Effects of Phytosterol Butyrate Ester on the Characteristics of Soybean Phosphatidylcholine Liposomes

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Abstract: The nutritional and structural properties of phytosterols (PS)/phytosterol esters (PEs) facilitate their use as substitutes for cholesterol in liposome encapsulation systems designed for oral drugs and health products. The purpose of this study was to determine the effect of phytosterol butyrate ester (PBE) on the properties of liposomes. PBE was encapsulated within liposomes (approximately 60 nm) prepared using soybean phosphatidylcholine using the thin-film hydration method. There was no significant change in the average particle diameter and zeta potential of these liposomal vesicles corresponding to the increasing amounts of encapsulated PBE. The incorporation of PBE increased the polydispersity index (PDI) independent of concentration. Additionally, we observed that the storage stability of PBE liposomes with uniform particle size and approximately spherical shape vesicle was better at low concentration. The results of Fourier-transform infrared (FTIR) spectroscopy and Raman spectroscopy showed that PBE was positioned at the water interface, which increased the order of hydrophobic alkyl chains in the lipid membranes. The incorporation of PBE led to an increase in the trans conformation of hydrophobic alkyl chain and consequently, the thermal stability of liposomes, which was confirmed by differential scanning calorimetry (DSC). The results of powder X-ray diffraction (XRD) analysis confirmed that PBE was present in an amorphous form in the liposomes. Additionally, the incorporation of PBE reduced the micropolarity of the lipid membrane. Thus, when preparing liposomes using thin-film hydration, the presence of PBE affected the characteristics of liposomes.

Key words: phytosterol butyrate ester, effect, characteristics, liposome

1 Introduction

There has been an increase in research investigating the role of liposomes in drug delivery and sustained release of bioactive compounds in the pharmaceutical, cosmetics, and food industries¹-². Liposomes have been widely used to incorporate essential oils⁴, vitamins⁵, minerals⁶, enzymes⁷, antioxidants⁸, antibacterial agents⁹, etc. Liposomes are constituted amphiphilic lipid/phospholipid molecules and are well known as spherical vesicles with an aqueous core. These vesicles have several advantages, such as high bioavailability, biodegradability, and biocompatibility⁵,¹⁰. The characteristics of liposomes depend on their composition, preparation method, and environmental conditions. Traditionally, cholesterol was added to the lipid membrane to improve the characteristics of the lipid bilayer¹¹-¹⁰. However, its use has been limited since it is contraindicated for cardiovascular patients.

Phytosterols (PS) and phytosterol esters (PEs) possess chemical structures similar to cholesterol and exhibit similar or better effects when incorporated into liposomes¹¹-¹⁶. Several studies have shown that β-sitosterol could significantly improve the stability of liposomes compared with cholesterol¹⁷,¹⁸. Another study showed that PS anchored to the membrane and exhibited better performance for membrane protection than gallic acid¹⁹. Previous studies also found that PEs could improve the stability of liposomes as well as the encapsulation efficiency of water-soluble compounds²⁰. As functional ingredients, PS and PEs have been shown to be effective in reducing the levels of total cholesterol and low-density lipoproteins, and thus, ar-
terosclerosis and cardiovascular diseases\textsuperscript{20,21}. Additionally, adequate intake of PS and PEs have been shown to effectively reduce serum LDL cholesterol by 10-15\%, thus effectively protecting the cardiovascular system\textsuperscript{22,23}. They also exhibit other health benefits, including anticancer\textsuperscript{24}, anti-inflammation\textsuperscript{25}, antioxidation\textsuperscript{26}, and neuroprotection\textsuperscript{27}. The nutritional and structural properties of PS/PEs make them promising substitutes for cholesterol in liposome encapsulation systems designed for oral drugs and health products.

Dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, and distearoyl phosphatidylcholine are generally used for the preparation and study of liposomes. However, natural soybean and egg lecithin are relatively abundant and cheap, and thus, are more suitable as food-grade liposomes. As an emulsifier, Tween-80 has been shown to stabilize the liposomes as well as increase their uniformity\textsuperscript{28}. A study showed that the liposome particle curvature was increased with the addition of Tween 80 surfactants, which reduced the liposomal size\textsuperscript{29}. Therefore, an economical and stable liposome could be prepared using natural phospholipids and tween-80 as membrane and auxiliary materials, respectively.

Phytosterol butyrate ester (PBE) possesses a steroidal structure as well as butyric acid, which is known to regulate intestinal health\textsuperscript{20}. There is a lack of published reports on the embedding of PBE in liposomes. In this study, we aimed to investigate the effects of different concentrations of PBE on the characteristics of liposomes.

2 Materials and Methods

2.1 Materials

Soybean phosphatidylcholine (SPC; 98\% purity) was purchased from Shenyang Tianfeng Biological Pharmaceutical Co. Ltd. (Liaoning, China). Phytosterols (97\% purity; 56\% \(\beta\)-sitosterol, 30\% stigmasterol, and 14\% campesterol) were purchased from Shanghai Yihe Biotechnology Co. Ltd. (Shanghai, China). Phytosterol butyrate ester (PBE) was prepared in the laboratory (97\% purity). \(\beta\)-sitosterol standard was purchased from Purufa Biotechnology Co. Ltd. (Chengdu, Sichuan, China, 99.99\% purity). Squalane was purchased from Shanghai Macklin Biochemical Co. Ltd. (Shanghai, China, 99\% purity). \(n\)-Hexane was chromatographically pure, and all other reagents used in this study were analytical grade.

2.2 Liposome preparation

The liposomes were prepared following the thin-film hydration method described by Mohan et al.\textsuperscript{37} with minor modifications. SPC (80 mg), tween-80 (40 mg), and PBE (2-8 mg) were added to a round-bottom flask, followed by the addition of 10 mL of organic solvent (chloroform:methanol = 2:1, v:v). The mixture was kept in the dark at room temperature for 15–30 min. The organic solvent was removed by rotary evaporation (gradient vacuum: 0.02 MP for 10 min; 0.04 MPa for 10 min; 0.06 MP for 10 min, water bath temperature 50\degree C) to form a uniform film on the wall of the round-bottom flask. Then, the flask was spun for an additional 30 min under a vacuum of 0.1 MPa. Next, 10 mL of 0.02 mol/L phosphate buffer solution (PBS, pH 7.4, 0.15 mol/L NaCl) was injected to hydrate the lipid membrane for 10 min, followed by sonication in an ice bath using an ultrasonic probe (200 W, 8 min, and 1 s on, 1 s off) (Ultrasonic Material Emulsifier, Shanghai Hannuo Instrument, China). The light-blue opalescent liposomes were obtained and stored at 4\degree C for further use. The liposomes without PBE were prepared by the same method. The final concentration of phospholipids was 8 mg/mL.

During the freeze-drying process, 10 mL of the newly prepared liposomal dispersions were placed in a 50-mL beaker and frozen at \(-40\degree C\) for 6 h. Lyophilization was performed in a vacuum freeze dryer (VFD-2000, Shanghai Bilang Instrument Manufacturing, China) operating at a vacuum level of 0.1 MPa and a starting cold-trap temperature of the vacuum pump of \(-40\degree C\). After 48 h, the freeze-dried samples were obtained at temperatures of \(-40\) to \(10\degree C\). All dried liposomes were powdery and slightly sticky.

2.3 The storage stability of PBE liposome

All liposomes were stored at 4\degree C, 25\degree C, and 37\degree C, respectively, and their stability was determined. The stability of liposomes was characterized via changes in particle size and PDI change. At 4\degree C, the samples were measured every 7 days. At 25\degree C, the samples were measured every 3 days. At 37\degree C, the samples were measured every day. Each sample has three parallel samples.

2.4 Characterization of liposomes

2.4.1 Average size, PDI, and zeta potential analysis

Dynamic light scattering (DLS) is a commonly used technique for measuring the diameter of particles exhibiting Brownian motion in a solution. The average particle size, PDI, and zeta potential were measured using DLS (Zetasizer Nano, ZS90, Malvern Instruments, Worcestershire, UK). The sample was diluted 100x with high-purity water and placed in the spectrometer immediately after dilution. Each sample was measured thrice at 25\degree C, and data were expressed as mean \pm standard deviation.

2.4.2 The encapsulation efficiency of phytosterols

The lipid content of liposomes was obtained by extracting lipids from liposomes using a chloroform-methanol extraction method as described by Wang and Acevedo\textsuperscript{30}. The PBE content was determined after saponification of lipid samples. We added 10 mL of KOH-ethanol solution (1.0 mol/L) to the lipid samples, placed it in a water bath at 80\degree C for reflux and condensation, magnetically stirred for 2 h, and...
cool it to room temperature. Next, we added saturated sodium chloride solution (20 mL), injected ethyl ether (15 mL), and transferred the mixture to the separation funnel and extracted thrice; all three extracts were combined. The ethyl ether extract was washed and neutralized with distilled water, and then a certain amount of anhydrous sodium sulfate was added to the ether extract for dehydration, followed by filtration and evaporation to remove the ether to obtain the saponifiable matter.

The following conditions were applied for Gas chromatography (GC-2010 Plus Gas chromatograph) analysis of PBE using DB-1HT chromatography column: FID temperature: 300°C; gas flow rate: hydrogen 30 mL/min, air 300 mL/min; carrier gas: nitrogen 25 mL/min; injection temperature: 300°C; split sampling, split ratio was 50:1; Column temperature: initially 180°C for 1 min, and was increased to 300°C at 30°C/min for 10 min; injection volume: 1.0 L; column pressure: 10 psi.

Squalane (100 mg) was accurately weighed in a 25 mL volumetric flask, and hexane was volumetric to scale and used as an internal standard solution. The β-sitosterol standard was measured and dissolved in n-hexane (50 mL), which was used as the standard storage liquid, β-sitosterol solutions (0, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 mL) were prepared and stored in 10 mL volumetric flasks, respectively. Squalane internal standard solution (1.0 mL) was added to each bottle, and then the volume was made up to 10 mL. The standard solution ranged in concentration from 0.1 to 1.6 mg/mL. The linear regression equation and correlation coefficient between β-sitosterol and internal standard were found to be A = 0.5395C + 0.0674 and R² = 0.9994. The encapsulation efficiency of PBE was calculated using β-sitosterol as the index.

2.4.3 Morphological analysis

The morphology of the liposomes was determined using transmission electron microscopy (TEM) with an HT7700 microscope (Hitachi, Japan). The liposome suspension was placed on a copper grid for 8 min and negatively stained using the phosphotungstic acid solution (2%) for 5 min. Excess solution was removed with filter paper, and the stained samples were air-dried and visualized. The micrographs were acquired using a voltage of 100 kV and 40,000 × magnification.

Atomic Force Microscopy (AFM) images were obtained under ambient conditions using the MFP-3D infinity Asylum system (Oxford Instruments, Britain). Surface morphology was measured in the tapping mode AFM at room temperature. A commercial silicon tip-cantilever with stiffness of approximately 26 Nm⁻¹ was used for height and phase imaging data. Scan frequency was 1.00 Hz in air. The liposome suspension was diluted 4000 times with ultra-pure water; a drop of the diluted liposome was placed on the silicon wafer for 15 min, and the excess liquid was absorbed with a filter paper. After keeping at room temperature for 15 min, the liposome samples were rinsed thrice with ultra-pure water and then placed at room temperature for 2 h and analyzed by AFM.

2.5 Fourier-transform infrared spectroscopy (FTIR) analysis

Fourier-transform infrared spectroscopy (FTIR) analysis was performed on the powdered purified product with a Spectrum TWO UATR (PerkinElmer, USA) in the range of 4000–400 cm⁻¹ using 16 scans at a resolution of 0.5 cm⁻¹. All spectra were an average of at least two samples. FTIR analysis was also performed on the liposome samples.

2.6 Raman analysis

Raman spectrometric analysis was done using a high-resolution laser confocal micro-Raman spectrometer from HORIBA JOBIN YVON SAS, France. The Nd:Yag laser was used for excitation at 532 nm. The laser power was 100 mW, and the test range was 400–3200 cm⁻¹.

2.7 Differential scanning calorimetry (DSC) analysis

Heating phase diagrams of liposome emulsion were acquired using DSC 8000 differential scanning calorimeter (PerkinElmer, USA). The experiments were performed in the temperature range of −30 to 90°C at a heating rate of 10°C/min under nitrogen flow at 40 mL/min. The DSC data were analyzed with the software supplied by the manufacturer, and the plots were drawn using Pyris version 13.

2.8 X-ray diffraction (XRD) analysis

The crystal state of freeze-dried SPC, PBE liposomes, and free PBE was analyzed by MiniFlex 600 (Rigaku, Tokyo, Japan). Diffraction patterns were recorded over a range of 20 angles from 5° to 60° at a speed of 0.15 s/step and a scan step size of 0.02° at a rate of 5°·min⁻¹. Samples were irradiated with Cu-Ka radiation at 30 mA and 40 kV.

2.9 Micropolarity of liposomes membrane

A pyrene fluorescence probe method was used for analyzing the micropolarity of the liposome membrane using a fluorospectrophotometer (Shimadzu RF-7000, Japan) [31]. Pyrene (304.00 mg) was dissolved in ethanol (50 mL), and the original solution of the fluorescent probe was prepared in a brown volumetric flask. The pyrene solution (0.012 mL) was added to a 50 mL beaker, blown with nitrogen, followed by the addition of 1.2 mL of different liposome emulsions. Next, the solutions were incubated in a water bath at 37°C and 95 rpm for 30 min, and then liposome emulsion (1.8 mL) was added to this mixture. The reaction was performed at room temperature. The excitation wavelength of the fluorescence spectrometer (Ex) was set at 334 nm, the Ex slit was set at 5 nm, and the emission (Em) slit was set at 1 nm. The intensity ratio (I1/I3) of peak 1 (approximately 375 nm) to peak 3 (approximately 385 nm) served...
as a reference for micropolarity analysis.

2.10 Data analysis

Data were analyzed using Analysis of variance (ANOVA) using the IBM SPSS21.0 Statistics software (IBM Analytics, Armonk, USA), followed by Duncan’s test at a significance level of \( p < 0.05 \). Each sample was tested in triplicates.

3 Results and Discussion

3.1 Physicochemical properties of SPC and PBE liposomes

Table 1 summarizes the results obtained for the physicochemical characterization of different liposome formulations prepared by the thin-film hydration method. The dispersions showed average diameters typically obtained for small vesicles with an average size of approximately 60 nm. However, we found no significant difference between PBE and SPC liposomes \( (p > 0.05) \) regarding particle size, which indicated that the incorporation of PBE had no significant effect on the particle size of the liposomes. Wang FC et al.\(^{30}\) found that the addition of phytosterol ester slightly reduced the particle size of liposomes, which was probably dependent on the type of phytosterol ester selected. The particle size of liposomes was ideal since, due to the Brownian motion of the nanocarriers, smaller sized particles have higher stability against gravity\(^{28,32}\). The absolute potential value of SPC and PBE liposomes was medium with a lack of significant difference between them \( (p > 0.05) \). The high negative zeta potential of liposomes was attributed to the presence of Tween-80, which could get adsorbed or incorporated into the liposomal bilayer and decrease the zeta potential by introducing a shift in the shear plane of the particle\(^{33,34}\).

The PDI value was <0.3, indicating a narrow distribution of the liposomes\(^{35}\). In this study, the incorporation of PBE in liposomes led to an increase \( (p < 0.05) \) in PDI. Thus, the addition of PBE slightly reduced the particle size of liposomes, while free PBE formed crystals with the larger particles in the system. Therefore, the PDI of PBE liposomes increased when the mean particle size of liposomes remained unchanged. Liposomes prepared with different concentrations of PBE had similar size distributions (<0.3). Figure 1(a) shows the size distribution of SPC and PBE liposomes. Compared with SPC liposomes, PBE liposomes had slightly smaller PDI (<200 nm) and slightly larger PDI (>2 μm). Thus, different liposome formulations prepared using the thin-film hydration method formed stable translucent suspensions (Fig. 1(b)).

| Sample          | Particle size (nm) | PDI         | Zeta potential (mV) | EE (%) |
|-----------------|--------------------|-------------|---------------------|--------|
| SPC LP          | 58.7 ± 3.4\(^a\)  | 0.15 ± 0.01\(^a\) | -16.4 ± 2.7\(^a\)  | -      |
| PBE/SPC (1/40) LP | 54.9 ± 4.6\(^a\)  | 0.22 ± 0.03\(^a\) | -15.8 ± 2.4\(^a\)  | 94.2 ± 1.2\(^a\) |
| PAE/SPC (1/20) LP | 57.9 ± 3.6\(^a\)  | 0.24 ± 0.03\(^b\) | -16.3 ± 1.9\(^a\)  | 93.7 ± 2.3\(^a\) |
| PBE/SPC (1/13) LP | 58.4 ± 2.1\(^a\)  | 0.25 ± 0.02\(^b\) | -15.3 ± 1.8\(^a\)  | 91.4 ± 2.6\(^a\) |
| PBE/SPC (1/10) LP | 60.1 ± 1.3\(^b\)  | 0.26 ± 0.01\(^b\) | -16.2 ± 3.5\(^a\)  | 90.3 ± 2.1\(^a\) |

Different letters (a, b) indicate significant differences \( (p < 0.05) \).

Values are the means ± standard deviation of three independent experiments.

Fig. 1 The size distribution (a) and transparent emulsion (b) of SPC and PBE liposomes.
The encapsulation efficiency of PBE in the liposome was found to be >90%, indicating that within this range (1/40–1/10), PBE could be effectively embedded in the lipid bilayer (Table 1). A previous study had shown a decrease in the encapsulation efficiency with an increase in the number of phytosterol esters in the presence of free phytosterols. This effect was attributed to the presence of free phytosterols, which could reduce the encapsulation efficiency of phytosterol esters. Cholesterol was also found to significantly reduce the encapsulation rate of fat-soluble substances with an increase in the amount of incorporation.

Since we found no significant difference in particle size and PDI for PBE liposomes at different concentrations (p > 0.05), SPC liposome (Fig. 2(a)) and PBE/SPC as 1/20 (Fig. 2(b)) liposomes were chosen to study the morphology of vesicles using TEM. The TEM images showed that the liposomes were uniformly distributed in the field of vision and were spherical without any evidence of coalescence. The particle size of liposomes was found to be consistent with that of DLS results.

3.2 Stability of liposomes

We determined the stability of liposomal suspensions at different temperatures. Figure 3 shows the average particle size and PDI of SPC and PBE liposomes at 4°C, 25°C, and 37°C. There was a significant increase in the average particle size of SPC liposomes at 4°C for 30 days, while there was no apparent change in different concentrations of PBE liposomes. However, at 25°C and 37°C for 18 and 7 days, respectively, the particle size of the liposome with 1/10 PBE/SPC showed a significant enhancement, followed by the SPC liposome. During the storage time, the PDI value increased gradually at 37°C and decreased slightly at 4°C and 25°C. The reasons for these changes are unclear, and further research is needed. Except for the liposome with PBE/SPC of 1/10, PDI of the remaining liposomes was found to be <0.3, indicating a relatively narrow particle size distribution.

Figure 4 shows the images of SPC at 4°C for 0 and 30 days and PBE (PBE/SPC: 1/10) liposomes at 25°C for 0 and 18 days obtained by AFM in the tapping mode. We observed significant dispersion of spherical particles of individual liposomes on a flat background on day 0. Liposomes with SPC and PBE/SPC of 1/10 at 4°C and 25°C showed membrane fusion and vesicle expansion during storage, indicating that these liposomes were unstable. Thus, liposomes with PBE/SPC of 1/40, 1/10, and 1/13 were significantly more stable than those with SPC and PBE/SPC of 1/10 at 4°C, 25°C, and 37°C. This implied that excessive PBE (PBE/SPC, 1/10) made the bilayer exceed the limit of the capacity, causing the loss of the stable structure of the phospholipid liposome. Thus, the liposomes with low concentrations of PBE had relatively good storage stability.

3.3 FT-IR analysis

Figure 5 shows the FT-IR spectra obtained for the SPC and PBE liposomes, which showed characteristic spectral features of phospholipid bilayers: the bands at approximately 2856 cm⁻¹ and 2924 cm⁻¹ were attributed to the symmetric and antisymmetric vibration corresponding to the -CH₂ group stretching, respectively; the peaks at approximately 1740 cm⁻¹ corresponded to the carbonyl (C=O) stretching frequency; the bands at 1090 cm⁻¹ and 1240 cm⁻¹ represented the symmetric and antisymmetric vibrations corresponding to the phosphate head group (PO₄⁻), respectively; the band at 970 cm⁻¹ represented the choline groups ((CH₃)₃N) vibration regions.

Regarding the polar region of SPC, FTIR spectra showed that the interaction of PBE with SPC liposomes led to a shift from 1246 cm⁻¹ to 1240, 1243, 1244, and 1244 cm⁻¹ in the phosphatidylcholine v₆ PO₄⁻ and a shift from 1094 cm⁻¹ to 1088, 1088, 1091, and 1091 cm⁻¹ in the phosphatidylcholine v₅ PO₄⁻ for PBE/SPC (1/40), PBE/SPC (1/20), PBE/SPC (1/13), and PBE/SPC (1/10) liposomes. Thus, the position of the PO₄⁻ stretching peak shifted to lower wavenumbers with an increase in hydrogen bonding of the phosphate groups with water or other compounds.
Since the carbonyl group of PBE could only act as the acceptor of H and not donor, it was unable to form hydrogen bonds with the phosphate group in the phospholipid molecule. Therefore, the presence of PBE only resulted in an increase in the hydration degree of the phosphate group. With an increase in PBE concentration, the hydration degree of phosphate groups slightly decreased, probably due to the presence of a large number of C=O groups in PBE that participated in hydrogen bond contention.

The carbonyl stretching vibration was identified at 1737 cm\(^{-1}\) in the SPC liposome. Considering the spectra obtained for the PBE (PBE/SPC (1/20), PBE/SPC (1/13), and PBE/SPC (1/10)) liposomes, we observed a minor decrease in the position of this peak, indicating that PBE increased the strength of the hydrogen bonding around the carbonyl groups of SPC membranes. Also, as the concentration of PBE increased, more C=O groups were introduced, resulting in the formation of more hydrogen bonds. There was no variation in the (CH\(_3\))\(_3\)N band in the SPC and PBE liposomes, suggesting that the hydration degree was not affected.

Additionally, we observed the presence of spectral peaks at 2856 and 2924 cm\(^{-1}\) in the SPC liposome, as well as in the spectra of PBE liposomes with a slightly lower shift at 2856 cm\(^{-1}\) and no modification at 2924 cm\(^{-1}\). These peaks were related to the symmetric and asymmetric CH\(_2\) stretching vibrations, and a decrease in their position indicated that the acyl chains of the phospholipids were more ordered, resulting from a decrease in the number of gauche conformers with respect to the trans conformers\(^{37, 38}\). The studied PBE might have enhanced the van der Waals forces between the alkyl chains, reducing the empty spaces in the
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Based on the results of the FTIR analysis, the PBE molecule was preliminarily determined to be located at the water interface and inserted into the phospholipid molecule.

3.4 Raman analysis

Raman spectroscopy provides powerful evidence to detect the changes in the environment of lipid hydrocarbon chains when external molecules are embedded into the lipid bilayers. Figure 6 shows that all spectral features of SPC and PBE liposomes were retained at room temperature. Herein, we investigated the structural changes in liposomes after the incorporation of PBE in a dose-dependent manner by observing the changes in the vital Raman bands in the C-H stretching vibration range \( \nu \) and the C-C stretching vibration range \( \nu \). The peaks at approximately 2850 and 2890 cm\(^{-1}\) corresponded to the symmetrical and asymmetrical vibrations of stretching of the C–H bond of the methylene groups, respectively. The peak at approximately 2930 cm\(^{-1}\) was attributed to the symmetrical vibration of stretching of the C–H bond of the final methyl group of the alkyl chain. The intensity ratios \( I_{2930}/I_{2890} \) and \( I_{2850}/I_{2890} \) are known to provide useful information on the interaction between acyl chains. The ratio of \( I_{2850}/I_{2890} \) has been used as an overall indicator of both intramolecular \( \langle \text{gauche} \rangle/\langle \text{trans} \rangle \) and intermolecular \( \langle \text{packing order} \rangle \) interactions in the phospholipid bilayer. The \( I_{2930}/I_{2890} \) ratio is known to be sensitive to subtle changes in the conformational order from rotations, kinks, twists, and bends in the hydrocarbon chain of lipids. In our study, Figure 6 shows the ratios of \( I_{2930}/I_{2890} \) and \( I_{2850}/I_{2890} \). The results showed that different concentrations of PBE did not cause significant changes in these ratios.

The peak at approximately 1130 cm\(^{-1}\) was attributed to the stretching vibration of the C–C bond for the \( \text{trans} \)-conformation of the alkyl chains, while the peak at approximately 1100 cm\(^{-1}\) was attributed to the stretching vibration of the C–C bond for the \( \text{gauche} \) conformation of the alkyl chains. The intensity ratio of these peaks, \( I_{1130}/I_{1100} \), provided information regarding the proportion between order and disorder that was present in the alkyl chain conformation. The longitudinal order parameter was slightly higher than that of the SPC liposomes with the incorporation of PBE, suggesting an increase in \( \langle \text{all-trans} \rangle \) bonds and a decrease in \( \langle \text{gauche} \rangle \) rotamers. The results could be interpreted as the steroid derivatives having an ordering effect upon the lipid acyl chains. The ratio at 1/40 and 1/20 of PBE/SPC showed no change but decreased slightly at 1/13 and 1/10. This indicated that the ordered effect of PBE on the alkyl chain was slightly reduced when PBE/SPC was \( \leq 1/20 \). The increase in the ratio of \( I_{1130}/I_{1100} \) even in the presence of small PBE content confirmed the marked sensitivity of liposomes to the presence of PBE. However, the measurement of the intensity ratios in the C-H stretching frequencies did not show any evidence for structural perturbation or ordering of the hydrocarbon chains due to the presence of PBE at 1/40, 1/20, 1/13, and 1/10 when using membrane model containing SPC. Since the intensity ratios in the C-C stretching frequencies were significantly affected by the incorporation of PBE, we concluded that most PBE were located parallel to the lipid hydrocarbon chains. Thus, the results of Raman spectroscopy were consistent with the predictions of the membrane model containing SPC.

Fig. 4 The images of the SPC at 4°C on 0(a) and 30(b) days and PBE (PBE/SPC: 1/10) liposomes at 25°C on 0(c) and 18(d) days using atomic force microscopy (AFM).

Fig. 5 FTIR spectra of SPC and PBE liposomes.
3.5 DSC analysis

In this study, DSC was used to investigate the phase transition behavior of SPC and PBE liposomes. In the DSC thermograms, the main phase transition showed the conversion of liposomes from the gel state to the liquid crystalline state, which provided information regarding the interaction between the exogenous substances and acyl chains of phospholipids. The changes in the main phase transition temperature ($T_m$) reflects the conformational transitions of the acyl chains. $T_m$ reflects the arrangement of the hydrophobic tail chain of the phospholipid molecule. The enthalpy change ($\Delta H$) provides information on the strengthening or weakening of non-covalent bonds, such as van der Waals forces in hydrophobic regions. Additionally, the width at half height ($\Delta T_{1/2}$) gives the information regarding the cooperativity of this conversion.

Figure 7 and Table 2 show the DSC results of SPC and PBE liposomes. The $T_m$ of SPC liposomes was 4.28°C, with that of FTIR.
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Table 2  The main phase transition temperature ($T_m$), enthalpy change ($\Delta H$), and the width at half height ($\Delta T_{1/2}$) of SPC and PBE liposomes.

|          | $T_m$ (°C) | $\Delta H$ (J/g) | $\Delta T_{1/2}$ (°C) |
|----------|------------|------------------|------------------------|
| SPC      | 4.28 ± 0.01$^a$ | 262.51 ± 0.12$^a$ | 4.58 ± 0.02$^a$       |
| PBE/SPC (1/40) | 4.18 ± 0.02$^b$ | 264.48 ± 0.23$^b$ | 4.70 ± 0.01$^a$       |
| PBE/SPC (1/20) | 4.82 ± 0.04$^b$ | 261.35 ± 0.31$^b$ | 5.02 ± 0.03$^b$       |
| PBE/SPC (1/13) | 4.93 ± 0.03$^{bc}$ | 259.47 ± 0.22$^c$ | 5.14 ± 0.01$^c$       |
| PBE/SPC (1/10) | 4.95 ± 0.02$^c$ | 260.48 ± 0.17$^c$ | 5.16 ± 0.02$^c$       |

Different letters (a, b, c) indicate significant differences ($p < 0.05$).

Values are the means ± standard deviation of three independent experiments.

which was higher than 0°C, inconsistent with the literature value of less than 0°C$^{40, 41}$.
This was probably due to the fact that Tween-80 increased the stability of the liposome$^{39}$, thus increasing the $T_m$. The peak shape was slightly asymmetrical with only a slight skewing towards higher temperatures, which was consistent with previous research$^{42, 43}$. Compared with SPC liposomes, no other peaks appeared in PBE liposomes, indicating that PBE did not disturb the liposome membrane structure by DSC measurement$^{44}$. These results were consistent with the results of Raman spectroscopy.

The addition of PBE resulted in a slight shift in $T_m$ to a higher temperature by about 0.5°C as the PBE concentration was increased except that PBE/SPC at 1/40 (Fig. 7 and Table 2). This result implied that PBE was incorporated into the hydrophobic part of the membrane and was consistent with the study by Wang FC$^{30}$. PBE entered the hydrophobic region of the liposome bilayer membrane, interacted with the phospholipid molecule tail chain, improved the order of the phospholipid hydrophobic tail chain, reduced its fluidity, and thus increased $T_m$. These results supported the information obtained from the FTIR and Raman analysis.

The exogenous substances could be classified as interstitial or substitutional impurities based on the changes in the $T_m$ and $\Delta H$ values$^{39, 45}$. Those impurities that can change $T_m$ and $\Delta H$ are called substitutional impurities, and those that change only $T_m$ but not $\Delta H$ are called interstitial impurities. PBE belongs to the latter category because it increases $T_m$ without affecting $\Delta H$. This could be explained that a superficial interaction between the proper concentration of PBE and SPC molecules and/or the intercalation of PBE molecules between the lipid chains increased the stability of the membrane. This result was supported by the increment of liposomal stability after the addition of PBE. This phenomenon was consistent with the literature$^{46}$.

The width at half height ($\Delta T_{1/2}$) of the main phase transition peak is an important calorimetric parameter. We also observed that the value of the $\Delta T_{1/2}$ slightly broadened (from 4.58°C to 5.16°C) with the addition of PBE into liposomes except for PBE/SPC at 1/40. This suggested that the addition of PBE resulted in a slight decrease in cooperativity between acyl chains of SPC membranes$^{37}$.

3.6 XRD analysis

Figure 8 shows the X-ray diffraction pattern for free PBE, PBE/SPC, and SPC liposomes. As expected, we found no characteristic peaks for SPC and PBE liposomes. The characteristic peaks of free PBE indicated that it had high crystallinity. The SPC liposomes used for XRD experiments were viscous powders. No characteristic peaks were found for SPC liposome without PBE, which indicated the presence of amorphous structure in the system. Under the influence of SPC, PBE had no characteristic peak in the liposomes. This observation was consistent with the results of previous studies$^{48, 49}$. Despite the incorporation of PBE, there were no corresponding crystal peaks in the liposome, which suggested changes in the physical state of PBE as well as the loss of crystallinity following encapsulation in liposomes. Similar results have been obtained for phytosterols liposomes in previous studies; for example, Wang et al.
confirmed that phytosterol esters existed in the lipid bilayer in an amorphous state after being encapsulated in liposomes\(^{30}\).

3.7 Micropolarity of liposome bilayer membrane

Pyrene as a fluorescent probe was embedded in the bilayer of liposomes to study the microenvironmental polarity. The ratio of pyrene fluorescence intensity at 373 nm to 384 nm (I\(_1/I_3\)) is known to be highly sensitive to microenvironmental polarity, and a decrease in the ratio is known to indicate a decrease in polarity\(^{50}\). The results showed a gradual decrease in the range 1/40-1/13 as PBE/SPC increased and a slight increase in 1/10 (Fig. 9). This finding suggested that the accumulation of PBE molecules in the phospholipid head group was likely to reduce the head-space and prevented water from permeating into the inner region of the membrane to decrease polarity. Additionally, the insertion of PBE increased the ordering of the lipid alkyl chain, thus reducing the water penetration. With a PBE/SPC ratio of 1/10, it was possible that too many PBE molecules altered the balance between the polar headgroup and the hydrophobic tail, resulting in an increase in micropolarity by allowing water to easily enter the membrane.

4 Conclusion

This study is the first report investigating the characteristics of PBE liposomes prepared by the thin-film hydration method. PBE showed positive effects on the characteristics of liposomes, such as improving the storage stability, increasing the order of hydrophobic alkyl chain, increasing the thermal stability, and decreasing the micropolarity of the membrane. Therefore, PBE could be used to construct stable liposomes at a suitable concentration.

Author Contributions

Gu, K. and Hou, L.: designed research; Hou, L.: performed research; Hou, L., Sun, X. and Pan, L.: contributed analytic tools; Hou, L., Sun, X. and Pan, L.: analyzed data; Hou, L.: wrote the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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