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Unequivalent Chemical Environment of Diene Groups in 1- and 2-Acyl Chains of Polymerizable Lipids Analyzed by Radical Polymerization[1)]

Hiroyuki OHNO, Shinji TAKEOKA, and Eishun TSUCHIDA*
Department of Applied Chemistry, Faculty of Science and Engineering,
Waseda University, Tokyo 160
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1,2-Di-*O*-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine is polymerized by radical initiators. An azobisisobutyronitrile (AIBN) and an azobis(2-amidinopropane) dihydrochloride (AAPD) are incorporated into hydrophobic membrane region of the lipid liposomes and an aqueous medium, respectively by sonication. The use of either AIBN or AAPD gives polymerization conversion of about 50% at 60 °C. This is not improved by the elongation of polymerization period at 60 °C or the increase of the amount of these radical initiators. The lyophilized lipids thus polymerized by either AIBN or AAPD are soluble in methanol suggesting the formation of linear polymers. Simultaneous polymerization by both AIBN and AAPD provides almost complete polymerization. AIBN and AAPD have been suggested to initiate radical polymerization of diene groups in 1- and 2-acyl chains, respectively. Potassium peroxodisulfate, another water-soluble radical initiator, yields polymerized liposomes with polymerization conversion of more than 80%. This corresponds to the polymerization of diene groups of not only 2- but also 1-acyl chains due to the hydroxyl radicals which are generated by the radial transfer in an aqueous medium. These different initiation reactions are attributed mainly to the unequivalent chemical environment of diene groups in 1- and 2-acyl chains.

Polymerization of assembled molecules is one of potent techniques to construct highly-ordered polymer structure. UV-irradiation is being used frequently to initiate polymerization of the assembled monomers as liposomes or monolayer membranes because of its convenience.[3-9)] This technique was quite effective to polymerize diacetylenic amphiphiles because of their high reactivity,[4-6)] but some problems raised for the polymerization of diene-containing amphiphiles.[2,10-12)] For example, a strong UV-light irradiation provided an average molecular weight of the diene-containing phospholipids around 5000.[12)] Liposomes with relatively lower degree of polymerization were analyzed to be stabilized only a little.[12)] On the other hand, gamma-ray irradiation is one of potent techniques to initiate in situ polymerization of lipids as liposomes.[11)] However this provides excellent stability of the polymerized liposomes than UV-irradiated ones, it is not commonly-usable method because of less availability of the gamma-ray source and careful treatments. Against this, radical initiators are very common reagents, and those have been found to effectively initiate polymerization of diene containing lipids and to improve membrane stability of the liposomes. This method is quite convenient to prepare polymerized liposomes with excellent stability.[13)] We succeeded the selective polymerization of diene groups of polymerizable amphiphiles as liposomes with water-soluble and -insoluble radical initiators.[12)] This finding may lead to finely regulated polymerization of the assembled molecules. It is therefore getting important to summarize the polymerization profile and characteristics of the selectively polymerized diene-containing lipids.

In this paper, relationship between structural characteristics of radical initiators and polymerization profile of diene-containing amphiphiles is discussed as well as physicochemical characteristics of the obtained polymerized liposomes.

Experimental

1,2-Di-*O*-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) was purchased from Nippon Oil & Fats Co. Ltd. and was characterized by thin-layer chromatography (Merck, silica-gel plates) with chloroform–methanol–water (65 : 35 : 5, by vol) as solvent before use.[11)] DODPC showing single spot with R_f value of 0.3 on TLC plate was used for further experiments. Azobisisobutyronitrile (AIBN) and azobis(2-amidinopropane) dihydrochloride (AAPD) were purchased from Tokyo Kasei Co. Ltd. Potassium peroxodisulfate ($K_2S_2O_8$) was purchased from Kanto Chem. Co. Ltd. AIBN was purified twice by recrystallization from dehydrated methanol. AAPD and $K_2S_2O_8$ were recrystallized twice from pure water. Crystals of these radical initiators were dried in vacuo. Triton X-100, polyethylene glycol mono-*p*-octylphenyl ether ($HO(CH_2CH_2O)_{10}C_6H_4C_8H_{17}$), was purchased from Tokyo Kasei Co. Ltd. and was used without further purification. Fluorescent grade 5(6)-carboxyfluorescein (CF) was purchased from Kodak Co. CF was used for the experiments without further purification.

Liposome Preparation. The polymerizable lipids (0.20 g) were dissolved in dehydrated chloroform and slowly evaporated in a rotated sample tube to prepare lipid thin film on the inner surface of the tube. Degassed pure water (20 ml) was then added to the tube. This was sonicated (Tomy Seiko, UR-200P) at 60 W for 10 min in water bath (20 °C) under nitrogen atmosphere to prepare single-walled small liposomes with an average radius of 15—25 nm.

Polymerization. The polymerizable lipids (0.20 g) were dissolved with 2.1 mg of AIBN (5.0 mol% to the polymerizable lipids) in chloroform and was slowly evaporated in a rotated sample tube. Degassed pure water (20 ml) was added to the tube. This was co-sonicated at 60 W for 10 min in water bath at 20 °C to prepare liposome suspension. Lipo-

somes containing different amount of AIBN were also prepared by the same method. Small amount of AIBN was decomposed by 10 min sonication at 60 W. Liposomes were incubated at 8 °C for 3 h to minimize the disordered molecular packing. This procedure provided single-walled liposomes with average radius of 40—70 nm. This liposome dispersion was polymerized under nitrogen atmosphere at 60 °C.

Liposome suspension was prepared and incubated by the same method as mentioned above. AAPD or potassium peroxodisulfate with 0.5—10 mol% to the polymerizable lipids, were then added to the suspensions to avoid decomposition of initiators by the sonication. Liposomes thus prepared were considered to have no AAPD molecule in the inner aqueous phase. Some of liposomes thus prepared were resonicated for 10 s to put AAPD molecules into an inner aqueous phase. Liposomes were polymerized at 60 °C for several hrs under nitrogen atmosphere.

A small amount of DODPC liposome suspension was periodically pipetted out from a sealed sample tube during polymerization. Accurately diluted liposome suspension was analyzed by UV spectrometry to quantitatively determine the polymerization conversion. Calculation of the polymerization conversion was based on the changes of molar extinction coefficient at 255 nm which corresponded to diene groups.[11]

Measurements. Polymerized liposome was stained by uranyl acetate and the suspension was dropped on carbon precoated copper grids and dried carefully to confirm their bilayer structure by TEM (JEOL-100CX). Relatively large liposome suspension (aqueous methanol solution) was dropped on copper sample rest. The liposomal structure was directly analyzed with scanning electron microscopy (JEOL-JSM T20). A laser particle analyzer (Coulter N4D, Coulter Electronics) was used to measure average radius of the polymerized liposomes with a theory of quasielastic light scattering and Stokes-Einstein equation. CF was incorporated into an inner aqueous phase of the liposomes by a similar manner as previously reported.[14] Liposomes containing 0.10 M (1 M=1 mol dm^{-3}) CF were isolated by gel permeation chromatography (Sepharose CL-4B). Diluted liposome suspensions were set into quartz cell for fluorescence measurement at settled temperature. The leakage of CF was followed as the increase of fluorescence intensity at 520 nm by fluorescence spectrometer (Hitachi HPF-4) with excitation beam of 330 nm. A 100% CF leakage was carried out as reference by the sonication of the liposome suspension in the presence of a small excess of Triton X-100.[11] Liposome suspension was prepared with D_2O instead of H_2O by the same manner as mentioned above. Polymerized liposome suspension was put into NMR sample tube with diameter of 5 mm. FT-NMR spectroscopy (JEOL FX-90Q) was used to characterize the segmental motion of the polymerized liposomes at different temperature.

Results and Discussion

Radical Polymerization Profile of DODPC as Liposomes. Polymerization of DODPC liposomes cause the decrease of spectral intensity at 255 nm which is attributed to the diene group.[11] This is essentially applicable to estimate the polymerization conversion. Figure 1 shows the polymerization conversion of

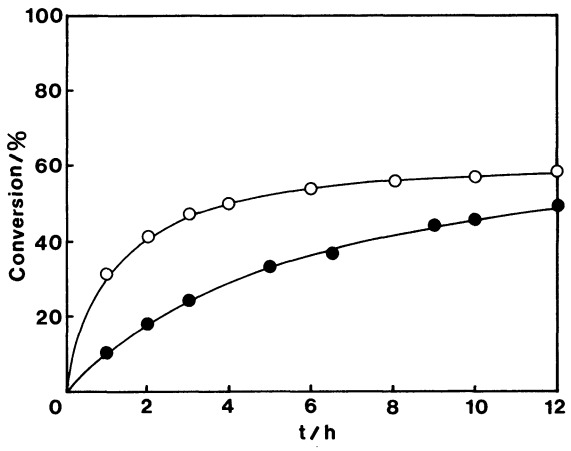

Fig. 1. Polymerization conversion of DODPC as liposomes. DODPC liposomes (10.0 g lipids dm^{-3} H_2O) were polymerized by AIBN (●) or AAPD (○) at 60 °C.

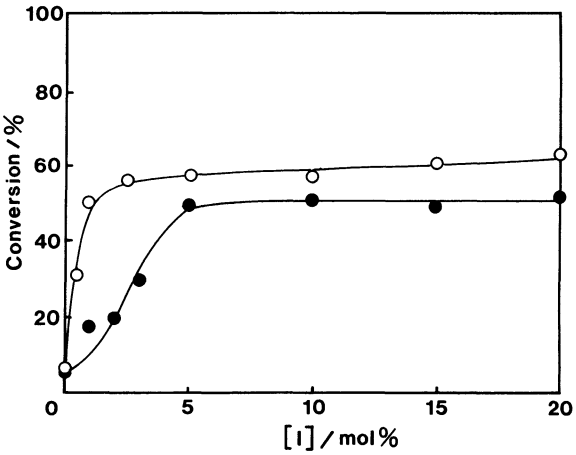

Fig. 2. Relationship between initial concentration of radical initiator and polymerization conversion of DODPC as liposomes (10 h at 60 °C). Liposomes were polymerized by AIBN (●) or AAPD (○).

DODPC lipids initiated by either AIBN or AAPD. The use of either AIBN or AAPD initiated the polymerization with final conversion of about 50% at 60 °C. This conversion was not improved by the elongation of the polymerization time. However certain improvement on the polymerization conversion was observed by further heating, this increase was almost the same as that by thermal polymerization. The increase of the amount of radical initiator did not improve it either as shown in Fig. 2. A thermal polymerization without radical initiator gave about 6—10% polymerization conversion after 12 h heating at 60 °C. AIBN with less than 0.5 mol% to the DODPC lipids was revealed not to participate the initiation of radical polymerization, because such a small amount of AIBN should be decomposed by 10 min sonication at liposome preparation.

The individual radical initiator (AIBN or AAPD) provided only 50% polymerization conversion, which

is important characteristic of DODPC lipids.[12] A 100% polymerization conversion was performed by simultaneous polymerization induced by both of these initiators. This tendency was firstly found by us for the polymerization of DODPC as liposomes.[12] This can be explained by the simple model considering different chemical environment of two diene groups bound to 1- and 2-acyl chains. According to the results of ^{2}D-NMR and X-ray analyses of lipid liposomes or crystals which were reported by Seelig et al.[15,16] and Hitchcock et al.[17] respectively, the glycerol segment was positioned perpendicular to the membrane plane. By applying this result simply to DODPC liposomes, the 2,4-diene group in 2-acyl chain is strongly considered to be faced an aqueous phase.[12] A molecular packing of the polymerizable lipid molecules in the bilayer membrane has already been discussed and the unequivalent characteristics of 1- and 2-acyl chains of the diacetylene-type amphiphiles have already been suggested by O'Brien[18] and Chapman.[19] As in their monomeric lipids, diacetylene groups were located deep in the hydrophobic region, they could not get direct evidence for the unequivalent reactivity of the 1-and 2-acyl chains of monomeric lipid. This unequivalent environment of diene groups was also demonstrated by us with 1-palmitoyl-2-(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (POPC).[2] POPC was a model lipid of DODPC and it was easily polymerized as liposomes by water-soluble radical initiator; AAPD, but hardly polymerized by AIBN. Namely, the 2,4-diene group in **2-acyl** chain must be exposed to the aqueous phase. The chemical environment of 2,4-diene groups in 2-acyl chain for POPC should be the same or quite similar to that for DODPC.

The unequivalent chemical environment of the 2,4-diene groups in 1- and 2-acyl chains was also clarified by comparing the polymerization manner with different radical initiators. For example, AAPD could initiate the radical polymerization of the DODPC liposomes which were preliminarily polymerized by AIBN as shown in Fig. 3. This means that the diene groups in 2-acyl chains are not initiated by AIBN radicals but are certainly initiated by AAPD radicals. The simultaneous initiation enabled polymerization with higher conversion than two-step initiation as shown in Fig. 3. This can be explained by the different penetration manner of AAPD through the membranes. The enough amount of AAPD molecules (not radicals) can penetrate the DODPC bilayer to initiate the polymerization of diene groups on 2-acyl chains which were faced the inner surface of the liposome at 60 °C. The penetration of AAPD molecules through bilayer membrane structure was supported by the following results. Some of DODPC liposome solutions containing AAPD in the outer aqueous phase were re-sonicated for 10 s to put AAPD molecules into an inner aqueous phase to compare the polymerization conversion with those for liposomes without re-sonication. There was no difference in the polymerization conversion between systems with and without re-sonication, suggesting that the enough amount of AAPD molecules did penetrate through the membrane at 60 °C. The penetration of radical initiators has already been analyzed to be governed by the temperature and there should be enough penetration to initiate the radical polymerization when temperatures were higher than the phase transition temperature.[12] However, covalent bonds of 1-acyl chains by AIBN-initiated polymerization restricted the permeation of AAPD molecules to cause the initiation less effective.

Similar improvement on the polymerization conversion was found by the addition of other water-soluble radical initiators. Potassium peroxodisulfate also initiated the radical polymerization of DODPC liposomes which were preliminarily polymerized by AIBN

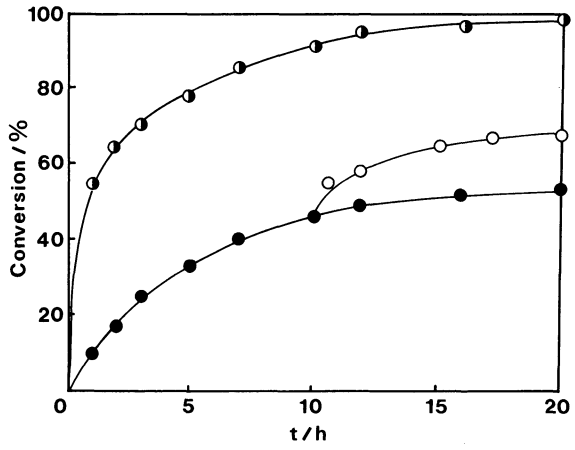

Fig. 3. Polymerization conversion of DODPC as liposomes at 60 °C. DODPC liposomes were polymerized by AIBN and AAPD simultaneously (◑), or by the addition of AAPD (○) to the DODPC liposomes which were pre-polymerized by AIBN (●) at 60 °C.

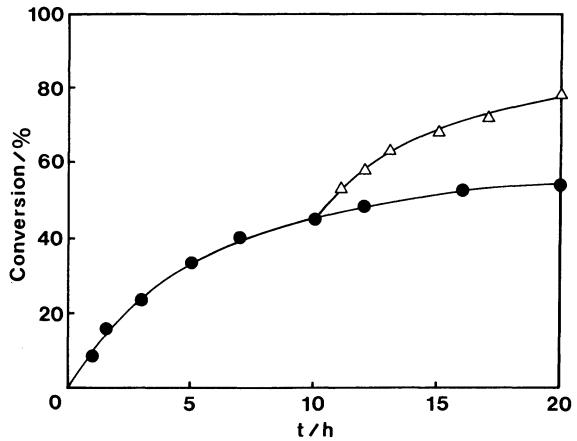

Fig. 4. Polymerization conversion of DODPC as liposomes at 60 °C. Polymerization was newly initiated by the addition of potassium peroxodisulfate (△) to the DODPC liposomes which were pre-polymerized by AIBN (●) at 60 °C.

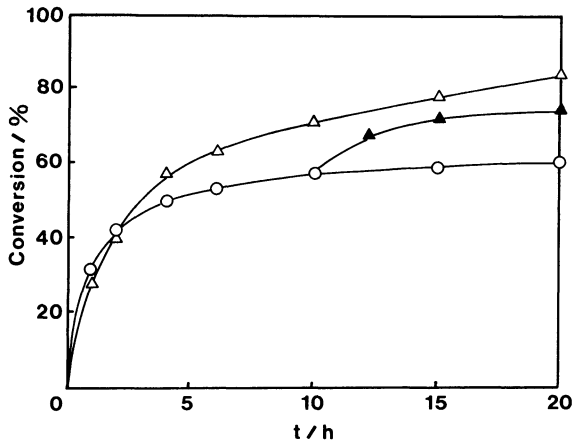

Fig. 5. Polymerization conversion of DODPC as liposomes at 60 °C. DODPC liposomes were polymerized by potassium peroxodisulfate (△), or by the addition of potassium peroxodisulfate (▲) to the DODPC liposomes which were pre-polymerized by AAPD (○) at 60 °C.

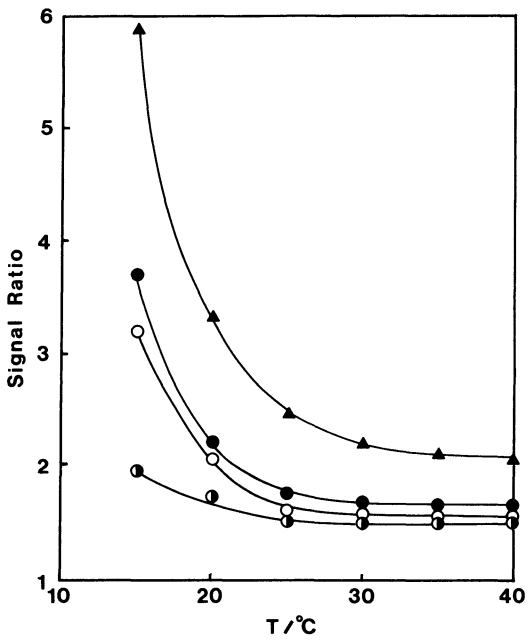

Fig. 6. Effect of temperature on the signal ratio of ^{1}H NMR peak for choline methyl protons to that for terminal methyl protons of polymerized DODPC liposomes. Liposomes were polymerized by; AIBN (●), AAPD (○), or both AIBN and AAPD simultaneously (◑). Data for monomeric DODPC liposomes were also shown as reference (▲).

as shown in Fig. 4. These data suggested that the polymerization reaction of diene groups in 1- and 2-acyl chains were independent with each other and no **intra**molecular polymerization should occur. It should be noted that the polymerization of DODPC liposomes with hydrophilic or hydrophobic radical initiators thus provided direct evidence for the different chemical environment and corresponding selective polymerization of the acyl chains of lipids as liposomes. A simple addition of potassium peroxodisulfate made the polymerization of DODPC liposomes to reach about 80—90% conversion as shown in Fig. 5. The DODPC liposomes which were prepolymerized by AAPD showed further increase in the polymerization conversion by the addition of potassium peroxodisulfate as also shown in Fig. 5. These results mean that not only diene groups in 2-acyl chains but also those in 1-acyl chains are polymerized by this radical initiator. Potassium peroxodisulfate newly initiated the radical polymerization of DODPC liposomes whereas AAPD did not. The diene groups in 2-acyl chains were considered to be polymerized by AAPD radicals, and no polymerization of other diene groups should be initiated by the further addition of AAPD. The decrease of absorption at 255 nm by the addition of potassium peroxodisulfate should therefore be attributed to the polymerization of diene groups in 1-acyl chains. The possibility for the attack of diene groups in the hydrophobic region could be explained by the size of freshly generated and transferred radicals. Potassium peroxodisulfate radicals were known to generate OH radicals through the radical transfer to water molecules.[20] As OH radicals were much smaller than AAPD radicals, the OH radicals might approach and attack the diene groups in 1-acyl chains.

The different polymerization conversion of DODPC liposomes was certainly observed beyond the experimental error between potassium peroxodisulfate-initiated polymerization (83%) and two-step one (73%) which was polymerized by AAPD first and the initiated by potassium peroxodisulfate as shown in Fig. 5. A simple polymerization of DODPC liposomes which was initiated by the addition of potassium peroxodisulfate made higher polymerization conversion than that for the two-step polymerization. This might be due to the tight covalent bonds on the surface of liposomes provided by the preliminary polymerization of diene groups on 2-acyl chains by AAPD. Some covalent bonds on the surface might restrict the attack of radicals from an aqueous medium to the diene groups in relatively hydrophobic region. A lower segmental motion of hydrophilic region in the DODPC liposomes pre-polymerized by AAPD was actually detected by the ^{1}H NMR measurements as shown in Fig. 6. This data supported the reduction of the radical attack frequency to the hydrophobic region.

A solution polymerization kinetics was applied to the radical polymerization as liposomes. It should be helpful to analyze the polymerization profile of DODPC especially to speculate the polymerization mechanism. Polymerization velocity was calculated from the initial slope of the polymerization conversion profiles as seen in Figs. 1 and 5. Typical polymerization velocity was summarized in Table 1 for each system. A thermal cleavage of AIBN and AAPD to produce radicals has already been analyzed and the

following equations have been empirically presented,[21,22]

$$\text{for AIBN: } k = 1.29\times10^{15}\, e^{-30800/RT}, \quad (1)$$

$$\text{for AAPD: } k = 3.77\times10^{15}\, e^{-30300/RT} \quad (2)$$

where k, R, and T mean the radical cleavage rate constant (s^{-1}), gas constant and absolute temperature, respectively. A radical cleavage of AAPD is faster than that of AIBN at the same temperature (see Eqs. 1 and 2), but the polymerization in liposome systems can not be compared directly. Because AIBN was dissolved only in the hydrophobic region of the liposomes and the local concentration was about 100 times higher than that for water-soluble one under the present experimental conditions. For example, the local concentration of AIBN in the hydrophobic region of the liposome was calculated to be 5.7×10^{-2} mol dm^{-3} at DODPC concentration of 1.0 wt% and AIBN/DODPC= 1/20 by mol. There was no significant difference in the polymerization velocity for the AIBN-added system at different liposome concentration if DODPC/AIBN mol ratio was set to be constant. The polymerization profile of AIBN-added liposome system was therefore analyzed to be similar to the bulk polymerization. On the other hand, the initial polymerization velocity for the water-soluble radical initiator added system was affected by their initial concentration. The polymerization profile seemed to be analyzable as the emulsion polymerization but these were not the same.

The initial polymerization velocity for AAPD-added system was larger than that for potassium peroxodisulfate-added system as shown in Table 1. This is mainly due to the different radical cleavage rate, and it for AAPD was larger than that for potassium peroxodisulfate. Furthermore peroxodisulfate anions were known to generate radicals and partially transfer them to water molecules to produce hydroxyl radicals.[20] The different polymerization conversion between AAPD- and potassium peroxodisulfate-added system was also explained by the different size of the effective radicals. Namely, small hydroxyl radicals can get in a bilayer membrane structure and initiate polymerization of diene groups in both 1- and 2-acyl chains. This was confirmed by the gamma-ray polymerization of DODPC liposomes.[11] The DODPC lipids provide their diene groups in unequivalent chemical environment due to molecular packing, this strongly suggests possibility of a selective polymerization with the use of suitable radical initiators.

Table 1. Initial Polymerization Velocity of DODPC Liposomes at 60 °C

Initiator	Radical cleavage rate	Polymerization rate
	s^{-1}	mol dm^{-3} s^{-1}
AIBN	8.42×10^{-6}	6.79×10^{-7}
AAPD	5.23×10^{-5}	3.20×10^{-6}
$K_2S_2O_8$	6.89×10^{-6}	1.65×10^{-6}

[DODPC]=1.0 wt%. [Initiator]/[DODPC]=1/20 by mol.

AIBN-initiated polymerization of DODPC was very different from others. One of characteristics of this system is higher local viscosity of the medium, i.e., liquid crystalline state of the hydrophobic region. The AIBN-initiated polymerization of DODPC as liposomes clearly showed the characteristics of the polymerization in viscous liquid-crystalline state. However the radical cleavage rate for AIBN was almost the same as that for potassium peroxodisulfate and local concentration of AIBN was 100 times higher, there was not significant difference in the polymerization velocity as summarized in Table 1. This less efficiency was well explained by the "Cage Effect".[23] The initiation efficiency for AIBN was calculated to be about 0.5—0.7 in ordinary organic solvent where apparent diffusion coefficient of radical pair was around 1×10^{-5} $cm^2 s^{-1}$. In a viscous medium, initiation efficiency of AIBN have already been analyzed by Smets to be getting smaller with increasing viscosity.[24] The liposomal membrane structure provides more viscous matrix, for example the diffusion coefficient of pyrene in dipalmitoylphsophatidylcholine at 60 °C was measured to be 2.0×10^{-7} $cm^2 s^{-1}$ by Sackmann et al.[25] Most of the generated radicals recombined in the matrix and a little radicals could initiate the polymerization of diene groups.

Stability of the Polymerized Liposomes. The leakage of fluorescent probes from an inner aqueous phase of the liposomes has frequently used to evaluate the membrane stability of liposomes. As the carboxyfluorescein (CF) affected the polymerization of DODPC or other monomeric amphiphiles,[26] CF was incorporated into liposomes which had already been polymerized previously.[14] The leakage of CF was extremely suppressed by the DODPC membrane structure which was polymerized by radical initiators as shown in Fig. 7.

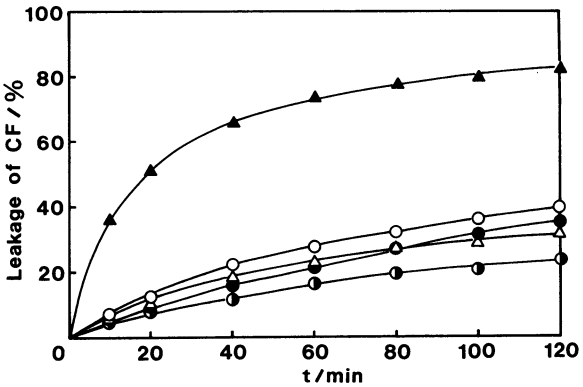

Fig. 7. Leakage of CF from inner aqueous phase of polymerized liposomes at 25 °C. Liposomes were polymerized by; AIBN (●), AAPD (○), potassium peroxodisulfate (△) or both AIBN and AAPD simultaneously (◑). Data for monomeric DODPC liposomes were also shown as reference (▲).

Polymerized liposomes with even 50% polymerization conversion showed excellent membrane stability. The 50% polymerization conversion means the complete polymerization of diene groups in either 1- or 2-acyl chains of lipids as liposome. If certain amount of lipids remained unpolymerized, these liposomes should be in the phase-separated state. For these systems, considerable CF leakage should be observed from the phase boundary.[27] Furthermore, phase separated polymerized liposomes could easily be detected by scanning electron microscopy.[28] These methods might be helpful to analyze the relationship between phase behavior of lipids and membrane stability.

The stability of the liposomes polymerized by both AIBN and AAPD was also examined through the changes of average diameter of the liposomes. The polymerized liposomes were agitated in an aqueous medium at 60 °C, and a small amount of the sample solution was periodically pipetted out and the number-average diameter was determined by a laser particle analyzer. It was confirmed that the polymerized liposomes were actually stable against heat-treating such as 60 °C for over 200 h. The excellent stability should be due to the larger average molecular weight of polymerized DODPC with highly crosslinked state. No diameter change was also found after several temperature cycling between freezing and melting of the polymerized liposomes. The process is well-known as "freeze thaw method" to prepare larger liposomes through liposomal fusion.[29] The polymerized liposomes were revealed to be extremely stable against heating and cooling. Scanning electron microscopy measurements also revealed that the polymerized DODPC liposomes were stable after organic solvent washing. Relatively large DODPC liposomes were polymerized by both AIBN and AAPD, or by potassium peroxodisulfate, and poured into aqueous methanol solution with final methanol content of about 70%. The polymerized liposomes were then characterized by SEM measurements to be stable enough even such a severe conditions. Figure 8 shows stereo-pair of the SEM picture of DODPC liposomes polymerized by potassium peroxodisulfate with polymerization conversion of 80%, and dispersed in an aqueous methanol solution. Spherical liposome structure with no damage was found suggesting that the DODPC liposomes polymerized by radical initiators had excellent membrane stability. Surface of the polymerized DODPC liposomes was smooth but wavy as shown in Fig. 8. For such large polymerized liposomes were generally wavy in surface especially for polymerized DODPC liposomes. This stereo view SEM method is useful to analyze structure for polymerized liposomes especially for the skeletonized ones.[28] The lyophilized DODPC liposomes polymerized by either AIBN or AAPD were stable but soluble in organic solvents such as methanol, ethanol or chloroform under agitation. No liposome structure was seen for these polymeric systems by SEM

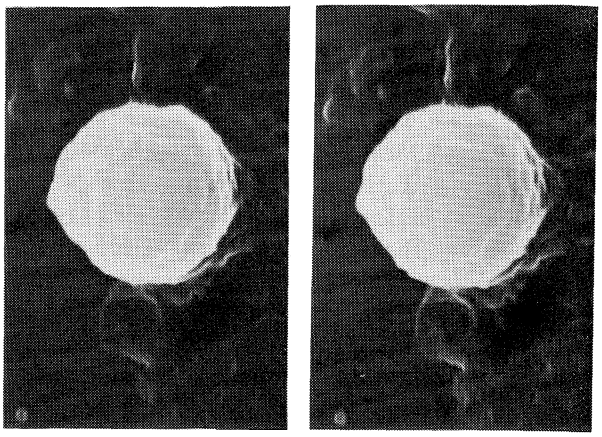

Fig. 8. Stereo pair of scanning electron micrograph of polymerized DODPC liposomes. Space bar indicates 2.0 μm.

observation. This fact is the proof of the formation of linear polymers, namely diene groups in 1- and 2-acyl chains can be polymerized selectively.

It was concluded that radical polymerization behaviors were effectively applied to analyze unequivalent chemical environment of diene groups in both acyl chains. Selective polymerization was also carried out by the suitable selection of the radical initiators.

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1) This forms part 3 of a series entitled "Polymerization of Diene-containing Lipids as Liposomes by Radical Initiators." Part 2.: Ref. 2.

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