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ORIGINAL CONTRIBUTION

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Interactions of hemoglobin with lecithin liposomes

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Abstract In this paper, the interaction of hemoglobin (Hb) with lecithin liposomes is studied by UV-vis spectroscopy, fluorescent spectroscopy, and transmission electron microscopy. The adsorption of Hb on liposomes is likely to be related to the hydrophobic interaction between Hb and liposomes, which brings about the increase of the microenvironmental polarity (I_1/I_3) and the decrease of the fluorescence polarization (P) of lecithin liposomes. These results are considered to be that the adsorption of Hb on liposomes makes the spaces between the lecithin molecules increase, and a temporary gap is consequently formed in the liposomal bilayer membranes. The leakage of aqueous-space marker from the liposomes is increased with the addition of Hb.

Keywords Lecithin liposome · Hemoglobin · Adsorption

Introduction

Liposomes are closed amphiphile membrane capsules consisting of single or multiple lamellae of noncovalently assembled amphiphilic molecules in aqueous media. Because the basic structure of the biological membrane is the phospholipid bilayer matrix, the study of self-assembling phospholipid molecules into bilayer structures has proven to be of great importance [1–4]. Liposomes are, in some ways, ideal drug carriers, being biodegradable and of minimal toxicity; however, there are drawbacks to their use in vivo. A part of the liposomal bilayer membrane is destroyed through the interaction with blood components. Therefore, it is important to know the stability and the pharmacokinetics of lipsome products in blood. Hemoglobin (Hb) is a tetrameric heme protein found in erythrocytes, where it is responsible for binding oxygen in the lung and transporting the bound oxygen throughout the body where it is used in aerobic metabolic pathways [5–7]. So, in our paper, we use Hb as a model protein in the blood and liposomes to investigate the interaction between Hb and liposomes. The adsorption of Hb on liposomes and the effect of the adsorption of Hb on the membrane characteristics are studied from standpoints of microenvironmental polarity, microfluidity, and permeability of liposomal bilayer membranes.

Experimental section

Reagents

The reagents used were lecithin (Microorganism Culture Medium Products Refinery of Haidian District, China), pyrene (Aldrich, 99 %), Hb (Shanghai Lizhu Dongfeng Biology Technique, China), calcein (Tianjing Kerme Chemical Reagent Development Center, China), 8-anilion-1-naphthalene sulfonic acid (ANS, Fluka, Switzerland, 97 %). The water used was distilled twice.

Preparation of liposomes

A chloroform solution of lecithin was placed in a 50-ml round-bottomed flask. Then the solvent was removed under vacuum at 30 °C by using a rotary evaporator. The resulting thin film of lecithin on the walls of the flask was dried under vacuum at room temperature for at least 2 h.

Then the dry lecithin 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 vesicles.

Measurement of UV-vis and fluorescence spectra

The UV-vis and fluorescence spectra were measured by using a UV-2501 spectrophotometer (Shimadzu, Japan) and an RF-5301 PC spectrophotometer (Shimadzu), respectively. All samples were measured at 25±0.1 °C.

Observation of the images of liposomes

The images of liposomes were obtained by transmission electron microscopy (TECNAI-12 TEM, Philips, the Netherlands). Before measurement, the samples were negative stained with 0.5 % uranyl acetate to enhance image quality and were deposited on films coated with Cu grids [8].

Measurement of adsorption amount of Hb on liposomes

Hb was dissolved in PBS solution. After being dissolved, Hb solutions with various concentrations were added to the liposome solution. After 1 h of mixing at 25±0.1 °C for reaching the equilibrium, the liposome solutions were ultracentrifuged (TGL-16 G, Shanghai Analytical Instrument Factory, China) at 10,000–15,000 rpm for 60 min and the upper clear solution was collected. Then, the amount of Hb adsorbed on the liposomes could be calculated by the absorbance of Hb at 405 nm according to the standard curve, which was obtained by plotting the absorbance of the Hb aqueous solution against the concentration. The absorbance was measured with a UV-2501 spectrophotometer, and distilled water was used as blanks.

Determination of the microenvironmental polarity in liposome

The microenvironmental polarity in lecithin liposome was determined by the stable-state fluorometry with pyrene (1.2×10^{-6} mol.L^{-1}) as probe. Pyrene shows five emission peaks when it is excited at 338 nm. The fluorescence intensity ratio (I_1/I_3) of the first peak (at about 373 nm) to the third peak (at about 384 nm) serves as a measure of the microenvironmental polarity where the probe exists. A larger I_1/I_3 ratio means greater microenvironmental polarity [9–11].

Measurement of the liposomal membrane fluidity

The solution of pyrene was added to the liposome suspensions and the final concentration of pyrene was 1.2×10^{-6} mol.L^{-1}. After the system was sonicated and mixed, the fluorescence polarization, P, of the probe was measured by inserting a polarization filter in the excitation and emission light paths of an RF-5301 PC fluorescence spectrophotometer. The emission wavelength was fixed at 373 nm, and the sample was excited at 338 nm. The emission intensities of the polarized light, parallel and perpendicular to the excitation-polarized light, $I_{\parallel}$ and $I_{\perp}$, were combined together to calculate the steady-state polarization P [9]:

$$P = \left[I_{\parallel} - I_{\perp}\right] / \left[I_{\parallel} + I_{\perp}\right] \quad (1)$$

Pyrene existed in the hydrophobic region of the liposomal bilayer membranes and the polarization P was thus able to indicate the mircoviscosity around pyrene and the change of the liposomal membrane fluidity.

Estimation of the hydrophobicity of liposomal bilayer membranes

The solution of ANS as a fluorescent probe was added to the liposome suspensions and the final concentration of ANS was 7.5×10^{-5} mol.L^{-1}. 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 [12, 13].

Measurement of the permeability of liposomes

Calcein was used as an aqueous marker entrapped in the liposomes. In a dilute solution, calcein's fluorescence intensity is in direct proportion to its concentration; however, when calcein's concentration is increased, or when it is entrapped in liposome, its fluorescence self-quenches completely. Thus, the permeability of a liposomal membrane can be determined by monitoring the fluorescence intensity of calcein in solutions [14]. The percentage of calcein leaked from the liposomes was calculated according to the following equation [14]:

$$Calcein\ leakage\,(\%) = 100\left[I_f - I_0\right] / \left[I_\infty - I_0\right] \quad (2)$$

where I_f is the fluorescence intensity at different times after the liposome suspension was prepared. The fluorescence intensity at the beginning (I_0) was determined as follows: calcein that possibly existed in the bulk phase was

quenched by the addition of $CoCl_2$ solution to the liposomal system, and the fluorescence intensity was recorded. I_∞ is the fluorescence intensity after the addition of Triton X-100 (final concentration 3 $g.kg^{-1}$) to destroy the liposomal membrane completely. The fluorescence intensity of calcein leaked from liposomes was measured using an RF-5301 PC fluorescence spectrophotometer. The excitation and monitoring wavelengths were 490 and 520 nm, respectively.

Results and discussions

The adsorption of Hb on liposomes

Figure 1 shows the morphological character of lecithin liposomes. The adsorption curve of Hb on liposomes is shown in Fig. 2, which is calculated by the characteristic absorbance of Hb at 405 nm according to Lambert–Beer law (Fig. 3) [15]. It can be seen from Fig. 2 that the adsorption amount of Hb on liposomes increases as the concentration of Hb increases.

Hb has a molecular weight of approximately 67,000 and contains two α and two β subunits, each of which has one redoxiron heme as its prosthetic group, the heme is located in crevices at the exterior of the subunit [5]. The absorption spectra of Hb in PBS aqueous solution and in the lecithin liposome system are shown in Fig. 4. The absorbance at 405 and 630 nm are the characteristic peaks of Hb, and the absorbance at 270 nm is due to the aromatic amino acids in peptide chains [15]. With the increase of lecithin liposome concentration, the absorbances at 405 and 630 nm drop gradually, while the maximum absorption wavelengths remain unchanged. This means that the heme is not exposed from the crevices at the exterior of the subunit.

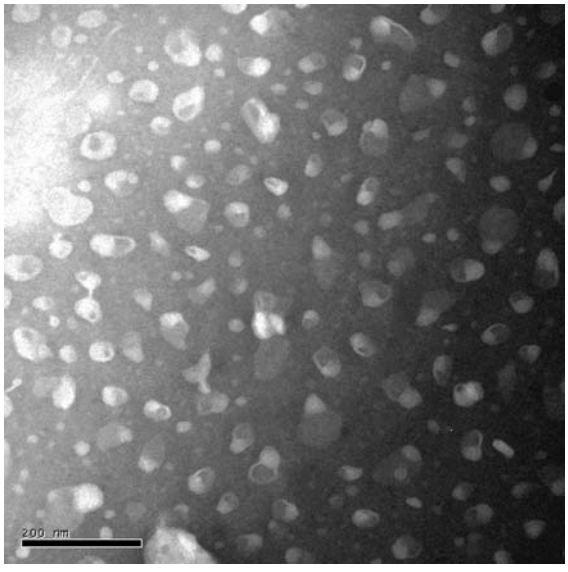

Fig. 1 TEM image of lectithin liposomes

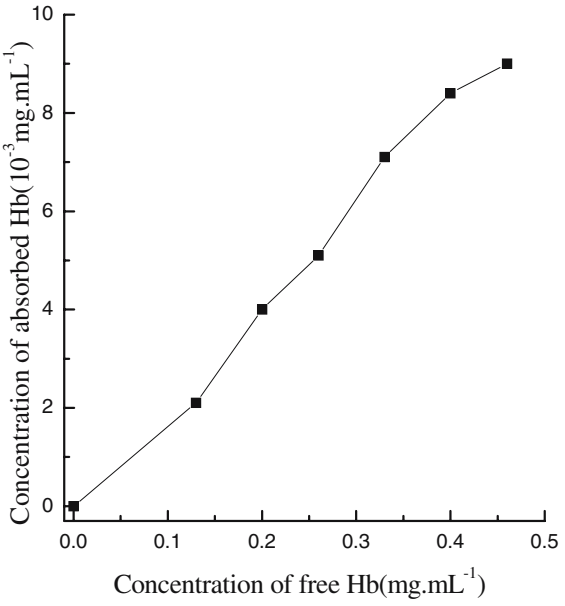

Fig. 2 Adsorption of Hb on liposomes. The lecithin concentration is 2.5×10^{-4} $g.mL^{-1}$

The increase of the absorbance at 270 nm is caused by the reversal of peptide chains, which can be verified by the fluorescence spectra of Hb in liposomes (Fig. 5). The fluorescence intensity of Hb is due mainly to tryptophan (Trp). Trp is buried in Hb and can be quenched by heme and the other amino acids [16], so the fluorescence intensity of Hb in water is very low. However, the fluorescence intensity of the emission peak at 330 nm is increased in the liposomal system. The adsorption of Hb on the liposomes can make the peptide chains reverse partially and decrease the quenching reaction of heme in Hb with

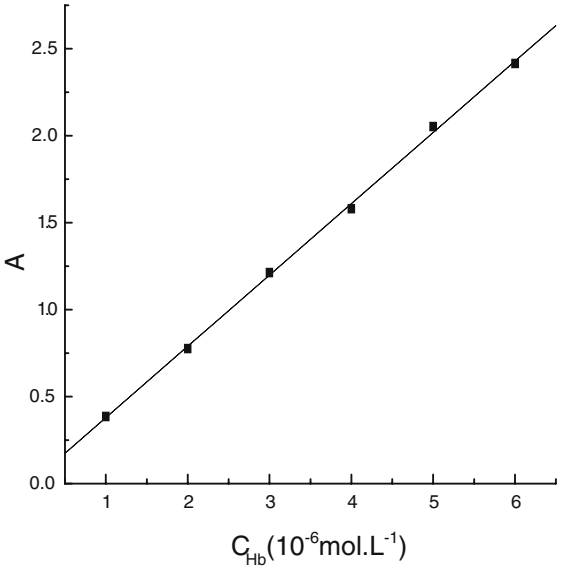

Fig. 3 The standard curve of absorption of Hb

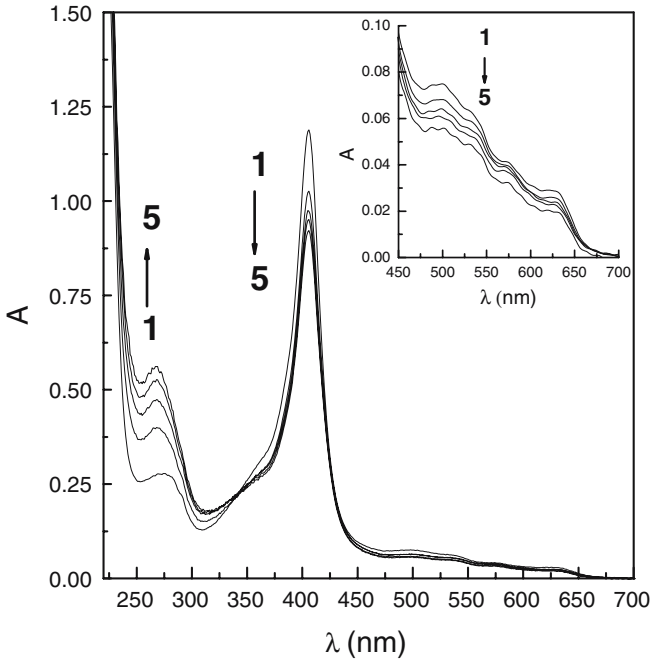

Fig. 4 The absorption spectra of Hb (3.0×10^{-6} mol.L^{-1}) in lecithin liposomes. Lecithin concentrations (g.mL^{-1}): *1*—0; *2*—1.0×10^{-4}; *3*—1.5×10^{-4}; *4*—2.0×10^{-4}; *5*—2.5×10^{-4}

Trp and other amino acids, so the fluorescence intensity of Hb increases. It can be included from Figs. 4 and 5 that the absorption of Hb on the liposomes does not obviously change the microenvironment of Hb, but only partially changes the folding of the peptides. It has been reported that albumin has high binding capacity to hydrophobic compounds and organics [17]. Thus, the adsorption of Hb on the neutral phospholipid bilayer matrix in the present study is mainly related to the hydrophobic interaction between Hb and liposomes. This conclusion is consistent with those of previous studies [12, 14].

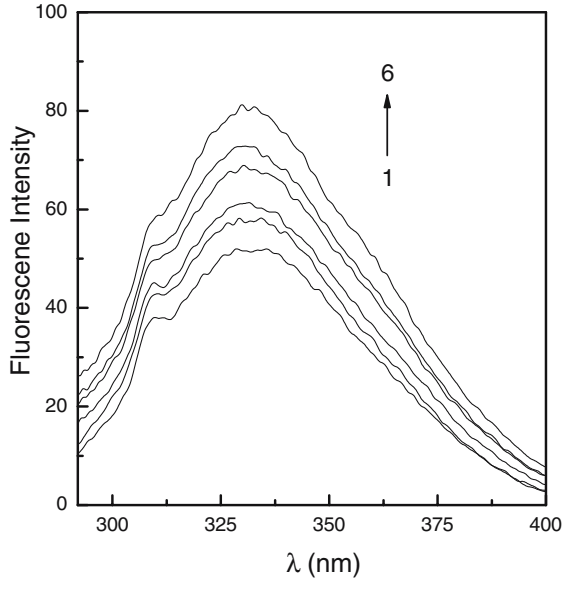

Fig. 5 The fluorescence spectra of Hb (3.0×10^{-6} mol.L^{-1}) in lecithin liposomes. Lecithin concentrations (g.mL^{-1}): *1*—0; *2*—0.5×10^{-4}; *3*—1.0×10^{-4}; *4*—1.5×10^{-4}; *5*—2.0×10^{-4}; *6*—2.5×10^{-4}

Microenvironmental polarity of lecithin liposome

The relationship between the value of I_1/I_3 (microenvironmental polarity) obtained by pyrene fluorescent method and the concentration of Hb is shown in Table 1. In the lecithin liposome, pyrene exists in the interphase of lecithin liposome, where a link forms between the polar group and hydrocarbon chain and the value of I_1/I_3 is 1.074 (sample 1, Table 1). When Hb is added, the value of I_1/I_3 is increased from 1.092 (sample 2) to 1.123 (sample 5), indicating that the Hb can be adsorbed on liposomes because of the hydrophobic interaction between Hb and liposomes. The adsorption of Hb on liposomes makes the spaces between the lecithin molecules increase and forms a temporary gap in the liposomal bilayer membranes, leading to the transferring of pyrene to the outside of the liposome and the increase of the microenvironmental polarity of pyrene.

Fluorescence polarization of lecithin liposome

In solutions with low viscosities, 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 [18]:

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

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

Table 1 Values of fluorescence microenvironmental polarity (I_1/I_3) and the fluorescence polarization (P) in lectithin liposomes 2.5×10^{-4} g.mL^{-1} with the addition of Hb

Samples	C_{Hb}/mol.L^{-1}	I_1/I_3	P
1	0	1.074	0.147
2	2.0×10^{-6}	1.092	0.142
3	3.0×10^{-6}	1.105	0.136
4	4.0×10^{-6}	1.114	0.130
5	5.0×10^{-6}	1.123	0.126

C_{Hb} concentration of Hb

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 fluidity of natural or artificial lipid bilayer membranes can be studied by the fluorescence polarization method using fluorescence probe [19].

In the present work, pyrene was used as fluorescence probe to study the fluidity of liposomal membranes because pyrene can easily enter the lipid bilayer. The fluorescence polarization of pyrene in lecithin liposome was measured with different Hb concentrations to characterize the effect of Hb on the liposomal membrane fluidity (Table 1). With the addition of Hb, the value of P is decreased from 0.147 to 0.126. This result is in accordance with the conclusion in "Microenvironmental polarity of lecithin liposome." The adsorption of Hb on liposomes makes the spaces between the lecithin molecules in lecithin liposome increased (discussed in "Microenvironmental polarity of lecithin liposome"), which would lead to the decrease of the microviscosity of pyrene and the increase of the rotation rate of pyrene. Thus, the liposomal membrane fluidity (P) is decreased when Hb is added.

Microhydrophobicity of lecithin liposome

The hydrophobic microenvironment in liposomal membranes was investigated by means of the ANS fluorescent method. ANS scarcely fluoresces in the aqueous bulk phase, while ANS strongly fluoresces when it is transferred to a hydrophobic environment [12]. The fluorescence intensities of ANS with Hb are shown in Fig. 6, from which it can be seen that the fluorescence intensity is decreased with increasing Hb concentration. This is due to the consequent decrease in the amount of ANS in liposomal bilayer membranes. The adsorption of Hb on liposomes makes the spaces between the lecithin molecules in lecithin liposome increase and brings more water molecules out to liposomal bilayer membranes. Thus, the addition of Hb decreases the hydrophobic degree of liposomal membranes.

The study of calcein release from lecithin liposomes

The time course of leakage of calcein from the liposomes in the presence of Hb was measured and the results are shown in Fig. 7. At 1 h, the percentages of calcein leaked from the liposomes with and without Hb (4.0×10^{-6} mol.L^{-1}) were 8.3 and 56.4 %, respectively. The adsorption of Hb on the liposomes' surfaces is in favor of the leakage of calcein, which indicates that the addition of Hb can bring out the

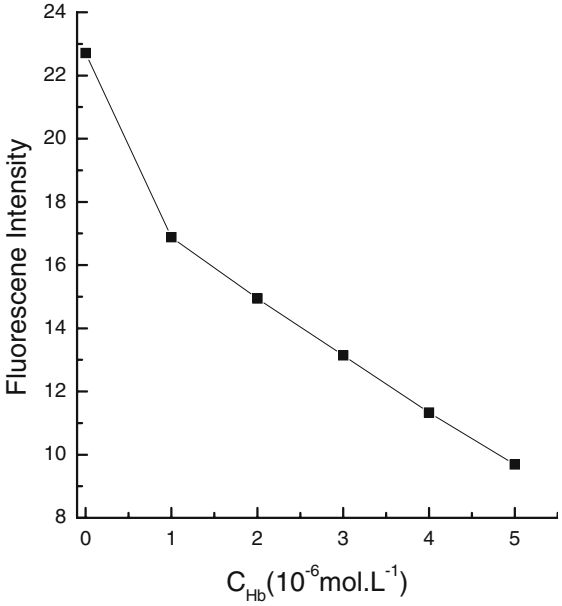

Fig. 6 Microhydrophobicity of liposome bilayer membranes by ANS fluorescent method. The lecithin concentration is 2.5×10^{-4} g.mL^{-1}

spaces between the lecithin molecules increased and affect the interaction between natural phospholipid molecules. So the stabilization of lecithin liposome is decreased and the permeability of liposomal bilayer membranes is increased. The barrier function of membranes containing constituent protein is actually lower than that of lipid membranes without protein [20]. It is suggested that the adsorption of

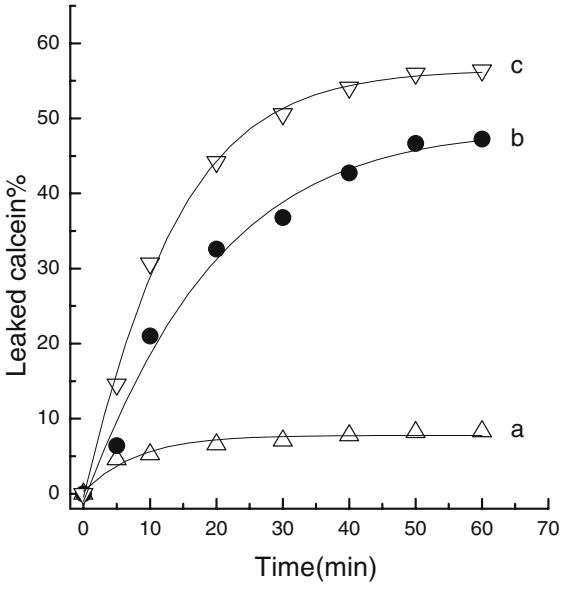

Fig. 7 Time course of calcein leakage from lecithin liposomes. The lecithin concentration is 2.5×10^{-4} g.mL^{-1}. Concentrations of Hb (mol.L^{-1}): **a** 0; **b** 2.0×10^{-6}; **c** 4.0×10^{-6}

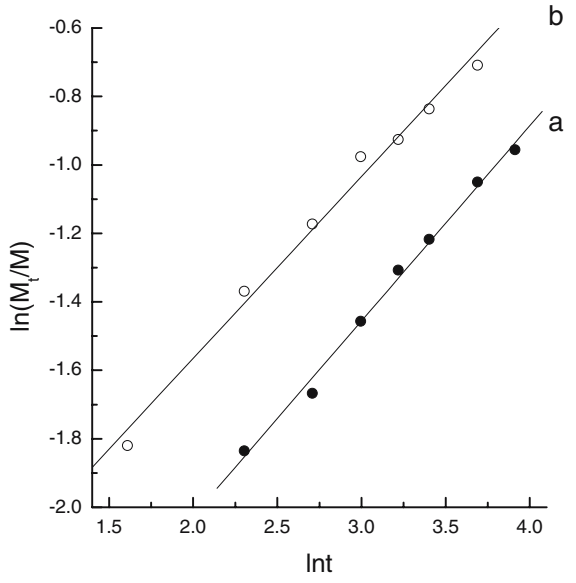

Fig. 8 The relations between ln (M_t/M) and ln$t$. Concentrations of Hb (mol.L^{-1}): **a** 2.0×10^{-6}; **b** 4.0×10^{-6}. The lecithin concentration is 2.5×10^{-4} g.mL^{-1}

protein on liposomes as drug carriers brings out a leakage of the drug in the circulation of liposomes in the blood.

The character of adsorption of Hb on liposomes was indicated indirectly with the velocity of calcein leakage from liposomes. The release dynamics parameters can be calculated as follows [21]:

$$\frac{M_t}{M} = k \cdot t^n \tag{4}$$

where M_t/M is the molar fraction of calcein leaked from liposome at different times, k is the release dynamics constant, and n is the exponent related to the release property of calcein.

Figure 8 shows a preferably linearity between ln (M_t/M) and ln$t$. The release dynamics constant k and the index gene n can be calculated from the intercept and the slope. The larger value of k means a more rapid velocity of calcein leakage from liposomes. When the value of n is 0.5, the course of the release follows Fick's law of diffusion, when n is 0, the course of the release is of zero-order release, and when $0.5<n<1$, the course is a mode of non-Fick's law of diffusion [22].

Table 2 The release dynamics constant and exponent of calcein leakage from liposomes in different concentrations of Hb

C_{Hb}/mol.L^{-1}	10^2k	n
2.0×10^{-6}	4.22	0.57
4.0×10^{-6}	7.24	0.53

C_{Hb} concentration of Hb

Table 2 shows that the release dynamics constant k of calcein is increased with the addition of Hb. When the concentration of Hb is 2.0×10^{-6} mol.L^{-1} and 4.0×10^{-6} mol.L^{-1}, the value of n is 0.57 and 0.53, respectively. The process of calcein leaked from liposomes can be considered as a mode of non-Fick's law of diffusion. The above results can be explained by the adsorption amount of Hb on liposomes. Because the higher adsorption amount of Hb can result in larger spaces between the lecithin molecules, this higher adsorption will decrease the barrier function of membranes and lead to more calcein being leaked from liposomes. When the adsorption amount of Hb on liposomes reaches saturation, the release of calcein comes to equilibrium.

Conclusions

In this paper, we show that Hb can be adsorbed on lecithin liposomes by the hydrophobic interaction between Hb and lecithin molecules, which results in an increase of the microenvironmental polarity (I_1/I_3) of pryene, a decrease of membrane fluidity (P), and microhydrophobicity of lecithin liposomes. With the increasing content of Hb, the permeability of the liposomal bilayer membranes increases. These results are considered to be due to the fact that the adsorption of Hb increases the spaces between the lecithin molecules, and a temporary gap is consequently formed in the liposomal bilayer membranes. It is suggested that the adsorption of protein in liposomes as drug carriers brings out a leakage of the drug in the circulation of liposomes in the blood.

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