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Formation and stability of the dispersed particles composed of retinoic acid, sesame oil and phosphatidylcholine

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Abstract

All *trans*-retinoic acid (RA) was dispersed by sonication with soybean phosphatidylcholine (PC). The particle size in the dispersion was increased to 240 nm up to the RA mol fraction range (X_{RA}) of 0.4. At $X_{RA} = 0.5$, the RA/PC mixture was difficult to disperse and the macroscopic oil/water phase separation was observed. On the other hand, by the addition of sesame oil (SO) to RA (molar ratio of RA:SO = 1:1), stable aqueous dispersions (diameter: 40–80 nm) were obtained in the mol fraction range RA and SO mixture (X_M) of 0.1–0.8. In order to clarify these dispersal mechanism, the dispersed particles were characterized and the interaction among RA, SO and PC was investigated using several physicochemical techniques. The trapped aqueous volume inside the RA/PC particles was determined using the aqueous space marker, calcein and it was increased with the addition of RA into small unilamellar vesicles of PC. On the other hand, that of RA/SO/PC particles was decreased remarkably with increase in X_M and the decline in the fraction of vesicular particles was also confirmed by fluorescence quenching of *N*-dansylhexadecylamine in the PC membrane by the addition of the quencher $CuSO_4$. These results indicate that the interaction of RA with PC bilayers and the structure of RA/PC mixture will be changed by the addition of SO.

Keywords: Retinoic acid; Soybean phosphatidylcholine; Sesame oil; Membrane; Structure

1. Introduction

Retinoic acid (RA) is effective in the treatment of acute promyelocytic leukemia and has been investigated for the potential in cancer chemeprevention and therapy (Weigand et al., 1993). To develop the use of this compound in these therapies, much effort has been focused on the elucidation of the molecular mechanisms for the effect of RA in these diseases and a number of pharmacokinetic studies have been carried out (Regazzi et al., 1997). In these studies, the fraction of dose absorbed of RA has been difficult to assess, since no intravenous formulation is available.

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RA is poorly water soluble (Han and Wiedmann, 1998) and in order to develop an injectable formulation of RA, we suspended RA in sesame oil (SO) and dispersed with soybean phosphatidylcholine (PC) using sonication. For the formation of the dispersion, it will be important to obtain the information on the interaction of RA with PC. RA is classified as a neutral lipid and the interaction of RA with phospholipids have been reported. Wassall et al. (1988) have reported that at least 20 mol% of RA can be incorporated into the phospholipid and decreased the membrane fluidity. Nastruzzi et al. (1990) have reported that at least 40 mol% of RA decreased the phase transition temperature of dipalmitoylphosphatidylcholine. Based upon these results, it can be concluded that RA have appreciable solubility in the phospholipid membranes.

Some neutral lipids, such as diglyceride (Das and Rand, 1986; Seddon, 1990), monoglyceride (Nilsson et al., 1991), menaquinone-4 (Handa et al., 1992), and α-tocopherol (Nakajima et al., 1990) have appreciable solubility in PC bilayers (>20 mol%) and the addition of a neutral lipid to the bilayers changes the hydrophilic–lipophilic balance and induces a phase transition from the bilayer to a hexagonal H_{II} or reversed cubic phase. On the other hand, neutral lipids, such as triglyceride (Tall and Small, 1980; Handa et al., 1990), ubiquinone-10 (Handa et al., 1991), α-tocopherol acetate (Asai and Watanabe, 1998), and SO (Asai and Watanabe, 1999) have low solubilities in PC bilayers (<5 mol%) and form droplets separated from the bilayers in an aqueous medium. The droplets of a neutral lipid are covered with a phospholipid monolayer and stabilized in an aqueous medium as emulsion particles. The surface monolayers of the droplets are in equilibrium with the bilayer (Handa et al., 1990, 1991).

In this study, in order to clarify the dispersal mechanism for RA with SO by PC and the interaction among them, we prepared dispersed particles by sonication and characterized them using several physicochemical techniques. The structure of RA/PC and RA/SO/PC particles was determined by dynamic light scattering, fluorescence quenching and analysis of the trapped aqueous volume inside the particles. The miscibility and solubility of RA/PC and RA/SO/PC were evaluated by surface monolayer techniques.

2. Materials and methods

2.1. Materials

All trans-retinoic acid (RA) was purchased from Sigma Chemicals Co., Ltd. (St Louis, Missouri, USA). Sesame oil (SO) was purchased from Takemoto-Yushi Co., Ltd. (Tokyo, Japan). Soybean phosphatidylcholine (PC) was purchased from Ajinomoto Co. Ltd. (Tokyo, Japan). Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and calcein(3,3′ - bis[*N*,*N* - bis(carboxymethyl)aminomethyl] - fluorescein) were purchased from Wako Pure Industrial Ltd. (Osaka, Japan). *N*-Dansylhexadecylamine (DSHA) was from Lambda Co., Ltd. (Graz, Austria).

2.2. Methods

2.2.1. Preparation of dispersed particle

RA and PC or RA, SO and PC were dissolved in chloroform and mixed. After evaporation of the solvent, water was added to give a final combined concentration of 5 mM. The mixtures were sonicated for 30 min under a stream of nitrogen gas at 50°C. A probe type sonicator, model UD-200 (Tomy Seiko Co., Ltd., Tokyo, Japan) was used at a power setting of 100 W.

2.2.2. Determination of particle size

Dynamic light scattering (DLS) measurements of the sonicated dispersions of RA and PC or RA, SO and PC were performed with a DLS-7000DL submicronanalyzer (Ohtsuka Electronics Co., Ltd., Osaka, Japan) at 25°C. The data were analyzed by the histogram method (Gulari et al., 1979) and the weight averaged particle sizes were evaluated.

2.2.3. Determination of the trapped volume inside the dispersed particles

Dried mixtures of RA and PC or RA, SO and PC were hydrated with a 70 mM calcein solution

instead of water for the preparation of the dispersion. Untrapped calcein was removed by gel filtration (Sephadex G-50). The volume of the calcein solution trapped in the dispersed particles was determined fluorometrically (Allen and Cleland, 1980) after solubilization of the lipid particles by the addition of 10% Triton X-100, and the aqueous volume trapped per mol of PC was evaluated. The PC in the dispersion was assayed by the method of Bartlett (Bartlett, 1959).

2.2.4. Measurements of spreading pressures

In order to evaluate the miscibility of RA, SO and PC in bulk phase, spreading pressures of the lipid mixtures were measured. RA, SO and PC were dissolved in chloroform and mixed. After evaporation of the solvent, the dried lipid mixtures were added to the distilled water in the surface tensiometer (Model CBVP-A3, Kyowa Kaimenkagaku Co., Ltd., Tokyo, Japan). The spreading pressures of the lipid mixtures at an air/water interface (surface pressures at the transition point from bilayer or solid states to monolayer) were obtained from the steady value of surface pressure at 6–8 h after addition of the lipid or the lipid mixtures to the water at 25°C. Details of the monolayer techniques have been described elsewhere (Handa et al., 1985; Nakagaki et al., 1985).

2.2.5. Fluorescence quenching

Fluorescence quenching techniques (Matsuzaki et al., 1991) were used to obtain information on structural changes (ratio of external to total (external plus internal) membrane) in the RA/SO/PC dispersed particles. In this study, $CuSO_4$ was used as a quencher for the DSHA fluorescence embedded in the lipid particles. RA/SO/PC dispersed particles containing 1 mol% of DSHA were titrated with small aliquots of 1 M $CuSO_4$. The fluorescence intensity I at 510 nm (with excitation at 330 nm) was measured as a function of the Cu^{2+} concentration $[Q]$. Assuming that only the fluorescence of the Cu^{2+} accessible DSHA is quenched according to the SternVolmer equation (Badley, 1976), one can estimate the exposed fraction of DSHA P, so that

$$I_0 \cdot [Q]/(I_0 - I) = (1/P) \cdot [Q] + 1/KP \quad (1)$$

where, I_0 is fluorescence intensity in the absence of the quencher, I the intensity after quenching by Cu^{2+}, $[Q]$ the concentration of Cu^{2+} and K the Stern-Volmer constant.

3. Results and discussion

3.1. Size and stability of dispersed particles from RA/PC or RA/SO/PC mixtures

Fig. 1 shows that the diameter of the dispersed particles as a function of RA mol fraction (X_{RA}) and the lipid mixtures (RA:SO = 1:1) mol fraction (X_M). UP to the $X_{RA} = 0.4$, particle size of RA/PC dispersions was increased to approximately 240 nm and at $X_{RA} = 0.5$ or greats, the mixtures were difficult to disperse and macroscopic oil/water phase separation was observed. On the other hand, RA/SO/PC mixture was observed more easily in the range of $X_M = 0$–0.9. Separation of the dispersion to oil/water phases was not observed in the dispersions of the RA/SO/PC mixture in the range of $X_M = 0$–0.8 within 72 h after preparation. However, at $X_M = 0.9$, the particle diameter was considerably larger at 130 nm, and the separation was observed 72 h after preparation. At $X_M = 0.95$, the particle diameter was 200 nm and the separation was detected within 24 h after preparation.

3.2. Aqueous space inside the dispersed particles

Fig. 2 shows the trapped volume of the particles per mol of PC at various X_{RA} and X_M. The trapped volumes of small unilamellar vesicles (diameter: 20–50 nm), large unilamellar vesicles (diameter: 200–1000 nm), and multilamellar vesicles (diameter: 400–3000 nm) have been estimated to be 0.2–0.5, 7–10, and 3–4 l mol^{-1}, respectively (Szoka and Papahadjopoulos, 1978). At $X_{RA} = O$, small unilamellar PC vesicles (diameter: 40 nm) had a trapped volume of 0.47 l mol^{-1}, which agrees with the reported value. The trapped volume of the dispersed particles of the RA/PC mixture was determined in the range of $X_{RA} = 0$–

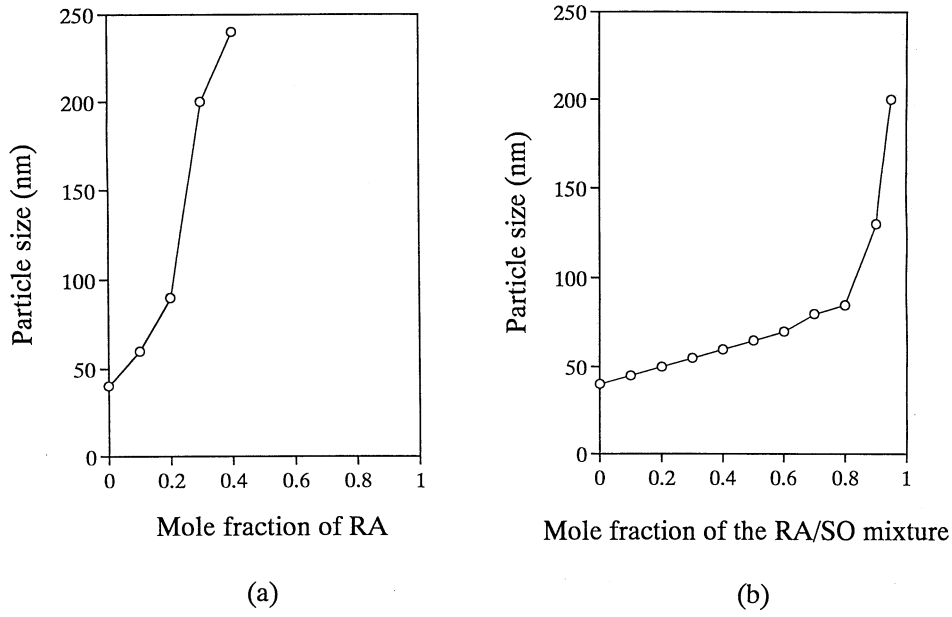

Fig. 1. (a) Weight-averaged diameter of dispersed particles represented as a function of mol fraction of RA (X_{RA}) in the mixture detemmined by dynamic light scattering (DLS) at 25°C. (b) Weight-averaged diameter of dispersed particles represented as a function of mol fraction of RA/SO mixture (X_M) determined by dynamic light scattering (DLS) at 25°C.

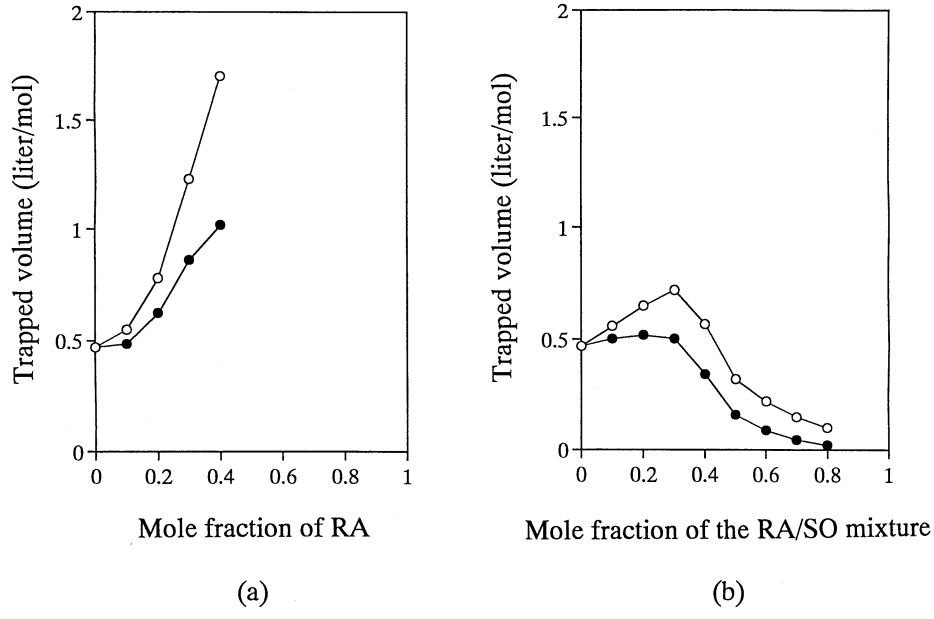

Fig. 2. (a) Trapped aqueous volume inside the dispersed particles represented as a function of the mol fraction of RA (X_{RA}) in the mixture. Volume of inner space per mol of PC (○---○), volume of inner space per total mol of the total lipids (RA + PC) (●---●). (b) Trapped aqueous volume inside the dispersed particles represented as a function of the mol fraction of RA/SO mixture (X_M). Volume of inner space per mol of PC (○---○), volume of inner space per total mol of total lipids (RA/SO + PC) (●---●).

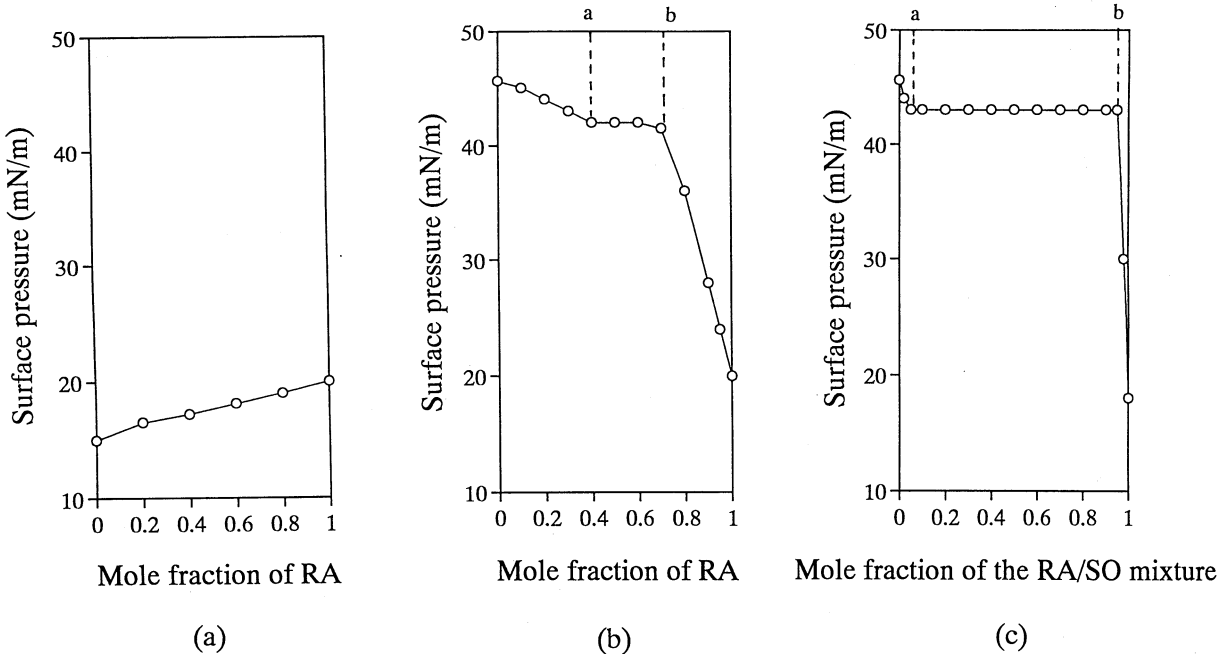

Fig. 3. (a) Spreading pressures of the RA and SO mixture in the presence of water at 25°C. (b) Spreading pressures of the RA and PC mixture in the presence of water at 25°C. The solubility of RA in PC is evaluated from the inflectional point, a, as the RA/SO mol fraction of approximately 0.4. The solubility of PC in the RA was evaluated from the inflection point, b, as the PC mol fraction of approximately 0.3. (c) Spreading pressures of the RA/SO and PC mixture in the presence of water at 25°C. The solubility of RA/SO in PC is evaluated from the inflectional point, a, as the RA/SO mol fraction of approximately 0.05. The solubility of PC in the RA/SO was evaluated from the inflection point, b, as the PC mol fraction of approximately 0.05.

0.4. The trapped volume of the dispersed particles of RA/PC mixture was increased with increase in X_{RA} and at $X_{RA} = 0.4$, the particle (diameter: 240 nm) had a trapped volume of 1.75 l mol^{-1}. These data indicate that the addition of RA to PC caused the change of the structure of the particles from small unilamellar vesicles to oligolamellar vesicles.

The trapped volume of the dispersed particles of the RA/SO/PC mixture was highest at $X_M = 0.3$ (trapped volume: 0.72 l mol^{-1}), then decreased sharply above $X_M = 0.4$. The trapped volume was also calculated on the basis of total mols of RA, SO and PC, and is represented in the same figure. The dramatic drop in the trapped volume indicates that the ratio of the liposomal structure in the dispersed particles decreased as a result of the addition of RA and SO to PC.

3.3. Miscibility of RA/SO, RA/PC or PA/SO/PC mixtures

Fig. 3 shows the spreading pressures of RA/SO,RA/PC and RA/SO/PC mixtures at 25°C. The spreading pressure of RA and SO were 20 mN m^{-1} and 15 mN m^{-1}, respectively (Fig. 3a). The spreading pressure of a lipid mixture is depend on the miscibility of the lipids (Nakagaki et al., 1985; Handa et al., 1990). The spreading pressure of RA and SO mixture varied with the mol fraction of RA in the lipid mixture. On the basis of the surface phase rule (Defy et al. 1966; Nakagaki et al., 1985), it was concluded that RA and SO were miscible. Fig. 3b shows the spreading pressures of RA and PC mixture. The solubility of RA in PC is evaluated from the inflectional point of spreading pressure, a, as the RA mol fraction of approx-

imately 0.4. The solubility of PC in the RA was evaluated from the inflection point for the spreading pressure, *b*, as the PC mol fraction of approximately 0.3.

The spreading pressure of hydrated PC (lamellar bilayers of PC) and RA/SO mixture (molar ratio of 1:1) were 45.6 mN m^{-1} and 18 mN m^{-1}, respectively (Fig. 3c). The spreading pressure of RA and PC mixture varied with the mol fraction of RA in the lipid mixture. On the other hand, that of RA/SO and PC mixture was decreased up to $X_M = 0.05$ and remained constant at 43 mN m^{-1} over the range of X_M 0.05–0.95. These results suggest that RA/SO and PC were partially miscible in the bulk phases (PC bilayer and RA/SO liquid phases). The solubility of RA/SO in PC is evaluated from the inflectional point of spreading pressure, *a*, as the RA/SO mol fraction of approximately 0.05. The solubility of PC in the RA/SO was evaluated from the inflection point for the spreading pressure, *b*, as the PC mol fraction of approximately 0.05. The monolayer–bilayer phase equilibrium for SO and PC has been reported (Asai and Watanabe, 1998) and SO and PC were partially miscible in the bulk phases (PC bilayer and SO liquid phases). The solubilities of SO in PC and PC in SO were 3 and 3 mol%, respectively (Asai and Watanabe, 1998). Probably, the addition of SO to RA will change the miscibility of RA to PC and prevent RA from the formation of hexagonal phase.

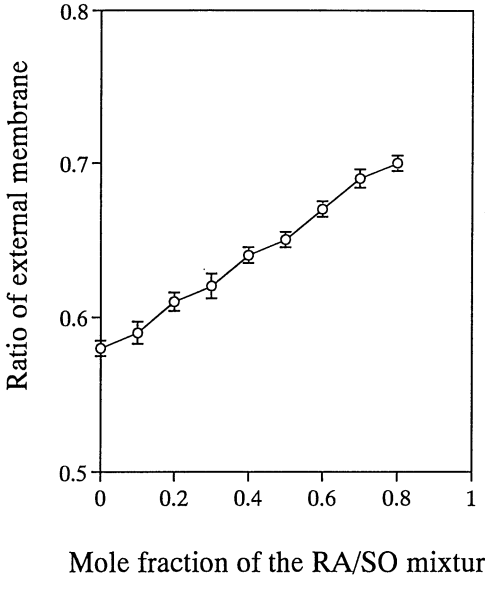

Fig. 4. Ratio of the external to total (external plus internal) membrane in the RA/SO and PC mixture detemmined by fluorescence quenching (probe: DSHA, quencher: Cu^{2+}, at 25°C) represented as a function of the mol fraction of RA/SO mixture (X_M). Each point represents the mean ± SEM of three samples.

3.4. Fluorescence quenching

In order to obtain the information of structural changes for the dispersed particles induced by the addition of RA and SO to PC bilayer other than determination of trapped volume, fluorescence quenching technique (probe: DSHA) was employed. The fluorescence characteristics of DSHA are known to be sensitive to the microenvironment around the probe, and the dansyl fluorophore is located in the vicinity of the glycerol backbone of the lipid bilayers (Iwamoto and Sunamoto, 1981). When the nonpenetrating fluorescence quencher $CuSO_4$ is added to RA/SO/PC dispersed particles, it only quenches the fluorescence of the DSHA in the outer aqueous phase. In the modified Stern-Volmer plot, the $I_0 \cdot [Q]/(I - I_0)$ versus $[Q]$ plots (the I values had been corrected for dilution) were linear. Fig. 4 shows the ratio of the external membrane to the total (external plus internal) (P) for RA/SO/PC dispersed particles as a function of X_M at 25°C. PC liposomes which served as a control had a P ratio of 0.58, which is in agreement with the molar ratio of PC molecules at the external membrane to total (external plus internal) surfaces of small unilamellar vesicles (Huang, 1969; Huang and Mason, 1978). The P value for the RA/SO/PC dispersed particles increased with increases in the X_M. These results suggest that an increase in X_M of the dispersed particles leads to a reduction in the fraction of PC which participates in the formation of the liposomal bilayers and an increase in the ratio of the PC monolayers for the formation of the emulsion particles.

3.5. Stability of sonicated particles in an aqueous medium

RA can be classified as a neutral lipid and forms monolayer with and without phospholipid.

A neutral lipid with limited solubility in PC bilayers (< 5 mol%) forms separate droplets of the excess neutral lipid. The droplets are covered with the PC monolayers in equilibrium with the bilayers and stabilized as emulsion particles in an aqueous medium. This kind of equilibrium has been observed in the dispersions composed of PC and triglyceride (Handa et al., 1990), ubiquinone-10 (Handa et al., 1991), α-tocopherol acetate (Asai and Watanabe, 1998), and SO (Asai and Watanabe, 1999). On the other hand, neutral lipids, such as diglyceride (Das and Rand, 1986; Seddon, 1990), monoglyceride (Nilsson et al., 1991), menaquinone-4 (Handa et al., 1992), and α-tocopherol (Nakajima et al., 1990; Yamamoto et al., 1994) have large solubility (> 20 mol%) in PC bilayers. The reversed topology of these phases causes rapid aggregation of dispersed particles and separation of the lipid mixture from the aqueous phase. The monolayer in equilibrium with the separate phase, containing a low fraction of PC, does not stabilize the lipidic phase as small particles in an aqueous medium (Handa et al., 1990, 1991). Up to $X_{RA} = 0.4$, the size of the RA/PC dispersed particles were increased to 240 nm and at $X_{RA} = 0.5$, the lipid mixture was not dispersed and an oil/water phase separation was observed. These results are caused by the formation of the reversed phase (hexagonal H_{II}) and rapid aggregation of the dispersed particles.

On the other hand, by the addition of SO to RA (molar ratio = 1:1), we could obtained the small dispersed particles (40–80 nm) in the range of X_M 0–0.8 and the oil/water phase separation was not observed. As mentioned above, SO has limited solubility in PC bilayer (5 mol%) and the addition of SO to RA will change the solubility of RA to PC bilayer (5 mol%, see Fig. 3). The RA/SO droplets will be covered with the PC monolayers in equilibrium with the bilayers and stabilized as emulsion particles in an aqueous medium.

However, when the PC content is less than the solubility in RA/SO (PC mol fraction less than about 0.05, see Fig. 3), the PC monolayer does not completely cover the hydrophobic RA/SO particle surfaces. At $X_M = 0.90$ or 0.95, separation into oil/water phases was observed after preparation, and the dispersions were not stable due to the lack of the surface monolayer of PC.

4. Conclusions

RA have appreciable solubility in PC bilayer (approximately 40 mol%) and the particle size in the dispersion was increased to 240 nm up to $X_{RA} = 0.4$. At $X_{RA} = 0.5$, the RA/PC mixture was difficult to disperse and the oil/water phase separation was observed. On the other hand, by the addition of soybean oil (SO) to RA (molar ratio of RA:SO = 1:1), the solubility of the lipid mixtures (RA and SO) in PC was decreased (approximately 5 mol%) and stable aqueous dispersions (diameter: 40–80 nm) were obtained in X_M range of 0.1–0.8. The solubility of the neutral lipid in PC bilayer is probably critically important to the production of the stably dispersed particles in aqueous media.

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