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Miscibility of mixed monolayer of L-α-di-(*cis*-9-octadecenoic acid) phosphatidylcholine and Rutin stearate at the air/water interface

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ABSTRACT

The research on the miscibility of the mixed monolayer of unsaturated phosphatide and flavonoids can reveal the mechanism of molecular interactions, which is important for bioactive mechanism of action of flavonoids. An amphiphilic rutin stearate (RS) was obtained by esterification of rutin with stearic acid catalyzed by immobilized Candida antarctica lipase B (Novozym 435), and the result suggested that the regioselectivity of the lipase-catalyzed esterification of rutin was specific at the $C_{4''}$ position of the rhamnose moiety. The mixed monolayer of L-α-di-(cis-9-octadecenoic acid) phosphatidylcholine (DOPC) and RS was spread at the air/water interface and its miscibility at subphase temperatures of 37 ℃ and 10 ℃ were investigated by Langmuir film balance. The two components of DOPC/RS mixed monolayer were miscible throughout the mixture composition range, and the better miscibility was obtained at higher subphase temperature, i.e., 37 ℃. At both subphase temperatures of 37 ℃ and 10 ℃, the condensing effect of RS was most prominent at the lower surface pressure. The condensing effect could be ascribed to the match of RS and DOPC molecular shapes during the monolayer formation, and the molecules of two components might occupy smaller areas.

Key words: Mixed monolayers; L-α-di-(*cis*-9-octadecenoic acid) phosphatidylcholine; Rutin stearate; Miscibility; Surface pressure-area isotherm; Molecular interaction

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INTRODUCTION

Flavonoids are widely distributed in plants and exhibit a wide range of biological activities, such as anticancer [1], anti-inflammatory [2,3], anti-virus [4], anticoagulant [5,6], anti-atherosclerosis [7], and inhibitory effects on low-density lipoprotein (LDL) oxidation [7-9]. The aglucon show more excellent antioxidation compared to glucoside because the former is more lipophilic and can partition preferentially into the hydrophobic core of the bio-membrane [10]. These results are confirmed by protecting LDL [11] and the complex of carotenoids and unsaturated fatty acids from oxidation [12, 13] by flavonoids. Unfortunately, the hydrophilic property of flavonoids glucosides (most of naturally occurring flavonoids), which dues to polyhydroxyl and saccharide groups, compresses their anti-oxidative activity. The research of Saija et al [14] showed that the appropriate hydrophile and lipophile balance value of flavonoids could provide the excellent resistance to oxidation and the ability of interacting with and crossing phospholipid membranes. Esterification of the hydroxyl of flavonoids glucosides by fatty acids is a solution to improve the hydrophile and lipophile balance values of flavonoids glucosides. In present paper, the lipophilic rutin stearate (RS) was obtained by acylation of rutin with stearic acid using the immobilized *Candida antarctica* lipase B (Novozym 435) as catalyst. RS was an amphiphilic molecule with rutin moieties as polar group and alkyl chain as hydrophobic groups (Fig.1a.). More opportune hydrophile and lipophile balance value and more prominent antioxidative activity may be expected for RS.

The absorption and metabolism of pharmaceuticals involve the interaction of pharmaceuticals and bio-membrane, which leads to the changes of structure and physio-properties of bio-membrane, such as permeation, fluidity. Lipid

monolayer is usually chosen as simple model of bio-membrane, so the investigation on interaction of flavonoids with lipid monolayer may be a simple as well as effective way to reveal the antioxidative activity mechanism of flavonoids. However, few papers have reported the interaction of flavonoids with lipid monolayer [15], but more with the lipid bilayer [10, 16-18]. This probably because flavonoids can not spread at air/water interface, as other conjugated aromatic molecules. However, amphiphilic flavonoids esters which were obtained by esterification could spread at air/water interface to form monolayer [15, 19].

In present paper, the monolayer of RS and the mixed monolayer of L-α-di-(*cis*-9-octadecenoic acid) phosphatidylcholine (DOPC) and RS was spread at the air/water interface, and the miscibility of DOPC/RS mixed monolayer at subphase temperatures of 37℃ and 10℃ had been investigated by Langmuir film balance to yield quantitative information on the nature of the molecular interaction, which could provide valuable insights to reveal the bioactive mechanism of action of flavonoids.

EXPERIMENTAL SECTION

Chemicals

L-α-di-(cis-9-octadecenoic acid) phosphatidylcholine (DOPC) was a commercial product (Fluka, 99%). Its formula is reported in Fig.1b. Rutin (≥95%) was purchased from Beijing Biochemical Reagent Company, China, and was used as obtained. Novozym 435 (immobilized lipase B from *Candida Antarctica*) (enzyme activity: 10470PLU/g) was obtained from Novo-Nordisk Co., China. Stearic acid, chloroform and methanol were analytical reagent and from Chengdu Kelong Chemical Plant, China. The *tert*-amyl alcohol was chemically pure and purchased from Beijing Chemical Plant, China. Molecular sieves 4Å were purchased from Sino-American Global Molecular Sieves Company, Shanghai, China. Silica gel (44-74 μm) was purchased from Qingdao Haiyang Chemical Plant, China. Sephadex LH-20 (25-100 μm) was obtained from Shanghai Chemical Plant, China.

Syntheses of rutin stearate

Rutin stearate (RS; Fig.1a.) was synthesized as follows: Novozym435 (340 mg) was added to a solution of rutin (762 mg) and stearic acid (1779 mg) in tert-amyl alcohol (85 ml). The suspension was heated to 60 ℃ and stirred at 300rpm with a mechanical stirrer. In order to control water content in the reaction medium, after 24h of reaction, molecular sieves 4Å were added to the reactor with 100 g/L. Molecular sieves were activated by heating at 150 ℃ for 24 h before used. Finally, the reaction was stopped and the enzyme filtered off. The solvent was removed in vacuum. The residue was chromatographed successively on silica gel [ethyl acetate/ethanol/water, 15: 1: 1(v/v/v)] and Sephadex LH-20 (methanol) to give pure rutin stearate, which is amorphous yellow powders.

The ^{1}H-NMR spectrum was measured on a Varian INOVA400 spectrometer operating at 400 MHz, and ^{13}C-NMR spectrum was measured on a Bruker AVANCE300 spectrometer operating at 300 MHz, using Me_4Si as internal standard.

Preparation of monolayer

Film balance measurements were performed using a KSV trough (KSV Instrument Ltd.) with a Wilhelmy type microbalance using a platinum plate. RS was dissolved in a mixture of chloroform and methanol with a volume ratio of 9:1. DOPC was dissolved in chloroform. The concentration of each solution was 1μmol/ml. The mixed solutions of DOPC and RS were successively prepared with RS molar fractions (X_{RS}) of 0.167, 0.25, 0.5, 0.75 and 0.833. 100μl of the mixed solutions and the pure components were randomly spread on the aqueous subphase. Following the evaporation of the solvent (15 min), the monolayer was compressed at a rate of 5 mm/min. Surface-pressure vs. molecular-area isotherms were recorded by film balance measurements. The experiments were performed at subphase temperatures of 37℃ and 10℃.

RESULTS AND DISCUSSION

Structure characterization of rutin stearate

Table 1 shows the selected ^{1}H-NMR data of rutin stearate and rutin. For the nuclear parent of flavone protons, no modification of chemical shift was observed. Only a downfield at 4.66 ppm for rutin stearate was observed in the spectrum of the glycoside moiety. So, the glycoside moiety of rutin is monoacylated.

Table 1 Selected ^{1}H-NMR data of rutin stearate and rutin

	rutin stearate ester	rutin
OH_5	——	12.48 (1H, s)
$H_{2'}$ and $H_{6'}$	7.53 (2H, m)	7.52 (2H, m)
$H_{5'}$	6.78 (1H, d, J = 9Hz)	6.80 (1H, d, J = 9Hz)
H_8	6.28 (1H, s)	6.36 (1H, s)
H_6	6.11 (1H, s)	6.18 (1H, s)
$H_{1''}$	5.32 (1H, d, J = 7.4Hz)	5.32 (1H, d, J = 7Hz)
$H_{4'''}$	4.66 (1H, t, J = 9Hz)	——
$H_{1'''}$	4.48 (1H, s)	4.42 (1H, s)
$H_{rhamnoglucosyl}$	3.73-2.65 (9H, m)	3.74-3.04 (10H, m)
Fatty chain $CH_2\alpha$	2.21-2.13 (2H, m)	——
Fatty chain $CH_2\beta$	1.5 (2H, m)	——
Fatty chain –	1.25-1.13 (26H, m)	——
Fatty chain CH_3	0.95 (3H, t, J = 7.0Hz)	——
CH_3 rhamnosyl	0.85 (3H, d, J = 6.7Hz)	1.01 (3H, d, J = 7.2Hz)

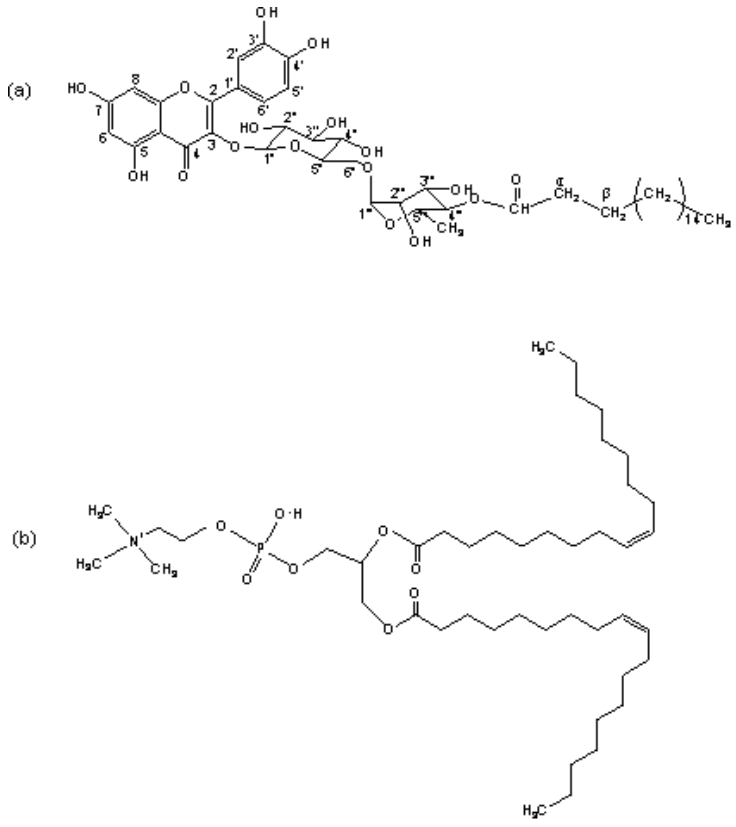


Fig.1 Chemical structures of rutin estarate (RS) (a) and L-α-di-(cis-9-octadecenoic acid)phosphatidylcholine (DOPC) (b)

The selectivity of *Candida Antarctica* lipase was precisely on the primary and a secondary OH of the glucose moiety and the secondary 4"'-OH of the rhamnose moiety [20, 21]. The precise position, on which the acylation took place, was obtained by means of ^{13}C-NMR spectrum of rutin stearate. The chemical shifts of C4" and C3" of glucose moiety were similar, and that was the same with C4"' and C3"' of rhamnose moiety in ^{13}C-NMR spectrum of rutin,

because their substitution conditions were similar. Whichever of them was acylated, the chemical shift would modify to high frequency. Selected ^{13}C-NMR data of RS (d_6-DMSO):δ (ppm) 177.5(C4), 174(C=O ester), 168.3(C7), 157.7(C9 or C2), 150.2(C4'), 133(C3), 122.5(C6'), 116.7(C5'), 77.9(C3"), 75.3(C5"), 74.7(C4"'), 71.6(C4" or C2"'), 70.9(C2"' or C4"), 69.3(C3"'), 67(C5"'), 34.7(fatty chain), 31.8(fatty chain), 29.7(fatty chain), 25.4(fatty chain), 25(fatty chain), 24.5(fatty chain), 23.7(fatty chain), 23.1(fatty chain), 18.5(fatty chain), 15.2(fatty chain), 12.1(fatty chain). From these data, it could be observed that $C_{4''}$ (δ 75.3 ppm) and $C_{3''}$ (δ 77.9 ppm) of glucose moiety showed similar chemical shifts, however, the chemical shift of $C_{4'''}$ (δ 74.7 ppm) of rhamnose moiety modified to high frenquency comparing with $C_{3'''}$ (δ 69.3 ppm) in ^{13}C-NMR spectrum of rutin stearate. So, the acylation takes place precisely on the secondary 4"'-OH of the rhamnose moiety (Fig.1a.).

Monolayer of RS and DOPC at air/water interface

Fig. 2 shows the surface pressure vs. mean molecular area (π-A) isotherms of DOPC/RS mixed monolayer at various mole fraction at 10℃ (a) and 37℃ (b), respectively.

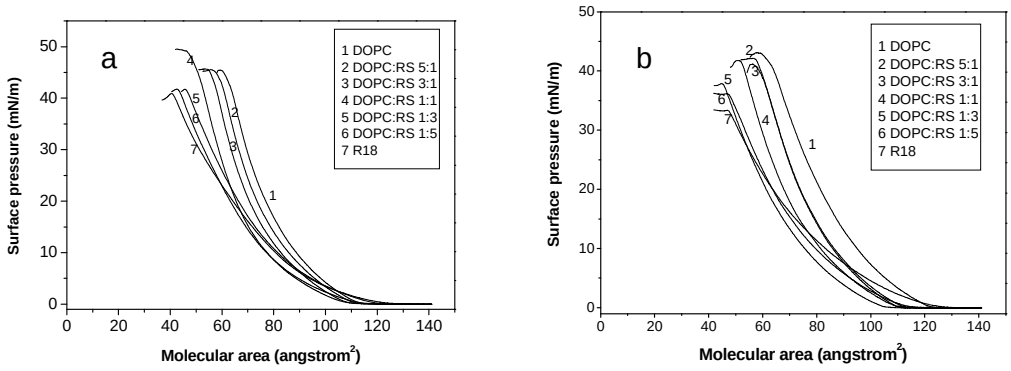
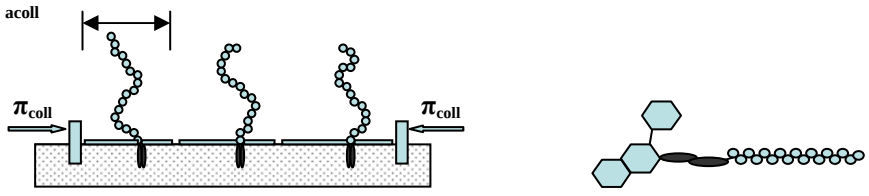


Fig. 2 Surface pressure vs. mean molecular area of DOPC/RS mixed monolayer at 10℃ (a) and 37 ℃ (b). The RS mole fraction was reported in the figure for each curve.

The isotherms indicated that RS was capable of forming monolayer with liquid-phase at the air/water interface. The intermolecular hydrogen bonds between molecules of water and rutin would lead to almost three of aromatic nucleus of rutin forming a large flat at air/water interface. The packing of the molecules in monolayer were affected by two different contributions. One was the hydrophobic interaction between the alkyl chains; the other was the interaction between the lateral surfaces of the polar groups. The packing of the RS seemed to be basically determined by the interaction between the lateral surfaces of the rutin molecules. Compared with the large polar groups, the alkyl chains was relatively short. Near to the collapse of the monolayer, the polar groups arranged tightly, while alkyl chains were still outside the range that was prerequisite for interaction between them, and then alkyl chains were orientational disorder. Thus, the isotherm of RS monolayer with liquid-expanded phase is observed.

The value of the molecular area corresponding to the collapse (a_{coll}) of the RS monolayer was 45 Å^2. This value equated the value found for the limiting areas per molecule (extrapolation of the linear parts on the isotherms to the abscissa) of quercetin palmitate molecules (QP) [15,19]. That indicated that when the RS monolayer collapsed, only the flavone nuclear parent contributed to a_{coll} value. We could expect the structures of the Langmuir monolayer of RS when collapsing. It is that glucose and rhamnose arrange close and parallely, and they pointed perpendicularly to the surface of the water, while alkyl chains point into air loosely (Fig. 3a).



(a) **(b)**

Fig. 3 Schemes illustrating the structures of rutin stearate (b) and the Langmuir monolayer of rutin stearate (a)

Pure DOPC also forms the liquid-phase monolayer. This might be explained in terms of the fact that, the cone-like structure which resulted from the two *cis*-double bonds of DOPC molecule leaded to the mismatch of molecular

shape and loose array of hydrophobe alky chains.

Miscibility of DOPC/RS mixed monolayer

From the isotherms of DOPC/RS mixed monolayer, it could be observed that at 10□, for $X_{RS} \leq 0.5$, the surface pressures at the collapse (π_{coll}) increased with the increase of X_{RS} and reached the maximum for $X_{RS} = 0.5$. At 37 □, the values of π_{coll} exhibited between those for the pure components and increased gradually with increasing proportion of DOPC. If two components of mixed monolayer were immiscible, there were two π_{coll} corresponding to the monolayer of two pure components [22]. Fig. 2 showed that there was a single π_{coll} for every isotherm at temperatures studied. Thus the two components are miscible throughout the mixture composition range.

The nature of the molecular interaction between the two components could be obtained quantitatively from a plot of the excess molecular area of mixing, A_{ex}, which was the difference in molecular area between the ideal value and the measured value, as a function of the mole fraction at a given pressure. A_{ex} could be given by Eq. [1].

$$A_{ex} = A_{12} - (X_1 A_1 + X_2 A_2) \quad [1]$$

Where A_{12} was molecular area of the mixed monolayer; A_1 and A_2 were the molecular areas of the monolayer of component 1 and component 2 at a given surface pressure π, respectively; and X_1 and X_2 were their corresponding mole fractions. With mixtures which both components exhibited ideal miscibility or complete immiscibility, the excess area was zero and the plot of A_{12} versus X_1 gave a straight line. Any deviation from the straight line indicated miscibility and non-ideality. With positive excess areas meaning greater cohesion between like molecules than the unlike components, that was, the interactions between the two components were repulsive, and negative excess areas indicating attractive forces between the unlike ones [23, 24].

Fig. 4 shows the variation of A_{ex} with X_{RS} at various surface pressures and temperatures

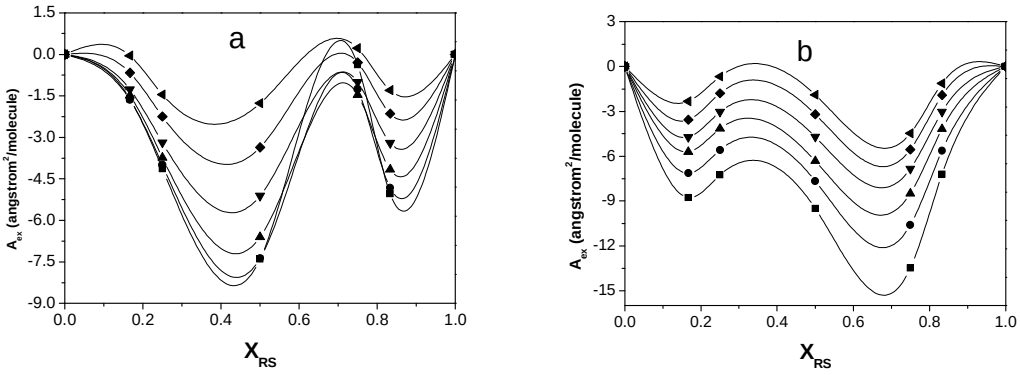


Fig. 4 Excess area, A_{ex}, as a function of the mole fraction of RS in DOPC/RS mixed monolayer at various surface pressures
10℃ (a), 37℃ (b)
■ 5 mN/m, ● 10 mN/m, ▲ 15mN/m, ▼ 20 mN/m, ◆25mN/m, ◄ 30 mN/m

From Fig. 4 (a), it could be seen that at 10℃, the values of A_{ex} of DOPC/RS mixed monolayer were negative for all mole fractions and surface pressures studied, with an exception for the case of π = 30mN/m, $X_{RS} = 0.75$, where A_{ex} = 0.22 Å^2. The greatest negative deviation from ideality was observed over the X_{RS} range from 0.38 to 0.44. These negative excess areas signify miscibility and intermolecular cohesive forces between DOPC and RS. The negative was more significant at the lower surface pressure, with the exception for the case of π = 5, 10 mN/m in the range of $0.56 < X_{RS} < 0.8$.

At 37℃, A_{ex} was negative for all mole fractions and surface pressures. This area condensing effect of RS was more significant at lower surface pressure. Regardless of the various surface pressures investigated, the condensing effect was most prominent at the same concentration of $X_{RS} \approx 0.68$, where the minimum of A_{ex} occurred.

At different temperatures, the condensing effect of RS on the DOPC monolayer was more significant at lower surface pressures. This implied that when the molecules of DOPC in monolayer interacted weakly, RS showed more condensation. At 10℃, positive A_{ex} values were observed at some X_{RS} and surface pressure. However, at 37℃, negative A_{ex} values showed attractive interaction between DOPC and RS molecules for all conditions studied. Thus,

the miscibility is more excellent at higher temperature. This fact was clearly due to the fact that higher subphase temperature allowed the lipid monolayer to stay in a fluid state which resulted to more contacting of DOPC and RS molecules and favored attractive forces between the unlike components. With the temperature increasing, the value of X_{RS} where the most negative value of A_{ex} occurred was increased and the value of A_{ex} was decreased. So, at high temperature, high mole fraction of RS can be miscible with DOPC and bring prominent condense effect.

The condensing effect of RS can be ascribed to the match of RS and DOPC molecular shapes during the monolayer formation. RS molecule has a large polar group and a hydrophobic chain, its three-dimensional shape was a positive cone at the air/water interface, which match just the inverted cone of DOPC at the air/water interface. So, the molecules of two components may occupy smaller areas.

CONCLUSION

The two components of DOPC/RS mixed monolayer were miscible throughout the mixture composition range, and the better miscibility was obtained at higher subphase temperature, i.e., 37℃. At both subphase temperatures of 37℃ and 10℃, the condensing effect of RS was most prominent at the lower surface pressure. The condensing effect could be ascribed to the match of RS and DOPC molecular shapes during the monolayer formation, and the molecules of two components might occupy smaller areas.

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