Polymerization of Lipid Bilayer Initiated by a Redox Initiator: Effect of Polymerization on Dyes in the Inner Water Phase

Etsuo HASEGAWA, Noboru KIMURA, Masanori HATASHITA, and Eishun TSUCHIDA*
Department of Applied Chemistry, Waseda University, Tokyo 169
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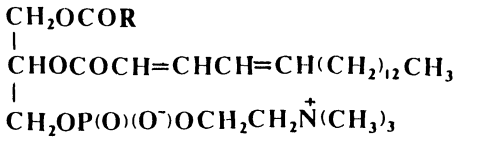
Synopsis. The polymerization of the liposomal lipid bilayers of phoshpolipids having one or two diene groups was studied by the use of a redox initiator ($NaHSO_3/K_2S_2O_8$) at low temperature (8 °C). The soluble and insoluble polymers were obtained, respectively. The membrane polymerization itself had small effect on the dye in the inner water phase.

Polymerization of lipids having polymerizable groups in liposomal bilayer was interesting to prepare physically stable model membranes.[1] For example, the polymerized liposomes having a porphinatoiron(II) complex was stable physically and could transport dioxygen in vitro and in vivo.[2] Polymerization of ene or yne residues was initiated mostly by UV irradiation,[3] by organic radical initiators (azobisisobutyronitrile, azobis(2-propanamine))[4] and by gamma ray radiation.[5] However, the degradation of incorporated materials such as drugs, biological compounds etc. was extensively induced during membrane polymerization, for example, by UV-irradiation. But, less attention has been paid to establish the mild conditions, for example, low temperature etc., inducing less degradation of solutes, although it has appeared another method to reduce the degradation of encapsulated compounds by incorporating them into the prepolymerized liposomes.[6,7] In this paper, the polymerization of liposomal bilayers of 1,2-bis[(2*E*,4*E*)-2,4-octadecadienoyl]-*sn*-glycero-3-phosphocholine[8] (**1**, T_c: 18 °C) or 1-palmitoyl-2-[(2*E*,4*E*)-2,4-octadecadienoyl]-*sn*-glycero-3-phosphocholine[9] (**2**, T_c: 28 °C) at low temperature was studied by using a redox initiator ($NaHSO_3/K_2S_2O_8$)[10] which was added into the outer water phase and the decolorization reaction of Methylene Blue in the inner water phase was studied to clarify the effect of polymerization on the solute.



1; R = $-CH=CHCH=CH(CH_2)_{12}CH_3$

2; R = $-(CH_2)_{14}CH_3$

Experimental

1,2-Bis[(2*E*,4*E*)-2,4-octadecadienoyl]-*sn*-glycero-3-phosphocholine (**1**) was purchased from Nippon Oil & Fats Co. (Tsukuba). 1-Palmitoyl-2-[(2*E*,4*E*)-2,4-octadecadienoyl]-*sn*-glycero-3-phosphocholine (**2**) was prepared.[9] Cholesterol (Wako Pure Chemicals Ind., Ltd., Tokyo) was recrystallized from methanol. Methylene Blue (Kanto Chem. Co., Inc., Tokyo) was recrystallized twice from ethanol. Benzene was distilled from calcium hydride. Tetrahydrofuran was distilled from sodium benzophenone under argon atmosphere. Lipid mixtures were freeze-dried from benzene prior to liposome preparation. Sepharose CL-20 was purchased from Pharmacia Fine Chem. Triton X-100 (polyoxyethylene octylphenyl ether) was purchased from Wako Chemicals Ind., Ltd.

The large unilamellar vesicles (LUVs) of **1** or **2** were prepared by extrusion method.[11] Freeze-dried powder (0.5 g) of **1** or **2** was suspended in 10 ml of distilled water and the mixture was vortexed with 2 ml of glass beads (diameter: 2—3 mm) under argon atmosphere at room temperature for 15 min. The suspention was extruded through the polycarbonate membrane (pore size: 2.0, 1.0, 0.6, 0.4, 0.2, and then 0.1 μm) to give the large unilamellar vesicles. An aqueous solution of Methylene Blue (0.3 mmol dm^{-3}) was encapsulated with the mixed lipid of **1** and cholesterol (molar ratio: 1/1) to prevent the leakage of the dye. Free Methylene Blue was removed by gel permeation chromatography on Sepharose CL-20 at 5 °C. The average diameters of these LUVs were determined by a quasi-elastic light scattering measurement (Coulter N4, Coulter Electronics Co.(U.S.A.)).

The polymerization of LUVs was initiated by the addition of $NaHSO_3/K_2S_2O_8$ (the molar ratio of $K_2S_2O_8$ to **1** or **2**: 0.05) under argon atmosphere at the low temperature (8 °C). The polymerization was followed by measuring the decrease of the UV absorption at 255 nm due to the diene chromophores of **1** or **2**.

The degree of polymerization was estimated by analyzing the membrane polymers of the LUV of **2** having only one polymerizable group in molecule. The lyophilized LUVs of **2** were methanolized in 20% HCl/abs methanol in an oil bath (100 °C) for 2 days.[12] After removing the solvent under reduced pressure, the residue was dissolved in chloroform, washed with water and then dried. The average molecular weights of the methanolized polymers were determined by high performance liquid chromatography with AD-803/AD-804/AD-80M/AD-802 GPC columns (Shouwa Denkou Co.), using tetrahydrofuran as solvent. The column was calibrated with polystyrene.

Results and Discussion

The sizes and shapes of liposomes prepared by the extrusion method were studied by a quasi-elastic light scattering and TEM measurements. These indicate the formation of LUVs with the average diameter 120 nm.

The polymerization yield of LUV of **1** initiated by the redox initiator mixture ($NaHSO_3/K_2S_2O_8$, the initial molar ratio of $K_2S_2O_8$ to **1**: 0.05) with times is shown in Fig. 1. The polymerization occurred and the rate increased with increasing the $NaHSO_3$ concentration where the initial concentration of $K_2S_2O_8$ was kept constant, while no disappearance of the UV absorption was induced by each components of the

redox initiator at the low temperature (8 °C). The polymerization yield reached at 32% after 20 h at 8 °C, but the further addition of the initiator caused further polymerization. The polymerization yield increased and saturated at 53% after multiple addition of the redox initiator. This saturation at 8 °C indicated that the outward lipids related to present polymerization as in the case of the water soluble and organic initiator.[9] The lyophilized liposomes (100 mg) were extracted by 25 ml of chloroform three times and the yield of insoluble powder was in agreement of the polymerization yield determined by UV measurements. This result shows that the polymerization was induced by the redox initiator mixture and the polymer was crosslinked. The polymerized LUV (the yield: 53%) obtained by the polymerization initiated by the redox initiator at 8 °C was stable against freeze-thawing (no change observed in particle size after freeze(−80 °C)-thawing(25 °C).

The methanolized polymers[13] of **2** were soluble in tetrahydrofuran. The weight- and number-averaged degrees of polymerization of the polymers were summarized in Table 1.[14] The polymerization at the high temperature gave the polymers of higher molecular weight with broader distribution.

The effect of membrane polymerization on soluted dissolved in the inner water phase of LUVs of **1** were then elucidated. Methylene Blue was used as a probe because the dye is found to be decolorized by various radicals including polymer growing ends.[15] Since the dye leaked slowly through the liposomal bilayer of **1** under the experimental conditions (5% after 24 h at 8 °C), the mixed lipid of **1** and cholesterol was used, where no leakage was found. The decolorization of the dye during the polymerization of LUVs was followed by measuring the absorbance at 664 nm. Because the dye forms aggregates (dimers and trimers) in water and their extinction coefficients are almost the same,[16] it could be calculated from the formation constants of the aggregates[17] the precise value of decolorization yield spectrophotometrically. The polymerization behavior and the decolorization of Methylene Blue were determined simultaneously on the LUV of **1** and cholesterol having Methylene Blue in its inner water phase. Figure 2 shows that the polymerization proceeded with almost the same rate as in the absence of Methylene Blue indicating no retardation of membrane polymerization by the inner phase dye. The amount of the dye bound to the outer surface of LUV was 0.13 mmol (g lipids)$^{-1}$, which is much smaller than that entrapped in the inner water phase (2.10 mmol (g lipids)$^{-1}$). The decolorization was caused with the same rate by $NaHSO_3$ alone. $K_2S_2O_8$ alone caused much less decolorization, and the decolorization amount (6 mol%) was the same of that of the dye bound to the outer surface of LUV.

The polymerized liposomes of **1** prepared at 8 °C by the 5 mol% redox initiator (polymerization yield: 30%) were stable against a high concentration (12 mM) of a surfactant (Triton X-100), while four mM surfactant completely decomposed non-polymerized liposomes of **1**. The stability was almost the same as that of the polymerized liposomes prepared by the polymeriza-

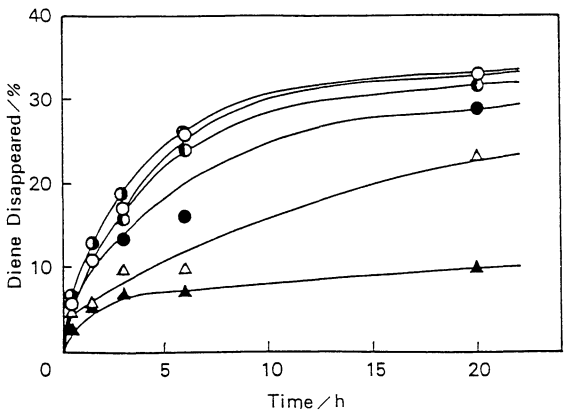


Fig. 1. Polymerization of LUVs composed of **1** at 8 °C. [**1**]=64 mM; $[K_2S_2O_8]/[\mathbf{1}]$=0.05 (constant); $[NaHSO_3]/[K_2S_2O_8]$=20 (◑) 15 (○), 10 (◐), 5 (●), 2 (△), 1 (▲).

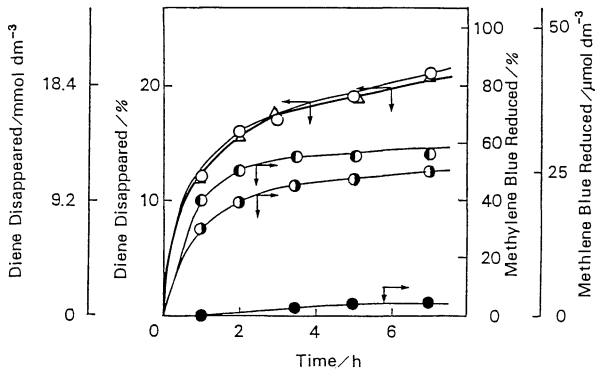


Fig. 2. Polymerization of LUVs composed of **1**-cholesterol (molar ratio: 1/1) having Methylene Blue at 8 °C. $[NaHSO_3]/[K_2S_2O_8]$=5.0 (○, ◑); $NaHSO_3$ alone (◐); $K_2S_2O_8$ alone (●); Polymerization of the LUVs having no Methylene Blue (△).

Table 1. Molecular Weight Analysis of Methanolized Polymer of **2** Polymerized with the Redox Initiator ($NaHSO_3/K_2S_2O_8$)[a]

Polymerization temperature	Polymerization yield[b]	$\overline{M}_n$ ($\overline{DP}_n$)[c]	$\overline{M}_w$ ($\overline{DP}_w$)[c]	$\overline{M}_w/\overline{M}_n$
°C	%	10^{-4}	10^{-4}	
8	53	0.78 (27)	1.16 (39)	1.49
35	91	1.33 (45)	7.22 (246)	5.43

a) The molar ratio of $K_2S_2O_8$ to **2**: 0.05; $[NaHSO_3]/[K_2S_2O_8]$=5. b) By UV. c) $\overline{M}_n$ and $\overline{M}_w$: number and weight average molecular weight; $\overline{DP}_n$ and $\overline{DP}_w$: number and weight average degree of polymerization.

tion initiated by 5 mol% azobis(2-propanamine) at 60 °C (polymerization yield: 50%).

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13) Anal. Found. C, 77.20; H, 11.82%. Calcd for $C_{19}H_{34}O_2$: C, 77.50; H, 11.64%; ^{1}H NMR ($CDCl_3$, TMS) δ=0.88 (t, $-CH_3$), 3.73 (s, $-OCH_3$) IR (KBr) 1740 cm^{-1}.

14) GPC Columns: AD-803/AD-804/AD-80M/AD-802 (Shouwa Dennkou Co.).

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