Effects of $\beta$-Sitosteryl Sulfate on the Hydration Behavior of Dipalmitoylphosphatidylcholine

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Abstract: We investigated the hydration behavior of dipalmitoylphosphatidylcholine (DPPC) bilayers containing sodium $\beta$-sitosteryl sulfate (PSO$_4$). PSO$_4$ was found to enhance hydration in the headgroup region of DPPC bilayers. Therefore, with the incorporation of PSO$_4$ into DPPC membranes, the amount of water required to reach the fully hydrated state was enhanced as indicated by the constant values of the main phase transition maximum ($T_{p1}$) and the bilayer repeat distance ($d$). For example, with the addition of 20 mol% of PSO$_4$, the saturation point was shifted to $\sim$70 wt% water compared to $\sim$40 wt% for pure DPPC and 47 wt% for DPPC-cholesterol. The effectiveness of PSO$_4$ in fluidizing the membrane and enhancing its hydration state can be useful in the pharmaceutical and cosmetic industries.

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

1 Introduction

Membrane hydration is important in biological systems. A fully hydrated biological membrane ensures the proper functioning of cells in the body. On the other hand, a lack of hydration can induce phase changes in the lipids and damage the membrane$^{1,2}$. In addition to its vital role in biological cells, membrane hydration is also important for developing membrane-based cosmetics and pharmaceuticals$^3$.

The presence of sterols in phosphatidylcholine (PC) membranes can induce changes in their phase structures. They can modify membrane fluidity and give rise to sterol-rich and sterol-poor domains$^4-6$. Previous studies have reported that the sterol-rich and sterol-poor phases retain different amounts of water. The temperature and fluidity of lipid mesophases also play key roles in membrane hydration$^7,8$.

Sterol sulfates are important derivatives of sterols, and some are found in living cells. Furthermore, they have been reported to possess antimicrobial properties$^9-11$. Among several sterol sulfates, only the effects of cholesterol sulfate (Chol-SO$_4$) on the hydration properties of PCs have been investigated thus far$^{10-12}$. In comparison with cholesterol, Chol-SO$_4$ is localized at a position in the bilayer that is closer to the hydrophilic layer. Furthermore, because of the presence of the negatively charged sulfate moiety, it retains more water molecules around itself and hence, enhances membrane hydration$^{10,12}$. At present, there are no studies on the behavior of $\beta$-sitosteryl sulfate (PSO$_4$) (Fig. 1).

PSO$_4$ is structurally similar to Chol-SO$_4$, except for the presence of an additional ethyl group in its aliphatic chain. Several studies have found that even minor changes in the structure of sterols either in the hydrophilic region or the hydrocarbon chain or rings can greatly affect their interaction with phospholipids$^{13,14}$. Therefore, the information obtained from studies of the PC-Chol-SO$_4$ system cannot be directly applied to the PC-PSO$_4$ system despite the structural similarities. Hence, a separate study of the

Abbreviations: DPPC = 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine or simply dipalmitoylphosphatidylcholine; DMPC = 1,2-Dimyristoyl-sn-glycero-3-phosphocholine or dimyristoylphosphatidylcholine; DPMC = 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; FF-TEM = Freeze-fracture Transmission Electron Microscopy; $d$ = Bilayer repeat distance; $T_m$ = Main phase transition maximum; $T_p$ = Pre-transition peak maximum; GIFT = Generalized Indirect Fourier Transformation.

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of ethyl acetate, the dried sample was dissolved in methanol by heating to 50°C. Finally, the sample was filtered, and pure PSO₄ was obtained from the filtrate by removing methanol via rotary evaporation at 70°C. PSO₄ was characterized by ¹H NMR spectroscopy, and the purity was estimated to be 95–98% by HPLC.

2.2 Sample preparation
Specific quantities of DPPC and PSO₄ were weighed to obtain mixtures with different compositions; the mole fraction of PSO₄ (x) ranged from 0 to 0.2. The mixtures were then dissolved in a solvent containing chloroform and methanol with a volume ratio of 3:1. The solvents were removed by passing a stream of nitrogen gas through the solution. The samples were then vacuum-dried. Water was added to obtain mixtures with different concentrations of lipids ranging from 10 to 80 wt%. The mixtures were then subjected to three cycles of annealing, and they were homogenized by heating at ~60°C (around 20°C above the main phase transition temperature, Tm) with vortexing and stirring. To ensure proper homogenization, the samples were also stirred with a spatula after cooling to room temperature. The test tubes containing these samples were then sealed tightly and incubated. Measurements of the equilibrated samples were taken within 2–3 weeks after sample preparation.

2.3 Polarized optical microscopy (POM)
Small amounts of samples were placed on clean and dry glass slides and carefully covered with coverslips in an inclined position with the help of a narrow spatula to avoid trapping any air bubbles. Then, they were gently pressed to achieve the appropriate thickness. The samples were observed using an IMT-2 microscope (Olympus Optical Co., Ltd.) equipped with a temperature control unit (Mats-1002RO; Tokai Hit Co., Ltd.) through crossed polarizers. The resulting birefringent textures were captured by a Moticam 2000 digital camera fitted on the eyepiece of the microscope. The textures were used to characterize lamellar phases and identify changes caused by the addition of PSO₄.

2.4 Differential scanning calorimetry (DSC)
DSC measurements were carried out using a Rigaku DSC-8230 instrument. The measurements were performed with continuous temperature scanning from 10°C to 85°C. Approximately 3.0–3.3 mg of samples were sealed in aluminum sample pans, and the heat flow was measured using alumina as a reference (scan rate = 1 Kmin⁻¹).

2.5 Small-angle X-ray scattering (SAXS)
Scattering experiments were carried out using a W 3830 X-ray generator (PANalytical Co., Ltd., Almelo, Netherlands), and diffraction patterns were recorded with a
SAXSess camera (Anton Paar Co., Ltd., Graz, Austria) in a line-collimation system. A semitransparent beam stop was used 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 a radiation of $\lambda = 0.154$ nm at 25°C for 20 min in vacuum, and the scattering pattern was detected with a 2D imaging plate detection system. A cyclone reader (PerkinElmer Inc., MA, USA) was used to read the scattering patterns that were converted to 1D profiles using SAXSquant software (Anton Paar Co., Ltd.). All data were normalized to the same incident primary beam intensity.

### 3 Results and Discussion

DSC, SAXS, and POM analyses were performed using DPPC samples containing different mole fractions of PSO$_4$ ranging from 0 to 0.5. The lipid concentrations of these samples ranged from 10 wt% to 80 wt%.

#### 3.1 Microscopic observation

POM was used to evaluate major differences in the hydration state of the membrane due to the addition of PSO$_4$. Changes in the hydration state were evaluated according to POM textures. As shown in Fig. 2 (top), Maltese crosses were observed for pure DPPC samples with ≥ 50 wt% water. When the amount of water was lower (e.g., 40 or 30 wt% water), white birefringent textures were formed. As reported by Yeagle and Kodama et al., approximately 48 wt% of water indicates the phase boundary, above which the material consists of a fully hydrated gel phase coexisting with excess free water. Below this boundary, the system is heterogeneous with the free-water phase and partially hydrated lipid molecules existing together. Therefore, our POM results indicated that excess free water plays a role in forming Maltese crosses. This is in agreement with the enhancement of the curvature of multilamellar vesicles (MLVs) by free water, which can retard light in a radial fashion, leading to the formation of the corresponding texture.

The incorporation of PSO$_4$ into DPPC could significantly change the texture. As shown in Fig. 1, Maltese crosses were absent with 10 mol% of PSO$_4$. As we previously reported, even with 1 mol% ($x = 0.01$) of PSO$_4$, Maltese crosses were mostly absent, converting the texture predominantly to a white birefringent texture of an ordered lamellar phase (gel or ripple gel). Maltese crosses re-

**Fig. 2** POM textures showing onset of Maltese crosses in the absence (top row) and presence (middle and bottom rows) of PSO$_4$. $x$ in each case is the mole fraction of PSO$_4$ and the % values refer to the wt% of lipid.
appeared in the 20 wt% lipid (80 wt% water) sample containing 10 mol% of sterol (Fig. 2, middle row). This was the same for the 10 wt% lipid sample (supplementary, Fig. F1). These observations indicate that the changes in textures caused by PSO were, at least partly, associated with its ability to promote the diffusion of free water into the membrane. However, there may be additional factors related to the presence of PSO that can also affect POM textures. Therefore, this result was insufficient for an accurate assessment of the effects of PSO on the hydration of DPPC. For a better understanding, we performed DSC and SAXS analyses using samples with different concentrations.

3.2 DSC and SAXS analyses

Figure 3 shows the DSC heating endotherms (rate = 1 Kmin⁻¹) for samples with various lipid concentrations and specific mole fractions of PSO₄. As shown in these thermograms, the T_m of the samples of each lipid composition (each mole fraction of PSO₄) was gradually decreased with decreasing lipid concentration until reaching a nearly constant value. Depending on their composition, samples with higher lipid concentrations contained an additional peak/shoulder at a higher temperature (indicated by * in the figures). These additional peaks may be attributed to the gel-to-liquid crystal phase transition of the partially hydrated lipid molecules, which are present at a high lipid concentration (low water) [1, 23].

Figure 4 shows the SAXS profiles obtained at 25°C for

**Fig. 3**  DSC heating endotherms (rate = 1 Kmin⁻¹) for the samples with various lipid concentrations and containing different quantities of PSO₄, x (mole fraction of PSO₄) = 0 (a), 0.1 (b), and 0.2 (c).
the same set of samples. The integral peak ratio demonstrated that the lipid molecules were arranged in a lamellar bilayer structure. The SAXS profiles of the samples containing 10 mol% (x = 0.1) of PSO, contained two sets of lamellar peaks, of which the first peak represents a modulated phase (Pβ; a rippled gel phase), and the second peak represents the liquid-ordered (Lo) phase10. The bilayer repeat distance (d) can be obtained from the SAXS profile for a given lipid sample using the relationship 
\[ d = \frac{2 \pi}{q} \]
where \( q \) is the scattering vector corresponding to the first Bragg peak for a given mesophase. As shown in the SAXS profiles, the d values were gradually increased with decreasing lipid concentration until a constant value was reached. The \( T_m \) and d data are summarized as a single plot in Fig. 5. As shown in Fig. 5, the \( T_m \) and d of pure DPPC reached a plateau with approximately 40 wt% of water (60 wt% lipid). This value (60 wt% lipid), which signifies the fully hydrated state of the membrane, was similar to the values reported by previous studies23, 29, 30. With the addition of 10 mol% of PSO, the plateau in both the \( T_m \) and d plots was shifted to 60 wt% water. In the SAXS profiles of this group of samples, two sets of lamellar peaks corresponding to the Pβ and Lo15 phases were obtained. A separate evaluation of the hydration states of these two phases was not possible using the available techniques; thus, we selected only the Pβ phase in this study. With 20 mol% of PSO, the plateaus in both plots were shifted to 70 wt% water. This large change in the limiting d value.

**Fig. 4**  SAXS patterns for the samples with various lipid concentrations and containing different quantities of PSO; x (mole fraction of PSO) = 0 (a), 0.1 (b), and 0.2 (c). Measurement temperature = 25°C.

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suggests enhanced membrane hydration\textsuperscript{10}, which may be attributed to the inherent properties of PSO\textsubscript{4}.

The gradual decrease in \(T_m\) with increasing water content in the presence of PSO\textsubscript{4} for samples with high lipid concentrations suggests that the phospholipid headgroups are no longer in a fully hydrated state at those concentrations. The modulation of hydration caused by PSO\textsubscript{4} can be explained by several studies. According to Scherer and Seeling\textsuperscript{31}, anionic species present in PC membranes can force the N\(^+\) end of the choline headgroup to reorient towards the membrane interior, hence resulting in reduced exposure to the water phase. This eventually leads to decreased headgroup hydration\textsuperscript{10, 31} and a higher \(T_m\) for the concentrated samples.

To obtain additional information on the modulation of hydration by PSO\textsubscript{4}, we evaluated the changes in the bilayer parameters of the membrane as a function of the mole fractions of PSO\textsubscript{4}. Thickness PDDFs (pair distance distribution function) were generated, and the approximate bilayer thicknesses \(d_l\) were calculated for the samples with 40 wt\% lipids using GIFT software (developed by O. Glatter) according to previously described methods\textsuperscript{22, 32}. As our samples had much higher lipid concentrations compared with concentrations commonly used, the \(d_l\) concentration effect was taken into consideration\textsuperscript{32} when generating PDDFs. The value of \(d_l\) (5.60 nm) obtained via this approach for pure DPPC was not significantly different from the value (5.2 nm) reported by Aburai \textit{et al.}\textsuperscript{22} and Fruhwirth \textit{et al.}\textsuperscript{33} for more dilute dispersions. If the latter value is considered as the standard \(d_l\) value for fully hydrated DPPC membranes, the error of our measurements would be 5.46 nm, which is not unreasonable. The variation in \(d_l\) as shown in Fig. 6 accurately demonstrated the effects of adding PSO\textsubscript{4} to the membrane. The \(d_l\) was increased with increasing sterol concentration. The \(d_l\) was also increased but to a lesser extent. An increase in the thickness parameters with the addition of sterols has also been reported previously\textsuperscript{14}. Specifically, in the case of sterol sulfates, the electrostatic repulsion between the charged surfaces arising from the presence of negatively charged sulfate moieties in the headgroup region also contributes to this change\textsuperscript{10}.

The difference between \(d\) and \(d_l\) is the water layer thickness \(d_w\). As shown in Fig. 6, with the addition of sterols,
$d_w$ was only slightly increased compared with $d_l$. This less-than-expected increase in $d_w$ for the 40 wt% lipid sample indicated that in the presence of PSO$_4$, more water molecules could occupy the space under the PC headgroups. This result is consistent with the role of PSO$_4$ as an efficient spacer between headgroups$^{11, 34}$. The DPPC bilayer reached the fully hydrated state at 60 wt% lipid (40 wt% water), and there was no excess water outside the bilayer at this concentration. Therefore, at lipid concentrations of 60 wt% or 50 wt%, the incorporation of PSO$_4$ into the PC bilayer could change it from a fully hydrated state to a partially hydrated state. The results suggest that this is not a process of dehydration but an increase in the demand for water in the PC headgroup region. Consequently, upon further diluting the mixture, a large amount of water could diffuse into the membrane from the outside. The hydration at higher dilutions may be attributed to the tendency of PSO$_4$ to bind water molecules via hydrogen bonding, thus retaining more water molecules around itself. This results in a bulkier sulfate moiety, which makes PSO$_4$ a better spacer$^{10}$. Accordingly, this could create favorable conditions for additional water molecules (at higher dilutions) to interact with the previously reoriented PC molecules, hence compensating for water deficiency. A schematic diagram of the changes in hydration is shown in Fig. 7.

Owing to the lipophilic properties of DPPC, the physical stability (against aggregation) of its liposomal particles cannot be sustained for a long time regardless of their fine dispersion in water. However, as a result of the incorporation of PSO$_4$ (e.g., $x = 0.2$) into the DPPC structure, around 70% of water can be retained with PSO$_4$ compared to around 40% of water retained without PSO$_4$. Accordingly, in comparison with the specific gravity of liposomal particles without PSO$_4$ (only DPPC), differences in the specific gravity of liposomal particles with PSO$_4$ between aqueous dispersion and bulk water should be greatly reduced. As a result, liposomal separation from the aqueous dispersion in the DPPC-PSO$_4$ system is expected to be much slower than.

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Fig. 7  Schematic drawing showing the modification of hydration of DPPC bilayer by PSO$_4$. 

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in the single DPPC system, indicating that liposomes with PSO₄ will be more stable than those without PSO₄; nevertheless, electrostatic repulsion is also a contributing factor. As shown in Fig. 5, an increase in PSO₄ concentration by 10 mol% shifted the saturation point upward by around 20%; however, this shift with 20 mol% of PSO₄ was only 30%. This less-than-expected difference in hydration caused by 10 and 20 mol% of PSO₄ demonstrated that the added sterol molecules were increasingly less effective than those added to pristine DPPC bilayers. The non-linear increase in d with increasing dilution (Fig. 5) may be attributed to the concentration-dependent interaction of sterol sulfate with PC (in contrast to the –OH sterols) [13].

Quantitatively, the increase in the d value caused by 20 mol% of PSO₄ is comparable to a similar increase caused by 30 mol% of Chol-SO₄ in DMPC [16]. This finding suggests that PSO₄ is more efficient in enhancing the hydration of PC membranes. The higher efficiency may be attributed to the presence of an extra alkyl group in PSO₄ that can further disrupt the van der Waals forces between PC molecules, hence allowing more water to occupy the space under the headgroups. The modulation of the hydration of the PC bilayer by PSO₄ can be useful for developing products including skincare cosmetics and liposomes as vehicles for pharmaceuticals.

4 Conclusion
The present study revealed that PSO₄ could significantly enhance the hydration of DPPC membranes mainly because of its capacity to retain water molecules via hydrogen bonding. The negatively charged sulfate moiyety can change the conformation of the choline groups in such a way as to move them away from the water layer. When water is limited, this conformational change will result in the partially hydrated state of the headgroups, thereby increasing the Tm. The distribution of water in the vicinity of the bilayer interface was also determined in this study. Our results indicated that in the presence of PSO₄, a large amount of water could occupy the space under the PC headgroups. As a result, a significant increase in d was observed only when there was excess water. Therefore, in addition to assessing the changes in the hydration of the DPPC bilayer following the incorporation of PSO₄, we also proposed a model to describe the distribution of water molecules in the vicinity of the PC headgroups. The findings may contribute to future studies of the sulfate derivatives of other sterols. The effects of 20 mol% of PSO₄ and 30 mol% of Chol-SO₄ on the hydration of the PC bilayer were similar; thus, PSO₄ would be more effective for hydration. The effectiveness of PSO₄ in enhancing the hydration state of the PC bilayer membranes can be useful in the development of pharmaceutical and cosmetic formulations.

**Supplementary Information**
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