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A DSC study of paeonol-encapsulated liposomes, comparison the effect of cholesterol and stigmasterol on the thermotropic phase behavior of liposomes

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Abstract Thermotropic phase behaviors of paeonol-encapsulated liposomes containing stigmasterol or cholesterol have been investigated by differential scanning calorimetry. We compared the thermotropic phase behavior of pure dipalmitoylphosphatidylcholine (DPPC) liposomes, sterol/DPPC liposomes, and paeonol/sterol/DPPC liposomes increasing the ratio of paeonol to sterol from 0 to 1, by analyzing the calorimetric parameters of main phase transition of liposomes including phase transition temperature (onset temperature and peak temperature) and phase transition cooperativity. The results showed that paeonol could incorporate into the hydrophobic region of DPPC, thus, decrease phase transition temperature of DPPC. Though stigmasterol interacts with DPPC less favorably than cholesterol, thermotropic phase behavior of paeonol/cholesterol/DPPC liposomes and that of paeonol/stigmasterol/DPPC liposomes are very similar. A phase separation occurred when the molar ratio of paeonol to sterol reached 1:1 in paeonol-encapsulated liposomes, where a paeonol-rich domain coexisted with a sterol-rich domain. The packing order of acyl chains of DPPC in sterol-rich domain is a little higher than that in paeonol-rich domain.

Keywords Paeonol-encapsulated liposomes · Differential scanning calorimetry (DSC) · Cholesterol · Stigmasterol · Phase separation

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Introduction

Paeonol (or peonol, ab. Pae, as shown in Fig. 1), 2-hydroxyl-4-methoxyacetophone, is one of the major components of Moutan cortex, which has been used as a tranquillizer and an antihypertensive. It has analgesic, antipyretic, and antibacterial properties and may be used in the treatment of arthritis and suppress ADP-or collagen-induced human blood platelet aggregation in a dose-dependent manner. Paeonol has also been shown to possess anti-inflammatory properties and to have a diuretic action [1–4].

The potential usefulness of liposomes as drug carriers has attracted considerable interest (for review, see [5]). These phospholipid vesicles are capable of encapsulating both hydrophobic and hydrophilic drugs. The drugs encapsulated in liposomes are sufficiently protected from enzymatic attack and immune recognition [6]. Paeonol is poorly soluble in water so that it is difficult to absorb and its bioavailability is low in vivo. Thus, it is significant to encapsulate paeonol into liposomes.

The amphiphilic molecules used for the preparation of liposomes are derived from or based on the structure of the lipids of biological membranes [5]. Generally speaking, phospholipids and sterols are typical amphiphilic molecules which are often used to prepare liposomes. As phosphatidylcholines (PCs) and cholesterol (shown in Fig. 1) are among the major phospholipids and major

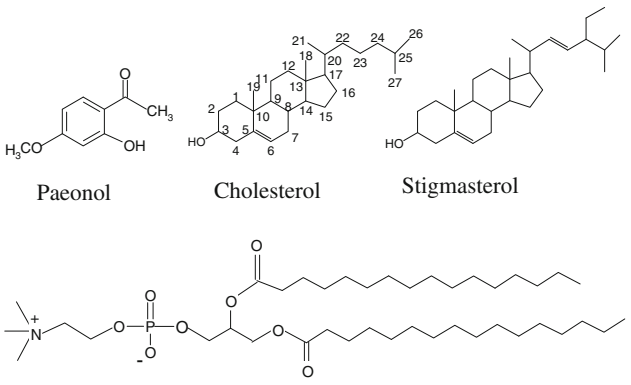

Fig. 1 Molecular structures of cholesterol, stigmasterol, paeonol, and DPPC

sterols presenting in plasma membranes, respectively [7], vesicles formed by PCs/cholesterol mixtures have been selected by many research groups as simple model systems of liposomes with or without drugs [8–11].

It is well known that high cholesterol increases the risk of many diseases including: coronary heart disease, stroke, and peripheral vascular disease. Thus, other sterols especially plant sterols such as stigmasterol (shown in Fig. 1) have been used to prepare liposomes. We have investigated the thermotropic phase behavior of stigmasterol/DPPC liposomes and constructed the binary phase diagram of this binary system employing differential scanning calorimetry (DSC), X-ray diffraction (XRD), and freeze-fracture electron microscopy (FFEM) techniques [12]. The results showed that the thermotropic phase behavior and binary phase diagram of cholesterol/DPPC and stigmasterol/DPPC liposomes are very similar. Muramatsu et al. [13] reported that the bioavailability of insulin-encapsulated liposomes containing stigmasterol is a little high than that containing cholesterol.

Phase behaviors such as phase transition and phase separation of liposomes have very important effects on the properties of liposomes such as stability, fluidity, and permeability. They play an important role in some vitro and vivo processes of drugs such as encapsulation, transferring, and release (for review, see [5, 14]). Phase behaviors of liposomes formed by PCs/sterol with or without drugs have been widely studied by many techniques such as DSC, XRD, and resonance energy transfer [8–11, 15–17], but there are few reports about phase behavior of paeonol-encapsulated liposomes no matter containing cholesterol or stigmasterol as yet.

DSC is a thermodynamic technique of great value in studies of lipid thermotropic phase behaviors of model phospholipid liposomes. DPPC has often been used as a phospholipid molecule of liposomes both because lecithins are the major components in mammalian membranes and also because they show a sharp and strong, thermotropic transition near the physiological temperature, arising from the phase transition from the gel to the liquid–crystal phase [18–20]. In this work, we employed DSC technique to compare the thermotropic phase behaviors of paeonol/cholesterol/DPPC and paeonol/stigmasterol/DPPC liposomes, mainly focused on phase transition and phase separation.

Experimental

Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and Paeonol (Pae), and 2-hydroxyl-4-methoxyacetophone were purchased from Sigma Chemicals (99%, St. Louis, MO, USA). Cholesterol and Stigmasterol were from MP Biomedicals Inc. (95%, Aurora, OH, Germany). They were used without further purification. All organic solvents were of analytical grade. Double deionized water with a resistivity of about 18.2 MΩ·cm was used for the preparation of buffers. Liposomes were prepared following Lichtenberger et al. [21]: paeonol/sterol/DPPC mixtures with designated molar ratios were dissolved in chloroform, dried under nitrogen, and then stored in vacuum overnight. The lipid films were hydrated with excess Tris–HCl buffers (50 mM Tris–HCl, 150 mM NaCl, and 0.1 mM $CaCl_2$, pH = 7.2) with repeated vortexing and heating to 60 °C and then stored at 4 °C at least for 24 h before experiments. The molar ratio of sterol to DPPC was 1:24 and that of paeonol to sterol was 0, 0.2:1, 0.4:1, 0.8:1, and 1:1, respectively.

Method

The calorimetry was performed using a Mettler-Toledo DSC821^e differential scanning calorimeter equipped with the high-sensitivity sensor HSS7, which has a temperature precision of ±0.1 °C and a heat flow precision of ±0.01mW. Usually 20 µl of sample was used in each scanning experiment with the scan rate of 0.5 °C min^{-1}. In addition, a scanning rate of 5 °C min^{-1} has also been applied to detect the low enthalpic pre-transition of DPPC in liposomes. All samples have been cooled from 65 to 20 °C slowly before measurement to achieve equilibrium. Measurements were repeated at least three times to insure reproducibility.

Deconvolution of the multicomponent DSC curves was carried out using PeakFit software (Aisn Software Inc). The baseline was created by the two-point linear method and peak type was Gauss + Lorentz.

Results and discussion

The pre and main phase transitions are often observed in PCs. The pre-transition, which corresponds to the conversion of a lamellar gel phase to a rippled gel phase ($\mathbf{L}_{\beta'}$ to $\mathbf{P}_{\beta'}$), is mainly related to the polar region of phospholipids; while the main phase transition, which reflects the change from a rippled gel phase to a liquid crystal phase ($\mathbf{P}_{\beta'}$ to $\mathbf{L}_{\alpha}$), is closely related to the acyl chains of phospholipid bilayers. Thus, the main phase transition of phospholipids can probe the interaction between the acyl chains of phospholipids and exogenous substances. Moreover, the half-height width (HHW) of its DSC peak is an index of the cooperativity of this conversion: the narrower the peak, the higher the cooperativity [22]. The calorimetric parameters of the main transition process, such as T_p (temperature of the peak maximum), T_{onset} (temperature at which the thermal effect starts), and HHW are the most sensitive measures of the physical properties of phospholipid liposomes, and their changes can be attributed to the packing of exogenous molecules within the hydrophobic interior of phospholipids array [22, 23].

Calorimetric parameters of phase transition of pure DPPC liposomes

Presented in Fig. 2 are DSC curves of cholesterol/DPPC (1:24, molar:molar) or stigmasterol/DPPC (1:24, molar:molar) liposomes containing different concentration of paeonol collected at a scan rate of 0.5 °C min^{-1}. To compare the phase behavior of paeonol/sterol/DPPC liposomes with that of pure DPPC liposomes, the DSC curve of pure DPPC liposomes is also shown in Fig. 2. In the absence of sterol and paeonol, DPPC liposomes display a low enthalpic pre-transition ($\mathbf{L}_{\beta'}$ to $\mathbf{P}_{\beta'}$) at 36.6 °C (T_p) and a sharp main transition ($\mathbf{P}_{\beta'}$ to $\mathbf{L}_{\alpha}$) at 42.7 °C (T_p). The phase transition temperatures are in good agreement with the published data [24, 25]. Compared with other liposomes, pure DPPC liposomes own the highest main transition temperature and its transition peak is the narrowest (the *HHW* equal to 0.5 °C).

As shown in Fig. 2, pre-transition of DPPC is undetectable for cholesterol/DPPC or stigmasterol/DPPC liposomes with or without paeonol at a scan rate of 0.5 °C min^{-1}. A fast scan rate of 5 °C min^{-1} was also used to detect pre-transition for these samples, but pre-transition still cannot be detected (data not shown). There are some other reports that pre-transition of PC liposomes is suppressed by adding low concentration sterols such as 5 mol% stigmasterol [16, 17], 5 mol% [16, 17], or 6 mol% [15] cholesterol, or low concentration drugs such as 5 mol% pirarubicin [22] and 2.5 mol% cortisone esters [25].

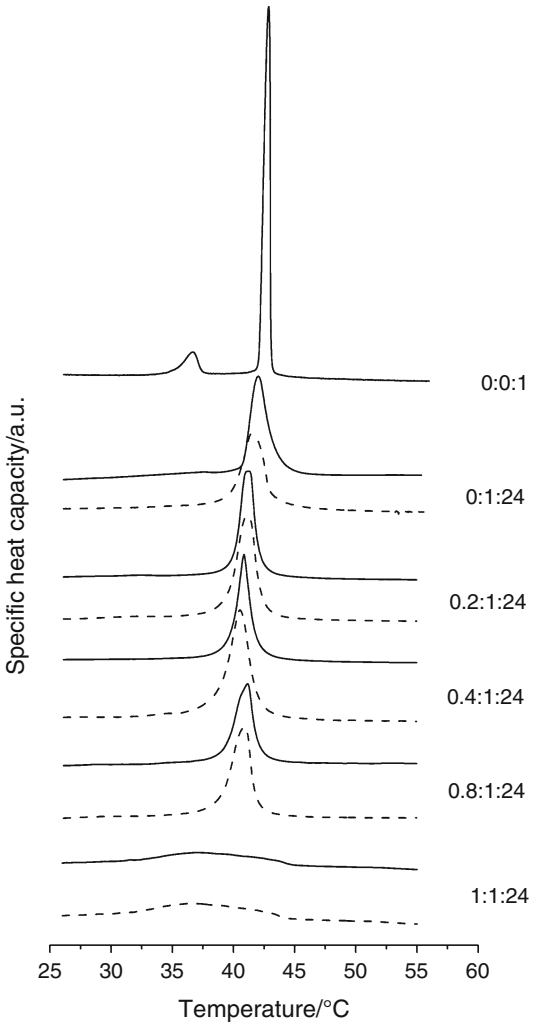

Fig. 2 DSC curves of paeonol/cholesterol/DPPC (*solid lines*) or paeonol/stigmasterol/DPPC (*dashed lines*) liposomes at different molar ratio of paeonol:sterol:DPPC collected at a scan rate of 0.5 °C min^{-1}

Thermotropic phase behavior of paeonol/sterol/DPPC liposomes

The calorimetric parameters of main transition of cholesterol/DPPC or stigmasterol/DPPC liposomes with or without paeonol at a scan rate of 0.5 °C min^{-1} are showed in Fig. 3. Data shown in Fig. 3 represents the average of at least three independent experiments and the error bar was determined based on the square root of the variance. Four conclusions could be drawn from Fig. 3:

First, paeonol could incorporate into the hydrophobic region of DPPC and interact with acyl chains of DPPC. As shown in Fig. 3a, the temperature of main transition (T_{onset} and T_p) of cholesterol/DPPC liposomes and stigmasterol/DPPC liposomes all decreased slightly with increasing the molar ratio of paeonol to sterol from 0 to 0.8, followed by a drastic decrease at the molar ratio of 1:1. Since the main

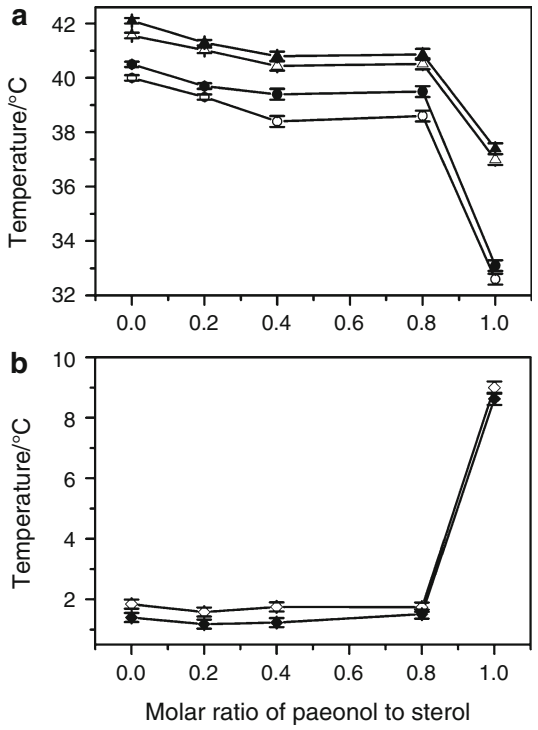

Fig. 3 The relationship between phase transition temperature (**a**), HHW (**b**) of paeonol/sterol/DPPC liposomes and molar ratio of paeonol to sterol. Legend for figure is as follows: T_{onset} for cholesterol (*filled circle*); T_{onset} for stigmasterol (*open circle*); T_p for cholesterol (*filled triangle*); T_p for stigmasterol (*open triangle*); HHW for cholesterol (*filled diamond*); and HHW for stigmasterol (*open diamond*). Each data point represents the average of at least three independent experiments

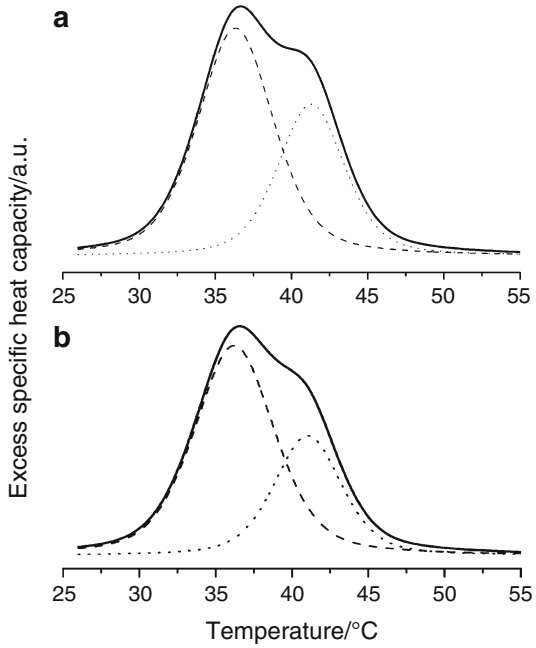

Fig. 4 The raw DSC curves (*solid lines*) of paeonol/sterol/DPPC liposomes (**a** is for cholesterol and **b** is for stigmasterol) at molar ratio of 1:1:24 (paeonol:sterol:DPPC) and deconvoluted curves (*dashed lines* and *dotted lines*)

phase transition is closely related to the acyl chains of DPPC, this result could imply that paeonol could incorporate into the hydrophobic region of DPPC and interact with acyl chains of DPPC. This could be interpreted by the fact that the molecule of paeonol has a hydrophobic and planar ring structure. Though paeonol owns a hydroxyl group, its ability to incorporate into the hydrophilic region of DPPC is poor because it is poorly soluble in water.

Second, stigmasterol interacts with DPPC a little less favorably than cholesterol. Figure 3a shows that the temperature of main transition (T_{onset} and T_p) of cholesterol/DPPC liposomes was slightly higher than that of stigmasterol/DPPC liposomes containing equal concentration of paeonol. A high transition temperature probably indicates the presence of relatively favorable, stabilizing interactions between sterols and phospholipids [17, 26]. Figure 3b shows that the cooperativety (inversely to the value of HHW) of the main phase transition of cholesterol/DPPC liposomes was a little higher than that of stigmasterol/DPPC liposomes containing equal concentration of paeonol. A high cooperativety of the main phase transition also means a relatively favorable, stabilizing interaction between sterols and phospholipids. Thus, no matter the results of phase transition temperature or the results of the cooperativety, all support the conclusion that cholesterol has a more favorable, stabilizing interaction with DPPC than stigmasterol. This conclusion could be explained as follows:

Compared with the molecular structure of cholesterol, the stigmasterol has an additional ethyl group at the position of C24 and a *trans* double bond between the position of C22 and C23. The additional ethyl group caused a less favorable and less stabilizing interaction with phospholipids duo to the steric hindrance, which suppressed the formation of ordered arrangements in phospholipids liposomes [17, 27]. As far as the double bond is concerned, some research groups have investigated the difference of ability to form ordered arrangements in phospholipid liposomes between stigmasteol and β-sitosterol [17, 27, 28]. β-Sitosterol is another plant sterol, which distinguishes stigmasterol only in the position between C22 and C23, where there is a single bond in β-sitosterol. Their research suggested that though the double bond may have caused a favorable interaction with phospholipids due to the decreased volume fluctuations in the sterol alkyl chain region, this effect is very limited. Therefore, the stabilizing effect of double bond of stigmasterol is lower than destabilizing effect of ethyl group of stigmasterol. Thus, the interaction of stigmasteol with DPPC is less favorable than that of cholesterol with DPPC though its double bond in the side chain is a benefit for its interaction with DPPC. In a

Table 1 Calorimetric parameters of coexisted pae-rich domain and sterol-rich domain of pae/sterol/DPPC liposomes at molar ration of 1:1:24 (pae:sterol:DPPC)

Sterol in liposomes	T_{onset} of Pae-rich domain/°C	T_{onset} of sterol-rich domain/°C	T_p of Pae-rich domain/°C	T_p of sterol-rich domain/°C	HHW of Pae-rich domain/°C	HHW of sterol-rich domain/°C
Cholesterol	31.1 ± 0.2	36.9 ± 0.3	36.3 ± 0.2	41.3 ± 0.2	5.1 ± 0.2	4.4 ± 0.3
Stigmasterol	30.8 ± 0.3	36.5 ± 0.2	36.2 ± 0.2	41.0 ± 0.2	5.4 ± 0.3	4.5 ± 0.2

Each data represents the deconvoluted results of at least three independent experiments

Langmuir monolayer film investigation of DPPC, Su et al. [29] found that stigmasterol interacted less effectively than cholesterol with DPPC monolayer membrane.

Third, thermotropic phase behavior of paeonol/cholesterol/DPPC liposomes and that of paeonol/stigmasterol/DPPC liposomes is very similar. As shown in Fig. 3a, the temperature of main transition (T_{onset} and T_p) of cholesterol/DPPC and stigmasterol/DPPC liposomes showed a very similar dependence on the molar ratio of paeonol to sterol. They decreased slightly with increasing the molar ratio of paeonol to sterol from 0 to 0.8, followed by a drastic decrease at molar ratio of 1:1. As shown in Fig. 3b, the values of HHW of cholesterol/DPPC and stigmasterol/DPPC liposomes showed a very similar dependence on the molar ratio of paeonol to sterol. They nearly remained constant when the molar ratio of paeonol to sterol was increased from o to 0.8, followed by a drastic increase at molar ratio of 1:1.

Finally, phase separation occurred in both paeonol/cholesterol/DPPC and paeonol/stigmasterol/DPPC liposomes when the molar ratio of paeonol to sterol reached 1:1. As shown in Fig. 3a and b, when the molar ratio of paeonol to sterol varied from 0 to 0.8, no matter whether the phase temperature (T_{onset} and T_p) or the value of *HHW* changed slightly but both calorimetric parameters changed drastically when the molar ratio reached 1:1. Peaks of main phase transition of paeonol/sterol/DPPC liposomes at molar ratio of 1:1:24 (paeonol:sterol:DPPC) shown in Fig. 2 were obscure due to the large scale of the figure. They have been showed in Fig. 4 (solid lines) clearly. They are much more broad and asymmetric than those at other molar ratios (shown in Fig. 2). Furthermore, they seemed to consist of two overlapped peaks, one peak was around 36 °C, and the other around 41 °C. This implied that there were two domains coexisted in these liposomes. That is to say, a phase separation occured when the molar ratio of paeonol to sterol reached 1:1 in paeonol/sterol/DPPC liposomes. One phase formed by sterol-rich domain coexisted with another phase formed by paeonol-rich domain. As the molecular structure of paeonol differs from that of sterols greatly (as shown in Fig. 1), there is no doubt that the interaction between DPPC with paeonol differs from that with sterols greatly. Thus, the thermal stability and the coorperativety of main transition between two domains differ greatly. Accordingly, the peaks of main transition appeared very broad and asymmetric.

Since the peak of main transition of liposomes at molar ratio of 1:1:24 (paeonol:sterol:DPPC) seemed to consist of two overlapped peaks around 36 and 41 °C, we deconvoluted it into two peaks around 36 °C (dashed lines of Fig. 4) and 41 °C (dotted lines of Fig. 4) employing Peakfit software and attributed them to paeonol-rich domain and sterol-rich domain, respectively. The reasons are as follows: compared with fused tetracyclic ring and branched extended side chain of cholesterol or stigmasterol, the molecule of paeonol only owns a single ring and does not own a side chain (as shown in Fig. 1). Thus, there is no doubt that stigmasterol and cholesterol have more favorable, stabilizing interactions with DPPC than paeonol because they could match DPPC molecules much better than paeonol. Thus, the deconvoluted peak at a lower temperature in Fig. 4 (dashed lines) should be attributed to paeonol-rich domain and another to sterol-rich domain. The calorimetric parameters of phase transition of these two domains are summarized in Table 1. As shown in Table 1, no matter onset temperature or peak temperature of sterol-rich domain is much higher than that of paeonol-rich domain. Data of Table 1 shows that the cooperativety (inversely to HHW) of peaks of paeonol-rich domain is lower than that of sterol-rich domain, and this was in accordance with our assignments of the two domains. The phase separation occurs when the molar ratio of paeonol to sterol reaches 1:1, which may mean that the concentration of paeonol in sterol-rich domain and that of sterol in paeonol-rich domain is nearly zero.

Conclusions

Thermotropic phase behaviors of paeonol-encapsulated liposomes containing stigmasterol or cholesterol have been investigated by DSC. Four conclusions could be drawn from the results of DSC experiments: First, paeonol could incorporate into the hydrophobic region of DPPC and interact with acyl chains of DPPC. Second, stigmasterol interacts with DPPC less favorably than cholesterol. Third, thermotropic phase behavior of paeonol/cholesterol/DPPC liposomes and that of paeonol/stigmasterol/DPPC

liposomes are very similar. Finally, phase separation occurred in both paeonol/cholesterol/DPPC and paeonol/stigmasterol/DPPC liposomes when the molar ratio of paeonol to sterol reached 1:1.

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