Effects of $\beta$-Sitosteryl Sulfate on the Phase Behavior and Hydration Properties of Distearoylphosphatidylcholine: a Comparison with Dipalmitoylphosphatidylcholine

Ananda Kafle$^1$, Takeshi Misono$^1$, Avinash Bhadani$^1$, Masaaki Akamatsu$^1$, Kenichi Sakai$^1$, Chihiro Kaise$^2$, Teruhisa Kaneko$^2$ and Hideki Sakai$^1$*

$^1$Department of Pure and Applied Chemistry, Faculty of Science and Technology, Tokyo University of Science, 2641-1 Yamazaki, Noda, Chiba 278-8510, JAPAN

$^2$L. V. M. C. Inc. Komagome-7-14-3, Toshima-ku, Tokyo 170-0003, JAPAN

Abstract: We have studied the phase behavior of distearoylphosphatidylcholine (DSPC) in the presence of sodium $\beta$-sitosteryl sulfate (PSO4). PSO4 was found to induce sterol-rich and sterol-poor domains in the DSPC membrane. These two domains constitute a fluid, liquid ordered ($L_\beta$) phase and a gel ($\beta$) phase. PSO4 was less miscible in DSPC than in a dipalmitoylphosphatidylcholine (DPPC) membrane, as evidenced by its tendency to separate from the bilayer at a concentration of 50 mol%. This lack of miscibility was attributed to the greater van der Waals forces between the PC hydrocarbon chains. In addition to affecting the phase behavior, PSO4 also enhanced the hydration of the membrane. Despite its weaker interaction with DSPC compared to DPPC, its tendency to fluidize this phospholipid and enhance its hydration can be useful in formulating cosmetics and pharmaceutical products.

Key words: $\beta$-sitosteryl sulfate, phosphatidylcholine, phase, SAXS, DSC

1 Introduction

Phosphatidylcholines (PC) are amphiphilic molecules having a pair of acyl chains linked to the phosphocholine head group. Their structure imparts them with amphiplicity and hence, in the presence of water, they form lamellar bilayers. PC bilayers are the building blocks of biological membranes. Depending on the temperature and the presence of other biomolecules, they can assume different structural forms known as phases. The phase behaviors of PCs are of prime importance in regulating the functions of biological membranes$^{1-6}$.

Saturated phospholipids (e.g., 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), DPPC, and DSPC) are widely used as model systems for understanding the behavior of biological membranes$^{7-9}$. When hydrated, they exhibit three main phases, namely, the gel phase ($L_\alpha$), the ripple phase ($P_b$), and the liquid crystalline ($L_n$) phase. The gel phase ($L_\alpha$) exists below a characteristic temperature known as the phase transition temperature. It is characterized by the presence of all-\textit{trans} acyl chains packed together to form a quasi-hexagonal lattice. The $L_n$ phase forms above the main transition temperature ($T_m$), and has disordered hydrocarbon chains. The $P_b$ phase forms upon heating the gel phase above the pre-transition temperature ($T_p$). The structure of this phase is based on a 2D monoclinic lattice, and consists both of gel-like and fluid-like sections within the same bilayer$^{10-13}$.

In addition to temperature, the presence of compounds such as sterols (Fig. 1) is another factor that plays an important role in modifying membrane phase behavior. These

Abbreviations: DPPC = 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine or simply dipalmitoylphosphatidylcholine; DMPC = 1,2-Dimyristoyl-sn-glycero-3-phosphocholine or dimyristoylphosphatidylcholine; DSPC = 1,2-Distearoyl-sn-glycero-3-phosphocholine or distearoylphosphatidylcholine; PSO$_4$ = Sodium $\beta$-sitosteryl sulfate; Chol-SO$_4$ = Cholesterol sulfate; DSC = Differential Scanning Calorimetry; SWAXS = Small and Wide Angle X-ray Scattering; POM = Polarized Optical Microscopy; $d$ = Bilayer repeat distance; $T_m$ = Main phase transition maximum; $T_p$ = Pre-transition peak maximum.

*Correspondence to: Hideki Sakai, Department of Pure and Applied Chemistry, Faculty of Science and Technology, Tokyo University of Science, 2641-1 Yamazaki, Noda, Chiba 278-8510, JAPAN
E-mail: hisakai@rs.noda.tus.ac.jp
Accepted November 9, 2017 (received for review August 16, 2017)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/   http://mc.manuscriptcentral.com/jocs
greater degree than cholesterol. They disorder the hydro-
membrane properties such as fluidity and permeability\(^2\)–\(^6\).
At moderate to high concentrations, sterols such as chole-
sterol, phytosterols, and cholesterol sulfate (Chol-SO\(_4\)) give
rise to a fluid phase known as the liquid ordered
molecular structures of some phosphatidylcholines
and sterols.

modifications lead to changes in biologically important
membrane properties such as fluidity and permeability\(^2\)–\(^6\).
A. Kafle, T. Misono, A. Bhadani et al.

The characteristics of the \(L_\alpha\) phase are intermediate
between those of the gel and liquid crystalline
phases. Thus, it resembles the gel phase in its chain conformation
and the \(L_\alpha\) phase in terms of lateral diffusion. In high-melt-
ing PCs containing fairly low or moderate concentrations of
sterol, this phase coexists with the gel phase. The behavior
at higher sterol concentrations, however, depends upon
the nature of both the phospholipid and the sterol, and on
the strength of the interactions between them. For
example, cholesterol, \(\beta\)-sitosterol, or stigmasterol convert
gel phase phosphatidylcholines into the \(L_\alpha\) phase (or \(L_\alpha +
L_\beta\)) at concentrations over 30-50 mol\%. However, other
phospholipids, e.g., phosphatidylethanolamine or phospha-
didylerine, which interact less strongly with those sterols,
are not fully converted to the fluid phase even at relatively
high sterol concentrations\(^2, 14, 15\).

Sterol sulfates are an important class of sterol deriva-
tives. They have been found to have antimicrobial poten-
tial\(^{16}\). Previous studies suggest that there are major differ-
ences between the interactions of PCs with \(-\text{OH}\) sterols
and with the sterol-sulfates. For example, both Chol-SO\(_4\)\(^3, 5, 17\)
and PSO\(_4\)\(^18\) lower the maxima of the differential scanning
calorimetry (DSC) phase transition peaks of PCs to a
greater degree than cholesterol. They disorder the hydro-
carbon chains of gel phase PCs more efficiently than the
respective \(-\text{OH}\) sterols (cholesterol, \(\beta\)-sitosterol), but are
weaker in ordering the chains in the fluid phase. Unlike the
\(-\text{OH}\) sterols, they also greatly decrease the \(T_m\) (transition-
peak maximum) of the \(L_\alpha \rightarrow L_\beta\) phase transition. The sterol
sulfates are located much closer to the water layer as com-
pared to cholesterol\(^3, 5, 17, 19, 20\). Faure et al.\(^{19}\) reported the
increased ability of Chol-SO\(_4\) to hydrate DMPC and DPPC
bilayers. We also observed similar behavior for PSO\(_4\) incor-
porated in the DPPC bilayer (Kafle, A.; Misono, T.; Bhadani,
A.; Akamatsu, M.; Sakai, K.; Kaise, C.; Kaneko, T. & Sakai,
H.; submitted manuscript). The differences between the
behavior of the sterol-SO\(_4\) derivatives and the sterols them-
sew themselves occur due to the presence of the bulky \(-\text{SO}_4^–\) moiety
in the sterol-SO\(_4\) compounds, which simultaneously makes
it more efficient as a spacer between the PC head groups
as well as a better water-binder\(^ {3, 17, 19}\).

Due to their behavior towards PCs, sterol sulfates may
provide new tools for understanding the molecular mecha-
nisms involved in sterol-phospholipid interactions\(^ {17}\).
Further, owing to their ability to disorder the PC hydro-
carbon chains to a greater extent than their parent sterols,
they have high prospects of being used in cosmetics and
pharmaceutical formulations in combination with saturat-
ed-chain phosphatidylcholines, which are more stable than
the natural, unsaturated PCs. Their capacity to enhance
membrane hydration can also be of use in cosmetics and
pharmaceutical products. Phytosterol sulfates have the ad-
ditional advantage of being derived from plant sources, and
thus causing less impact on health and the environment.
In fact, PSO\(_4\) is already in use in skincare cosmetics as a ker-
atinocyte differentiation regulator\(^ {11, 21}\). Despite these merits,
no sufficient studies have been performed so far on the in-
teraction between phytosterol sulfates and phosphatidyl-
cholines. Previously, we carried out the first such study on
DPPC-PSO\(_4\) mixtures\(^ {18}\). In the present paper, we discuss
the phase behavior and hydration properties of DSPC in
the presence of PSO\(_4\) and compare them with the results
during the study\(^ {18}\).

2 Materials and Methods
2.1 Materials

DSPC of 99\% purity was supplied by NOF Corporation,
Japan and was used without further purification. PSO\(_4\) was
supplied by LVMC Inc., Tokyo, Japan. The as-received
sample was first dissolved in a solvent consisting of a 1:1
mixture of hexane and acetone by heating at 50°C. The
resulting material was filtered and dissolved in ethyl acetate
at 50°C with occasional sonication. The resulting com-
pound in ethyl acetate was filtered and dried in a rotary
 evaporator at 70°C for 30 minutes. After the removal of
ethyl acetate, the dried sample was dissolved in methanol
by heating at 50°C. Finally, the solution was filtered, and
PSO\(_4\) was obtained from the filtrate by removing the meth-
anol in a rotary evaporator at 70°C. The resulting sterol
isolated from the non-sulfate impurities by this method
was used for the study\(^ {18}\).
Phase Behavior and Hydration Properties of DSPC-β-sitosteryl Sulfate Membranes

2.2 Sample preparation
Appropriate quantities of DSPC and sodium β-sitosteryl sulfate were weighed to obtain mixtures of varying compositions such that the mole fraction of PSO$_4$ ($x$) varied from 0 to 0.5. The mixtures were then dissolved in a solvent containing chloroform and methanol in a ratio of 3:1 by volume. The solvents were removed by passing a flow of nitrogen gas through the solution. The samples were then vacuum-dried. Water was added to obtain mixtures with different wt% of lipids. Subsequently, the mixtures were subjected to three cycles of annealing and then homogenization by heating at ~74°C (approximately 20°C above $T_m$) with vortexing and stirring. To ensure proper homogenization, the samples were also stirred with a spatula after being cooled to room temperature. The test tubes containing these samples were then sealed tightly and incubated. Measurements were completed within 2-3 weeks of sample preparation. Samples with a lipid concentration of 40 wt% were used to investigate the phase behavior, while samples with lipid concentrations of 30 and 20 wt% were used to assess the hydration properties.

2.3 Polarized optical microscopy (POM)
Small volumes of the samples were trapped between a glass slide and a coverslip, and then gently pressed to achieve an appropriate thickness. The samples were observed under an IMT-2 microscope (Olympus Optical Co. Ltd.) through crossed polarizers, and the resulting birefringent textures were transferred to a computer by means of a Moticam 2000 digital camera fitted on the eyepiece. The textures were used to characterize the lamellar phases as well as to observe the changes brought about by the addition of PSO$_4$.

2.4 Differential scanning calorimetry
DSC measurements were performed using a Rigaku DSC-8230 instrument, with temperature scan range of 10°C to 85°C. About 3.0-3.3 mg of each sample was sealed in an aluminum sample pan, and the heat flow was measured using alumina as a reference. Two heating and cooling cycles were performed for each sample at rates of 3 and 1 K min$^{-1}$. The 1 K min$^{-1}$ run was used to study the general phase transition behaviors, and the 3 K min$^{-1}$ run was used to study the variation of the pre-transition behavior.

2.5 Small and wide angle x-ray scattering (SWAXS)
Scattering experiments were carried out using a W 3890 X-ray generator (PANalytical Co., Ltd., Almedo, Netherlands) and diffraction patterns were recorded using a SAXSess camera (Anton Paar Co., Ltd., Graz, Australia) in a line collimation system. A semitransparent beam stop was available to attenuate the beam. The samples were placed in a vacuum-proof metallic cell between Mylar windows, and the cell was tightened at both ends. Each sample was exposed to radiation of $\lambda = 0.154$ nm for 20 minutes in a vacuum, and the scattering pattern was detected on a 2D imaging plate. A cyclone reader (PerkinElmer Inc., Massachusetts, USA) was used to read the scattering patterns, which were converted to 1D profiles using SAXSquant software (Anton Paar Co., Ltd.). All data were normalized to the same incident primary beam intensity.$^{[18, 23]}

3 Results
3.1 Polarized optical microscopy (POM)
The POM textures obtained at 25°C for the 40 wt% lipid DSPC-PSO$_4$ samples with varying sterol contents are shown in Fig. 2a. The "Maltese crosses" texture for the $x = 0$ sample is consistent with a lamellar phase. Upon addition of 0.05 mole fraction of PSO$_4$, the texture takes the form of "white birefringence", which is similar to the gel-phase texture for partially hydrated DPPC bilayers (e.g., DPPC containing ≤ 40 wt% water)$^{[24, 25]}$. The presence of water outside the bilayer membrane enhances the curved regions in multilamellar vesicles (MLVs), giving rise to the defects structures known as "confocal domains"$^{[26]}$. These confocal domains are responsible for the formation of the "Maltese crosses" by hydrated PCs in the gel phase. Thus, this drastic change in texture from "Maltese crosses" to "white birefringence" can be attributed, at least partly, to the intake of free water into the membrane caused by PSO$_4$. Similar birefringence was observed for the samples with $x = 0.1$ and 0.15.

Upon further diluting the lipid mixture, "Maltese crosses" were again observed in the 20 wt% lipid (80 wt% water)/sample containing 10 mol% of the sterol (Fig. 2b). This indicates that in the case of a highly diluted mixture, the large amount of water subdues the effects of PSO$_4$. A similar observation was made in our previous study on the DPPC–PSO$_4$ system (Kafle, A.; Misono, T.; Bhadani, A.; Akamatsu, M.; Sakai, K.; Kaise, C.; Kaneko, T. & Sakai, H.; submitted manuscript), and was further confirmed using DSC and SAXS results (in addition to the POM textures). Therefore, we suggest that PSO$_4$ enhances the hydration of the membrane composed of DSPC.

In addition to providing fundamental information regarding membrane hydration, the POM textures also provide information about the change in fluidity of the lipid bilayers, and hence the changes in its phases (if any)$^{[27]}$. For samples containing a PSO$_4$ mole fraction of 0.2 or higher, oily streak textures were formed. The conversion of an existing gel-phase texture into oily streaks is an indication of the change of this phase into a fluid phase (Fig. 2a, $x = 0.2$ to 0.5)$^{[27, 28]}$. Thus, we conclude that the addition of PSO$_4$ into the DSPC membrane progressively converts its gel phase into a fluid phase.

3.2 Differential scanning calorimetry (DSC)

The DSC heating endotherms for the 40 wt% DSPC-PSO₄ samples are shown in Fig. 3a. The pre- and main transition temperatures of pure DSPC of 51.2°C and 54.6°C, respectively, are consistent with the reported values of 51.0°C and 54.9°C for fully hydrated DSPC²⁹. The pre-transition temperature (Tₚ) represents the L⁰ → Pβ phase transition, whereas the main transition peak represents the Pβ → Lα transition⁵,3⁰.

Similar to DPPC, the pre-transition peak persisted for sterol concentrations of up to 5 mol% (x = 0.05). As shown in Fig. 3b, the decrease of approximately 10°C in the pre-transition temperature with the addition of 5 mol% of sterol is comparable to the corresponding change in the case of DPPC. In addition to its temperature, the enthalpy of the pre-transition is also greatly decreased in the presence of this amount of sterol.

The addition of ≥ 10 mol% PSO₄ gives rise to thermograms containing multiple peaks (Fig. 3a). This is evident in the endotherms for the x = 0.1, 0.15, and 0.2 samples, each of which shows three overlapping peaks. The middle, sharp peak (green arrow) in each case corresponds to the usual main-phase transition peak (as can be seen by comparison with the corresponding Tm peaks for the preceding curves).

In several PC-sterol mixtures previously studied by other researchers⁵,⁶,3¹–3⁴, as well as in our own study on DPPC-PSO₄, the DSC thermograms have multiple components, which are successfully separated using a mathematical operation known as deconvolution. The thermogram for x = 0.075 in the current study, which is apparently a single, asymmetric peak, can also be deconvoluted (Fig. 4) by fitting with a combined Gaussian-Lorenzian fit. It has previously been established that the broad component corresponds to the sterol-rich domains and the sharp component corresponds to the sterol-poor domains in the mixture⁵,3⁰–3². In the literature, the former is frequently assigned as a liquid ordered phase (L₀) and the latter, as a
Phase Behavior and Hydration Properties of DSPC-β-sitosteryl Sulfate Membranes


437

Fig. 3 DSC heating thermograms (scan rate = 1-Kmin⁻¹) for 40 wt% DPPC-PSO₄ (a) and change in pre-transition peaks with the addition of PSO₄ (scan rate = 3Kmin⁻¹) (b). x in each case is the mole fraction of PSO₄. The black, green and red arrows in (a) represent respectively, the sterol-rich fraction, fully hydrated sterol-poor fraction and less-hydrated sterol-poor fraction. Only pre-transition peaks have been depicted in (b).

Fig. 4 DSC thermograms for the 40 wt% lipid mixtures containing 7.5 mol% of PSO₄. The broad components correspond to the cholesterol-rich and sharp components correspond to cholesterol-poor fractions.

gel phase (e.g. L₀). In the case of the DSPC samples with sterol concentrations of 10 mol% or higher, the component peaks are clearly separated from each other without requiring deconvolution. The broad component representing the L₀→Lα transition in this case is located on the left side (black arrow) of the sharp component.

These thermograms show that the sharp component is affected to a lesser extent than the analogous component in the case of DPPC, while the broad component (black arrows) is still strongly affected. The unequal influence of PSO₄ on the two components of the DSC peak leads to a clear separation of these two components.

A third peak is also present on the right side of the sharp peak (red arrow). The position and appearance of this peak is comparable to the 'high-temperature shoulder' arising from the presence of partially hydrated lipid molecules in DPPC containing <48% of water. Therefore, this peak may arise due to modification of the hydration of the bilayer by PSO₄. The samples with x = 0.15 and 0.2 also have a similar composition of peaks, except that the sharp (sterol-poor, fully hydrated, middle peak, which represents the L₀→Lα transition) gradually diminishes, while the peak on the right side becomes more prominent. The peak co-
responding to the sterol-rich fraction \((L_o\) phase, black arrow) is further diminished at \(x = 0.3\), and this peak, as well as the middle peak, disappears completely at \(x = 0.4\). The right-most peak (red arrow) is much less affected by the PSO\(_4\) concentration for the compositions \(x = 0.1\) to 0.5. As a result, there is almost no change in the temperature of this peak until \(x = 0.4\). A change of approximately 2 degrees at \(x > 0.5\) is attributed to the temperature-induced dissolution of the phase-separated PSO\(_4\) in the gradually fluidizing membrane\(^{37}\). Thus, the single peak that persisted at a concentration of \(x = 0.5\) is the same as the shoulder peak observed on the right side of the sharp peak in the \(x = 0.1\) sample. This indicates that the fraction of the lipid responsible for this peak does not readily interact with the sterol. The resistance of the third peak against the addition of PSO\(_4\) can be attributed to the fact that in a partially hydrated state the PC headgroups are tightly bound to each other by intermolecular forces\(^{30,33}\), and hence tend to force the PSO\(_4\) molecules out. According to previous studies\(^{30,33}\), the persistence of such a DSC peak even at high sterol concentrations is due to poor miscibility of the sterol in the bilayer. The small, sharp peak near the broad phase transition peak in the thermograms for \(x = 0.4\) and 0.5 was attributed to the transition of a fraction of the lipid mixture consisting of a large excess of PSO\(_4\) with traces of the PC. Though reproducible (refer to Fig. 1S, supplementary materials), we do not think it is a major transition, and it has been excluded from the rest of the discussion.

### 3.3 Small and wide angle x-ray scattering (SWAXS)

The SAXS and WAXS profiles for the 40 wt% lipid samples obtained at 25°C are given in Fig. 5. The bilayer repeat distance of 6.83 nm \((q = 0.92\) nm\(^{-1}\)) for the pure DSPC membrane in agreement with the previously reported value\(^{39}\). The broadening of the peaks indicates the coexistence of more than one phase. However, unlike in the case of DPPC-PSO\(_4\) mixtures, no evidence for the presence of a modulated (ripple) phase \((P\beta)\) was obtained at room temperature. The lack of formation of the \(P\beta\) phase may be due to the stronger van der Waals forces between the hydrophobic chains of the DSPC molecules, which caused partial resistance to the disordering of the hydrocarbon chains in the presence of small amounts of PSO\(_4\) (in sterol-poor domains).

The WAXS profile for the \(x = 0\) sample features a sharp peak at \(q = 15\) nm\(^{-1}\) with an adjoining shoulder, indicating a \(L_o\) gel phase\(^{14,40}\). The same is true in the case of \(x = 0.05\). The existence of a \(L_o\) phase in the presence of 5 mol% of the sterol is in agreement with the persistence of a pre-transition in the DSC for this sample. The peak intensities decrease as the concentration of PSO\(_4\) increases from \(x = 0.15\) to 0.4, indicating that the hydrocarbon chains become increasingly disordered. Unlike in the case of DPPC, however, the peaks do not disappear completely even at

![Fig. 5 SAXS (a) and WAXS (b) profiles (25°C) for 40 wt% lipid samples containing DPPC-PSO\(_4\).](image-url)
higher concentrations of PSO₄, indicating that a complete transition to a fluid phase is not achieved. At x = 0.5, the separation of PSO₄ crystals was observed. As shown in Fig. 5, the two peaks located to the left of the gel phase peak in the WAXS profile (indicated in the figure) and the peak at q = 1.44 nm⁻¹ in the SAXS profile are due to the phase-separated PSO₄. These PSO₄ peaks were identified by obtaining the scattering patterns for pure, hydrated PSO₄ (x = 1) and comparing the obtained profile with that of the lipid mixture. This separation of PSO₄ was not observed in our previous study on DPPC-PSO₄. Thus, the SAXS and WAXS profiles, as well as the DSC results, indicate that PSO₄ interacts less strongly with DSPC than with DPPC, and that it is crystallized out from the membrane at higher sterol concentrations.

4 Discussion

The binary phase diagram drawn based on the above results is shown in Fig. 6a. In this phase diagram, the $L_β$ $→$ $L_α$ + $L_α$ boundary was chosen based on the disappearance of the DSC pre-transition peak, the transformation of the $L_β$ phase WAXS peak into a peak characteristic of the $L_α$ phase, and the tendency of the DSC main peak to split into broad and sharp components. Unlike the $L_β$ phase, the $L_α$ phase of DSPC did not convert into a $P_β$ phase due to the lack of a tilt with respect to the bilayer normal. The top, horizontal phase boundary, which shows the thermal changes, was derived from the DSC main transition peaks as well as the phase changes indicated by the SWAXS and POM data. Only temperatures representing the overall transition of the coexisting lamellar phases ($L_α$ and $L_α$) into the $L_α$ phase were considered in this simplified phase diagram, instead of including the temperatures corresponding to the individual peak components. The phase diagram for DPPC-PSO₄ from our previous study has also been presented in Fig. 6 for the sake of comparison.

We observed the following differences between the two phase diagrams:
- PSO₄ completely fluidized the DPPC membrane but not the DSPC membrane.
- The decrease in $T_m$ with the addition of PSO₄ was greater in the DPPC membrane than in the DSPC membrane.
- The incorporation of PSO₄ induced a modulated phase ($P_β$) in the DPPC membrane, but not in the DSPC membrane.
- PSO₄ precipitated out from the DSPC membrane above x = 0.4, but not from the DPPC membrane.

Our microscopic study showed that with the progressive addition of PSO₄ to the DSPC bilayer, the membrane gradually changed from the gel phase into a fluid phase. Lamellar vesicles form “Maltese cross” textures in the presence of excess water. Excess water contributes to the formation of these textures by inducing membrane curvature. Thus, from the formation of “Maltese crosses” (Figs. 2a and b), it can be concluded that the presence of PSO₄ (10 mol% in this case) shifts the “hydration saturation” point of DSPC to a lower lipid concentration. In this way, the POM textures also indicate that the hydration of the membrane is
enhanced when PSO₄ is added. This result agrees with our previous similar study on DPPC-PSO₄.

The $T_m$ (main phase transition) peak for the sample with $x = 0.075$, which does not contain any obvious indication of multiple peaks, can be deconvoluted into a broad and a sharp component. In this case, as in DPPC, the broad component lies to the left side of the sharp component. The same is true for the samples with higher sterol concentrations ($x = 0.1$ to $0.3$), except that at these concentrations, the broad component is clearly separated from the sharp component without a need for deconvolution. This can be attributed to the fact that PSO₄ affects the sterol-rich fraction to a greater extent than the sterol-poor fraction.

The third peak (shown with red arrows in Fig. 3) in the endotherms of the samples with $x = 0.1$ and higher arises from the change in the hydration of the headgroups caused by PSO₄. According to previous studies, the intercalation of anionic species in the phosphatidylcholine bilayer reorients the headgroups, causing them to face towards the membrane interior, i.e., away from the water layer. That means the reoriented PC molecules are relatively less hydrated as compared to others. However, in the case of the fluid phase domain, this may not be true since the headgroups are less tightly packed, and a portion of the inter-bilayer water molecules can occupy the space under the headgroups. From this, it is expected that even the reoriented DSPC molecules remain well hydrated. In addition to the change in the conformation of the headgroups, another factor that leads to a heterogeneously hydrated interface is the high tendency of the sulfate moiety in the PSO₄ molecule to accumulate water. Thus, a large amount of water is accumulated near the PSO₄ molecules. All of these phenomena lead to an increased demand for water within the membrane. We previously investigated this increased demand for water in the DPPC-PSO₄ system (Kafle, A.; Misono, T.; Bhadani, A.; Akamatsu, M.; Sakai, K.; Kaise, C.; Kaneko, T., and Sakai, H, submitted manuscript). Previously, Faure et al. also carried out a similar study on the DMPC-CholSO₄ (cholesterol sulfate) system. The enhanced hydration of the phosphatidylcholine membranes can be beneficial in formulating cosmetics, such as moisturizing lotions, as well as higher-efficiency drug vehicles.

As can be seen in Fig. 3a, the three peaks are affected differently by the increase in the quantity of PSO₄. The leftmost or the broad component (PSO₄ rich, $L_e$ phase, shown with black arrow) is affected the most, in terms of transition temperature and cooperativity. The larger temperature shift of this fraction, which corresponds to the sterol-rich domain, is due to the presence of a larger number of water molecules accumulated by PSO₄ as well as the tendency of PSO₄ to strongly lower the $L_e$ to $L_a$ phase transition temperature of PCs. The middle peak is a fraction of the sharp component (representing the fully hydrated gel ($L_g$) phase) arising from sterol-poor domains in the mixture, which is similar to the sharp components observed in several other PC-sterol combinations, including the mixture of DPPC and PSO₄.

When sterol-rich and sterol-poor domains form after the addition of water to the vacuum-dried lipid mixtures, a larger fraction of water passes into the sterol-rich domain due to the high tendency of PSO₄ to bind water. Thus, the remaining portion, i.e., the PSO₄-poor fraction, is no longer a homogeneous gel phase coexisting with the excess free water. Instead, it exists as a mixture of an ‘excess-water-gel’ phase and partially hydrated lipid molecules of the kind described by Kodama et al. and Yeagle. A schematic diagram of the modification of the hydration of the DSPC membrane by PSO₄ is depicted in Fig. 7.

Our study shows that PSO₄ is less miscible in DSPC than in DPPC. Consequently, the DSC $T_m$ peak is not eliminated even by the addition of 50 mol% of PSO₄, and the scattering experiments show peaks characteristic of free PSO₄ in both the small and wide-angle regions. The poorer miscibility of PSO₄ in DSPC compared to DPPC can be attributed to the increased van der Waals attraction forces between the hydrocarbon groups in the DSPC molecule. Due to its poor miscibility, PSO₄ can be expected to move away from the DSPC molecules in the bilayer, and eventually precipitate, at sufficiently high sterol concentrations (e.g., $x = 0.5$). Furthermore, such hydrocarbon chains are more disordered in the fluid phase than in the gel phase. Sterol sulfates in particular lead to greater disordering than their parent sterols (−OH sterols). Since this configuration is more favorable for accommodating the molecules of PSO₄, a much larger fraction of the sterol is present in this domain rather than in the tightly bound sterol-poor domain (gel phase). This may be the reason for the jump in transition temperature between $x = 0.2$ and 0.3 samples.

Thus, this study shows that PSO₄ is less miscible in DSPC than in DPPC. However, its effects on the fluidity and hydration properties are comparable in both cases. DSPC is a major component in commercially important phospholipids such as hydrogenated soy lecithin and egg lecithin. Therefore, the information obtained from DSPC could be useful for combining PSO₄ with these lecithins in cosmetics or pharmaceutical formulations. The tendency of PSO₄ to partially fluidize the PC membranes and enhance their hydration could be useful in a number of ways for the design of skincare cosmetics and drug delivery systems, as described in the following paragraph.

When formulating skin-care products such as moisturizers, an important parameter to consider is the capacity of the formulation to supply moisture to the skin, as well as to prevent the loss of moisture from the skin. This study shows that the amount of inter-bilayer water in the PC membrane can be enhanced by the addition of PSO₄ to the membranes. When applied to the skin, a formulation consisting of these lipids will be placed either on the surface of...
the skin, or in the gaps formed due to the loss of natural intercellular lipids. It will then continue to act as a reservoir of water for the skin cells. The ability to enhance hydration is also important in drug delivery systems, since it will ultimately result in an increased aqueous volume in the liposomes required for carrying drugs. Furthermore, it can also improve the dispersibility of the liposomal particles by decreasing their densities. Similarly, the coexistence of gel and fluid phases in the membrane can also be beneficial. The fluid \( L_\beta \) fraction of the membrane induced by PSO\(_4\) is suitable for percutaneous transport of drugs and cosmetic ingredients due to the higher elasticity of the vesicles in this phase. On the other hand, the rigid, solid-like gel phase will continue to function as a reservoir for moisture as well as a protective coating to prevent the loss of moisture from the skin\(^{47}\). Furthermore, it has also been claimed by previous researchers\(^{48}\) that dispersions consisting of solid lipid particles dispersed in another lipid in a fluid phase are more suitable for preparations such as medicinal dosage forms, which contain lipids at high concentrations with a minimal amount of water.

5 Conclusion

Thus, based on the experimental results, we conclude that PSO\(_4\) significantly affects the phase behavior and hydration properties of the DSPC membrane. At moderate to high concentrations, PSO\(_4\) fluidizes the membrane and enhances the demand for water within it. PSO\(_4\) greatly decreases the transition temperature of the sterol-rich fraction, and to a lesser extent, that of the “excess-water-gel” phase. However, it does not significantly affect the fraction of the lipid molecules that are reoriented by the PSO\(_4\) molecules.

Despite its tendency to fluidize the membrane and enhance its hydration, the behavior of PSO\(_4\) in the DSPC membrane is significantly different than in the DPPC membrane. These differences include its inability to completely fluidize DSPC, such that the \( L_\beta \) phase persists even at a
high concentration of PSO₄. It is less miscible in the DSPC membrane than in the DPPC membrane because of the stronger van der Waals forces between the PC molecules. The poorer miscibility of PSO₄ in the DSPC membrane is responsible for its inability to completely convert the membrane to a fluid phase.

However, despite its poorer miscibility, PSO₄ can still find applications in cosmetics and pharmaceutical formulations containing DSPC (e.g., in the form of hydrogenated soy lecithin), owing to its capacity to fluidize the membranes and enhance their hydration.

**Supplementary Information**
This material is available free of charge via the Internet at http://dx.doi.org/jos.67.10.5650/jos.ess.17182

**References**
23) Aburai, K.; Ogura, T.; Hyodo, R.; Sakai, H.; Abe, M.; Glatter, O. Location of cholesterol in liposomes by using small-angle X-ray scattering (SAXS) data and the
Phase Behavior and Hydration Properties of DSPC-β-sitosteryl Sulfate Membranes


