# Cholesterol Suppresses Pressure-induced Interdigitation of Dipalmitoylphosphatidylcholine Bilayer Membrane

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We investigated the effect of cholesterol on the pressure-induced interdigitation of dipalmitoylphosphatidylcholine (DPPC) bilayer membrane by fluorescence technique using 6-propionyl-2-(dimethylamino)naphthalene (Prodan) as a probe. The interdigitation pressure (IP), minimum pressure required for the bilayer interdigitation, was elevated with increasing cholesterol composition ($C_{ch}$) up to ca. 2 mol %. Further increase of $C_{ch}$ did not give any effect on the IP value. Finally, the interdigitation could not be observed at $C_{ch} = 8$ mol %. Constructed pressure–$C_{ch}$ phase diagram at 50 °C suggests that the retardation and the abolition of the interdigitation are attributable to an interference effect and eventual formation of ordered molecular orientation induced by cholesterol.

Phospholipids form self-assembled bilayer membranes in an aqueous environment, which have long been utilized as models for biomembrane. In some studies the application of high pressure to model membranes has been attempted in association with pressure-adaptation of deep-sea organisms. Braganza and Worcester[1] have revealed for the dipalmitoylphosphatidylcholine (DPPC) bilayer that high pressure induces the interdigitated structure (i.e., $L_\beta I$ phase), that is, non-bilayer structure where two opposite monolayers constituting a bilayer interpenetrate mutually. We also have studied on the bilayer phase behavior of various kinds of phospholipids including the pressure-induced interdigitation by constructing temperature–pressure phase diagrams.[2–5] Recently, increasing use has been made of binary and ternary lipid membranes containing cholesterol as model membranes. This is because cholesterol is one of major components of biomembrane and its effect on the membrane structure and properties is considered to be closely related to the nature of cell membranes. However, as far as we know, there has been no attempt to clarify how cholesterol affects the pressure-induced bilayer interdigitation. In this paper, we report our recent finding of the effect of cholesterol on the pressure-induced interdigitation for DPPC–cholesterol binary membrane revealed by fluorescence technique.

DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), cholesterol (5-cholesten-3$\beta$-ol) and fluorescent probe Prodan [6-propionyl-2-(dimethylamino)naphthalene] were purchased from Avanti Polar Lipids Inc., Sigma-Aldrich Co., and Molecular Probes Inc., respectively, and used without further purification. A homogeneous aqueous suspension of the DPPC–cholesterol binary vesicle was prepared as follows. A mixture of appropriate amounts of DPPC/chloroform, cholesterol/chloroform, and Prodan/ethanol solutions was thoroughly dried in vacuum, and the resulting film was suspended in a given amount of double-distilled water with vortex and sonication. The concentrations of DPPC and Prodan were fixed at 4.0 and $2 \times 10^{-3}$ mmol kg$^{-1}$ (i.e., molar ratio of DPPC:Prodan = 2000:1), and the cholesterol composition ($C_{ch}$) against DPPC was varied from 0 to ca. 12 mol %. Fluorescence spectra of Prodan (excitation wavelength = 361 nm) were obtained at 50 °C and different pressures using a fluorescence spectrophotometer (Hitachi Model F-2500) equipped with high-pressure cell (Teramecs PCI-400). The pressure was varied in a stepwise manner by ca. 20 MPa using a hand-operated hydraulic pump and monitored with a Heise gauge (resolution of 0.5 MPa). The analysis of the spectra was done by attached software of FL-solutions.

We have previously demonstrated that the barotropic bilayer phase transitions can be monitored by the wavelength at the maximum intensity ($\lambda_{max}$) in the Prodan fluorescence spectra ($F(\lambda)$): the $\lambda_{max}$ shift from 480 to 430 nm corresponds to the main transition from the liquid crystalline ($L_\alpha$) phase to the ripple gel ($P_\beta'$) phase and the shift from 430 to ca. 500 nm does to the $P_\beta'/L_\beta I$ phase transition (i.e., the interdigitation).[6] Further, our recent report[5] showed that the second derivatives (i.e., $F''(\lambda) = \partial^2 F(\lambda)/\partial\lambda^2$) of the fluorescence spectra are useful for the determination of the bilayer phase transitions, especially the bilayer interdigitation. Figure 1 shows $F''(\lambda)$ vs. $\lambda$ curves of the DPPC bilayer containing 0.99 mol % cholesterol obtained at 50 °C and pressures from 82 to 201 MPa. In the pressure range, the $\lambda_{max}$ value, appearing as the wavelength at minimum intensity in each $F''(\lambda)$ curve, was constant at 430 nm, but the intensity $F''(430)$ became weaker with increasing pressure and a minor peak appeared at 497 nm above 146.5 MPa. The weakening of the peak at 430 nm and the appearance of the minor component at 497 nm are attributable to the bilayer phase transition from the $P_\beta'$ phase to the $L_\beta I$ phase. The inset in Figure 1 shows the pressure dependence of the ratio of $F''(497)/F''(430)$. The dependence exhibited a discontinuous change at ca. 140 MPa, indicating that the binary bilayer membrane underwent the interdigitation at the pressure. This pressure is rather higher than the interdigitation pressure (IP) of the pure DPPC bilayer membrane at 50 °C (ca. 125 MPa).[2]

Similar observations were carried out for various DPPC–cholesterol binary bilayers with different $C_{ch}$ values and the relation between IP and $C_{ch}$ was examined. The result is given in a pressure–$C_{ch}$ phase diagram in Figure 2. The diagram includes also the main-transition (i.e., the $L_\alpha/P_\beta'$ transition) pressure, which was determined by a similar way using the pressure dependence of the ratio $F''(480)/F''(430)$. Possible phase boundaries were drawn on the basis of the phase rule and by referring to

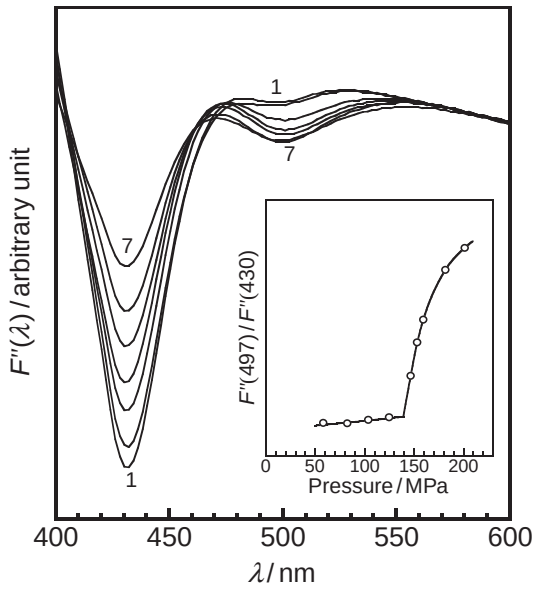


**Figure 1.** Second derivatives ($F''(\lambda)$) of Prodan fluorescence spectra vs. wavelength ($\lambda$) curves for DPPC binary bilayer membrane containing 0.99 mol % cholesterol at 50 °C and different pressures: 1, 82; 2, 124.5; 3, 146.5; 4, 153; 5, 159; 6, 181.5; and 7, 201 MPa. The peaks at 430 and 497 nm are attributable to the bilayer phase states of $P_{\beta'}$ and $L_\beta I$. The inset shows pressure dependence of $F''(497)/F''(430)$.

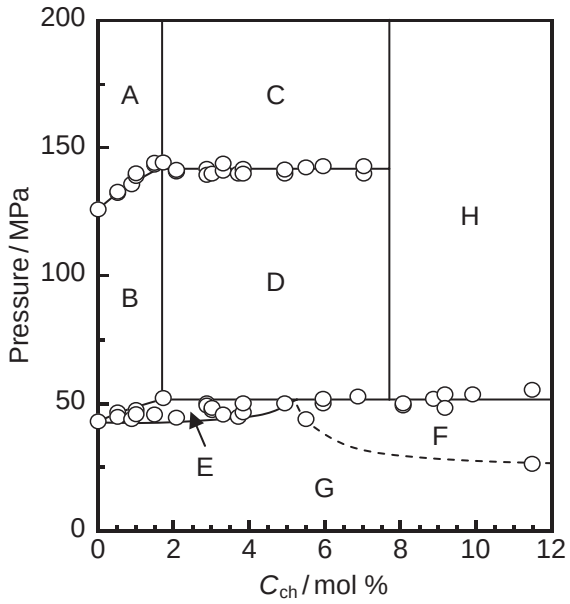


**Figure 2.** Pressure–cholesterol composition ($C_{ch}$) phase diagram of DPPC–cholesterol bilayer membrane at 50 °C. Phase assignment to each area designated by an alphabet is as follows: A, $L_\beta I$; B, $P_{\beta'}$; C, $L_\beta I + L_\beta(1{:}12)$; D, $P_{\beta'} + L_\beta(1{:}12)$; E, $P_{\beta'} + L_\alpha$; F, $L_\alpha + L_\beta(1{:}6)$; G, $L_\alpha$; H, $L_\beta(1{:}12) + L_\beta(1{:}6)$.

the temperature–$C_{ch}$ phase diagram under atmospheric pressure[7] and regular distributions of cholesterol based on a hexagonal lattice.[8] As shown in the diagram, the IP value increased with increasing $C_{ch}$ up to 2 mol % and remained almost constant at ca. 145 MPa for 2 mol % $< C_{ch} <$ 8 mol %, and finally the bilayer interdigitation itself was abolished at $C_{ch} >$ 8 mol %. The initial increase of the IP value means that the presence of slight amount of cholesterol causes significant retardation of the bilayer interdigitation. The succeeding isobaric transition can be explained from the viewpoint of two-phase coexistence. In the $C_{ch}$-region of 2–8 mol % the binary bilayer membrane contains two different phase states: one is the state of the binary bilayer formed at $C_{ch} = 2$ mol % and the other is that at $C_{ch} = 8$ mol %. The former state can undergo the barotropic $P_{\beta'}/L_\beta I$ phase transition at ca. 145 MPa whereas the latter cannot. Since varying $C_{ch}$ corresponds to the change of the proportion of both states, the bilayer interdigitation always occurs at the constant pressure in this $C_{ch}$-region. Finally, the bilayer interdigitation was abolished at $C_{ch} = 8$ mol %. The composition is very close to 7.7 mol %, which corresponds to that of the binary bilayer membrane of which molecular packing can be represented on the hexagonal lattice by the entire occupation with a unique type of units, each of which is composed of a cholesterol molecule at the center and 12 surrounding DPPC molecules within the next-nearest neighbor sites. This indicates that the interference effect by one cholesterol molecule, on average, works on 12 DPPC molecules around it. This consideration is supported by the fact that an isopleth line lies at almost the same composition also in the temperature–$C_{ch}$ phase diagram under atmospheric pressure as a phase boundary between different gel phases.[7]

We assigned the bilayer phase to each area in the diagram as described in the caption of Figure 2. It must be noted that the phase assignment includes some presumptions based on known cholesterol effects and the temperature–$C_{ch}$ phase diagram under atmospheric pressure. First, the phase state of the binary membrane at 7.7 mol % was assigned as $L_\beta(1{:}12)$ considering that cholesterol tends to induce an $L_\beta$ state, namely ordered conformation of the hydrocarbon chains of its adjacent phospholipid molecules[9] and molecular orientation perpendicular to bilayer surface.[10,11] Second, the $L_\beta(1{:}6)$ phase was presumed to exist at $C_{ch} = 14.3$ mol % from the coherence with the phase behavior under atmospheric pressure.[12] Third, as for the barotropic main transition, the eutectic phase behavior, including the miscibility of cholesterol and DPPC in $C_{ch} \leq 2$ mol %, was also inferred from the topology of the temperature–$C_{ch}$ phase diagram. In this regard, further investigation is required for the exact determination of phase boundaries, especially the liquidus line represented by a dashed line, which is based on less experimental evidence in this report.

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