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

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Abstract: We have studied the phase behavior of distearoylphosphatidylcholine (DSPC) in the presence of sodium β -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_o) phase and a gel (L_β) 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: β -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 amphiphilicity 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 membranes¹⁻⁶⁾.

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 membranes⁷⁻⁹⁾. When hydrated, they exhibit three main phases, namely, the gel phase (L_β '), the ripple phase (P_β '), and the liquid crystalline (L_α) phase. The gel phase (L_β ') exists below a characteristic temperature known as the phase transition temperature. It is characterized by the presence of all-*trans* acyl chains packed together to

form a quasi-hexagonal lattice. The L_α phase forms above the main transition temperature (T_m), and has disordered hydrocarbon chains. The P_β ' 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 bilayer¹⁰⁻¹³⁾.

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; PSO₄ = Sodium β -sitosteryl sulfate; Chol-SO₄ = 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.

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Fig. 1 Molecular structures of some phosphatidylcholines and sterols.

modifications lead to changes in biologically important membrane properties such as fluidity and permeability^{2–6}. At moderate to high concentrations, sterols such as cholesterol, phytosterols, and cholesterol sulfate (Chol- SO_4) give rise to a fluid phase known as the liquid ordered (L_o) phase. The characteristics of the L_o phase are intermediate between those of the gel and liquid crystalline (L_a) phases. Thus, it resembles the gel phase in its chain conformation and the L_a phase in terms of lateral diffusion. In high-melting 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, β -sitosterol, or stigmasterol convert gel phase phosphatidylcholines into the L_o phase (or $L_o + L_a$) at concentrations over 30–50 mol%. However, other phospholipids, e.g., phosphatidylethanolamine or phosphatidylserine, 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 derivatives. They have been found to have antimicrobial potential¹⁶. Previous studies suggest that there are major differences between the interactions of PCs with $-OH$ 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 hydrocarbon chains of gel phase PCs more efficiently than the respective $-OH$ sterols (cholesterol, β -sitosterol), but are weaker in ordering the chains in the fluid phase. Unlike the $-OH$ sterols, they also greatly decrease the T_m (transition-peak maximum) of the $L_o \rightarrow L_a$ phase transition. The sterol sulfates are located much closer to the water layer as compared 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 incorporated 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 themselves occur due to the presence of the bulky $-SO_4^-$ 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 mechanisms involved in sterol-phospholipid interactions¹⁷. Further, owing to their ability to disorder the PC hydrocarbon chains to a greater extent than their parent sterols, they have high prospects of being used in cosmetics and pharmaceutical formulations in combination with saturated-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 additional 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 keratinocyte differentiation regulator^{21, 22}. Despite these merits, no sufficient studies have been performed so far on the interaction between phytosterol sulfates and phosphatidylcholines. Previously, we carried out the first such study on DPPC- PSO_4 mixtures¹⁸. 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 for DPPC.

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 compound 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 methanol in a rotary evaporator at 70°C. The resulting sterol isolated from the non-sulfate impurities by this method was used for the study¹⁸.

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 $\text{PSO}_4(x)$ 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 $\sim 74^\circ\text{C}$ (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 3830 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 $\lambda = 0.154\text{ nm}$ 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 $\leq 40\text{ wt\%}$ water)^{24, 25}. The presence of water outside the bilayer membrane enhances the curved regions in multilamellar vesicles (MLVs), giving rise to the defect 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.

a.

b.

Fig. 2 POM Textures at 25°C for the samples containing 40 wt% lipids (a) and 20 and 30 wt% lipids plus 10 mol% PSO_4 (b) x in each case is the mole fraction of PSO_4 .

3.2 Differential scanning calorimetry (DSC)

The DSC heating endotherms for the 40 wt% DSPC- PSO_4 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 (T_p) represents the $L_\beta' \rightarrow P_\beta'$ phase transition, whereas the main transition peak represents the $P_\beta' \rightarrow L_\alpha$ transition^{5, 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% PSO_4 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 T_m peaks for the preceding curves).

In several PC-sterol mixtures previously studied by other researchers^{5, 6, 31–34)}, as well as in our own study on DPPC- PSO_4 ¹⁸⁾, 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-Lorentzian 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 mixture^{5, 30–32)}. In the literature, the former is frequently assigned as a liquid ordered phase (L_o) and the latter, as a

Fig. 3 DSC heating thermograms (scan rate = 1-Kmin^{-1}) for 40 wt% DPPC- PSO_4 (a) and change in pre-transition peaks with the addition of PSO_4 (scan rate = 3Kmin^{-1}) (b). x in each case is the mole fraction of PSO_4 . 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 PSO_4 . The broad components correspond to the cholesterol-rich and sharp components correspond to cholesterol-poor fractions.

gel phase (e.g. L_β). 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 $L_o \rightarrow L_\alpha$ 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 DPPC¹⁸⁾, while the broad component (black arrows) is still strongly affected. The unequal influence of PSO_4 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^{35, 36)}. Therefore, this peak may arise due to modification of the hydration of the bilayer by PSO_4 . 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 $L_\beta \rightarrow L_\alpha$ transition) gradually diminishes, while the peak on the right side becomes more prominent. The peak cor-

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 membrane³⁷. 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 forces³⁸, 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 ($q=0.92 \text{ nm}^{-1}$) for the pure DSPC membrane is in agreement with the previously reported value³⁹. 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_β) was obtained at room temperature. The lack of formation of the P_β 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 $q \approx 15 \text{ nm}^{-1}$ with an adjoining shoulder, indicating a L_β' gel phase^{14,40}. The same is true in the case of $x=0.05$. The existence of a L_β' 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 .

higher concentrations of PSO_4 , indicating that a complete transition to a fluid phase is not achieved. At $x=0.5$, the separation of PSO_4 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 $q = 1.44 \text{ nm}^{-1}$ in the SAXS profile are due to the phase-separated PSO_4 . These PSO_4 peaks were identified by obtaining the scattering patterns for pure, hydrated PSO_4 ($x=1$) and comparing the obtained profile with that of the lipid mixture. This separation of PSO_4 was not observed in our previous study on DPPC- PSO_4 . Thus, the SAXS and WAXS profiles, as well as the DSC results, indicate that PSO_4 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 $L_{\beta}' \rightarrow L_{\beta} + L_o$ 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_{β} phase, and the tendency of the DSC main peak to split into broad and sharp components. Unlike the L_{β}' phase, the L_{β} phase of DSPC did not convert into a P_{β}' phase due to the lack of a tilt with respect to the bilayer normal^{41, 42}. 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_o and L_{β}) into the L_{α} phase were considered in this simplified^{43, 44} phase diagram, instead of including the temperatures corresponding to the individual peak components. The phase diagram for DPPC- PSO_4 from our previous study¹⁸) has also been presented in Fig. 6 for the sake of comparison.

We observed the following differences between the two phase diagrams:

- PSO_4 completely fluidized the DPPC membrane but not the DSPC membrane.
- The decrease in T_m with the addition of PSO_4 was greater in the DPPC membrane than in the DSPC membrane.
- The incorporation of PSO_4 induced a modulated phase (P_{β}) in the DPPC membrane, but not in the DSPC membrane.
- PSO_4 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 PSO_4 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 curvature⁴⁵). Thus, from the formation of "Maltese crosses" (Figs. 2a and b), it can be concluded that the presence of PSO_4 (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

Fig. 6 Binary phase diagram for 40 wt% lipid DSPC- PSO_4 mixture (a) and similar diagram for DPPC- PSO_4 mixture (b). Figure b was taken from Kafle *et al.*¹⁸) for comparison.

enhanced when PSO_4 is added. This result agrees with our previous similar study on DPPC- PSO_4 .

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 PSO_4 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 PSO_4 . According to previous studies^{19, 46)}, 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 re-oriented 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 PSO_4 molecule to accumulate water. Thus, a large amount of water is accumulated near the PSO_4 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- PSO_4 system (Kafle, A.; Misono, T.; Bhadani, A.; Akamatsu, M.; Sakai, K.; Kaise, C.; Kaneko, T., and Sakai, H, submitted manuscript). Previously, Faure *et al.*¹⁹⁾ also carried out a similar study on the DMPC-Chol SO_4 (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 PSO_4 . The leftmost or the broad component (PSO_4 rich, L_o 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 PSO_4 ^{18, 19)} as well as the tendency of PSO_4 to strongly lower the $L_o \rightarrow L_\alpha$ phase transition temperature of PCs⁵⁾. The middle peak is a fraction of the sharp component (representing the fully hydrated gel (L_β) 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 PSO_4 ^{5, 31, 32)}.

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 PSO_4 to bind water. Thus, the remaining portion, i.e., the PSO_4 -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.*³⁵⁾ and Yeagle³⁶⁾. A schematic diagram of the modification of the hydration of the DSPC membrane by PSO_4 is depicted in Fig. 7.

Our study shows that PSO_4 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 PSO_4 , and the scattering experiments show peaks characteristic of free PSO_4 in both the small and wide-angle regions. The poorer miscibility of PSO_4 in DSPC compared to DPPC can be attributed to the increased van der Waals attraction forces between the hydrocarbon groups in the DSPC molecule³⁰⁾. Due to its poor miscibility, PSO_4 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 ($-\text{OH}$ sterols). Since this configuration is more favorable for accommodating the molecules of PSO_4 , 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 PSO_4 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 PSO_4 with these lecithins in cosmetics or pharmaceutical formulations. The tendency of PSO_4 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 PSO_4 to the membranes. When applied to the skin, a formulation consisting of these lipids will be placed either on the surface of

Fig. 7 Schematic diagram showing the modification of hydration of DSPC bilayer by PSO_4 .

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_o) 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 skin⁴⁷⁾. Furthermore, it has also been claimed by previous researchers⁴⁸⁾ 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_β phase persists even at a

high concentration of PSO_4 . 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 PSO_4 in the DSPC membrane is responsible for its inability to completely convert the membrane to a fluid phase.

However, despite its poorer miscibility, PSO_4 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

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