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# Phase Separation of Polymerized Lipids in Hybrid Liposomes

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## Summary

Hybrid liposomes were prepared from the mixture of dipalmitoyl phosphatidylcholine (DPPC) and di-2,4-octadecadiene phosphatidylcholine (DODPC), and their phase separation behaviours was analyzed. In hybrid liposome systems, two transition temperatures (18 and 41°C for DODPC and DPPC, respectively) were clearly observed by means of DSC and fluorescence techniques. These two transition temperatures were also found after polymerization of DODPC molecules in hybrid liposomes by the UV irradiation. These strongly suggest that DPPC and DODPC molecules do not dissolve well but form clusters in the membrane. This phase separation was directly confirmed by freeze-fracture TEM technique.

## Introduction

In recent years, polymerized liposomes collect keen interests due to their unusual characteristics. Especially, polymerizable lipids provide extremely thin polymeric films as well as liposomes having excellent mechanical strength. In hybrid liposome systems, several physico-chemical characteristics could be controlled by mixing ratio of different lipids. It is therefore important to analyze the phase transition behaviour and phase separation of the hybrid liposomes. It has already revealed that some structural characteristics of lipids such as structures of head groups and hydrophobic alkyl chains, hydrophobic-hydrophilic balance and specific interactions with the added third components [DEMEL et al., 1967; CHAPMAN et al., 1973] In the present paper, we report the cluster formation of the hybrid liposomes and freezing of phase separation by the polymerization.

## Experimental

### Materials

L-α-dipalmitoyl phosphatidylcholine (DPPC) was purchased from Sigma and was used without further purification. Di-2,4-octadecadiene phosphatidylcholine (DODPC) was the gift from Nippon Oil & Fats Co., Ltd. Both lipids were characterized by thin-layer chromatography before use. 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Kanto Chem. Co., Ltd. and was used without further purification.

Small unilamellar liposomes were prepared by sonication method (Tomy Seiko Co. Ltd.) as reported previously [OHNO et al., 1981]. A part of the obtained hybrid liposomes were kept on the incubation at room temperature to prepare larger liposomes through liposomal fusion.

Liposomes containing polymerizable lipids (hybrid liposomes) were irradiated in quartz cuvette with 150W high-pressure mercury lamp at room temperature for 2 hr. Polymerization process was confirmed by the dis-

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appearance of the characteristic absorption band (255 nm) which is assigned to the diene groups.

Measurements

Surface area-surface pressure isotherms of the hybrid monolayer membrane on water air interface were measured by the custom-made trough with Wilhelmy pickup.

Small unilamellar liposomes were labeled with DPH by sonication at 60W for 1 min. There the labeled liposomes were incubated at 50°C for at least 1 hr. The molecular motion of the fluorescent probe in liposomes were alalyzed by means of fluorescence depolarization spectrometry (JASCO FP-550). Light with wavelength of 366 nm was used for the excitation of the incorporated DPH, and the emission intensity from DPH was measured at 430 nm. The fluorescence anisotropy ($r_s$) was evaluated from the fluorescence intensities polarized parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the direction of the polarized excitation beam (Eq. 1).

$$r_s = (I_{//} - I_{\perp}) / (I_{//} + 2I_{\perp}) \quad (1)$$

The fluorescence anisotropy corresponds to the segmental motion of the membrane-composing molecules and the increase of $r_s$ value corresponds to the reduction of segmental motion of lipid alkyl chains.

For the preparation of freeze-fractured samples, a small volume of the phospholipids dispersions was pipetted and put into a specimen holder at 30°C for 30 min. The sample was rapidly frozen in liquid Freon-12 at liquid nitrogen temperature, and was transferred to liquid nitrogen. The sample was fractured and shadowed with platinum-carbon at -110°C in a Hitachi HFZ-1 freeze-fracture apparatus. Replicas were floated onto hypochlorous acid (HClO) and cleaned by distilled water. The replica was then collected on 300 mesh grids and obserbed by an electron microscopy.

Results and discussion

Formation of closed liposomes is generally confirmed by $^1$H-NMR spectrometry with $Eu(NO_3)_3$ as shift reagent [OHNO et al., 1981 (a)]. Radius of the hybrid liposomes prepared in this study was calculated as about 400 A from the intensity ratio of the splitted two signals of choline methyl groups faced on inside and outside liposomes.

Fluorescence measurements give informations about segmental motion of the fluorescent probe incorporated into lipid bilayer [OHNO et al., 1981 (b, c), SEKI et al., 1984]. Diphenyl hexatriene (DPH) is known to be incorporated in the center of the hydrophobic hydrocarbon region of the bilayer membrane. Fig. 1 reflects the segmental motion of hydrophobic alkyl chains in DPPC-DODPC hybrid liposomes. The segmental motion drastically changed at 18°C. This temperature corresponds to the gel to liquid crystalline phase transition temperature of DODPC liposomes. In hybrid liposome systems, this transition temperature ($T_t$) shifted to 20°C, and it is being ambiguous with the polymerization. However there is a $T_t$ of DODPC, the polymerization of diene groups suppress segmental motion of alkyl chains considerably. As the polymerizable diene groups locate relatively near from hydrophilic head group, alkyl chains are considered to have segmental motion in some extent even after polymerization. In hybrid liposomes systems, two $T_t$ were observed corresponding to that of DODPC and DPPC, respectively. These results strongly suggent that DODPC lipids show clusters in hybrid liposomes, and they behave independently in liposomes. Two $T_t$ were also observed in the polymerized hybrid liposomes, i.e., polymerized DODPC are considered to be surrounded by DPPC molecules to form clusters.

Phase separation of this hybrid liposomes was also investigated by surface area-surface pressure isotherm. These isotherms were measured for several hybrid lipid systems with different composition. The average area per molecule under constant surface pressure of 30 dyn/cm was the function

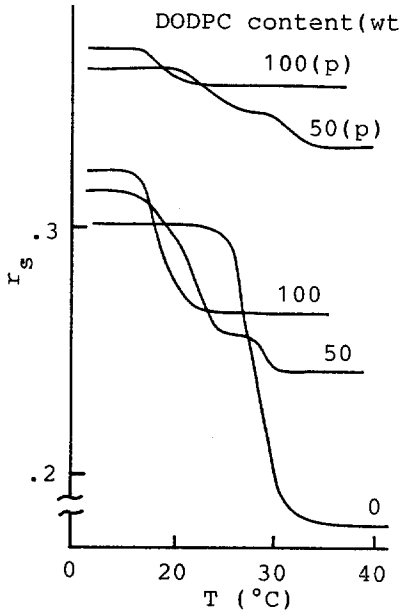


Fig. 1

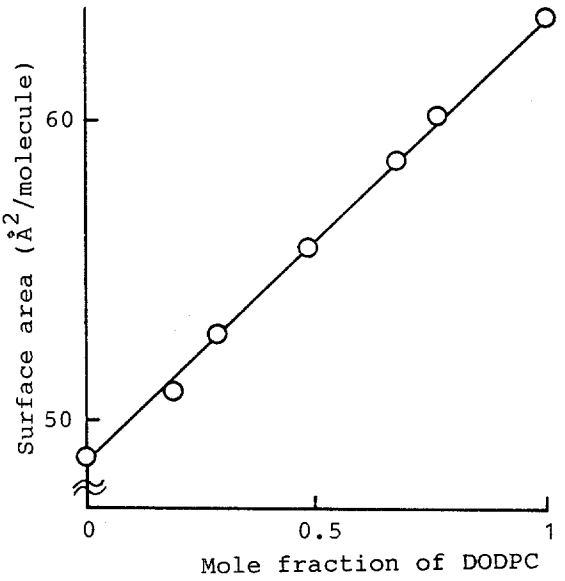


Fig. 2

Fig. 1 Temperature Dependence on the Phase Transition of Liposomes. (p): Polymerized liposomes.

Fig. 2 DODPC Fraction Dependence on the Average Surface Area of the Hybrid Monolayer Membranes at 30 dyn/cm at 20 °C.

of lipid composition as shown in Fig. 2. If two different kind of lipids do not dissolve homogeneously with each other and they tend to form clusters in the monolayer lipid membranes on water-air interface, the average area under certain surface pressure should show a linear relation with lipid composition just like Fig. 2. This therefore indicates clear phase separation in this hybrid lipid membrane. This pahse separation can be directly confirmed by the freeze-fracture electron microscopy [INOKO et at., 1980, OHKI et al., 1981]. It is well known that lipid membrane shows a band or terrace structure at gel state and they show a jumble or a smooth pattern at higher temperature than $T_t$. Fig. 3 shows electron micrographs of replicas for freeze-fractured membranes prepared at 30°C. DODPC (b) and DPPC (c) liposomes clearly show fluid and solid phases, respectively. In hybrid liposomes, fluid and solid phases coexisted in unilamellar liposome systems at 30°C as shown in Fig. 3 (a).

As phase separation of lipids in hybrid liposomes should be applied to create higher-ordered novel reactive domain or organization of functional matrix, basic knowledges on the formation and regulation of phase separation hebaviour of membrane-composing molecules should be collected.

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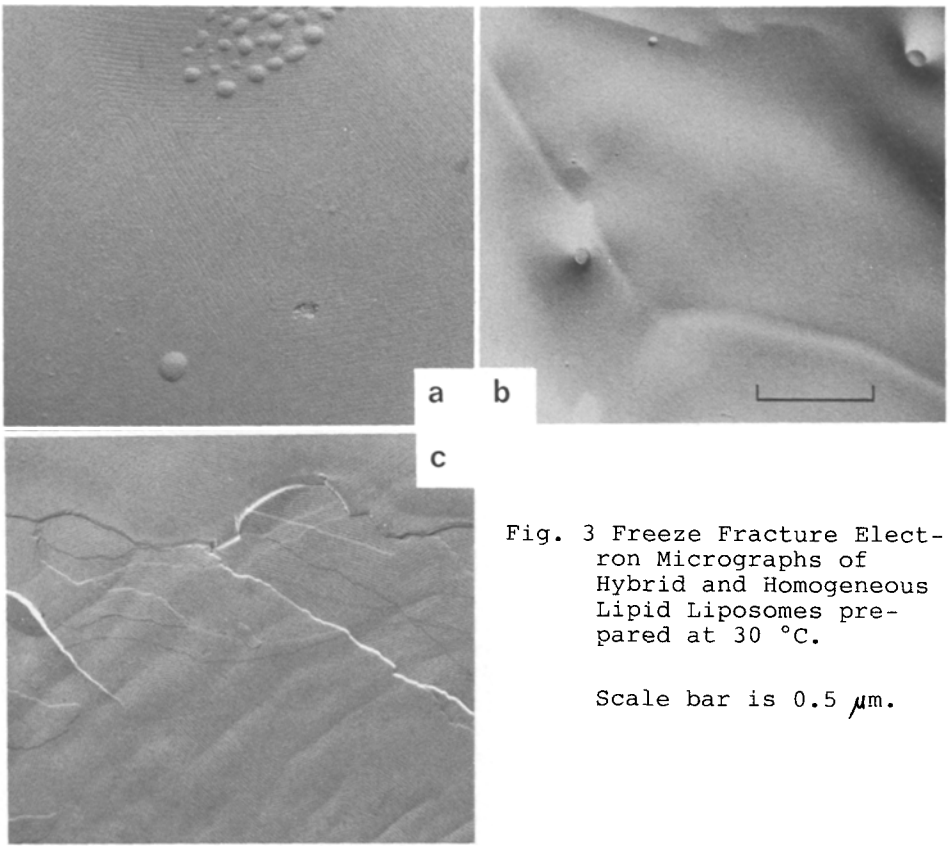


Fig. 3 Freeze Fracture Electron Micrographs of Hybrid and Homogeneous Lipid Liposomes prepared at 30 °C.

Scale bar is 0.5 μm.

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