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Studies on molecular interactions between puerarin and PC liposomes

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Fluorescence emission spectra, FTIR spectra, zeta potential measurements, and *ab initio* quantum calculation are used to study the interaction between puerarin and membranes composed of egg phosphatidylcholine (PC) liposome. The hydrophobic interactions cause the puerarin molecule to partition into lipid bilayers with its B-ring, and favor the displacement of acid-base equilibrium of puerarin towards the base form. Due to the hydrogen bond formation between the puerarin hydroxyl groups and polar groups of PC molecules on the water/membrane interface, puerarin can easily intercalate into the organized structure of phospholipids and modulate the membrane function. Our results reveal that the liposome membrane integrity is significantly higher compared with that of empty liposome.

puerarin, phosphatidylcholine, liposome, drug encapsulation

The flavonoids are a large group of naturally occurring compounds and they are widely distributed in the plant kingdom and are especially ubiquitous in vegetables, berries and fruits[1,2]. In recent decades, this class of compounds has attracted substantial attention due to the assumed link between its antioxidant properties and its beneficial effects on health[3,4]. Some flavonoids have been suggested to be potential anticancer agents promoting apoptosis in tumor cells[5]. The amphipathic character of flavonoids allows them to partition into or bind lipid bilayer. Several studies on flavonoid-bimembrane interactions have been reported. For example, Saija et al.[6] showed that flavonoid incorporation into liposomal membranes altered the barrier functions of the bilayer. William et al.[7] studied the interactions of genistein and related isoflavones with lipid micelles, and suggested that the incorporation of daidzein into phosphatidylcholine liposomes promoted aggregation and precipitated formation.

Puerarin is a naturally occurring isoflavone C-glycoside and isolated from pueraria lobota[8]. Its chemical name is 8-*β*-*D*-glucopyransyl-7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one (Figure 1). The biomedical effects of puerarin, which have been experimentally or clinically demonstrated[9−11], include the improvement of blood circulation, prevention of cardiovascular diseases, control of alcoholism, and treatment for arrhythmia[12,13]. Here, we tested the binding capacity of puerarin to liposome and investigated if the interactions of puerarin with membrane could affect membrane physical properties.

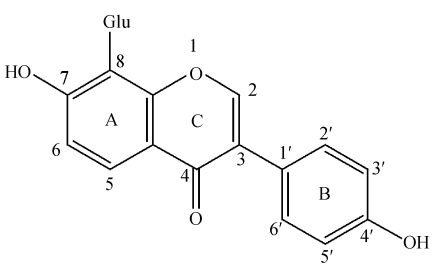

Figure 1 The structure of puerarin.

1 Materials and method

1.1 Chemical

The regents used were egg-PC (Microorganism Culture

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Medium Products Refinery of Haidian District, Beijing, China), pyrene (Sigma-Aldrich Chemical Co., 99%), calcein (Tianjin Kerme Chemical Reagent Development Center, China), 8-anilion-1-naphthalene sulfonic acid (ANS, Acros Organics, 97%). Puerarin (HPLC > 98%) being pharmaceutical purity grade was kindly provided by Nanjing Chemical Reagent Plant (China). Water used was distilled twice.

1.2 Preparation of liposomes

A chloroform solution of PC was placed in a 50 mL round-bottomed flask. Then the solvent was removed under vacuum at 30℃ by using a rotary evaporator. The resulting thin film of PC on the walls of the flask was dried under vacuum at room temperature for at least 2 h. Then the dry PC film was dispersed in phosphate-buffered saline (PBS, pH = 7.4) and was sonicated for 2 h using a CQ250 water bath-type sonicator (220 V, 50 Hz, Shanghai Ultrasonic Wave Instrument Works, China) to obtain liposomes. After the solution was centrifuged for 10 min at 3500 r/min, the supernatant containing small unilamellar vesicles was used for further examinations.

1.3 Observation of the images of liposomes

The images of liposomes were obtained by transmission electron microscopy (TECNAI-12 TEM, Philips, Netherlands). Before measurement, the samples were negatively stained with 0.5% uranyl acetate to enhance image quality and were deposited on films coated with Cu grids.

1.4 Measurement of UV-vis and FTIR spectra

The UV-vis spectra were measured by using a UV-2550 spectrophotometer (Shimadzu, Japan). All samples were measured at 25±0.1℃. FTIR spectra of puerarin in the PC liposomes were recorded with a Brucker Equinox 55 FTIR spectrometer. Single-beam IR spectra were the result of about 32 co-added interferograms and ranged from 800 to 3000 cm^{-1} with a spectral resolution of 4 cm^{-1}.

1.5 Zeta potential measurement

Zeta potential measurements were conducted using the JS-94H microelectrophoretic zetasizer (East China Normal University, China). Five zeta potential measurements were collected for each solution and the results averaged.

1.6 Partitioning of puerarin into PC liposome

Methods based on fluorescence quenching were used to determine the partition coefficient (given as K_d) of puerarin to bilayer model membrane systems. The methods were originally based on procedures described by Verkman[14] and van Dijk et al.[15]. Partition coefficients were determined, assuming the Stern-Volmer equation, from the fluorescence intensity of probe as a function of test compound concentration:

$$\frac{1}{(F_0/F)-1}=\frac{K_d}{[PC]_{tot}}\frac{1}{[Puerarin]}\frac{1}{[PC]_{tot}}, \quad (1)$$

where F and F_0 are the fluorescence intensity observed in the presence and absence of quencher, respectively. A straight line was obtained from a plot $1/((F_0/F)-1)$ against $1/[Puerarin]$, which gave K_d values as a slope divided by an intercept. K_d values were obtained by gradual addition of puerarin into vesicles with a fluorescent probe. Here, pyrene was used as a probe for the determination of partition coefficient of puerarin to PC liposomes.

1.7 *Ab initio* quantum calculations

Ab initio quantum calculations of puerarin were carried out at the level of B3LYP/6-31 G(d) with the GAUSSIAN 03 program.

1.8 Measurement of membrane fluidity

The solution of pyrene was added to the liposome suspension and the final concentration of pyrene was 1.2×10^{-6} mol/L. After the system was sonicated and mixed, the fluorescence polarization, P, of probe was measured by an RF-5301PC fluorescence spectrophotometer (Shimadzu). The emission wavelength was fixed at 373 nm, and the sample was excited at 338 nm. The emission intensity of the polarized light, parallel and perpendicular to the excitation-polarized light, $I_{\parallel}$ and $I_{\perp}$, was combined together to calculate the steady-state polarization P[16]:

$$P=[I_{\parallel}-I_{\perp}]/[I_{\parallel}+I_{\perp}]. \quad (2)$$

1.9 Measurements of membrane hydrophobicity

The solution of ANS as a fluorescent probe was added to the liposome suspension and the final concentration of ANS was 7.5×10^{-5} mol/L. After the system was sonicated and mixed, the fluorescence intensity of ANS was recorded to characterize the change of liposomal membrane hydrophobicity. The wavelengths of excitation and emission are 350 and 480 nm, respectively[17].

1.10 Measurements of membrane permeability

Calcein was used as an aqueous marker entrapped in the

liposomes. The percentage of calcein leaking from the liposomes was calculated according to the following equation[18]:

Calcein leakage (%) = $100[I_f - I_0]/[I_\infty - I_0]$, (3)

where I_f was the fluorescence intensity at different time after the liposome suspension was prepared. The fluorescence intensity at the beginning (I_0) was determined as follows: calcein possibly existing in the bulk phase was quenched by the addition of $CoCl_2$ solution to the liposomal system, and the fluorescence intensity was recorded. I_∞ was the fluorescence intensity after the addition of Triton X-100 (final concentration 3 g/kg) to destroy the liposomal membrane completely. The fluorescence intensity of calcein leaking from liposomes was measured using an RF-5301PC fluorescence spectrophotometer. The excitation and monitoring wavelengths were 490 and 520 nm, respectively.

2 Results and discussion

2.1 The affinity of puerarin for PC liposome

Figure 2 shows the morphological character of lecithin liposomes and the mean liposome diameters ranges from 50 to 100 nm. Puerarin possesses a hydrophobic character and is a weak acid, having two ionizable moieties and the pK_1 and pK_2 values are 6.91 and 9.93 respectively in aqueous solutions[19]. At physiological pH (pH = 7.4), about 75% of puerarin molecules are negatively charged. Figure 3 represents the absorption spectra of puerarin in the PC liposomes. In liposomal solutions, the acid and base forms of puerarin exhibit different trends in their absorption spectra. The absorbance of acid form at 306 nm decreases, giving increase to the absorbance of base form at 345 nm[19]. The result shows that the puerarin molecule undergoes the deprotonation reaction in PC liposome system. It is also clear that puerarin can partition into the liposomal membrane, because the microenvironment around puerarin in lecithin liposome is much less polar than in PBS aqueous solution and the former research had illuminated that the reduction of polarity favors the deprotonation reaction of drug.

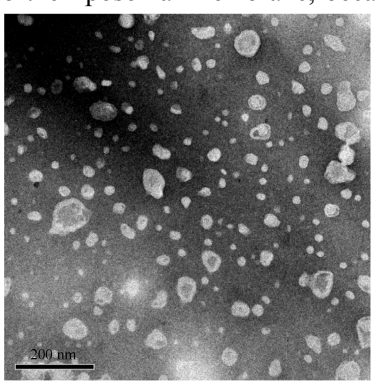

Figure 2 TEM image of lecithin liposomes. PC concentration: 0.25 mg/mL.

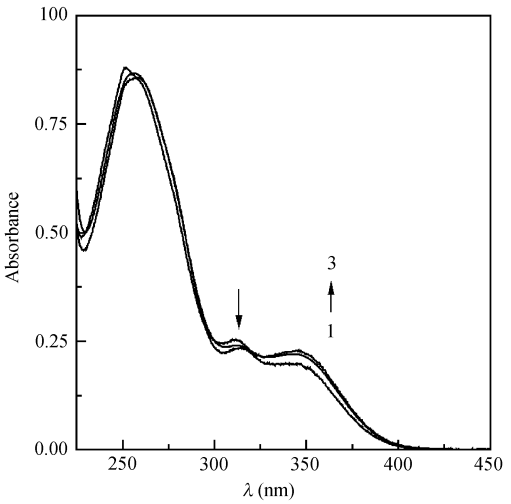

Figure 3 The absorption of puerarin (3.0×10^{-5} mol/L) in PC liopsomes. PC concentrations (mg/mL): 1, 0; 2, 0.10; 3, 0.25.

Table 1 reveals the liposomes made of PC process negative charges at pH = 7.4, consistent with the reported one in ref. [20], which indicates that there is a weak electrostatic repulsive force between PC liposomes at pH = 7.4. This experiment also demonstrates that the addition of puerarin into bilayer membrane increases the negative zeta potential from –17.5 to –32.2 mV as the concentration of puerarin increases. The association of puerarin with membranes increases the electrostatic repulsive force because puerarin is negatively charged molecule, whose presence in the liposomes enhances the negative charge of the phosphate group. Methods based on the fluorescence quenching are used to determinate the partition coefficient of puerarin into PC liposomes. The partitioning does not directly imply partitioning into the hydrophobic core of the membrane, but rather tests the puerarin association with membranes and the partition coefficient obtained is K_d = 55.8 μmol/L (data not shown). In fact, the membrane partition experiments were reported by Pia Vuorela[21], who concluded that

Table 1 The change of fluorescence polarization (P) and zeta potential (ζ) of PC liposome (0.25 mg/mL) with the addition of puerarin at 25℃

Samples	$C_{puerarin}$ (mol/L)	ζ (mv)	P
1	0	–17.5	0.119
2	1.0×10^{-5}	–24.6	0.124
3	2.0×10^{-5}	–27.8	0.132
4	3.0×10^{-5}	–30.1	0.141
5	4.0×10^{-5}	–32.2	0.154

when the number of free OH groups in the flavonoid was increased, stronger interactions with liposomal membrane were measured. In our study, puerarin molecule has aromatic rings and two free OH groups, its solubility in water is low and it has the capacity to incorporate in the hydrophobic region of liposome.

The optimization of the puerarin molecule by *ab initio* quantum chemical calculations can give the information about the localization part of puerarin in liposomes. The results show that the stable structures of puerarins with different charges are not planar (Figure 4), but with the B-ring connected to the C-ring by a single C—C bond around which rotation can occur, and the B-ring deviates with 36.83°[19], 33.44° and 35.86° from the planarity for neutral, monoanionic and dianionic forms, respectively. In addition, the presence of a sugar moiety renders puerarin less hydrophobic and more bulky. This fact may result in steric hindrance of its incorporation into the hydrophobic regions of liposome. So, judging from its optimized structure, we induce that puerarin is very likely to partition into the PC liposome with its B-ring (Figure 1). The FTIR spectra observations will further provide confirmative evidence for the location position of puerarin. With the spectra range of 1400 to 2300 cm^{-1}, the very spectra we are interested in appear (Figure 5). For the free puerarin molecule, the two bands located at 1627 and 1588 cm^{-1} are the stretching of C═O and the phenyl skeleton, respectively (curve 1 in Figure 5). The original v (C═O) band at 1627 cm^{-1} of the puerarin is an expected value for the mechanical coupling between C═O and C═C stretching. The addition of the PC li- posomes makes the v (C═O) band of puerarin shift to a lower frequency with increasing intensity and cover phenyl skeleton stretching vibration

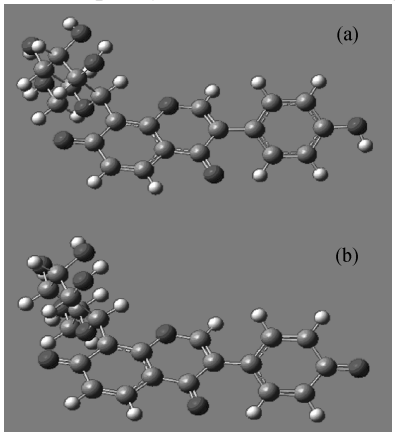

Figure 4 Optimized structure of puerarin molecule. (a) Monoanionic (HPu^{-}) form; (b) dianionic form (Pu^{2-}) of puerarin.

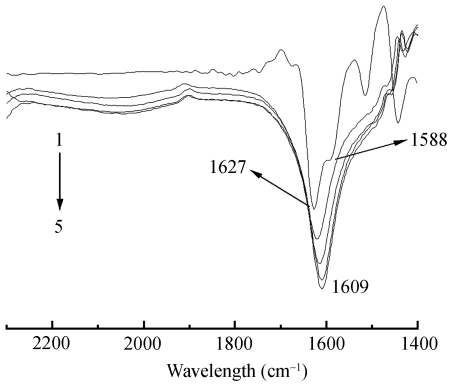

Figure 5 FTIR spectra of puerarin (5.0×10^{-3} mol/L) in PC liposome. PC concentration (mg/mL): 1, 0; 2, 0.10; 3, 0.15; 4, 0.20; 5, 0.25.

band at 1588 cm^{-1} and eventually form a broad band centered at 1609 cm^{-1} (curves 2—5 in Figure 5). As we know, with the extension of π conjugation and the increase of the planarity of the whole puerarin molecule, the C═O stretching vibration will move to a lower frequency and the band inten- sity will be greatly enhanced[22]. With the B-ring of puerarin molecule solubilized in liposome, the rotation of B-ring is limited and the planarity of the whole molecule is increased, eventually leading to the above change of the molecule vibration in the puerarin. So the result does show that the puerarin molecules can partition itself into the hydrophobic region of membranes with its B-ring.

2.2 Effect of puerarin on liposome membrane physical properties

Membrane function is of vital importance to normal processes and can be affected by a wide range of factors. One such factor is the modulation of membrane function by dietary components such as vitamin E, vitamin D, vitamin C, β-carotene, and flavonoids. In the following study, we investigate the effect of puerarin on liposome membrane physical properties. In solutions with low viscosity, or in non-vitreous state solutions, the rotation of molecular luminophores can change the orientation of the transition moment in the lifetime of the excited state. This induces the oscillator system, which can emit fluorescence deviating from the initial direction and produce fluorescence depolarization. The fluorescence depolarization is given by the following expression known as the Perrin formula[20]:

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + KT\tau/\eta V), \quad (4)$$

where P_0 is the limit polarization value when there is no molecular rotation or no interaction between the luminescent molecules and the adjacent ones (such as energy

transfer or migration) in the system, K and T are the Boltzmann constant and the absolute temperature, respectively, τ is the fluorescence lifetime, V is the molecular volume, and η is the environmental viscosity of the luminescent molecules. Thus, if we know the values of P_0 and τ and estimate the volume of the luminescent molecules, the viscosity of the media surrounding the probe can be calculated according to the measured P value. Hence, the steady-state polarization P offers an index to microfluidity, because the fluorescence polarization P has a relationship with the molecular rotation velocity and the microviscosity of the surroundings. The larger the value of P is, the smaller the microfluidity will be. In the present work, pyrene is used as fluorescence probe to study the fluidity of liposomal membranes because pyrene can easily enter the lipid bilayer and can probe the microfluidity in the hydrocarbon region of bilayer membranes near the bilayer center.

The effect of puerarin on the microfluidity of liposomal bilayer membranes is shown in Table 1. The addition of puerarin signification increases in the polarization values, which indicate that the incorporation of puerarin into the PC liposomes bilayer leads to the drop of the liposomal membrane fluidity. As discussed above, the addition of puerarin with membranes increases the electrostatic repulsive force between the phosphate groups. It seems that the membrane integrity and stability would decrease as the electrostatic repulsive force increases, and the puerarin incorporation seems to increase liposomal membrane fluidity and this contradicts the results shown in Table 1. Hence, the results obtained can only be explained by interactions between the puerarin hydroxyl groups and polar heads groups of PC molecules on the water/membrane interface. Flavonoids are known to have varying degrees of hydroxylation, showing that hydroxyl groups have a high capacity to form hydrogen bonds[23]. According to our results, the membrane fluidity is apparently decreased by the interfacial hydrogen bonding network present on the water/membrane interface, which indicates that the membrane integrity and stability are enhanced with the addition of puerarin.

The hydrophobic microenviroment in liposomal membranes is investigated by means of ANS fluorescent method. ANS scarcely fluoresces in aqueous bulk phase, while ANS strongly fluoresces when it is transferred to a hydrophobic environment[17]. The fluorescence intensity of ANS with puerarin is shown in Figure 6, from which it can be seen that the fluorescence intensity is increased with increasing puerarin concentration, which indicates that the addition of puerarin can increase the hydrophobic degree of liposomal membranes. As discussed above, the interfacial hydrogen bonding network present on the water/membrane interface can increase the integrity and stability of liposome membrane. So, the association of puerarin with PC liposome makes the spaces between the lecithin molecules in lecithin liposome decrease and causes the consequent decrease in the amount of water molecules in liposomal bilayer membranes. So, the hydrophobic degree of liposomal membranes is increased.

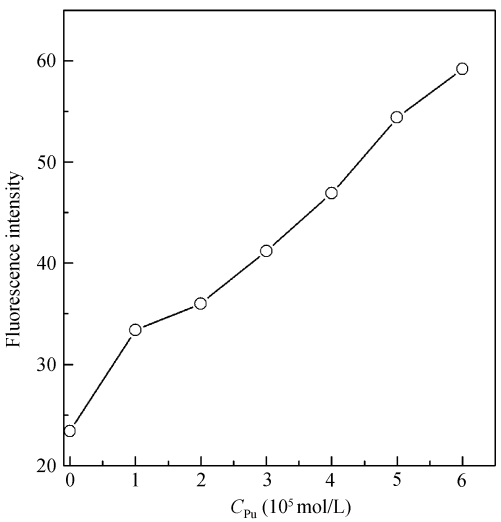

Figure 6 Microhydrophobicity of liposome bilayer membranes by ANS fluorescent method. PC concentration is 0.25 mg/mL.

The isoflavone-induced perturbing of liposomal membranes is studied fluorometrically by observing calcein release from PC liposome. Calcein release from liposome has proven to be a good method to investigate the ability for detergents and other amphipathic molecules to perturb membranes. The time course of leakage of calcein from the liposome in the presence of puerarin is measured and the results are shown in Figure 7. Within 1 h, the percentages of calcein leaked from the liposomes with and without puerarin (5.0×10^{-5} mol/L) are 13.3% and 8.3%. The results show that the permeability of liposome is decreased and it is clear that the incorporation of puerarin in liposome significantly stabilized the liposomes. These changes may be resulting in the change in orientation of phosphatidycholine head groups at the surface of liposomes. The formation interfacial hydrogen bonding network present on the water/membrane interface enhances the membrane integrity and the stability of liposome is enhanced. So, the per-

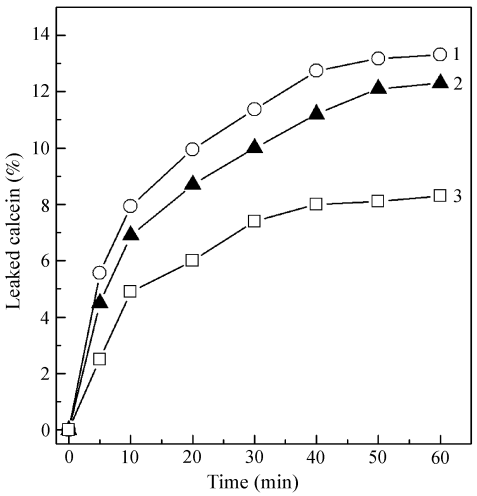

Figure 7 Time course of calcein leakage from PC liposome. PC concentration is 0.25 mg/mL. Concentrations of puerarin (mol/L): 1, 0; 2, 3.0×10^{-5}; 3, 5.0×10^{-5}.

meability of liposome is decreased with the addition of puerarin.

3 Conclusions

Summarizing the results of this study, we may conclude that puerarin molecule can partition itself into the hydrophobic region of membranes with its B-ring. On the other hand, the interfacial hydrogen bonding network could be present on the water/membrane. The two modes of interactions between puerarin and PC liposome coexist and modify their surface charge and membrane integrity. As a consequence, puerarin can accumulate at the membranes' surface, both outside and inside the membrane. Through this kind of interaction, we suggest that the selected isoflavone help maintain membranes' integrity by forming the hydrogen bonding network present on water/membrane interface. Finally, given the present results, it is reasonable to speculate that isoflavone-induced changes in membrane structure may also modulate the response of cells to signaling molecules.

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