Thermotropic Phase Behavior of Hydrogenated Soybean Phosphatidylcholine–Cholesterol Binary Liposome Membrane

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By combination of differential scanning calorimetry (DSC) and fluorescence spectroscopy of 6-propionyl-2-(dimethylamino)naphthalene (Prodan), we elucidated the thermotropic phase behavior of hydrogenated soybean phosphatidylcholine (HSPC)–cholesterol binary liposome membrane which has similar lipid composition to Doxil®, the widely used liposome product in treatment of various tumors. We found that the characteristic points at cholesterol mole fraction ($X_{\text{ch}}$) of 0.023 and 0.077 correspond to the hexagonal lattice, in which cholesterol molecules are considered to be regularly distributed in all regions of HSPC lipid bilayer with 1:42 and 1:12 units, respectively, as static averaged structures. Apparent endothermic peak disappeared at $X_{\text{ch}}=0.40$ in the DSC thermograms, indicating the existence of single liquid ordered phase at $X_{\text{ch}}>0.40$. In addition, fluorescence measurements of Prodan and its lauroyl derivative in poly(ethylene glycol) (PEG)-modified liposomes indicated that PEG modification has a negligible effect on the phase behavior of HSPC–cholesterol binary liposome membrane. These results may provide useful information in developing novel liposome products whose stability and encapsulated drug release are controlled.

Key words liposome; cholesterol; phase diagram; differential scanning calorimetry; Prodan

Liposomes are used as drug delivery systems because of their high biocompatibility and ability to encapsulate a large amount of pharmaceuticals inside the vesicle. Thermodynamic states of liposome membrane are considered as an important factor in terms of particle stability and encapsulated drug release. Therefore, phase transition temperatures and phase states of lipid membranes are important physiocochemical index in assessing the liposomal product.

Liposomes are mainly composed of phospholipids in the lamellar phase such as dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and hydrogenated soybean phosphatidylcholine (HSPC). Because all these lipids have saturated fatty acids, they have relatively high phase transition temperatures and therefore, are used in preparation of stable liposomes. The commercial liposomal products such as Doxil® and Ambisome® are composed of HSPC and cholesterol in which the incorporation of cholesterol into the lipid bilayer modulates the liposome membrane fluidity. Poly(ethylene glycol) (PEG)-lipid is also a fundamental component in Doxil® to extend the circulation time in plasma. In theory, HSPC and cholesterol can be either domain segregated, randomly distributed, or regularly distributed depending upon the energy function. However, the relationship between the phase states of HSPC–cholesterol liposome membrane and lateral distribution of cholesterol in the membrane has not been fully elucidated. Optimal modulation of miscibility between lipid molecules is of fundamental importance to control drug release from liposomes.

The phase diagram for lipid mixtures shows several different regions of phase coexistence, including lamellar gel ($L_{\text{g}}$ or $L_{\text{p}}$), ripple gel ($P_{\text{g}}$), liquid crystalline ($L_{\text{c}}$) and liquid-ordered ($L_{\text{o}}$) phases. At the pretransition, the flat $L_{\text{g}}$ phase, where acyl chains of phospholipid molecule in all-trans conformation are tilted at about $30^\circ$ to the membrane normal, changes to the wavy $P_{\text{g}}$ phase. At the main transition, the $P_{\text{g}}$ phase changes to the fluid-like $L_{\text{c}}$ phase, where the acyl chains are disordered due to high degree of trans–gauche isomerization and do not show any tilt. The $L_{\text{c}}$ phase, which is characterized by high conformational order in the hydrocarbon chains of phospholipid like the gel state and relatively fast lateral diffusion, appears when cholesterol content in the membrane increases. Identification and characterization of respective phases and their location in the compositional diagram are important to understand the behavior of lipid mixtures.

In the present study, we examined the thermotropic phase behavior of HSPC–cholesterol binary liposome membrane by high-sensitivity differential scanning calorimetry (DSC) and fluorescence spectroscopy using 6-propionyl-2-(dimethylamino)naphthalene (Prodan). Prodan molecule is located in the surface region of lipid membrane, providing critical information about the microscopic change near the hydrophilic surface of the membrane. In terms of interpretation of the phase behavior, we explained the lateral distribution of cholesterol molecules in the binary liposome membrane based on a modified superlattice model. We also investigated the effect of PEG modification on the phase diagram of the HSPC–cholesterol binary liposome membrane.

Experimental

Materials HSPC was kindly provided from NOF corporation (Tokyo, Japan). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). N-(Carbomethoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG2000) was obtained from NOF corporation. Prodan and 6-lauroyl-2-(dimethylamino)-naphthalene (Laurdan) were from Life Technologies (Carlsbad, CA). The authors declare no conflict of interest.

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Liposome Preparation

For multilamellar vesicle (MLV) preparation used in DSC measurements, the mixture of HSPC and cholesterol was dissolved in a chloroform–methanol mixture (2:1, v/v) and dried to a thin film under a stream of nitrogen gas, followed by incubation overnight under vacuum to remove residual solvent. The thin lipid film was hydrated with 10 mM Tris–HCl buffer (pH 7.4) in a water bath above 60°C.

In fluorescence measurements, the mixture of HSPC and cholesterol containing 0.2 mol% of Prodan or 1 mol% of Laurdan was used for liposome preparation and the liposome suspension was passed through a Mini-extruder (Avanti, Alabaster, AL, U.S.A.) equipped with 0.2 µm polycarbonate filter. For PEG-modified liposome, the mixture of HSPC, cholesterol, and DSPE-PEG2000 was premixed in a chloroform–methanol solution and PEG-liposome was prepared similarly as described above. The phospholipid concentration was determined by the Bartlett method. 17)

DSC Measurements

DSC measurements were carried out by a high-sensitivity differential scanning calorimeter MicroCal MCS DSC. The heating rate was 45°C/h and the temperature range was from 30 to 70°C. The midpoint temperature and the enthalpy associated with phase transitions were determined from endothermic peaks in the thermograms using MicroCal ORIGIN software.

Fluorescence Studies

All fluorescence measurements were performed using a Hitachi F-4500 fluorescence spectrophotometer. The excitation wavelengths for Prodan and Laurdan were 361 and 350 nm, respectively, and the emission spectra were recorded from 370 to 600 nm. The generalized polarization (GP) values for Prodan and Laurdan were calculated from the equation $GP = (I_B - I_R)/(I_B + I_R)$, where $I_B$ and $I_R$ are the emission intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum, respectively. 18)

Results

DSC Measurements for HSPC–Cholesterol Binary Liposome Membrane

Figure 1A shows DSC thermograms of HSPC–cholesterol liposome membrane at different $X_{ch}$ from 0 to 0.40 $X_{ch}$=0 (1), 0.05 (2), 0.15 (3), 0.25 (4), 0.35 (5), 0.40 (6). (B) DSC Thermograms in the Vicinity of the Pretransition Temperature $X_{ch}$=0 (1), 0.01 (2), 0.02 (3), 0.03 (4). (C) Enthalpy Change of HSPC–Cholesterol Liposome Membrane as a Function of $X_{ch}$ for the Main Transition Peak

Fig. 1. (A) DSC Thermograms of HSPC–Cholesterol Liposome Membrane at Different $X_{ch}$ from 0 to 0.40 $X_{ch}$=0 (1), 0.05 (2), 0.15 (3), 0.25 (4), 0.35 (5), 0.40 (6) (B) DSC Thermograms in the Vicinity of the Pretransition Temperature $X_{ch}$=0 (1), 0.01 (2), 0.02 (3), 0.03 (4) (C) Enthalpy Change of HSPC–Cholesterol Liposome Membrane as a Function of $X_{ch}$ for the Main Transition Peak

Fig. 2. (A) Fluorescence Spectra of Prodan in HSPC Liposome Membrane ($X_{ch}$=0) from 38 to 64°C (B) Temperature Dependence of Wavelength at Maximum Fluorescence Intensity and GP Value of Prodan for HSPC Liposome Membrane

Prodan concentration was 2 µM.

Fig. 2. (A) Fluorescence Spectra of Prodan in HSPC Liposome Membrane ($X_{ch}$=0) from 38 to 64°C (B) Temperature Dependence of Wavelength at Maximum Fluorescence Intensity and GP Value of Prodan for HSPC Liposome Membrane

Prodan concentration was 2 µM.
early with increasing \( X \) and reached zero at about \( \lambda \) from 38 to 64°C. It is known that the wavelength of fluorescence spectra in HSPC liposome membrane at temperatures

Membrane

phase.

that all HSPC molecules are conformationally restrained and \( X \)-othermic peak disappears at about \( \lambda \).

The \( \Delta H \) values became zero at \( X \).

In Fig. 1C, the enthalpy change (\( \Delta H \)) for the main transition is plotted as a function of \( X_{ch} \). The \( \Delta H \) values decreased linearly with increasing \( X_{ch} \) and reached zero at about \( X_{ch}=0.40 \). The \( \Delta H \) is attributable to the required energy for the trans–gauche conformational change of the hydrocarbon chains of phospholipids (i.e., chain melting) in the liposome membrane. The incorporation of cholesterol into the gel phase increases the fluidity of liposome membrane, resulting in the decrease in \( \Delta H \) of the main transition.\(^{19} \)

Taking into consideration that endothermic peak disappears at about \( X_{ch}=0.40 \), it is suggested that all HSPC molecules are conformationally restrained and form the \( L_o \) phase at \( X_{ch}>0.40 \). As for the pretransition, the \( \Delta H \) values became zero at \( X_{ch}>ca. 0.02 \) (Fig. 1B), corresponding to the conversion of both \( L_p^{\alpha} \) and \( P_p^{\beta} \) phases to the \( L_p^{\beta} \) phase.

Prodan Fluorescence Measurements for HSPC Liposome Membrane Figure 2A shows the changes in \( \lambda_{max} \) or GP value of Prodan in HSPC liposome with temperature, which were constructed on the basis of the spectra shown in Fig. 2A. The \( \lambda_{max} \) values of 440 and 490nm correspond to the gel and liquid crystalline phases of liposome membrane, respectively.\(^{15} \)

A two-step change in \( \lambda_{max} \) with increasing temperature was seen: the first step is from \( ca. 440 \) to \( ca. 470 nm \) at 45–47°C and the second from \( ca. 470 \) to \( ca. 490 \)nm at 50–53°C. Such a two-step change was also seen in the GP value and is expected to result from the change in the vertical location of Prodan molecules with the thermotropic phase transition of liposome membrane; that is, the pretransition from the \( L_p^{\alpha} \) to the \( P_p^{\beta} \) phase and the main transition from the \( P_p^{\beta} \) to the \( L_p^{\beta} \) phase.\(^{20} \)

We found that the lower and higher temperatures for abrupt changes in \( \lambda_{max} \) or GP value of Prodan are close to those in DSC thermograms showing the pre- and main transitions at 47.8°C and 53.6°C, respectively (Fig. 1A). These results indicate that the phase state of the liposome membrane can be detected by the change in \( \lambda_{max} \) or GP value of Prodan embedded in the membrane surface.

Prodan Fluorescence Measurements for HSPC–Cholesterol Binary Liposome Membrane Figure 3A shows the temperature dependence of Prodan \( \lambda_{max} \) for HSPC–cholesterol binary liposome membrane. The pretransition became obscure at \( X_{ch}=0.05 \) and the stepwise shift of \( \lambda_{max} \) disappeared at \( X_{ch}=0.30 \). During this range of \( X_{ch} \), the continuous increase in temperature for abrupt change in \( \lambda_{max} \) was observed with increasing \( X_{ch} \). For example, at \( X_{ch}=0.25 \), at least about 20°C rise in temperature was needed for the \( \lambda_{max} \) value to reach the plateau region at 490nm from 440nm. At \( X_{ch}\geq0.30 \), no significant increase in \( \lambda_{max} \) was observed (data not shown). In contrast, GP values for HSPC–cholesterol binary liposome membrane gradually decreased with increasing temperature (Fig. 3B). Interestingly, the temperature at which the gradual decrease in the GP value starts is almost constant independent of \( X_{ch} \) and close to the transition temperature observed in the DSC measurements (Fig. 1A).

Phase Diagram of HSPC–Cholesterol Binary Liposome Membrane In Fig. 4, the phase transition temperatures determined from DSC measurements (closed circle) and the temperatures at which Prodan \( \lambda_{max} \) reaches about 490nm (open square) indicative of the completion of the transition to...
the \( L_\beta \) phase are plotted as a function of \( X_{ch} \) to construct the temperature–\( X_{ch} \) phase diagram for HSPC–cholesterol binary liposome membrane. As seen from the shape of the phase diagram, the range between both main-transition temperatures determined from the DSC measurements and the Prodan \( \lambda_{\text{max}} \) behavior arises from the two-phase coexistence of the gel and \( L_\beta \) phases or the \( L_o \) and \( L_\alpha \) phases. Note that we use a term of \( L_o \) phase as a synonym of liquid disordered (\( L_o \)) phase, which is generally used for representing the phase state of binary or ternary membranes containing cholesterol.

We assigned the phase states for each area in the phase diagram (Fig. 4) on the basis of the above consideration and also thermodynamics of solid–liquid equilibria as follows: (I) \( L_\beta \), (II) \( L_\beta \pm P_{F_\beta} \), (III) \( L_\beta \pm P_{F_\alpha} \), (IV) \( L_\beta \pm L_{F_\beta} + L_{F_\alpha} \), (V) \( L_o \pm L_\beta \pm L_{F_\beta} + L_{F_\alpha} \), (VI) \( L_\beta \pm L_{F_\beta} + L_{F_\alpha} \), (VII) \( L_o \pm L_{F_\beta} + L_{F_\alpha} \), (VI) \( L_o \pm L_{F_\beta} + L_{F_\alpha} \) (IX) \( L_\beta \pm L_{F_\beta} + L_{F_\alpha} \) (X) \( L_o \). The explanation on the phase assignment is given in the following section. In these assignments, \( L_\beta \) (1:42) phase is a gel phase composed of a single type of unit which includes one cholesterol molecule and 42 surrounding HSPC molecules within the next–next–nearest neighbor sites (Fig. 5A). \( L_o \) (1:12) phase is similarly depicted as one cholesterol molecule and 12 surrounding HSPC molecules within the next–next nearest neighbor sites (Fig. 5B). In these figures, an open and a closed circles on each lattice point represent an HSPC (\( i.e. \), DSPC or DPPC) and a cholesterol molecules, respectively. Here, we should note that these schematic illustrations are given only to explain the range of cholesterol effects presumed as static averaged structures from bulk thermodynamic properties in terms of the formation of several types of complexes with different stoichiometries, and not to claim that rigid and highly ordered structures are formed within the HSPC–cholesterol bilayer depending on \( X_{ch} \). The phase diagram indicates that four kinds of phase separation can occur in the membranes below the main transition temperature and at \( X_{ch} < 0.40 \) (\( i.e. \), areas III, IV, VI, IX in Fig. 4). This means that the respective phase-separation regions differ in the combination of coexisting two phases depending upon \( X_{ch} \). For example, at \( X_{ch} = 0.05 \) in area VI, the present liposome membrane consists of the two different types of regions, namely \( L_\beta \) (1:42)-phase region and \( L_o \) (1:12)-phase region.

**Effect of PEG Modification on Prodan and Laurdan Fluorescence in Liposomes** To investigate the effect of PEG modification on the phase diagram of HSPC–cholesterol binary liposome membrane, the fluorescence measurements using Prodan were conducted for PEG-modified liposomes containing 5.6 mol% PEG-lipid. This composition is similar to that in Doxil®. The temperatures at which the \( \lambda_{\text{max}} \) of Prodan in PEG-modified liposomes or unmodified liposomes begins to rise from about 440 nm and reaches about 490 nm are shown in Table 1. The temperature dependence of Prodan \( \lambda_{\text{max}} \) for PEG-liposomes is similar to that for unmodified liposomes, indicating that the PEG modification has an insignificant effect on the phase behavior of HSPC–cholesterol bilayer liposome membrane.

Laurdan, a fluorescent probe with the same fluorophore as Prodan, is similarly sensitive to the polarity of its environment. These two probes differ only in the length of their acyl residue: Laurdan has a lauroyl chain, composed of 12 carbon atoms, while Prodan has a propionyl chain. Thus, as compared to Prodan, Laurdan is tightly anchored in the hydrophobic core by the cooperative van der Waals interactions between the lauroyl chain and lipid hydrocarbon chains. Thus, the GP values for each probe allow a quantitative estimation of local membrane polarity, related to local membrane fluidity. As shown in Fig. 6A, the GP values for Prodan in the PEG-modified liposomes were significantly lower than those for unmodified liposomes at all temperatures, suggesting that the incorporation of PEG-lipids into the liposome membranes increases the membrane fluidity of the hydrophilic region in

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**Table 1. Effects of PEG Modification on the Transition Temperature of Onset (ca. 440 nm) and Completion (ca. 490 nm) in Prodan Fluorescence**

<table>
<thead>
<tr>
<th>( X_{ch} )</th>
<th>DSPE-PEG2000 0 mol%</th>
<th>DSPE-PEG2000 5.6 mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Onset temperature (°C)</td>
<td>Completion temperature (°C)</td>
</tr>
<tr>
<td>0.06</td>
<td>50.5</td>
<td>54.6</td>
</tr>
<tr>
<td>0.10</td>
<td>50.1</td>
<td>57.9</td>
</tr>
<tr>
<td>0.25</td>
<td>54.3</td>
<td>68.4</td>
</tr>
</tbody>
</table>
which Prodan is located. In contrast, it is only above the main transition temperature that the GP values for Laurdan in the PEG-liposomes were lower than those in the unmodified liposomes (Fig. 6B). This result is consistent with the hypothesis that a part of PEG-chain inserts into the fluid-phase phospholipid bilayers.

Discussion

In this study, we elucidated the thermotropic phase behavior of HSPC–cholesterol binary liposome membrane by combination of the Prodan fluorescence and DSC measurements. In addition, by use of the superlattice model, we theoretically explained the interaction with HSPC and cholesterol molecules in gel phases.

We constructed the comprehensive phase diagram of HSPC–cholesterol binary liposome membrane (Fig. 4) by plotting the phase transition temperature from the DSC thermograms (Fig. 1) as well as the temperature at which the Prodan 

![](https://example.com/Fig_4.png)

which is consistent with the previous study which demonstrated that the main transition temperature of the liposomes composed of DPPC and HSPC can be controlled by the molar ratio of the two phospholipids.

At \(X_{ch}=0.40\), which is a cholesterol composition similar to that in Doxil®, no endothermic peak was observed in DSC thermograms (Fig. 1A), indicating that only the single \(L_o\) phase exists at \(X_{ch}>0.40\). This agrees with the fact that Doxil® is thermally stable liposome composed of the \(L_o\) phase. In contrast, the appearance of the single \(L_o\) phase in the DSPC–cholesterol and the DSPC–cholesterol binary liposome membranes was observed at \(X_{ch}>0.30\) and 0.50, respectively. These results indicate that the requisite cholesterol contents for the appearance of the single \(L_o\) phase differs depending upon the sort of the constituent phospholipid.

The phase diagram of the HSPC–cholesterol bilayer membrane shown in Fig. 4 may seem to be complicated but is very similar to a typical solid–liquid phase diagram for a eutectic binary mixture which exhibits congruent melting behavior (e.g. benzene–aniline binary mixture). In general, the presence of a congruent melting point indicates that the two components react to produce a new solid compound with a specific stoichiometry, and its stoichiometry well corresponds to the composition of the congruent melting point. Taking this into consideration, the fact that the congruent melting point is observed at \(X_{ch}=0.077\) for the HSPC–cholesterol bilayer suggests that a cholesterol molecule interacts with 12 HSPC molecules around the cholesterol molecule to form a 1:12-complex of cholesterol and HSPCs (Fig. 5B). This is also explained reasonably by the superlattice view proposed by Somerharju et al. That is, when the bilayer surface is entirely occupied with the units (1:12) composed of one cholesterol molecule and 12 surrounding HSPC molecules within the next–next nearest neighbor sites, the \(X_{ch}\) value equals to 0.077 (Fig. 5B).

This \(X_{ch}\) value is close to the composition of the congruent melting point on the phase diagram (Fig. 4). Similarly, another characteristic composition of \(X_{ch}\) ca. 0.02 corresponding to the composition at which the pretransition is abolished (Fig. 1B), can be well explained by the unit (1:42) in terms of the superlattice view, as shown in Fig. 5A. The abolition of the pretransition is generally thought to be attributed to the induction of an \(L_g\)-like molecular orientation by the presence of cholesterol, in which phospholipid molecules are arranged almost parallel to the bilayer normal. Therefore, this cholesterol effect of inducing such non-tilted molecular orientation is presumed to prevail among 42 HSPC molecules around a cholesterol molecule on average. Finally, the phase assignment as described above was derived from the consideration on the identification of each phase state, including \(L_o\) phase, and from the general thermodynamic knowledge on the solid–liquid phase equilibria for a eutectic mixture exhibiting congruent melting behavior. For example, it is understandable that all the looped areas above the eutectic temperature on the phase diagram correspond to the two-phase regions composed of a solid and a liquid phase, and also that all the solid-phase regions below the eutectic temperature are basically two-phase regions made of two different types of solids, because all the components including a new compound composed of the two components of the mixture are immiscible in a solid state.

As shown in Table 1, the PEG modification has no effect on the Prodan \(\lambda_{max}\), indicating that the thermotropic phase behavior of the PEG-liposome membrane is similar to that of the unmodified liposome membrane. However, recent molecular dynamics simulations and Langmuir monolayer measurements suggested that PEG-lipids may insert their polyether moiety...
into the fluid phase phospholipid bilayers.\textsuperscript{25} In this regard, the quantitative assessment of liposome membrane fluidity by using the GP values for Prodan (Fig. 6A) indicates that the hydrophilic region in liposome membrane appears to be somewhat perturbed by incorporated PEG-lipids. In addition, Fig. 6B suggests that some of PEG polymers interact with the glycerol backbone region of the liposome membrane above the main transition temperature, which is consistent with the finding that the PEG chain triggers the release of drug in liposomal lumen above the transition temperature.\textsuperscript{29}

In summary, the phase diagram of the HSPC–cholesterol binary liposome membrane was successfully obtained by combination of the high-sensitivity DSC and Prodan fluorescence measurements. In the phase diagram, the lipid miscibility and the characteristic compositions were explained by the hexagonal lattice model. The present results may provide useful information on the development of novel liposome products whose stability and encapsulated drug release are controlled.

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