



The interaction of paeonol with DPPC liposomes

Differential scanning calorimetry and synchrotron X-ray diffraction studies

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Abstract

Differential scanning calorimetry and synchrotron X-ray diffraction techniques have been employed to investigate the interaction of paeonol with DPPC liposomes and to investigate the location of paeonol molecules in liposomes. The results showed that the location of the paeonol molecules in liposomes is concentration dependent. When the concentration of the paeonol is not more than 5 mol%, both main transition temperature and transition enthalpy of liposomes decrease with increasing the concentration of paeonol, indicating that paeonol molecules incorporate into the hydrophobic region of DPPC molecules. When the concentration of the paeonol is between 5 and 15 mol%, additional paeonol molecules will incorporate into the hydrophilic region of the DPPC molecules and interact with its polar groups, resulting in an increase tendency of the main transition enthalpy. When the concentration of the paeonol is more than 15 mol%, both main transition temperature and transition enthalpy of liposomes decrease with increasing the concentration of paeonol, indicating that the additional paeonol molecules locate at the region of hydrocarbon chains of DPPC again. Calorimetric data show that the main transition temperature of all paeonol/DPPC liposomes is lower than that of pure DPPC liposomes but the enthalpy of liposomes containing more than 10 mol% paeonol is higher than that of pure DPPC liposome, which is related to the nonsynchronous change of the head and tail part of DPPC molecules during the main transition. This study will play an important role in the further investigation of interaction of drugs with biomimetic membranes and in the further study of phase transition mechanisms of DPPC liposome.

Keywords Paeonol · Liposomes · Encapsulating location · DSC · Synchrotron X-ray diffraction · Phase transition mechanism

Abbreviations

DPPC	Dipalmitoylphosphatidylcholine
XRD	X-ray diffraction
DSC	Differential scanning calorimetry
SAXS	Small angle X-ray scattering
WAXS	Wide angle X-ray scattering
$L_{\beta'}$	Lamellar-gel phase

$P_{\beta'}$	Rippled gel phase
L_{α}	Liquid-crystal phase
T_p	Temperature of the peak maximum of DSC peak
$\Delta T_{1/2}$	The half-height width of DSC peak

Introduction

Paeonol (as shown in Fig. 1), 2'-hydroxy-4'-methoxyacetophenone, is a major phenolic component of the root cortex Moutan peony (Chinese name: Mudan, which is the national flower of China) [1], which has been used extensively as traditional Chinese medicine (TCM) for treating various diseases [2]. Paeonol has been shown to possess the biological activities of anti-aggregatory, antioxidant, anxiolytic-like and anti-inflammatory functions [1]. Moreover, it has been reported that paeonol is able to exert an anti-cancer effect on human colorectal cancer cells through

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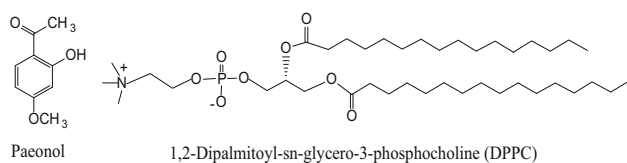


Fig. 1 Molecular structures of paeonol and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)

inhibition of PGE2 synthesis and COX-2 expression [3], and to protect endotoxin-induced acute kidney injury by inhibiting TLR4-NF- κ B signal pathway [4]. However, paeonol is poorly soluble in water thus its bioavailability in vivo is low, which limits the application of paeonol in clinical trials [5, 6].

Liposome is a self-assembling structure of lipid dispersion in water, which has been employed in encapsulation and effective delivery of various drugs, including antibiotics, antineoplastics, steroids, soluble bronchodilators, nucleotides and so on [7]. As a drug delivery system, liposome has many advantages, such as increasing drug capacity, facile surface decoration, good biodegradability properties, and targeting and protection of entrapped agents [8]. Thus it is significant to encapsulate paeonol into liposomes.

The amphiphilic molecules for the preparation of liposomes are derived from or based on the structure of the lipids of biological membranes [9]. Phospholipids are frequently used as raw materials for liposomes. Among the phospholipids forming bilayer, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, as shown in Fig. 1), which is a major component of lecithins, is perhaps the most intensively studied one. On the one hand, DPPC is an abundant phospholipid present in the human body and accounts for 10–20% of the phosphatidylcholine content of brain myelin and erythrocyte membranes [10]. On the other hand, it shows a sharp and strong thermotropic transition near the physiological temperature [11]. Some spectroscopy-based techniques, such as sum frequency generation (SFG) vibrational spectroscopy, attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, and various fluorescence-based techniques (steady-state and time-resolved spectroscopy, confocal microscopy and flow cytometry), have been used to

Previous studies have shown that paeonol molecules can be encapsulated in DPPC liposomes [6]. As an amphiphilic substance, paeonol molecules may be encapsulated in the hydrophobic region or in the hydrophilic region of DPPC liposomes. The relationship between the location of the paeonol molecules in liposomes and their concentration can not be found in the literature so far. Some spectroscopy-based techniques, such as attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy [12], sum frequency generation (SFG) vibrational spectroscopy [12–14], and fluorescence-based techniques [15], have been used to investigate drug-membranes interaction.

Differential scanning calorimetry (DSC) technique is a highly sensitive technique to study the thermotropic properties of many different biological macromolecules [16], and synchrotron X-ray diffraction (XRD) technique is often used to determine their structures [17]. These techniques have been widely applied to lipid-based drug delivery systems and drug interactions with biomimetic membranes [18–20]. In this study, we employed DSC and synchrotron XRD techniques to study the interaction of paeonol with DPPC liposomes and to investigate the relationship between the location of the paeonol molecules in liposomes and their concentration. This study will play an important role in the further investigation of interaction of drugs with biomimetic membranes and in the further study of phase transition mechanisms of DPPC liposome.

Experimental

Samples preparation

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and paeonol, 2-hydroxyl-4-methoxyacetophone were purchased from Sigma Chemicals (99%, St. Louis, MO, USA). They were used without further purification. All organic solvents were of analytical grade. Double deionized water with a resistivity of about 18.2 M Ω cm was used for the preparation of buffers. Paeonol/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 (pH = 7.4) with repeated vortexing and heating-cooling between 60 and 20 °C for at least three times. The molar percentage of paeonol (x) was 0, 5, 10, 15, 20, 25, respectively.

Differential scanning calorimetry

The calorimetry was performed using a Mettler-Toledo DSC3 differential scanning calorimeter equipped with the high-sensitivity sensor HSS8⁺. Usually 20 μ l of sample was used in each scanning experiment with the scanning rate of 1 °C min⁻¹. In addition, a fast scanning rate of 5 °C min⁻¹ has also been applied to detect the low enthalpic pre-transition when $x = 15, 20,$ and 25 mol%. All samples have been cooled from 60 to 20 °C slowly before measurement to achieve equilibrium.

Synchrotron X-ray diffraction

Synchrotron wide-angle X-ray scattering (WAXS) and small-angle X-ray scattering (SAXS) experiments were performed at the beamline 1W2A of the Beijing

Synchrotron Radiation Facility (BSRF) ($\lambda = 1.54 \text{ \AA}$), China. The distance from sample to detector was 246.0 mm for WAXS and 1617.0 mm for SAXS, respectively. A standard silver behenate sample was used for the calibration of diffraction spacings following Hatta et al. [21]. A Linkam thermal stage (Linkam Scientific Instruments, the United Kingdom) was used for temperature control ($\pm 0.1 \text{ }^\circ\text{C}$). The heating rate is $1 \text{ }^\circ\text{C min}^{-1}$. X-ray scattering intensity patterns were recorded during 120 s exposure of the samples to the synchrotron beam. The X-ray diffraction intensity data were analyzed using the program Fit2D (<http://www.esrf.eu/computing/scientific/FIT2D/>). The scattered intensity was reported as a function of the reciprocal spacings (s):

$$s = q/2\pi \quad (1)$$

$$q = 4\pi \sin \theta/\lambda \quad (2)$$

where q is the scattering vector, θ is half the scattering angle and λ the wavelength [21]. From the value of reciprocal spacings (s), the repeat distances, d ($d = 1/s$) were calculated.

Results and discussion

As temperature is increased from room temperature, DPPC is known to display a low enthalpic pretransition (from the gel phase $L_{\beta'}$ to the rippled gel phase $P_{\beta'}$) and a sharp main transition (from the rippled gel phase $P_{\beta'}$ to the liquid-crystalline phase L_{α}) [22–25]. The main transition is closely related to the acyl chains of phosphatidylcholines bilayers. Thus the main transition can be used to probe the interaction between the acyl chains of phosphatidylcholines and exogenous substances [6]. Transition temperature T_p (temperature of the peak maximum of DSC peak) and enthalpy ΔH are important calorimetric parameters, which can be used to predict the influence of exogenous molecules on the structure and properties of liposomes. Synchrotron WAXS technique is often used to study the molecular interactions among liposomes [26]. The WAXS data can reflect the carbon–carbon packing information in the hydrocarbon chains [24, 25] and SAXS yielded information on long range bilayer organization [17] or the periodicity of the ripple phase of DPPC multibilayers [29].

The effect of paeonol on the pre-transition of DPPC liposomes

Presented in Fig. 2 are DSC curves of pure DPPC and paeonol/DPPC liposomes recorded at a scanning rate of 1 or $5 \text{ }^\circ\text{C min}^{-1}$. The DSC curve of the pure DPPC liposome displays a first thermal transition at $36.6 \text{ }^\circ\text{C}$, corresponding

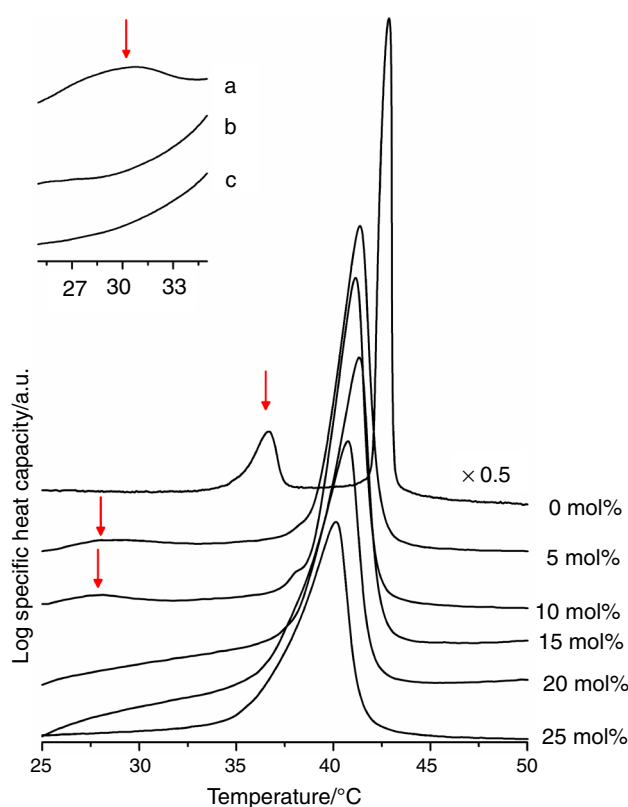


Fig. 2 DSC curves of paeonol/DPPC liposomes encapsulating different molar percentage of paeonol collected at a scan rate of $1 \text{ }^\circ\text{C min}^{-1}$ (the heat flow of pure DPPC liposome has been reduced by half to show the DSC curves of other samples more clearly). Inset shows the DSC curves of pre-transition of paeonol/DPPC liposomes collected at a fast scan rate of $5 \text{ }^\circ\text{C min}^{-1}$. The concentrations of paeonol are: 15 mol% (a), 20 mol% (b), and 25 mol% (c)

to the pre-transition ($L_{\beta'}$ to $P_{\beta'}$), and a second transition at $42.8 \text{ }^\circ\text{C}$, corresponding to the main transition ($P_{\beta'}$ to L_{α}). These data are determined by the peak temperatures of the DSC curve following McMullen and McElhaney [27], and are in good agreement with the published data [6, 28–30]. Moreover, compared with paeonol/DPPC liposomes, the main transition temperature of the pure DPPC is the highest and its transition peak is the narrowest.

As shown in the Fig. 2, the pre-transition cannot be found in DPPC liposomes containing more than 15 mol% paeonol. This indicates that these liposomes exist as rippled gel phase before heating, thus they do not undergo pre-transition any longer during heating process. This interpretation will be proved by the results of the synchrotron WAXS and SAXS experiments, described as below.

In order to determine the phase state of these liposomes, we must first know the difference between the synchrotron WAXS patterns of the gel phase $L_{\beta'}$ and that of the rippled gel phase $P_{\beta'}$. This difference can be typically demonstrated from the synchrotron WAXS patterns of pure DPPC

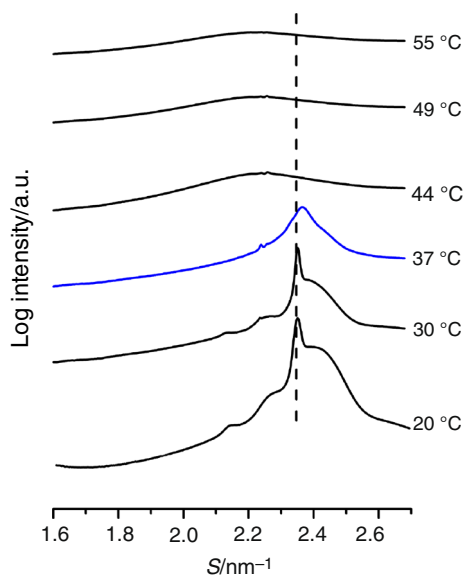


Fig. 3 Synchrotron WAXS patterns of pure DPPC liposomes at different temperature collected at a scanning rate of $1\text{ }^{\circ}\text{C min}^{-1}$

liposomes at different temperature. Presented in Fig. 3 are synchrotron WAXS patterns of pure DPPC liposomes at different temperature collected at a heating rate of $1\text{ }^{\circ}\text{C min}^{-1}$. The d -spacing ($d = 1/s$) of pure DPPC liposomes shows a slight decrease from 0.428 nm of $L_{\beta'}$ phase (20 and 30 $^{\circ}\text{C}$) to 0.422 nm of $P_{\beta'}$ phase (37 $^{\circ}\text{C}$), then shows a drastic increase to 0.456 nm of L_{α} phase (44, 49, and 55 $^{\circ}\text{C}$). These data are in good agreement with published data [23, 31, 32]. The XRD features of $P_{\beta'}$ phase are significantly different from that of $L_{\beta'}$ phase. As shown in Fig. 3, the diffraction patterns of the WAXS spectra of pure DPPC at 20 and 37 $^{\circ}\text{C}$ represent the characteristics of the $L_{\beta'}$ phase and those of the $P_{\beta'}$ phase, respectively. By contrast with the WAXS pattern of $L_{\beta'}$ phase, the intensity of diffraction of $P_{\beta'}$ phase becomes lower and the diffraction peak tends to be more symmetric.

Presented in Fig. 4 are synchrotron WAXS patterns of paeonol/DPPC liposomes collected at 20 $^{\circ}\text{C}$. The reciprocal spacings (s) and d -spacings (d) are listed in Table 1. As shown in Fig. 4, the symmetric WAXS patterns of DPPC liposomes when $x \geq 20$ mol% strongly suggest that these liposomes exist as rippled phase. Furthermore, as shown in Table 1, the d -spacings of paeonol/DPPC liposomes when $x \geq 20$ mol% are 0.422 nm, which is in good agreement with the d value of pure DPPC liposome at 37 $^{\circ}\text{C}$, indicating that these liposomes exist as rippled gel phase. As a result, they do not undergo pre-transition any longer during heating process. These results are consistent with above DSC results.

Our SAXS data also provide important information of the rippled gel phase among liposomes when $x \geq 20$ mol%

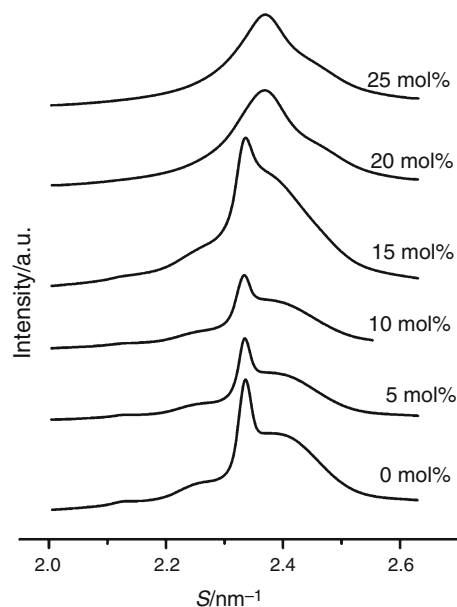


Fig. 4 Synchrotron WAXS patterns of paeonol/DPPC liposomes encapsulating different molar percentage of paeonol at 20 $^{\circ}\text{C}$

Table 1 The reciprocal spacings (s) and d -spacings (d) of WAXS of paeonol/DPPC liposomes at 20 $^{\circ}\text{C}$

$x/\text{mol}\%$	s/nm^{-1}	d/nm
0	2.335	0.428
5	2.335	0.428
10	2.335	0.428
15	2.335	0.428
20	2.370	0.422
25	2.370	0.422

x is the molar percentage of paeonol in paeonol/DPPC liposomes

at 20 $^{\circ}\text{C}$. It is well known that pure DPPC liposome at 20 and at 37 $^{\circ}\text{C}$ exists as gel phase and rippled gel phase, respectively. As shown in Fig. 5, the SAXS pattern appearing in the lower angle (indicated by an arrow) to the first-order of the lamellar repeat for pure DPPC liposome at 37 $^{\circ}\text{C}$ is a typical representation of the rippled gel phase and reflects the periodicity of the ripple [29]. As shown in Fig. 5, this typical diffraction peak (indicated by arrows) of the rippled gel phase also can be seen from the SAXS patterns of the paeonol/DPPC liposomes at 20 $^{\circ}\text{C}$ when $x \geq 20$ mol%. That is to say, rippled gel phase exists among liposomes at 20 $^{\circ}\text{C}$ when $x \geq 20$ mol%.

There are some other reports that incorporating 5 mol% pirarubicin [33] or 2.5 mol% cortisone esters [30] into DPPC liposomes or incorporating 26 mol% (10 w/w%) lidocaine [34] into DMPC liposomes can suppress the pre-transition of PCs liposomes. Encapsulating sterols such as

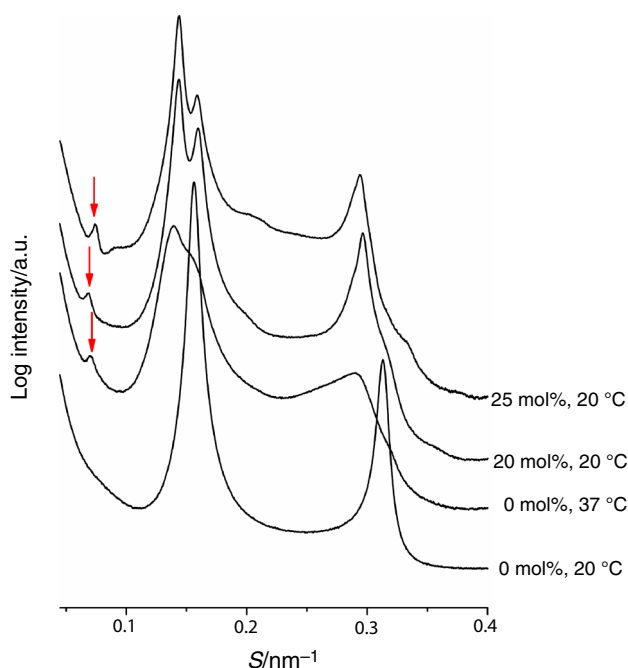


Fig. 5 Synchrotron SAXS patterns of pure DPPC liposomes and paeonol/DPPC liposomes

3.6–6 mol% stigmasterol or cholesterol into PC liposomes also can suppress the pre-transition [6, 29, 35–37].

The concentration dependence of the location of the paeonol molecules in liposomes

As an amphiphilic substance, paeonol molecules may be encapsulated in the hydrophobic region or in the hydrophilic region of DPPC liposomes. In order to investigate the location of the paeonol molecules in liposomes, it is necessary to analyze the effect of paeonol on the main transition of DPPC liposomes. The relationships between the calorimetric parameters of the main transition and the molar percentage of paeonol among liposomes are shown in Fig. 6. Based on these data, we can analyze the concentration dependence of the location of the paeonol molecules in liposomes, described as below.

As shown in Fig. 6, both transition temperature and transition enthalpy of the main transition decrease with increasing the concentration of paeonol when $x \leq 5$ mol%. Since the main transition is closely related to the acyl chains of DPPC, the decreases of the temperature and enthalpy indicate that paeonol molecules incorporate into the hydrophobic region of DPPC liposome when $x \leq 5$ mol%, as shown in schematic Fig. 7. Perhaps the reason why the temperature and enthalpy decrease with increasing the concentration of paeonol is that the incorporation of the paeonol weakens the molecular interaction

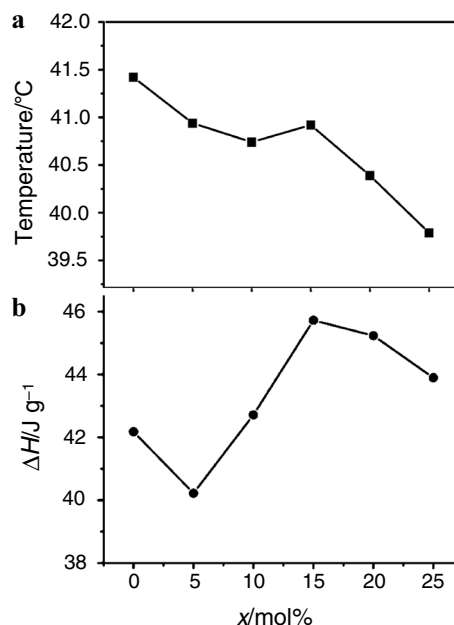


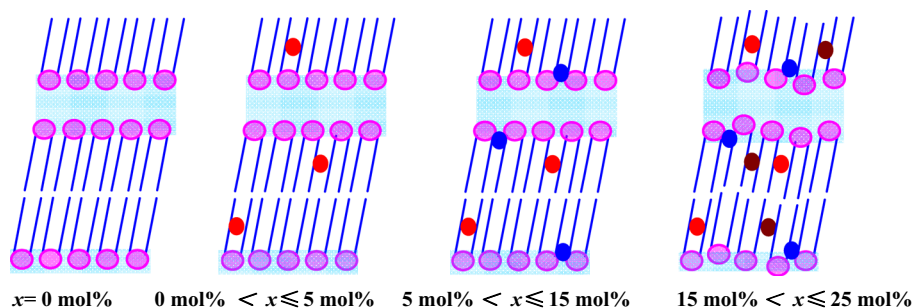
Fig. 6 The relationship between the peak temperatures (a), the enthalpy changes (b) of the main transition and the molar percentage of paeonol among liposomes

between hydrophobic chains of DPPC molecules. Many research groups have reported that the incorporation of the exogenous molecules into the hydrophobic region of DPPC liposome could weaken the molecular interaction between hydrophobic chains, followed by a decrease of the transition temperature or/and the transition enthalpy of the main transition [5, 6, 38].

As shown in Fig. 6a, the transition temperature remains nearly unchanged at the concentration of paeonol between 5 and 15 mol%, indicating that additional paeonol molecules do not incorporate into the hydrophobic chains of DPPC molecules any more at this concentration range. Surprisingly, as shown in Fig. 6b, the transition enthalpy increases gradually with increasing the concentration of paeonol at this concentration range. These data imply that additional paeonol molecules incorporate into the hydrophilic region of the DPPC molecules, as shown in schematic Fig. 7. This can be explained as follows. As shown in Fig. 1, paeonol molecule only owns a benzene ring but owns many polar groups, such as phenolic hydroxyl and keto-carbonyl, thus it can incorporate into the hydrophilic region of the DPPC and interact with its polar groups. During the process of main phase transition, it is necessary for DPPC molecules to absorb heat to overcome the molecular interaction between the polar groups of paeonol and the polar groups of the hydrophilic region of DPPC molecules.

As shown in Fig. 6, the transition temperature and the transition enthalpy show a downward trend once more

Fig. 7 The schematic drawing of the concentration dependence of the location of the paeonol molecules in liposomes at 20 °C



when the concentration of the paeonol exceeded 15 mol%, indicating that the additional paeonol molecules locate in the region of hydrocarbon chain again, as shown in schematic Fig. 7. Our WAXS data also support this conclusion. As described in the previous section, when the concentration of the paeonol exceeds 15 mol% at 20 °C, WAXS data indicate that the phase state of liposome will change from gel phase to rippled gel phase, which means that a large number of drug molecules incorporate into the hydrophobic region of DPPC liposome.

Figure 6 shows another surprising result: the transition temperature of all paeonol/DPPC liposomes is lower than that of pure DPPC liposomes, however, the transition enthalpy of paeonol/DPPC liposomes is higher than that of pure DPPC liposomes when $x \geq 10\%$. This surprising result may be related to the nonsynchronous change of the head and tail part of DPPC molecules during the main transition. Wu et al. [24] has reported that the change of the head and tail part of DPPC molecules during the phase transition is nonsynchronous and it is tail part of DPPC molecules that triggers the process of the phase transition. Accordingly, the temperature of the phase transition mainly depends on the surroundings of the tail part of DPPC molecules. As a result, the transition temperature of all paeonol/DPPC liposomes is lower than that of pure DPPC liposomes because there are paeonol molecules at the tail part of DPPC molecules among all paeonol/DPPC liposomes (as shown in schematic Fig. 7). However, the transition enthalpy involves the overall heat which DPPC molecules absorb during the phase transition, which depends not only on the surroundings of the tail part of DPPC molecules, but also on the surroundings of their hydrophilic region. Though incorporating paeonol molecules into the hydrophobic region of the DPPC will decrease the enthalpy of the phase transition, incorporating paeonol molecules into the hydrophilic region will increase the enthalpy due to the molecular interaction between the polar groups of paeonol and the polar groups of the hydrophilic region of DPPC molecules. That is to say, paeonol molecules located at the hydrophilic region exert a contrary effect on the transition enthalpy compared to those

located at the hydrophobic region. The fact that the transition enthalpy of paeonol/DPPC liposomes when $x \geq 10\%$ is higher than that of pure DPPC liposomes indicates that paeonol molecules located at the hydrophilic region play the dominating role at this concentration range.

Conclusions

DSC and synchrotron XRD techniques have been employed to investigate the interaction of paeonol with DPPC liposomes and to investigate the location of paeonol molecules in liposomes. The results showed that the location of the paeonol molecules in liposomes is concentration dependent. When the concentration of the paeonol is not more than 5 mol%, both main transition temperature and transition enthalpy of liposomes decrease with increasing concentration of paeonol, indicating that paeonol molecules incorporate into the hydrophobic region of DPPC molecules. When the concentration of the paeonol is between 5 and 15 mol%, additional paeonol molecules will incorporate into the hydrophilic region of the DPPC molecules and interact with its polar groups. In this concentration range, the transition temperature remains nearly unchanged but the transition enthalpy increases gradually with increasing the concentration of paeonol. When the concentration of the paeonol is more than 15 mol%, the additional paeonol molecules will locate in the region of hydrocarbon chain again. The calorimetric data of this study are related to the nonsynchronous change of the head and tail part of DPPC molecules during the main transition. This study will play an important role in the further investigation of interaction of drugs with biomimetic membranes and in the further study of phase transition mechanisms of DPPC liposome.

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