As the fusion velocity was very fast when these were incubated at 4 °C, it was not possible to control the liposome radius through fusion. This process also produced a relatively broad size distribution. On the other hand, the average radius of DODPC liposomes increased from 15 to 42 nm upon incubation at 8 °C for 12 h, as shown in Figure 1. This suggests that the incubation at 8 °C is effective in controlling liposome size. In contrast, incubation at 20 °C, which is slightly higher than T_c, provided no fusion and the average radius was almost the same as that for freshly prepared liposomes.

There are several reports on the effect of liposome size on lipid packing. Lipid packing affects the cooperative phase transition behavior of lipids in a bilayer membrane structure. Toyoshima and his co-workers have already observed the effect of average size on the phase transition behavior of dipalmitoyl phosphatidylcholine liposomes using fluorescence measurements.[9] Structural disorder for freshly prepared small liposomes was also suggested by DSC measurements. Freshly prepared liposomes showed less cooperative phase transition in DSC diagrams, suggesting disordered membrane structure.[7] On the other hand, large multilamellar DODPC liposomes showed a cooperative phase transition at 21 °C by DSC measurement.[10] The appearance of a distinct phase transition is generally accepted as a proof of ordered lipid packing and cooperative phase transition. DSC measurements showed phase transition behavior during a temperature scan but the results provide little information concerning the extent of the phase transition. Therefore, ^1H NMR spectrometry was also applied to analyze the phase transition behavior. The main phase transition temperature of large DODPC liposomes was found to be 16 °C by ^1H NMR spectrometry. DODPC liposome suspensions were incubated for 12 h at 4, 8, or 20 °C, and ^1H NMR spectra were recorded at different temperatures ranging from 10 to 30 °C. Above the main phase transition temperature, these incubated liposomes showed signals attributed to hydrophobic methylene and terminal methyl protons of acyl chains at 1.28 and 0.85 ppm, respectively. Liposome size, i.e., the lipid packing, affected the phase transition behavior of DODPC liposomes. Figure 2 shows the ratio of the signal intensity for the methylene protons to that for the hydrophilic choline methyl protons (3.20 ppm) at different temperatures for liposomes with different average radii. As the proton signals for hydrophilic choline methyl groups also show temperature dependence, the signal ratio for protons of hydrophobic and those of hydrophilic groups is useful for detecting phase behavior in liposomes. Larger

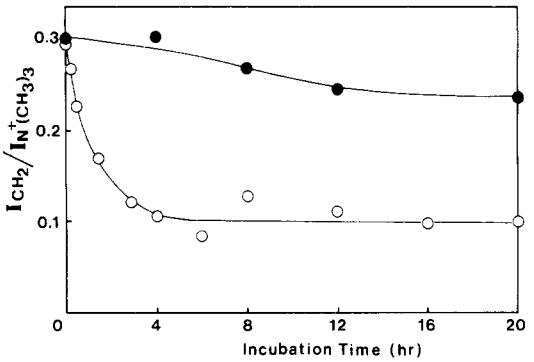

Figure 3. Effect of incubation time and temperature on the intensity ratio of hydrophobic methylene proton signal to choline methyl proton signal at 16 °C: (○) incubated at 8 °C for 12 h (average radius = 42 nm); (●) incubated at 20 °C for 12 h (average radius = 14 nm).

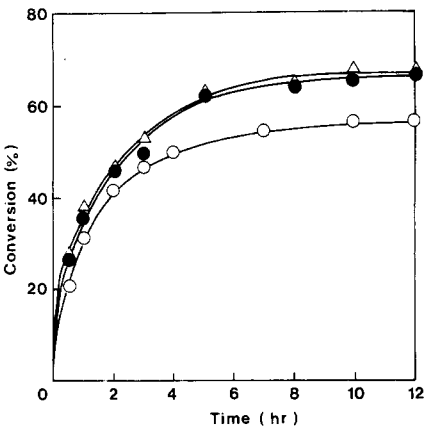

Figure 4. Polymerization conversion of DODPC liposomes initiated by AAPD of 5.0 mol % to the DODPC lipids: (△) freshly prepared by sonication (average radius = 14 nm); (●) incubated at 20 °C for 12 h (average radius = 14 nm); (○) incubated at 8 °C for 12 h (average radius = 42 nm).

signal ratio means higher segmental motion of the acyl chains. Segmental motion of hydrophobic acyl chains of large DODPC liposomes, provided by the incubation at 4 °C, starts increasing at 16 °C ($=T_c$). On the other hand, freshly prepared DODPC liposomes having an average radius of 14 nm show ^1H NMR signals assigned to hydrophobic methylene and terminal methyl protons even below 16 °C. The lipid packing in such small liposomes is believed to be considerably distorted due to their large curvature and to allow increased segmental motion of the acyl chains. Incubation at 8 °C for 12 h provided liposomes with an average radius of 42 nm, and the phase transition behavior of these liposomes was found to be between that of freshly prepared liposomes and those incubated at 4 °C for 12 h. Since the difference in the intensity ratio between fresh and incubated liposomes at 8 °C reached a maximum at around 16 °C, the ^1H NMR measurements were taken at 16 °C to ensure maximum effect of incubation on lipid packing.

Figure 3 shows the effect of incubation time on the ratio of signal intensity of hydrophobic methylene protons to that of choline methyl protons of DODPC liposomes at 16 °C. Incubation at 20 °C effects slightly the ratio of signal intensity of the hydrophobic methylene protons to that of the choline methyl protons of DODPC liposomes. Liposomes freshly prepared by sonication have transient irregular lipid packing, and this transient irregular lipid packing may be corrected without changing the average radius by incubation at 20 °C. On the other hand, the signal intensity ratio changed considerably with incubation at 8 °C. The lipid packing is known to depend essentially on the size of the liposomes.[11] It has already been shown that incubation at a temperature lower than T_c induces liposomal fusion, and reduction of the signal intensity ratio is therefore attributed to improved lipid packing.[6-8]

In the next stage, we examined the effect of lipid packing on the polymerization conversion of polymerizable lipids as liposomes. DODPC liposomes were polymerized by AAPD radicals which were generated from an aqueous phase at 60 °C. The liposome suspension was periodically pipetted from a sealed sample tube during polymerization, and accurately diluted samples were analyzed with UV spectrometry to quantitatively analyze the polymerization conversion. The spectral change at 255 nm was followed to determine the polymerization conversion for DODPC. Polymerization of DODPC liposomes which were fused by incubation at 8 °C for 12 h was completed by heating at 60 °C for 12 h and the conversion reached a constant value (56%), as shown in Figure 4. Heat treatment at 60 °C without a radical initiator gave about 8% polymerization conversion after 12 h. This means that the diene groups in the 2-acyl chains of large DODPC liposomes were polymerized selectively. On the other hand, freshly prepared small DODPC liposomes were polymerized by AAPD radicals and the polymerization conversion reached about 70%. For small DODPC liposomes, AAPD radicals can attack not only diene group in 2-acyl chains but also some of diene groups in 1-acyl chains of DODPC lipids in the outer half of the bilayer membranes. This nonselective polymerization should occur only for the outer half of the membrane and never for the inner half because the unusual contact of water molecules with diene groups in the 1-acyl chains of outer-facing lipids is induced by the large positive curvature. For the inner half of the bilayer, head groups were densely packed and little contact of water molecules with the diene groups in the 1-acyl chains should occur. Incubation at 8 °C for 12 h provided the DODPC liposomes with an average radius of 42 nm (see Figure 1). Polymerization conversion was reduced to 56% by incubation at 8 °C but not at 20 °C, as seen in Figure 4. As mentioned above, the transient irregular lipid packing was corrected by incubation at 20 °C without changing the average radius of the liposomes. These liposomes, incubated at 20 °C, still show excess polymerization, suggesting that the transient irregular lipid packing is not a major factor in inducing excess polymerization. This is believed to be attributed to the small size of the liposomes, i.e., large curvature. Figure 3 suggests that the lipid packing evolved into a more favorable form and that a cooperative phase transition was therefore seen in the DODPC liposomes by incubation at 8 °C for more than 5 h. Thus there is almost no difference in the radical attack from the aqueous phase between DODPC liposomes with an average radius of 35 nm and those of 42 nm or more.

It is necessary to check the effect of concentration of radical initiators on the polymerization conversion.[1] Figure 5 shows the relationship between polymerization conversion of DODPC liposomes and the molar ratio of AAPD and DODPC for liposomes incubated at 8 °C for 12 h. About 1 mol % AAPD is required to polymerize almost all of the diene groups in the 2-acyl chains of DODPC lipids as liposomes. The polymerization conversion slightly increased with the amount of AAPD, and reached a constant value (56%) at 3 mol %, as shown in Figure 5. Taking the contribution of thermal initiated polymerization (8%) into account, selective polymerization was carried out by the addition of AAPD. Little AAPD concentration dependence on the polymerization conversion could be observed. The DODPC liposomes freshly prepared by

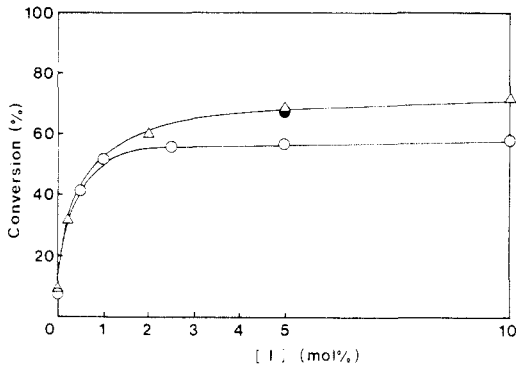

Figure 5. Effect of AAPD concentration on the polymerization conversion of DODPC as liposomes. Polymerization was taken under nitrogen atmosphere for 12 h at 60 °C: (△) freshly prepared by sonication (average radius = 14 nm); (●) incubated at 20 °C for 12 h (average radius = 14 nm); (○) incubated at 8 °C for 12 h (average radius = 42 nm).

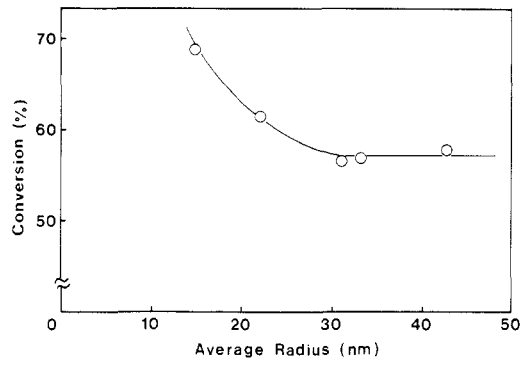

Figure 6. Relationship between average radius of the DODPC liposomes and observed polymerization conversion initiated by the AAPD at 60 °C for 12 h; [AAPD] = 5 mol % to the DODPC lipids.

sonication always show a higher polymerization conversion than the incubated ones, as shown in Figure 4 (△). Small DODPC liposomes, incubated at 20 °C for 12 h, also show higher polymerization conversion as shown in Figure 4 (●). This increase in polymerization conversion was believed to be due to the disordered lipid packing in the small liposomes.

Third, the effect of average radius for relatively small DODPC liposomes on the polymerization profile, especially on the polymerization conversion, was analyzed. DODPC liposome suspensions were prepared by sonication and incubated at 8 °C. Increase in the radius was produced by incubation at temperatures lower than T_c, for example at 8 °C, as shown in Figure 1. Sample suspensions were periodically pipetted and polymerized in the same manner as mentioned above. Figure 6 shows the relationship between an average radius of DODPC liposomes and polymerization conversion after reaction at 60 °C for 12 h. The point to be analyzed is the effect of curvature of liposomes with average radius ranging from 15 to 35 nm on the polymerization. AAPD radicals initiated radical polymerization of diene groups not only in the 2-acyl chains but also in the 1-acyl chains of lipids facing the outer aqueous phase partially. The lipids in the inner half of the lipid bilayer of the liposomes were tightly packed and no AAPD radical was able to invade and to initiate radical polymerization of diene groups in the 1-acyl chains. The overall polymerization conversion reached about 70%; i.e., all of the diene groups in the 2-acyl chains and some of those in the 1-acyl chains were polymerized. As a ratio of the numbers of lipids in the outer and inner half of lipid bilayer membrane is about 2 for small liposomes with average radius of 15 nm,[6] this excess conversion (70%) corresponds to the polymerization of about 60% of diene groups bound in 1-acyl chains of lipids facing the outer aqueous phase. The attack of AAPD radicals on the diene groups in 1-acyl chains was suppressed by the less constrained lipid packing in liposomes with larger average radius, as shown in Figure 6. It is clear that the average radius of more than 35 nm is necessary for the regulated polymerization of diene groups as liposomes when AAPD was used as the initiator as shown in Figure 6. That of 35 nm is also necessary to realize cooperative phase transition, as shown in Figure 3. It should be noted, however, that the polymerization of membrane components is an excellent method to stabilize the membrane structure, but the polymerization should be carried out under suitable conditions because it sometimes reflects the molecular packing of the component molecules. As liposome size is believed to affect polymerization, it is expected that the liposome size also influences, the average molecular weight of the lipid polymers, membrane stability, and so on. These questions will be reported on in the near future.

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Registry No. DODPC (homopolymer), 108916-62-7.

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