Polymerization of Diene-Containing Lipids as Liposomes by Radical Initiators. 6:[1] Polymerization of 1,3-Bis(2,4-octadecadienoyl)-*rac*-glycero-2-phosphorylcholine

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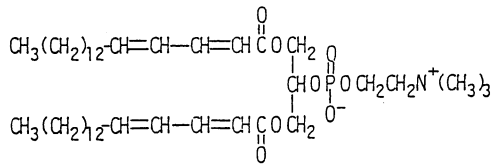
1,3-Bis(2,4-octadecadienoyl)-*rac*-glycero-2-phosphorylcholine (1,3-DPC), polymerizable lipid which contains diene groups in both 1- and 3-acyl chains, was polymerized as liposomes in an aqueous medium. Polymerization was initiated by water-insoluble azobis(isobutyronitrile) (AIBN) or water-soluble azobis(2-amidinopropane) dihydrochloride (AAPD). AIBN was mixed with the monomeric lipids and the mixture was dispersed in an aqueous medium by sonication to facilitate transfer of AIBN into monomeric lipid liposomes. On the other hand, AAPD was simply added to the liposome suspension. The 1,3-DPC liposomes were easily polymerized by the addition of either water-soluble AAPD or water-insoluble AIBN to give polymerization conversion of over 80% in both cases. No selective polymerization of diene groups in the different acyl chains was found in 1,3-DPC liposomes whereas the selective polymerization was clearly seen in 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC). Polymerized 1,3-DPC liposomes were not soluble in organic solvent suggesting the formation of crosslinked bilayer structure. The obtained results suggested that the diene groups in both 1- and 3-acyl chains were almost equivalent and located in the interface between an aqueous phase and hydrophobic region. Polymerized liposomes thus prepared were stable against heating, detergent attack and even methanol washing. Copolymerization of mixed liposomes composed of 1,3-DPC and DODPC (5 : 1) revealed that the AIBN or AAPD initiated polymerization conversion of DODPC reached about 68%. 1,3-DPC is considered to be radical propagation transfer between diene groups in 1- and 2-acyl chains of DODPC during polymerization.

Liposomes and the lipid assemblies have attracted attention as one of potent candidates for the basic materials for the coming molecular devices. However, they are generally not so stable and usually cause aggregation and fusion.[2,3] Polymerizable amphiphiles have been synthesized as major components to construct stable bilayer membrane.[4-13] In our previous paper,[14] small unilamellar liposomes composed of 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC), which contains one diene group in each acyl chain, were polymerized by radical initiators. It was revealed that the polymerization profile was significantly dependent on the characteristics of the applied radical initiators.[1] However this monomeric lipid had polymerizable groups in the same position (2,4-diene) in two acyl chains, polymerization of diene groups bound to the 1-acyl chains was selectively initiated by AIBN which was incorporated into a hydrophobic region of the bilayer membrane. On the contrary, the diene groups in the 2-acyl chains were polymerized by the addition of water-soluble radical initiators suggesting that the diene groups in 2-acyl chains faced an aqueous phase. Selective initiation of diene groups bound to 2-acyl chains by AAPD was clearly demonstrated by the model reaction using 1-palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (POPC).[15] The polymerization of this POPC was initiated only by the addition of AAPD and no effect was found when AIBN was added to them.[15] We reported the unique method to evaluate the chemical environment of polymerizable groups in the lipid liposomes with their polymerization manner initiated by different radical initiators.[14,16] In the present paper, polymerizable lipid containing one diene group in 1- and 3-acyl chains was polymerized as liposomes by two different radical initiators to clarify the chemical environment of diene groups and the effects of the molecular packing of polymerizable lipids on the polymerization profile as liposomes.

Experimental

1,3-Bis(2,4-octadecadienoyl)-*rac*-glycero-2-phosphorylcholine (1,3-DPC) was synthesized according to the method as reported previously.[17] The purity was confirmed by thin layer chromatography (Merck, silica gel). TLC plates were developed with chloroform/methanol/water (65 : 35 : 5 by vol) to show a single spot with R_f value of 0.3.



1,3-DPC

Azobis(isobutyronitrile) (AIBN) and azobis(2-amidinopropane) dihydrochloride (AAPD) were purchased from Tokyo Kasei Co. AIBN and AAPD were purified twice by recrystallization from methanol and water, respectively. Triton X-100, polyethylene glycol mono-*p*-octylphenyl ether, was purchased from Tokyo Kasei Co. Ltd. and used without further purification. Fluorescent grade 5(6)-carboxyfluorescein (CF) was purchased from Kodak Co. and used

without further purification. Chloroform, methanol, and water were distilled twice before use.

Preparation of Liposomes. A total of 0.200 g of monomeric amphiphiles was dissolved in dehydrated chloroform and was slowly evaporated in a rotated sample tube to prepare thin lipid film on the inner surface of the tube.[14,18] Distilled water was then added to the tube to set the lipid concentration 1.0 wt%. Small unilamellar liposome suspension was prepared by sonication (Tip-type Tomy Seiko UR-200P) at 60 W for 20 min under nitrogen atmosphere. The freshly prepared liposomes were incubated in a refrigerator to prepare larger unilamellar liposomes with an average diameter of 75 nm. This process minimizes the structural defects and irregular molecular packing.[19]

Polymerization of Liposomes Initiated by Organic Free Radicals. Free radical initiated polymerization of 1,3-DPC was carried out by a similar manner as mentioned previously.[14] A total of 0.200 g of 1,3-DPC was dissolved with 2.1 mg of AIBN (5.0 mol% to the monomeric lipids) in chloroform and slowly evaporated in a rotated sample tube. Degassed distilled water (20.0 ml) was added to the tube. Liposome suspension was prepared by sonication method as mentioned above. These liposomes were polymerized at 60 °C under nitrogen atmosphere. The polymerization conversion was successively analyzed by the decrease in the absorption intensity at 243 nm for diene groups.

On the other hand, AAPD was added to the liposome suspension to avoid the decomposition of AAPD by sonication.[15] Liposomes were polymerized at 60 °C for several hours under nitrogen atmosphere. AAPD was added to the liposomes which had already been polymerized by AIBN. These were then re-polymerized to check the possibility of selective polymerization.[14]

UV Spectroscopic Analysis of the Polymerization. A small amount of the liposome suspension was periodically withdrawn with a pipette from the sample tube during polymerization. Accurately diluted sample solution was analyzed by UV spectrometry to quantitatively determine the polymerization conversion. Spectral change at 243 nm corresponding to the diene groups of 1,3-DPC as liposomes in an aqueous solution was analyzed to determine the polymerization conversion of 1,3-DPC.

Transmission Electron Microscopy. Polymerized liposome suspension was dropped on copper grids, and they were stained with uranyl acetate. The dried sample grids were analyzed to confirm their bilayer structure by TEM (JEOL-100CX).

Light-Scattering Measurement. A liposome suspension (concentration; 0.100 g/100 cm^3) was placed in a round cell of 10 mm diameter. Methanol was added to the suspension and the scattered-light intensities (90°) were subsequently recorded with apparatus (Union Giken LS-601) at 25 °C to evaluate stability of the polymerized liposomes upon addition of organic solvent. He-Ne laser with wavelength of 632.8 nm was used as the light source.

Fluorescein Leakage. 0.100 mol·l^{-1} of CF was incorporated into an inner aqueous phase of the polymerized liposomes at 60 °C for 100 h.[20] Then CF-containing liposome suspension was passed through gel permeation chromatography column (Sepharose CL-4B 30 mm ϕ, 600 mm h) to expel an excess CF dissolved in an outer aqueous phase. The leakage of CF was determined as the increase of fluorescence intensity at 520 nm by fluorescence spectrometer (JASCO FP-550) with excitation beam at 330 nm. A 100% CF leakage was carried out by the sonication of CF containing liposomes with the existence of Triton X-100.

Results and Discussion

Polymerization and Stability of 1,3-DPC Liposomes. 1,3-Bis(2,4-octadecadienoyl)-*rac*-glycero-2-phosphorylcholine (1,3-DPC) was dispersed in an aqueous medium, and small unilamellar liposomes were prepared by sonication under N_2 atmosphere. Preparation of closed small liposomes was confirmed by TEM and ^{1}H NMR measurements.[21] A DSC measurement revealed that the 1,3-DPC formed liposomes with gel-to-liquid crystalline phase transition temperature(T_c) of 28 °C. The T_c was also determined with ^{1}H NMR spectrometry. Proton signals attributed to hydrophobic methylene and terminal methyl groups appeared at temperatures higher than 28 °C, suggesting the formation of liposome structure. Figure 1 shows the ^{1}H NMR spectra for 1,3-DPC liposomes in deuterium oxide at 50 °C in the absence and presence of Eu^{3+}, a shift reagent. The proton signals attributed to hydrophilic choline methyl groups were split by the addition of 3.8 m mol·l^{-1} $Eu(NO_3)_3$. This is the proof of the closed liposome structure because Eu^{3+} interacted with polar head groups of lipids facing outer aqueous phase to shift its signal to higher magnetic field as shown in Fig. 1 (b). The ratio of the split signal intensities showed no change for over 60 min suggesting no Eu^{3+} leakage through the membrane. The average radius of the liposomes can be estimated from the intensity ratio of the split choline methyl proton signals.[20] The average radius of the liposomes analyzed in this experiment was calculated to be about

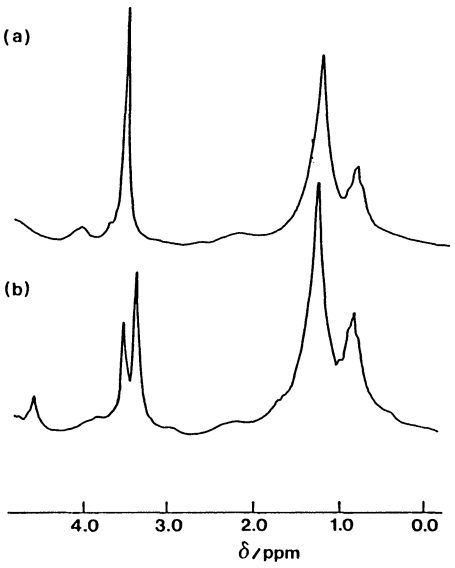


Fig. 1. ^{1}H NMR spectra of 1,3-DPC liposomes in D_2O at 50 °C in (a) absence or (b) presence of Eu^{3+}. [1,3-DPC]=10.0 g lipids dm^{-3} D_2O, [Eu^{3+}]=3.8 mmol·l^{-1}.

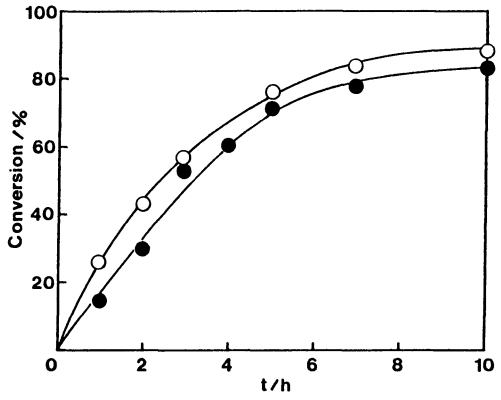


Fig. 2. Polymerization conversion of 1,3-DPC as liposomes at 60°C. 1,3-DPC liposomes (10.0 g lipids dm^{-3} H_2O) were polymerized by AIBN (●) or AAPD (○).

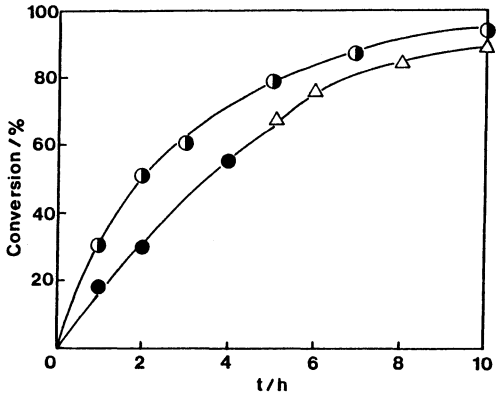


Fig. 3. Polymerization conversion of 1,3-DPC as liposomes. 1,3-DPC liposomes were polymerized by AIBN and AAPD simultaneously (◑), or by the addition of AAPD (△) to the DODPC liposomes which were pre-polymerized by AIBN (●) at 60°C for 5 h.

210 nm.

The AIBN and AAPD are potential azo compounds to initiate radical polymerization of lipids as liposomes.[9,10] AIBN and AAPD generate free radicals in hydrophobic region and aqueous region of lipid liposomes, respectively. It is known that polymerization profile reflects the acyl chain packing and chemical environment of diene group.[16] The 1,3-DPC liposomes were also polymerized by either one or both of these radical initiators to analyze chemical environment of diene groups. Figure 2 shows the results on the polymerization of 1,3-DPC as liposomes initiated by either AIBN or AAPD. Both AIBN and AAPD initiated radical polymerization of diene groups in 1,3-DPC liposomes and the polymerization conversion reached about 80% in both cases. It is previously reported that AIBN (5 mol% to the monomeric lipids) initiates radical polymerization of 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) for only 50%.[14,16] A simultaneous use of both AIBN and AAPD provided polymerization of DODPC in almost 100%. For DODPC liposomes, the diene groups bound in 1-acyl chains and those in 2-acyl chains were selectively polymerized by AIBN and AAPD radicals because of their non-equivalent chemical environment[16] Namely, diene groups in 2-acyl chains are considered to face the aqueous phase and they are polymerized by water-soluble radical initiators. And those in 1-acyl chains are located in the hydrophobic region and therefore polymerization should be initiated by the radical attack from hydrophobic region. 1,3-DPC however showed high polymerization conversion regardless the solubility of radical initiators, suggested that the chemical environment of diene groups in 1- and 3-acyl chains was almost equivalent. Furthermore they are located in the interface between the aqueous phase and hydrophobic region. One could also explain the result by assuming incomplete lipid packing which enabled the attack of radical initiators from both region. This was however denied by the ^1H NMR data as shown in Fig. 1, in which no leakage of Eu^{3+} was found.

We must note the following results for spectroscopic analysis of the polymerizable lipids as liposomes. Both 1,3-DPC and DODPC show the maximum UV absorbance at 265 nm attributed to diene groups in chloroform solution. The maximum UV absorbance of 1,3-DPC shifted to 243 nm when they formed liposomes in an aqueous phase. Against this, DODPC liposome suspension showed the maximum UV absorbance at 255 nm.[18] These different λ_{max} can be discussed in terms of different acyl chain packing. The maximum absorbance was found at 243 nm for both 1,3-DPC and DODPC below the phase transition temperature and it shifted to 255 nm beyond it. In the present work, polymerization conversion was carried out by UV spectrometry. The solution temperature for measurement was about 20°C and it was therefore lower than the T_c of 1,3-DPC. Anyway, spectral shift is strongly suggested to reflect the molecular packing of the polymerizable lipid as liposomes. Detailed results will be published elsewhere.

A simultaneous use of AIBN and AAPD did not improve the polymerization conversion of 1,3-DPC liposomes as shown in Fig. 3. There is little contribution of AAPD addition to the 1,3-DPC liposomes which has been previously initiated by AIBN radicals (Fig. 3 ●→△). This is considerably different from the polymerization profile for DODPC liposomes. These data also support equivalent chemical environment of the diene groups in 1- and 3-acyl chains for 1,3-DPC and their radical polymerization can be initiated by radical attack from both hydrophobic and hydrophilic phases. It can be concluded that 1,3-DPC packed more symmetrically than DODPC. No difference was found in the polymerization profile of 1,3-DPC with radical initiators employed in the present experiments. How-

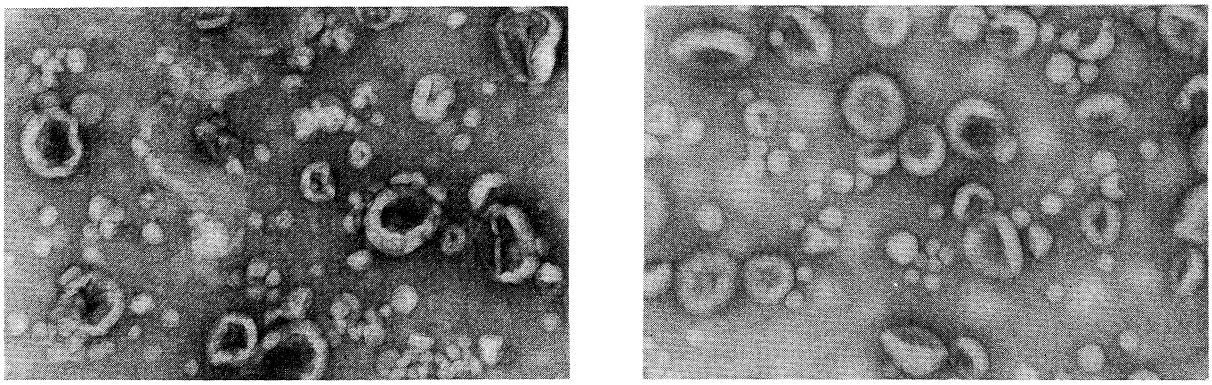


Fig. 4. Transmission electron micrographs of 1,3-DPC as liposomes (a) before and (b) after polymerization initiated with AIBN and AAPD simultaneously. Space bar indicates 0.2 μm.

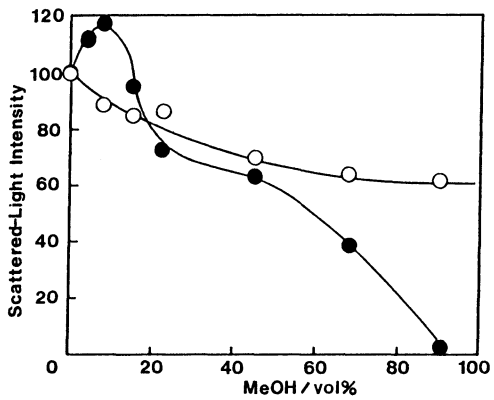


Fig. 5. Effect of methanol on scattered-light intensity for 1,3-DPC liposomes at 25°C. Liposomes were monomeric (●), or polymerized by AIBN and AAPD simultaneously (○).

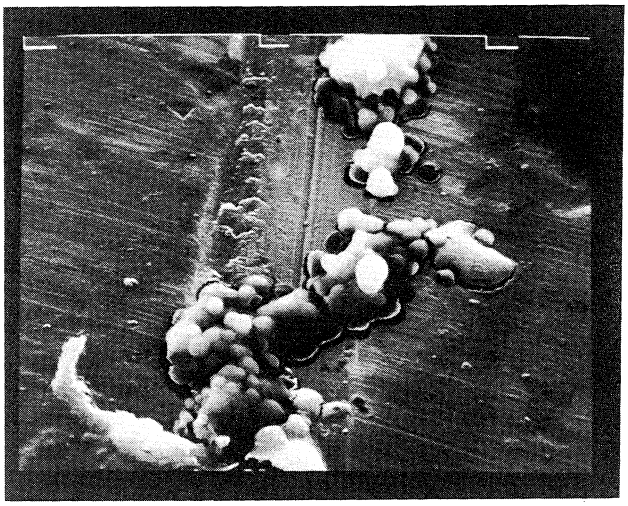


Fig. 6. Scanning electron micrograph of 1,3-DPC liposomes polymerized by AIBN and AAPD. Space bar indicates 5 μm. Sample was dried after washing with methanol.

ever, 1,3-DPC cannot be packed completely symmetrically but slight difference should be detected by other techniques such as small angle X-ray scattering.

The liposome structures of 1,3-DPC before and after polymerization were analyzed with TEM measurements. Figure 4 shows the TEM picture for the polymerized 1,3-DPC liposomes. Bilayer liposome structure is clearly seen in the micrographs for both monomeric and polymerized ones. The outlines of polymerized liposomes are more smooth than those of monomeric ones. It indicates that polymeric liposomes are more stable and maintain their spherical structures perfectly after dryness. This structure is found to be kept even after methanol washing. The effect of added methanol content on the scattered-light intensity from 1,3-DPC liposome suspension is depicted in Fig. 5. Monomeric liposomes showed decrease in the scattered light intensity, which may be attributed to the structural change such as destruction of liposomes structure with the addition of methanol. Nevertheless only a little intensity change was found for the polymerized one suggesting a stable membrane structure. Liposome structure was also observed with SEM even after washing large polymerized 1,3-DPC liposomes with pure methanol. In this experiment, giant 1,3-DPC liposomes were prepared by hydration method.[13] Polymerized 1,3-DPC liposomes with average diameter of about 1 μm were filtered on a Millipore membrane filter with average pore size of 0.05 μm. They were washed with pure methanol for several times, and their liposome structure was directly monitored with SEM after Au sputtering as shown in Fig. 6. Although they were aggregated, it was found that liposome structure was clearly maintained almost completely. This stable membrane structure was also confirmed by solubility test. The 1,3-DPC liposomes, polymerized with appropriate radical initiators, were insoluble in any organic solvents. This insolubility also supported the formation of crosslinking between

polymer chains, i.e., their polymerization underwent intermolecularly and no intramolecular polymerization occurred. In other words, diene group in 1-acyl chain does not form covalent bonding with that in 3-acyl chain of the same lipid monomer, but it may attack diene groups in not only 1-acyl chains but also 3-acyl chains of the neighboring lipid monomers. Related discussions on this polymerization manner will be mentioned later.

The leakage measurement of carboxyfluorescein (CF) was commonly used to evaluate the membrane structure or stability of the liposomes.[8,20] Figure 7 shows the leakage of CF from polymerized 1,3-DPC liposomes. Those from DODPC liposomes before and after polymerization were also plotted as references. Preparation of the polymerized liposomes containing CF molecules in their inner aqueous phase has already been mentioned elsewhere.[20] The CF leakage data for monomeric 1,3-DPC was not measured consistently because their liposomes were unstable and easily aggregated in solution under the conditions for this experiment. There was a certain temperature dependence of CF leakage from liposomes. No CF leakage was found from these polymerized liposomes at room temperature, and these measurements were therefore made at 50 °C to accelerate CF leakage. The CF leakage rate from an inner aqueous phase of the DODPC liposomes was considerably decreased by the polymerization, and almost the same CF leakage rate was found for the polymerized 1,3-DPC liposomes. It was demonstrated that radical polymerization of 1,3-DPC provided very stable membrane structure which was almost equivalent for that of polymerized DODPC liposomes.

Copolymerization of 1,3-DPC with DODPC in Mixed Liposomes. From the results obtained in the previous section, it was expected that the existence of 1,3-DPC might disturb selective polymerization manner and might induce further polymerization of DODPC as liposomes. Chloroform solution of 1,3-DPC was mixed with that of DODPC, and their mixed liposomes were prepared through the same procedure as mentioned above. Polymerization of the mixed liposomes was also analyzed by UV spectroscopy. Figure 8 shows the polymerization conversion for the DODPC/1,3-DPC (5/1 by mol) mixed liposomes initiated by AIBN (5.0 mol% to the total lipid monomers) at 60 °C. If 1,3-DPC and DODPC did not affect the polymerization manner with each other in the mixed liposome, theoretical polymerization conversion could be calculated from the results of their individual polymerization manner. For liposomes with average radius of 30 nm or more, AIBN initiated the polymerization of 1,3-DPC and DODPC liposomes to reach the polymerization conversion of about 80% (see Fig. 2) and 50%,[14] respectively at 60 °C for 12 h. Since we have collected kinetic data on the polymerization conversion of those lipid liposomes, the average polymerization conversion for the mixed liposome system can be calculated as a simple summation of the individual polymerization conversion at every stage of the polymerization. The calculated polymerization conversion was depicted in Fig. 8. The experimental data on the polymerization conversion (open circles in Fig. 8) was always higher than the calculated one. For example, the polymerization conversion reached 68% was actually found for the mixed liposomes after 12 h. heating at 60 °C in the presence of AIBN whereas the calculated value was only 55%. When the increase in the polymerization conversion was assumed to be due to the newly polymerized diene groups in 2-acyl chains of DODPC lipids, 31% of the diene groups in the 2-acyl chains of DODPC were calculated to be polymerized. The polymerized mixed liposomes obtained were stable against organic solvent washing and no channel or hole was formed by the skeletonization.[13] These results strongly suggest the following points: Firstly,

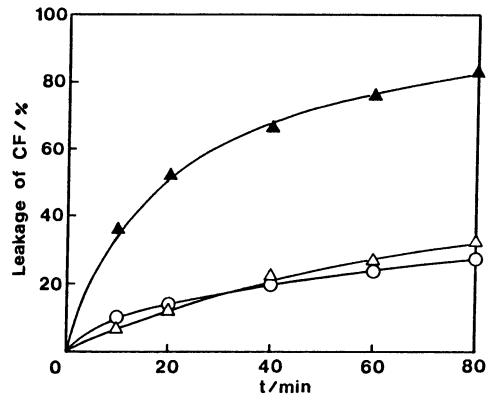


Fig. 7. Leakage of CF from inner aqueous phase of 1,3-DPC liposomes polymerized by both AIBN and AAPD simultaneously (○) at 50 °C. Monomeric (▲) and polymerized (△) DODPC liposomes were also analyzed under the same condition as references. No CF leakage was found at 30 °C.

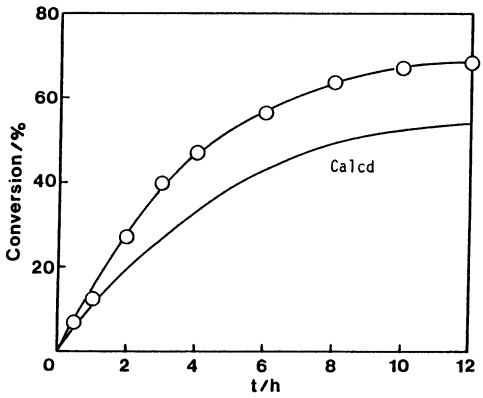


Fig. 8. Polymerization conversion of DODPC/1,3-DPC (5/1 by mol) mixed liposomes. Liposomes were polymerized by AIBN. Solid curve was obtained by calculation from polymerization conversions of DODPC[14] and 1,3-DPC liposomes.

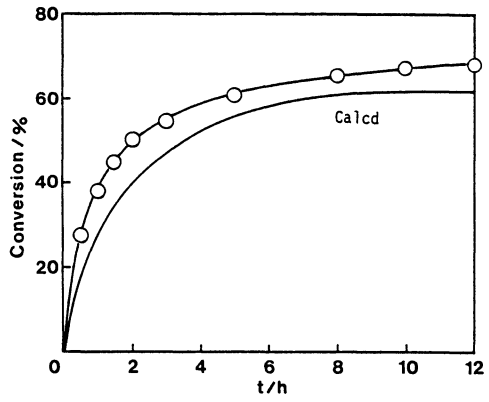


Fig. 9. Polymerization conversion of DODPC/1,3-DPC (5/1 by mol) mixed liposomes. Liposomes were polymerized by AAPD. Solid curve was obtained by calculation from polymerization conversions of DODPC[14] and 1,3-DPC liposomes.

1,3-DPC and DODPC were miscible with each other in the mixed liposomes and no phase-separated structure might be formed at 60 °C. Secondly, 1,3-DPC and DODPC lipids were copolymerized to form highly crosslinked ultrathin polymer membrane. And finally, terminal radicals on the 1,3-DPC in the copolymers might attack the diene groups in 2-acyl chains of DODPC which were never attacked by AIBN radicals in liposome systems.[14] Especially, the final point is very interesting and important. The diene groups in 2-acyl chains of DODPC lipids can be polymerized by the addition of AIBN in the presence of 1,3-DPC. The detailed study on the relationship between the improved polymerization conversion and composition of the mixed liposomes is now in progress.

It was previously reported that AAPD, a water-soluble radical initiator, was able to initiate radical polymerization of diene groups in 2-acyl chains of DODPC as liposomes.[14] The effect of 1,3-DPC on the polymerization of DODPC initiated by AAPD was also analyzed. A slight improvement was also found as shown in Fig. 9. About 16% of the diene groups in 1-acyl chains of DODPC were polymerized by AAPD under coexistence of 1,3-DPC. It is important that the polymerization conversion of 1,3-DPC/DODPC mixed liposomes reached a constant value, 68% regardless the employed radical initiators. This suggests that 1,3-DPC lipids certainly play a significant role to improve polymerization conversion as crosslinking agents.

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