Impact of Doping a Phytosteryl Sulfate on the Properties of Liposomes Made of Saturated and Unsaturated Phosphatidylcholines

Risa Tanaka¹, Ananda Kafle¹*, Masaaki Akamatsu¹, Avinash Bhadani², Kenichi Sakai¹,², Chihiro Kaise²,³, Teruhisa Kaneko²,³, and Hideki Sakai¹,²*

¹ Department of Pure and Applied Chemistry, Faculty of Science and Technology, Tokyo University of Science, 2641-Yamazaki, Noda, Chiba, 278-8510, JAPAN
² Research Institute for Science and Technology, Tokyo University of Science, 2641-Yamazaki, Noda, Chiba, 278-8510, JAPAN
³ L. V. M. C. Inc. Komagome-7-14-3, Toshima-ku, Tokyo, 170-0003, JAPAN

Abstract: The size, dispersibility, and fluidity of DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), POPC (1-palmitoy-2-oleoyl-sn-glycero-3-phosphocholine), and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) liposomes doped with β-sitosteryl sulfate (PSO₄) were comparatively studied. In all three types of liposomes, PSO₄ reduced sizes and enhanced the negative values of the ζ-potential. However, the effect on sizes quantitatively differed in the three cases in a manner dependent on their phase behaviors. PSO₄ rigidified each type of membrane in its liquid crystalline phase and fluidized the gel phase. It enhanced the glucose trapping efficiency (TE) of both DPPC and DOPC liposomes. The TE of DPPC first increased with the increasing concentration of PSO₄, then decreased gradually. On the other hand, in the case of DOPC, the TE increased significantly upon addition of PSO₄, then remained nearly constant. Though the exact dependence of TE on the PSO₄ concentration differed in the two cases, its effect, in each case, was more than the effect of β-sitosterol (POH). The ability of PSO₄ for reducing the size and enhancing dispersibility and TE of liposomes can be useful for preparing cosmetics and pharmaceutical formulations.

Key words: trapping efficiency, β-sitosteryl sulfate, phosphatidylcholine, liposome, DPPC, DOPC, POPC

Introduction

Phospholipids (Fig. 1) are amphiphilic molecules which possess a hydrophilic headgroup and a lipophilic tail consisting of one or two acyl chains. When mixed with water, the lipid molecules undergo self-assembly forming lamellar bilayers. These bilayers form the structural backbone of biological membranes, and therefore, are studied as models for understanding the membrane phenomenon⁴⁻⁶. At low temperatures, they exist in a significantly rigid structure known as gel phase. At temperatures above a threshold called main phase transition temperature (Tm), they exist in the fluid, liquid crystalline phase. In dilute dispersions, these membranes take the form of spherical vesicles known as liposomes (Fig. 1). Liposomes are widely used as drug carriers in advanced drug delivery systems (DDS)⁴,⁶. Liposomes consist of one or more concentric lipid bilayers that enclose water at the center as well as between successive bilayers⁶,⁷. Water-soluble drugs are dissolved in the aqueous core of liposomes and transported into the body. Similarly, lipophilic drugs are loaded in liposome’s hydrophobic tail region. Biocompatibility of phospholipids makes them a popular choice for using in DDS. They are also used in skincare cosmetics⁴⁻⁸.

Steroids constitute a class of organic compounds possessing a characteristic arrangement of four fused ring systems. The members of this family having an –OH functional group are called sterols. Among numerous steroids found in organisms, cholesterol in animals and phytosterols in plants serve as important constituents in biomembranes¹⁰,¹¹. Cholesterol sulfate is also present in various animal tissues¹²,¹³. The major function of sterols in the membrane is to optimize its fluidity and permeability. By locating themselves between phospholipid molecules in the membrane, sterols can fluidize the membrane if it is initially in a gel phase and rigidify if it is in the liquid crystalline phase¹⁴⁻¹⁶. In liposomes too, sterols are used to achieve

*Correspondence to: Ananda Kafle, Department of Pure and Applied Chemistry, Faculty of Science and Technology, Tokyo University of Science, 2641-Yamazaki, Noda, Chiba, 278-8510, JAPAN; Hideki Sakai, Department of Pure and Applied Chemistry, Faculty of Science and Technology, Tokyo University of Science, 2641-Yamazaki, Noda, Chiba, 278-8510, JAPAN.
E-mail: hisakai@rs.tus.ac.jp (HS); anandakafle@rs.tus.ac.jp (AK) ORCID ID: Kenichi Sakai (https://orcid.org/0000-0003-4038-8954)

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β-particles against agglutination also improves dispersibility and stability of the liposomal pharmaceutical formulations. However, recently, cholesterol is being used for this purpose in cosmetic and pharmaceutical formulations. We found that cholesterol is found to transmit a fatal mammalian disease known as prion disease. Therefore, an alternative to this sterol is necessary.

Previously, we carried out studies on the systems consisting of phospholipids and plant sterols. We found that β-sitosteryl sulfate, a derivative of β-sitosterol, efficiently fluidizes and enhances hydration of the membranes made of saturated phosphatidylcholines (PCs). It also improves dispersibility and stability of the liposomal particles against agglutination. These properties of β-sitosteryl sulfate are valuable for the preparation of chemically and physically stable liposomes from saturated phosphatidylcholines. Further, being a plant derived steroid, it is more health-friendly than cholesterol and is safer towards diseases such as prion disease. Though in our previous studies we shed light on some important aspects of the roles of PSO₄ in membranes made of saturated PCs, no study has yet been carried out in another important property, the trapping efficiency of such liposomes, which is instrumental for effective performance of a drug carrier. Further, though saturated phospholipids are better for preparing chemically stable liposomes, phospholipids with unsaturated hydrocarbon chains are also preferred frequently for their higher fluidity and flexibility. Despite this, no study has so far been carried out on the effects of PSO₄ on the properties of the unsaturated PCs such as DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine). In this paper, we are reporting a comparative study on the size, dispersibility, fluidity, and trapping efficiency of the DPPC and DOPC liposomes containing PSO₄.

2 Materials and Methods

2.1 Materials

DPPC, DOPC, and POPC (each 99% pure) were purchased from the NOF Corporation, Japan, and were used without further purification. PSO₄ and one of the samples of β-sitosterol (POH) were obtained from LVMC Inc., Tokyo, Japan, and purified as described elsewhere. Another POH sample was purchased from Sigma. The fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH, >98% purity) was obtained from Sigma-Aldrich. The coloring reagent used for UV-Vis spectrophotometry was purchased from Wako Pure Chemicals Industries Ltd.

2.2 Sample preparation

For the preparation of samples for the measurement of size, fluidity, and ζ-potential, appropriate quantities of DPPC, DOPC or POPC, and PSO₄ were weighed to obtain mixtures of varying compositions in which the mole fraction of PSO₄ (x) ranged from 0 to 0.5. The mixtures were then dissolved in a solvent containing, respectively, 3 and 1 parts by volume of chloroform and methanol. A current of N₂ gas was passed into each sample taken in a 60 mL vial until whole of the solvent was removed. While passing N₂, the vial was gently rotated so that a thin, uniform lipid film was deposited along its inner walls. The vial was then placed in a vacuum desiccator to remove any remaining traces of the solvent. Ultrapure water was warmed at 62°C (~20°C above the Tₘ of DPPC) and added to the dry lipid film so as to obtain a suspension with 2 mmol/L⁻¹ solid concentration. The suspensions were vortex-mixed for about 15 min at this temperature. The multilamellar vesicles (MLVs) thus obtained were used in further experiments. For DSC measurement and the measurement of trapping efficiencies (TE), the sample concentration used was 10 mmol/L⁻¹ instead of 2 mmol/L⁻¹. For the measurement of TE, 0.027 M glucose solution was used to hydrate lipids instead of water.

2.3 Particle size and zeta-potential

Dynamic light scattering (photon correlation spectroscopy) technique was used to measure particle size distribu-
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2.4 Fluorescence measurements

A thin film of 1,6-diphenyl-1,3,5-hexatriene (DPH) dissolved in chloroform was deposited along the inner walls of a glass vial and dried with a current of N₂ followed by 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 stored in dark overnight at 25°C. The fluorescence intensities were measured at different temperatures within the range of 25 to 45°C using RF-5000 spectrofluorometer (Shimadzu Co., Ltd). The emitted light (wavelength = 450 nm) polarized parallel and perpendicular to the excitation radiation (wavelength = 350 nm) was used for these measurements. From the obtained fluorescence intensities, the fluorescence anisotropy (r) values were calculated. Fluorescence anisotropy (r) for highly turbid samples were corrected for light scattering effects using a method described elsewhere.

2.5 Trapping efficiency

For the evaluation of the glucose-trapping efficiency (glucose-TE or simply TE), liposomes of 10 mM lipid concentration were prepared with 0, 0.05, 0.075, 0.1, 0.15, 0.2, 0.3, 0.4 and 0.5 mole fractions (x) of PSO₄. For this, the dry lipid film deposited on a vial, as described above, was hydrated with 0.027 M glucose solution followed by vortex mixing at 62°C. Dialysis technique was used to remove the unentrapped glucose molecules. For dialysis, 1 mL of the liposome of each composition was filled in a cellulose tube and sealed at both ends with two clips (Supplementary Information). The tubes were then placed in a 0.014 M NaCl solution precooled at ~3-5°C for 2 hours. The salt solution was stirred with the help of a magnetic stirrer at this temperature for 11 hours. In this duration, the salt solution was changed at intervals of 1 h, 1 h, 2 h, 3 h and 4 h. 50 μL of liposome solution was taken out from the cellulose tubes and treated with 150 μL volume of ethanol. 80 μL of the ethanolic solution, which is equivalent to 20 μL of the liposome, was taken out into another vial. 3 mL of an assay containing glucose oxidase (GOD), 4-aminopyridine and phenol, was added into this vial, which was warmed for 5 minutes and then shaken for about 30 sec. The absorbances of the glucose-containing samples were measured with the help of a UV-Vis spectrophotometer (Cary 5484, Agilent Technologies, Singapore). From the absorbances obtained, the glucose concentrations were calculated based on the calibration curve. The glucose-trapping efficiency was calculated as follows:

\[
\text{Trapping efficiency (TE)}(\%) = \frac{\text{Amount of glucose in liposomes}}{\text{Glucose of the whole system}} \times 100
\]

Release of glucose as a function of storage period was also evaluated. Before storage, N₂ gas was passed into each vial to avoid any biodegradation of glucose.

3 Results and Discussion

3.1 Sample appearance

Figure 2 shows the images of the liposome samples made from DPPC, DOPC and POPC with varying amounts of PSO₄. Each of the pure PCs forms an opaque, white suspension. Precipitation occurs by the next day in DPPC suspension whereas the other two remain well dispersed for several days. The early precipitation in DPPC sample agrees with our previous study. While the addition of PSO₄ reduces the turbidity of each suspension, the effect is highest in DPPC than the other two.

3.2 Size and ζ-potential

Figure 3 shows the hydrodynamic diameter (D_h) and ζ-potential of the DPPC and DOPC liposomes as a function of the mole fraction (x) of PSO₄. As shown in this figure, the addition of 0.075 mole fraction of PSO₄ reduces the D_h of the DPPC liposomes by about 4 times. The size further decreases with successive additions of PSO₄. This decrease in size upon addition of PSO₄ has been attributed to the introduction of negative surface charge in the liposome by PSO₄. This negative charge results in an electrostatic repulsion among liposomal particles necessitating an increased vesicle curvature and higher translational entropy. The requirements for the greater vesicle curvature and the higher translational entropy lead to the formation of a larger number of smaller-sized liposomes. A similar behavior was observed in our previous studies on 2 mM and 5 mM DPPC liposomes. The sizes of the DOPC and POPC liposomes are much smaller than that of the DPPC liposom...
some. This can be attributed to the smaller number of bilayers present in the DOPC and POPC liposome which are in the liquid crystalline (L_α) phase compared to that in the DPPC which is in a gel (L_β) phase. The increased surface area and decreased shear rigidity in the L_α phase bilayers are responsible for the smaller number of bilayers in this phase. As the addition of PSO4 leads to a less strong perturbation of the liquid crystalline DOPC and POPC membrane compared to the gel phase DPPC membrane, the impact of adding PSO4 into these bilayers is accordingly less. For the same reason, the size of the DPPC liposome decreases more rapidly with the addition of PSO4 than the other two.

The negative values of the ζ-potentials for the three pure lipid samples are in the order POPC > DPPC > DOPC, which is in agreement with the previous reports. These increase dramatically with the addition of 0.075 mole fraction of PSO4. This change in the value of ζ-potential is a consequence of the increased negative surface charge of the vesicles owing to the introduction of PSO4 in the membrane. However, instead of decreasing continuously, the ζ-potential of the liposomes remained almost constant upon successive addition of PSO4. There was no evidence for the formation of a micelle or a bicelle in the system. The observed tendency in the effect of PSO4 on the ζ-potential of the PC liposomes is explained as follows. The PSO4 molecules incorporated in the PC bilayers increase not only the number of charged groups in the vicinity of the bilayer interface but also the spacing between headgroups. This phenomenon reduces the number of molecules per unit area, which leads to a decrease in the surface charge density, thus resulting in a lower value of ζ-potential than expected. Moreover, PSO4 gives rise to a corrugation along the membrane surface. In the case of DPPC, such corrugation is repeated at regular intervals in a well-defined bilayer ripple phase. Though such a well-defined phase has not so far been reported in the case of DOPC, it is expectable that in this case too, PSO4 introduces such irregularities in membrane surface. The corrugated charged surfaces act as an obstacle for the free movement of the solvent layer and restricts its mobility, resulting in a reduced ζ-potential. Of course, the contribution from the lowered degree of dissociation of PSO4 also cannot be neglected in this context.

3.3 Phase transition

DSC heating endotherms of the DPPC-PSO4 and DOPC-
PSO₄ liposomes are shown in Fig. 4. In the case of the POPC-systems, the phase transition peaks were masked by the intense ice-melting peak located near 0°C because of the closeness of the two, and has not been shown here. The main transition temperatures (Tₘ) of −17°C and 42.1°C, respectively for DOPC and DPPC are consistent with the previously reported values. The large difference in Tₘ of the two PCs is due to the loose packing of the cis-hydrocarbon chains in DOPC bilayers compared to the all-trans chains in DPPC. The pre-transition peak of DPPC exists as a small, diffused peak located at 37.3°C. The pre-transition temperature (Tₚ) is a characteristic of the bilayers made of saturated PCs. It represents the transition from the gel (Lₑ) phase to the rippled (Pₑ) phase. In the case of DPPC, this phase forms when the concentration of water is above 18 wt%.

In its gel phase, the Rₚₑ decreases with increasing x indicating that PSO₄ fluidizes such membrane. Slight rigidification of the Lₑ phase membranes is observed in both cases.

3.4 Membrane fluidity

Figure 5 shows the DPH fluorescence anisotropy (rₑ) for DPPC-PSO₄, DOPC-PSO₄, and POPC-PSO₄ liposomes at 25°C and 45°C. For DPPC in its gel phase, rₑ decreases with increasing x indicating that PSO₄ fluidizes the membrane. Slight rigidification of the Lₑ phase membranes is observed in both cases.

In its gel phase occurs due to the disordering effect of PSO₄ on the hydrocarbon chain of the lipid molecules. At 45°C, on the other hand, the rₑ increases with increasing x indicating an ordering effect of PSO₄ in the membrane in its liquid crystalline phase. Similar ordering effect of PSO₄ was observed with DOPC and POPC at room temperature for the same reason as the ordering of the DPPC membrane in the liquid crystalline (Lₑ) phase. According to the previous reports, the ordering effect of the membrane steroids arises from their unique structural characteristics and the tendency of the phospholipid molecules to occupy fixed positions around the steroid molecules. Our previous studies have revealed that PSO₄, like cholesterol and other phytosterols, has both fluidizing and rigidifying effects on the PC membranes depending on whether the membrane is in a gel or a liquid crystalline phase. However, it has a higher fluidizing effect compared to other sterols.
mainly owing to its bulky sulfate moiety.

3.5 Trapping efficiency

Glucose trapping efficiencies (TE) of DPPC and DOPC liposomes containing 0 to 0.5 mole fraction of PSO₄ were evaluated. The effect of PSO₄ was compared with the effect of β-sitosterol (POH). To evaluate the effect, if any, of the isomeric composition and the origin of products in the role played by steroids, we used two different samples of POH, obtained respectively from LVMC Inc. and Sigma. The steroid sample obtained from Sigma was in the form of a mixture of β-sitosterol (POH) and PSO₄. We isolated the two components from the mixture and used separately for glucose trapping efficiency experiments. The β-sitosterol sample obtained from Sigma, on the other hand, was used as is. The trapping efficiencies were determined with the help of UV-Visible spectrophotometry of the dialyzed liposome samples hydrated with glucose solution.

UV-Vis Spectrophotometry

Absorbances of the solutions treated with GOD assay (Fig. S3, Supplementary Information) were measured using a UV-visible spectrophotometer at λ_max = 505 nm. Concentration of the trapped glucose was determined in each solution against a calibration curve plotted using standard glucose solutions provided by the supplier of the GOD assay. The values of the trapping efficiencies (TE) of the DPPC and DOPC liposomes with and without steroids are given in Table 1. The TE for pure DPPC liposomes equal to 1.8%, is comparable to that obtained by Otake et al.⁴².

The data on Table 1 show that PSO₄ enhances the TE of DPPC liposomes much more significantly than POH. For example, POH at a concentration of x = 0.1 increases the TE of pure DPPC liposome from 1.8% to ~3.5% while the same amount of PSO₄ enhances it to ~12%. The liposome of pure DOPC has a trapping efficiency (TE) of approx. 2.5% which is slightly higher than the TE of DPPC (1.8). The slightly higher TE of the DOPC liposome can be attributed to its longer hydrocarbon chains (by two carbon units) compared to DPPC and the presence of two double bonds⁴³,⁴⁴. Upon addition of 0.05 mole fraction of PSO₄, the TE increases to 9.3%. It shows that PSO₄ enhances TE of liposomes regardless of whether the phospholipid is saturated or unsaturated. A notable difference between the DPPC and DOPC liposomes, however, is that in the case of DPPC, the trapping efficiency first increases with the increasing concentration of PSO₄ and then decreases, while in the case of DOPC, once PSO₄ is incorporated, the TE remains nearly same regardless of the concentration of PSO₄. This result can be explained on the basis of the variation of the size and the membrane properties of the vesicles caused by the addition of PSO₄.

As can be seen in Table 1, the size of pure DPPC liposomes is over three times bigger than the size of the DOPC liposomes. It’s because of the fact that the MLV of DPPC, which is in the gel phase, possesses a larger number of bilayers compared to the DOPC MLV. Thus, it can be said that the TE is higher for the system consisting of a larger number of vesicles with fewer bilayers than the system consisting of a smaller number of vesicles with a larger number of bilayers. The TE of the DOPC liposomes containing PSO₄ are uniform throughout the whole composition range. This can be attributed to the uniform sizes of the vesicles and a smaller effect of PSO₄ on the membrane fluidity (PSO₄ slightly rigidifies the DOPC MLVs which are initially in a liquid crystalline phase). Phases are also uniformly the Lₒ and Lₒ (both fluid phases) phases in the case of the DOPC-PSO₄ compared to the DPPC-PSO₄ liposomes.

### Table 1  Trapping of glucose by the DPPC-POH and DPPC-PSO₄ liposomes.

<table>
<thead>
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<th>x</th>
<th>Size (nm)*</th>
<th>Trapping efficiency (%)</th>
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<tr>
<td></td>
<td>DPPC-POH</td>
<td>DPC-POH</td>
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<tr>
<td></td>
<td>Sigma</td>
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<tr>
<td>0</td>
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<tr>
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<tr>
<td>0.4</td>
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<tr>
<td>0.5</td>
<td>6641</td>
<td>3100</td>
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</tbody>
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*Note: The sizes plotted in Table 1 correspond to the 10 mM liposomal dispersions prepared in 0.027 M glucose solution.
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3.6 Leakage of glucose

The DPPC and DOPC liposomes exhibited significant differences in their behavior regarding leakage of glucose as a function of storage period. As shown in Fig. 6, there is no significant leakage of glucose from the DOPC liposomes upon storage for over four days. This tendency is different from the corresponding DPPC systems in which the glucose TE decreased significantly upon storage. This can be attributed to the fact that in DOPC liposomes which undergo the Lo → Lα (fluid to fluid) transition upon addition of PSO4, there is much less structural perturbation upon addition of PSO4, whereas in DPPC which undergoes a gel → fluid phase transition. Higher the structural perturbation, higher can be the number of defects in the bilayer leading to a higher leakiness.

4 Conclusions

Effects of β-sitosteryl sulfate (PSO4) on the liposomal properties of saturated, mono-unsaturated, and di-unsaturated phosphatidylcholines were studied comparatively. PSO4 reduced the sizes of the liposomal particles of each of DPPC (saturated), POPC (monounsaturated), and DOPC (di-unsaturated). However, the decrease in size of DPPC was the most significant. It also enhanced the ζ-potentials of both saturated and unsaturated liposomes, thus ensuring an enhanced dispersibility compared to the liposomes obtained from pure PCs. The negative surface charge induced by PSO4 is responsible for the observed effects on both the size and ζ-potential. In both cases, PSO4 rigidified the membranes in their fluid phase. Fluidization of the gel phase DPPC membrane was also observed. It also significantly enhanced glucose trapping efficiencies of both DOPC and DPPC liposomes. However, the two showed significant differences in the trend of variation of TE with PSO4 concentration. In the case of DOPC, when a small amount of PSO4 is added, the TE increases once and then remains unaffected by further addition. But in the case of DPPC, TE first rises and then gradually drops with increasing PSO4 concentrations. This difference is attributed to the uniformity in phase structure of DOPC regardless of the concentration of PSO4 compared to DPPC which undergoes various phase changes. The TE also depends on the particle sizes of liposomes, and hence, the diversity observed in the sizes of DPPC liposomes upon addition of PSO4 is also reflected in its TE. POH, due to its lack of capacity for enhancing membrane hydration, has far less effect than PSO4. The ability of PSO4 for reducing liposomal sizes, and enhancing dispersibility, stability, and trapping efficiencies is useful for formulating cosmetics and drug delivery systems.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess21035

References


J. Oleo Sci.


7. Wiacek, A.E. Influence of dipalmitoylphosphatidylcholine (or dioleoylphosphatidylcholine) and phospholipase A2 enzyme on the properties of emulsions. *J. Colloid Interface Sci.* **373**, 75-83 (2012).


29. Chibowski, E.; Szczes, A. Zeta potential and surface charge of DPPC and DOPC liposomes in the presence...
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