Effects of β-Sitosteryl Sulfate on the Properties of DPPC Liposomes

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Abstract: The effect of β-sitosteryl sulfate (PSO₄) on the liposomal size, stability, fluidity, and dispersibility of DPPC liposomes prepared by vortex mixing, bath-sonication, and probe-sonication has been studied. PSO₄ significantly decreases the particle size of the multilamellar liposomes (MLVs). The sizes of the vortex-mixed and the bath-sonicated liposomes vary as a function of PSO₄ concentration. On the other hand, PSO₄ has only little effect on the particle sizes of probe sonicated liposomes. In some cases, the liposomal stability at higher PSO₄ concentrations depends on the preparation method. PSO₄ improves the dispersibility of the DPPC liposomes and enhances their hydration. It also increases the fluidity of the liposomes prepared by each method. Our results suggest that liposomes consisting of DPPC and PSO₄ can be suitable as a cosmetic or pharmaceutical ingredient for the effective delivery of the active components into the body.

Key words: β-sitosteryl sulfate, hydrodynamic diameter, corrugation, ζ-potential, fluorescence anisotropy

1 Introduction

Liposomes are spherical vesicles prepared from phospholipids. A sterol, conventionally cholesterol, is often added to achieve the desired fluidity, permeability, and stability in liposomes. Properties such as biocompatibility, optimum hydrophilicity, and size tunability make liposomes a promising system for drug delivery¹. The fundamental properties of an efficient liposomal formulation include appropriate particle size, fluidity and elasticity, drug-trapping and releasing efficiency, and the level of hydration². High physical and chemical stabilities are the key prerequisites for a good quality liposomal formulation. Changes such as agglutination, precipitation, rupture, or chemical degradation of the liposomal particles may lead to their reduced efficiency or even health hazards. Steroid additives play important roles in stabilizing liposomes. They also facilitate the processes of drug release and their removal from the body²-⁵.

Conventionally, phosphatidylcholines (PCs) obtained from natural sources and containing unsaturated hydrocarbon chains are used for preparing liposomal formulations. These PCs exist in a liquid crystalline (L.ToolStripItem) phase even at temperatures much below room temperature. Cholesterol is added into PC membranes to optimize their fluidity, stability, and permeability. While these liposomes perform their functions efficiently, an important limitation is their low chemical stability, since the unsaturated hydrocarbon chains present in these lipids are vulnerable to oxidative damages⁶,⁷. Further, in recent times, their inefficacy in drug delivery across the hepatic and cardiac tissues, and toxic effects on the surrounding cells during cure of cancer have been reported⁸. In this sense, as well as for the proper absorption of the liposomes, particularly in certain types of cancer cells, saturated PCs with main phase transition temperatures (Tₘ) higher than the physiological temperature (~37°C) are thought to be better. However, a high rigidity in the liposomes obtained from saturated PCs is a hindrance for drug delivery. Such liposomes are also hard to eliminate from the body after their application. Conventionally, cholesterol is being used to increase their fluidity.

Abbreviations: DPPC = 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine or simply dipalmitoylphosphatidylincholine; PSO₄ = Sodium β-sitosteryl sulfate; Tₘ = Main phase transition temperature; MLV = Multilamellar Vesicle; DLS = Dynamic Light Scattering; DPH = 1,6-Diphenyl-1,3,5-hexatriene; ANS = 1-Anilinonaphthalene-8-sulfonic acid.

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However, reported cases of the fatal infection known as bovine spongiform encephalopathy (BSE) that can be spread by cholesterol, has further driven the need for identifying a substitute for cholesterol in formulations. Thus, it is important to find alternatives for the combination of PC and sterol, so as to obtain stable formulations with appropriate fluidity.

In our previous studies, we reported the strong capacity of PSO₄ for fluidizing the phosphatidylcholine membranes (either completely or partially, depending on the length of the hydrocarbon chains). It also enhances the hydration along the membrane headgroup region. These properties of PSO₄ can be useful for the preparation of liposomes with appropriate fluidity and an increased aqueous core available for entrapping drugs. Thus, based on their potential applications in formulating cosmetic and pharmaceutical products, it is important to investigate the properties of liposomes consisting of saturated PCs and PSO₄.

2 Materials and methods

2.1 Materials

DPPC (99% pure) was a kind gift from the NOF Corporation, Japan and was used without further purification. PSO₄ was obtained from LVMC Inc., Tokyo, Japan, and purified as described elsewhere. The fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH, >98% purity) was obtained from Sigma-Aldrich. 1-Anilinonaphthalene-8-sulfonic acid (ANS) probe was purchased from MP Biomedicals, LLC, Solon, OH.

2.2 Sample preparation

Appropriate quantities of DPPC and PSO₄ were weighed to obtain mixtures of varying compositions such that the mole fraction of PSO₄ (x) varied from 0 to 0.5. The mixtures were then dissolved in a solvent containing chloroform and methanol in the volume ratio 3:1. A slow stream of N₂ gas was passed into each sample taken in a 60 mL vial so as to remove the solvents. In this process, the vial was gently rotated so that a thin, uniform lipid film was deposited along the inner walls. Any remaining traces of the solvents were removed by vacuum desiccation. Ultrapure water was added to the dry lipid film at a temperature of 62°C (~20°C above the T_m of DPPC) so that the final lipid concentration was 2 mM. The suspensions were vortex-mixed for about 15 min at this temperature. The MLVs thus obtained were used in further experiments. The second set of samples was prepared by sonicating a portion of these MLVs on a bath-type sonicator for about 7.5 h (labeled as bath-sonicated samples). The third set was prepared by sonicating the MLVs for 15 minutes using a probe-type sonicator (labeled as probe-sonicated samples).

2.3 Particle size and zeta-potential

Particle size distribution was determined by the dynamic light scattering (photocorrelation spectroscopy) technique using a Litesizer 500 instrument (Anton Paar, Graz, Austria). A plastic cuvette containing about 1 mL of the suspension was placed in the sample holder. Measurements were performed at 25°C with laser light of wavelength 658 nm produced by a single-frequency laser diode, providing 40 mW. It was possible to select detection angles of 15°, 90°, or 175°, depending upon the transmittance of the sample. Fluctuations in the scattered light intensity caused by the moving vesicles in solution were analyzed and the parameters including optical density, size distribution, and mean hydrodynamic diameter were calculated using the Kalliope software. The same instrument was used to measure the z-potentials. For this, about 350 μL of the sample was filled in an Omega cuvette made from polycarbonate and provided with two gold electrodes. The measurements were carried out in the voltage range 0.1–200 V, automatically set by the instrument.

2.4 Fluorescence measurements

A thin film of 1,6-diphenyl-1,3,5-hexatriene dissolved in chloroform was deposited along the inner walls of a glass vial. The solvent was removed by passing a stream of N₂ gas followed by vacuum desiccation. The liposome solution, warmed at 62°C, was added to the film so that the final DPH concentration was 300 times less than the lipid concentration of the liposome. The material was vortex mixed and kept overnight in the dark at 25°C. The samples of each composition were labeled in the same manner. The fluorescence intensities were measured at different temperatures in the range 25–45°C using RF-5000 spectrophotometer (Shimadzu Co., Ltd). The emitted light polarized parallel and perpendicular to the excitation radiation was used for these measurements. The excitation and emission
wavelengths were 350 nm and 450 nm, respectively. From the obtained fluorescence intensities, the fluorescence anisotropy (r) values were calculated. Labeling of the membrane with the ANS (1-anilinonaphthalene-8-sulfonic acid) probe was carried out by a similar method using its ethanolic solution (0.1 mM). The excitation and emission wavelengths chosen in this case were 380 and 465 nm, respectively. r for highly turbid samples were corrected for light scattering effects using a method described elsewhere.

2.5 Negative staining electron microscopy
A small amount of sample was dropped on a copper grid, and phosphotungstic acid (1 wt%) was added to it as a negative stain. The excess amount of the sample and stain was removed by absorbing in a filter paper. Then the grid was dried for one day and observed using a Hitachi Hitech H7650 electron microscope.

3 Results and discussion
3.1 Sample appearance and turbidity
Figure 1 shows the appearance of the MLVs and the sonicated samples as a function of the mole fractions (x) of PSO4. The MLVs of pure DPPC formed an opaque, white suspension. The addition of 0.05 mole fraction of PSO4 dramatically decreased the turbidity of the suspension (Fig. 1, top row). Further addition of PSO4 slightly increased the clarity of the samples and transient suspensions were obtained for all compositions up to x = 0.5. In agreement with the visual observations, the absorbance of the samples abruptly decreased upon the addition of 5 mol% (x = 0.05) of PSO4 (Fig. 2). It decreased slightly up to x = 0.1, and then remained nearly constant until x = 0.3, above which it increased again. These changes in the turbidity of the samples show that PSO4 significantly reduces the size of the liposomes.

Bath-sonication did not cause a significant change in the turbidity of pure DPPC liposomes, whereas the turbidity of the PSO4-containing samples further decreased. The bath-sonicated samples with x = 0.075–0.2 were clear, whereas those with x > 0.2 were slightly turbid.

Among the probe-sonicated samples, the pure DPPC suspension appeared slightly hazy while the rest of the samples were clear. As with the MLVs and bath-sonicated liposomes, the visual characteristics of the probe-sonicated liposomes are reflected in the absorbance data plotted in Fig. 2, the x = 0 sample has slightly higher turbidity than all other samples containing PSO4.

3.2 Size and dispersion stability
Figure 3 (A) shows the particle sizes of MLVs plotted as a function of the storage duration. The particle sizes of the MLVs decrease greatly upon the addition of PSO4; for example, the incorporation of just 5 mol% (x = 0.05) of PSO4 decreases the vesicle size by a factor > 6. For 7.5 mol% (x = 0.075), the size is comparable to that of the 5 mol% sample. The size further decreases between x = 0.075 and x = 0.1. Until x = 0.3, the size slightly decreases, above which it again increases significantly. A similar variation in size was also observed in vesicles with 5 mM lipid concentration (Fig. S1, Supplementary Material). This shows that extremely small vesicles can be obtained by using a moderate concentration of PSO4.

Bath sonication for ~7.5 h does not significantly reduce the size of the liposomes formed by pure DPPC (Fig. 3 (B)). On the other hand, the sizes of the liposomes containing up to x = 0.2 of PSO4 decrease significantly. While the bath-sonicated liposomes with x < 0.3 are highly stable, those with higher x are unstable and precipitate upon storage for a few days. As shown in Fig. 3 (B), the pure DPPC liposomes treated in this manner precipitate soon after their preparation indicating a lower stability.

Exposure to strong ultrasonic energy during probe-sonication breaks down each individual vesicle to a smaller size regardless of the amount of PSO4 present in it. Consequently, the particle size of the liposomes of pure DPPC is less than 100 nm when measured one day after sample preparation. The particle sizes for the rest of the samples are ~60 nm for PSO4 content up to x = 0.3 and ~70 nm for x = 0.4 and 0.5. As illustrated in Fig. 3 (A) and 3 (C), both the MLV and probe-sonicated samples containing PSO4 are stable towards coagulation for at least 15 days. The Dh of the probe-sonicated liposomes decreased slightly by the next day, as compared to the values recorded soon after sample preparation. This decrease can be attributed to the deformation of the liposomes caused by the strong ultrasonic energy applied to them in the fluid phase. However, any deformation caused was only temporary and the liposomes later regained their initial shape, as observed in the negative staining electron micrograph (Fig. 4). The Dh values of the liposomes one day after their preparation are given in Table 1. As with the bath-sonicated samples, in the case of the MLVs as well as the probe-sonicated samples, the Dh of pure DPPC shows less stability than those containing PSO4. Among the samples containing PSO4, the Dh for the x = 0.5 sample increases slightly after 10 days indicating the presence of bigger particles.

3.3 Zeta-potential
The ζ-potential for pure DPPC in each group of samples has a value close to zero. The addition of PSO4 led to a significant increase in the negative value of the ζ-potential caused by the increase in surface charge of the liposomes. It can be easily understood that the enhanced dispersibility of the liposomal particles is a consequence of the mutual repulsion among the particles. The variation of ζ-potential as a function of PSO4 concentration is plotted in Fig. 5.
However, contrary to our expectations, the $\zeta$-potential of the liposomes remained almost constant above $x' > 0.075$. There was no evidence for the formation of a micelle or a bicelle, and the observed constancy of the value of $\zeta$-potential is explained as follows. The PSO$_4$ molecules incorporated in the DPPC bilayer increases the number of charged groups in the interface as well as the spacing between the headgroups. This phenomenon reduces the number of molecules per unit area, which leads to a decrease in the surface charge density, and hence results in a lower value of $\zeta$-potential than expected. Furthermore, a corrugation is introduced by PSO$_4$ along the membrane surface (e.g., in the $P_{β}$ phase). Corrugated charged surfaces tend to act as an obstacle for the solvent layer and impose extra restrictions on its mobility, resulting in a reduced $\zeta$-potential.

Fig. 1 Appearance of DPPC-PSO$_4$ liposomes: upper row—prepared by vortex mixing without sonication, middle row—bath-sonication (7.5 h) after vortex mixing, bottom row—probe sonicated (15 min) after vortex mixing.

Fig. 2 The absorbance of the liposomes as a function of PSO$_4$ concentration.

Fig. 3 Hydrodynamic diameter ($D_h$) of the liposomes of different compositions as a function of storage time: (A) MLVs, (B) bath-sonicated, and (C) probe-sonicated.
3.4 DPH fluorescence anisotropy

The fluorescence anisotropy (r) of a material is given by:

\[
r = \frac{I_0 - I_r}{I_0 + 2I_r}
\]

where: \(I_0\) = Fluorescence intensity when the polarizers are aligned parallel to each other.

\(I_r\) = Fluorescence intensity when the polarizers are aligned perpendicular to each other.

Fluorescence anisotropy (r) measurements yield an estimate of the fluidity and order of the molecules in a membrane. A decrease in r indicates an increase in fluidity. Figure 6(A) shows DPH fluorescence anisotropies (r<sub>dpf</sub>) for the MLVs, bath-sonicated, and probe-sonicated samples, measured at 25°C. As shown, at 25°C the r for the MLVs first decreases significantly upon addition of 0.05 mole fraction of PSO<sub>4</sub> and then does not change much until \(x = 0.2\). Above that, it decreases more rapidly indicating an increase in fluidity of the membrane at higher concentrations of PSO<sub>4</sub>. The increase in fluidity above \(x = 0.2\) is consistent with the phase diagram for the DPPC-PSO<sub>4</sub> system as reported in our previous paper<sup>7</sup>. Although each of the mixtures with 0.05 ≤ \(x\) ≤ 0.3 contains a fluid phase (\(L_m\)) coexisting with a modulated phase (\(P_r\)) and overall fluidity of the mixture increases with increasing PSO<sub>4</sub> concentration<sup>7</sup>, a marked change in the value of r was not observed with the addition of PSO<sub>4</sub>. However, though it appears contrary to the actual behavior of the DPPC-PSO<sub>4</sub> mixtures, this observation is not usual since the fluorescence measurement performed using DPH as a probe gives the anisotropy of the whole system rather than that of the individual phases. The results agree with our previous findings for the highly concentrated DPPC-PSO<sub>4</sub> system. As compared to the DPPC-cholesterol system<sup>18</sup>, however, the increase in fluidity upon the addition of sterol at a mole fraction greater than 0.2 is higher with PSO<sub>4</sub>. It shows that the capacity of PSO<sub>4</sub> to disorder the PC hydrocarbon chains at room temperature is higher than that of cholesterol. The behaviors of the probe-sonicated and bath-sonicated samples are also comparable to the behavior of the MLVs. At 45°C (a temperature well above the \(T_m\) of DPPC), PSO<sub>4</sub>, like other sterols, also shows a tendency towards ordering of hydrocarbon chains (Fig. 6(B), and Fig. S2 in Supplementary Material). The decrease in the temperature dependence of membrane fluidity with increasing concentration of PSO<sub>4</sub> is illustrated in Fig. 7. It is seen that for \(x = 0\) and \(0.2\), there is a sharp decrease in \(r_{dpf}\) near the \(T_m\), whereas for \(x = 0.3\), the decrease is uniform. This is because of the presence of the gel phase, \(L_g\) or \(P_r\), in \(x = 0\) and \(x = 0.2\) samples and its absence in the \(x = 0.3\) sample<sup>7</sup>.

ANS fluorescence anisotropy (r<sub>ans</sub>) was used to evaluate the fluidity and hydration<sup>20</sup> along the headgroup region of a bilayer membrane. r<sub>ans</sub> data for the MLVs are plotted in Fig. 8. Despite the expected disorder in the headgroup region caused by the addition of PSO<sub>4</sub>, the r<sub>ans</sub> of the MLVs increase at room temperature instead of decreasing. Such a change in the r<sub>ans</sub> has been attributed to the enhanced hydration of the membrane headgroup region which restricts the rotational freedom of the probe molecules. A similar effect of the enhanced hydration has been reported previously by Mohapatra and Mishra<sup>20</sup> in DPPC membranes containing bile salt. This enhancement of hydration of DPPC by PSO<sub>4</sub> is also consistent with our previous reports<sup>8,9</sup>. For the sonicated samples, a comparable tendency was observed (data not shown) but the r value was very low; this could be due to poor incorporation of the probe in the bilayer because of the extremely small sizes of the liposomes and their high curvature.

3.5 Effects of PSO<sub>4</sub>

The size of the MLVs first decrease upon the addition of PSO<sub>4</sub> and then increase for \(x ≥ 0.3\) (Fig. 9). The initial de-

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Table 1  Hydrodynamic diameters (D<sub>H</sub>) of the liposomes studied.

| Mole fraction of PSO<sub>4</sub> (x) | Hydrodynamic diameter (D<sub>H</sub>) (nm) |
|------------------------------------|-----------------------------------------|
|                                    | MLV | Bath sonicated | Probe sonicated |
| 0                                  | 4435 | 2954 | 85 |
| 0.05                               | 689  | 126  | 57 |
| 0.075                              | 746  | 41   | 63 |
| 0.1                                | 280  | 37   | 58 |
| 0.2                                | 238  | 170  | 63 |
| 0.3                                | 218  | 299  | 65 |
| 0.4                                | 637  | 283  | 66 |
| 0.5                                | 808  | 417  | 72 |

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Fig. 4  Negative staining electron micrograph of the probe-sonicated liposome containing 7.5 mol% of PSO<sub>4</sub>.
crease can be attributed to the electrostatic repulsion that necessitates an increased vesicle curvature and higher translational entropy that leads to the formation of a larger number of smaller-sized liposomes. This behavior of MLVs with a net surface charge has been reported previously\(^{21-23}\).

The increase in vesicle size above \( x = 0.3 \), however, can be attributed to the decrease in membrane curvature caused by the PSO\(_4\) molecules\(^{24, 25}\).

The spontaneous dramatic decrease in vesicle size, particularly in the case of samples with \( x = 0.1-0.3 \), is of interest from the practical viewpoint. It has been suggested that the liposomes larger than 100 nm are easily available for sequestration by the phagocytes present in the body. Thus, we believe that these MLVs can even be used directly in formulations without any further breakdown of the particles. While their intravenous administration may be limited by their bigger sizes (~200 nm), they can still be appropriate for topical applications\(^{26, 27}\). To the best of our knowledge, no previous report demonstrates such a dramatic decrease in liposomal sizes without a significant external perturbation\(^{28, 29}\). Even in the case of the PC-cholesterol liposomes, achieving the diameters of a few hundred nanometers requires either sonication or extrusion techniques, regardless of whether or not an ionic species is employed as a dispersing agent\(^{2, 11, 13}\). Thus, using PSO\(_4\) industrially in the manufacture of liposomes can reduce the extra cost of performing sonication or extrusion. Further, since the liposomes prepared with PSO\(_4\) are formed nearly spontaneously, they can be expected to have higher stability against coagulation. The highly negative values of \( \zeta \)-potential achieved by the addition of PSO\(_4\) further enhances the stability. As a result, all three types of liposomes containing PSO\(_4\) were found to be more stable than those without it.

Though several anionic and cationic species such as stearylamine and dicetylphosphate are available for en-

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**Fig. 5** Variation of \( \zeta \)-potentials of different types of liposomes as a function of the PSO\(_4\) concentration, measured at 25°C.

**Fig. 6** (A) DPH fluorescence anisotropy \( (r_{\text{dph}}) \) of the liposomes as a function of PSO\(_4\) concentration, measured at 25°C. (B) Comparative effect of PSO\(_4\) on the fluidity of the MLVs at 25°C and 45°C.

**Fig. 7** Temperature dependence of \( r_{\text{dph}} \) for samples with \( x = 0, 0.2, \) and 0.3.
DPPC-β-Sitosteryl Sulfate Liposomes

Enhancing the dispersibility of liposomes, they do not decrease the size of liposomes as effectively as PSO₄. Moreover, they are also found to have toxic effects in mammals under certain circumstances. PSO₄ also enhances the fluidity and hydration of the membranes significantly. Given all these beneficial characteristics of PSO₄, it can act as an efficient as well as a healthy/environment-friendly alternative to cholesterol for use in cosmetic and pharmaceutical formulations. A schematic diagram illustrating the above-mentioned effects of PSO₄ on the particle size and dispersibility of the DPPC liposomes is summarized in Fig. 9.

4 Conclusion

Based on the current study, we conclude that PSO₄ enhances the dispersibility of DPPC liposomal particles. It also reduces the size of the liposomes to a larger extent than cholesterol. As evident from the fluorescence anisotropy data, its capacity for fluidizing the membrane at room temperature is higher than that of cholesterol. The dispersion stability is also enhanced by a moderate concentration of PSO₄. Sonication techniques can be used to obtain liposomes of sizes about 100 nm or less. However, a prolonged exposure of the liposomes to bath sonication leads to precipitation of PSO₄ from the membrane when it is present in a significantly high concentration. The efficiency of PSO₄ in dispersing the liposomal particles as well as reducing the particle size and stabilizing the dispersion could make it an excellent alternative to cholesterol for use in cosmetic and pharmaceutical formulations.

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Supporting Information
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