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Formation of the dispersed particles composed of retinol and phosphatidylchiline

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Abstract

The purpose of this study was to investigate the dispersal mechanism of retinol (Vitamin A, VA) into phospholipid. VA was dispersed with soybean phosphatidylcholine (PC) using sonication and the dispersal mechanism was evaluated by characterizing the dispersed particles using dynamic light scattering, fluorescence spectroscopy and surface monolayer techniques. The dispersions in the VA mole fraction range of 0.1–0.7 were stable at room temperature for 3 days. A limited amount of VA was incorporated into PC bilayer membranes (approximately 3 mol%). The excess VA separated from the PC bilayers was stabilized as emulsion particles by the PC surface monolayer. When the PC content was less than the solubility in VA (mole fraction of VA: more than 0.8), the PC monolayer did not completely cover the hydrophobic VA particle surfaces. In the case, the particle size increased drastically and the separation into oil/water occurred. The miscibility between VA and PC and the lipid composition were critically important for the stability of the dispersed particles (coexistence of emulsion particles (surface monolayer of PC + core of VA) with vesicular particles (bilayer)) of the lipid mixtures.

Keywords: Retinol; Soybean phosphatidylcholine; Miscibility; Stability; Monolayer–bilayer equilibrium

1. Introduction

The retinoids (Vitamin A and derivatives)—retinol (Vitamin A), retinal (Vitamin A aldehyde), and retinoic acid (Vitamin A acid)—form a homologous series of lipid soluble molecules that are essential for the maintenance of health of an organism (DeLuca and Shapiro, 1982). Although they vary in structure only at polar end, their biological function differs markedly. Retinol promotes reproduction, retinal is necessary for vision while retinoic acid, which does not support either of the two aforementioned process, is active in growth promotion. The amphiphilic nature of the retinoids suggest that they will locate membranes that may constitute a site of action. Indeed, retinal is, together with phospholipid and protein, a major component in visual receptor membranes.

A number of physical techniques, including nuclear magnetic resonance (Boeck and Zidovetzki, 1988), electron spin resonance, and fluorescence spectroscopy (Wassall et al., 1988) have been used to investigate the interaction of retinoids with phosphatidylcholine. These studies suggest that the influence of retinoic acid differs from retinol and retinal. Retinoic acid can be incorporated into the phospholipid up to 20 mol% and increases the membrane fluidity. On the other hand, the latter two retinoids have limited solubility in the membrane (less than 1 mol%) and does not affect the fluidity.

In this study, we focused on all-*trans*-retinol (Vitamin A, VA) in the retinoids. In order to clarify the

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interaction between VA and phospholipid, we prepared the dispersed particles of VA and soybean phosphatidylcholine (PC), VA/PC particles, by sonication and characterized them to investigate the dispersal mechanism using several physicochemical techniques. The structure of VA/PC particles was determined by dynamic light scattering (DLS), fluorescence quenching, and analysis of the trapped aqueous volume inside the particles. The miscibility and solubility of VA and PC were evaluated by surface monolayer techniques.

2. Materials and methods

2.1. Materials

All-*trans*-retinol (Vitamin A, VA) was purchased from Sigma Co., Ltd. (St. Louis, Missouri). VA was purified by alumina column chromatography. The solvent was gradually changed from petroleum ether to chloroform and the eluted fractions were assayed by TLC (solvent: chloroform/methanol = 95/5). Occasionally samples were analyzed by thin-layer chromatography to monitor stability and it was found that there was no appreciable alteration as a consequence of the experiment. Soybean phosphatidylcholine was purchased from Ajinomoto Co., Ltd. (Tokyo, Japan). Calcein (3,3′-bis[*N*,*N*-bis(carboxylmethyl)aminomethyl]-fluorescein) was from Dojin (Kumamoto, Japan). Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was 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 particles

VA and PC were dissolved in chloroform. After evaporation of the solvent, water was added to give a final combined concentration of VA and PC of 5 mM. The mixtures were sonicated for 30 min under a steam of nitrogen at 70 °C. A probe type sonicator, model UD-200 (Tomy Seiko Co., Ltd., Tokyo, Japan) was used at a power setting of 100 W. After cooled to room temperature, the dispersion was filled into ampoules and the nitrogen gas was filled into the empty head space.

2.2.2. Determination of particle size

DLS measurements of the sonicated dispersions of VA/PC particles were performed with a DLS-7000DL submicron analyzer (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

A dried mixture of VA and PC was 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 mole of PC was evaluated. The PC in the dispersion was assayed by the method of Bartelett (Bartelett, 1959).

2.2.4. Fluorescence quenching

Fluorescence quenching techniques were used to obtain an information on structural changes (ratio of the number of the molecules of external to total (external plus internal) membrane) in the VA/PC dispersed particles. Fluorescence quenching techniques have been previously described (Matsuzaki et al., 1991). In this study, $CuSO_4$ was used as a quencher for the DSHA fluorescence embedded in the lipid particles. VA/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 Stern–Volmer equation (Badley, 1976), one can estimate the exposed fraction of DSHA, P, so that

$$\frac{I_0[Q]}{I_0 - I} = \frac{1}{P}[Q] + \frac{1}{KP} \quad (1)$$

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

2.2.5. Measurements of collapse and spreading pressures

Monolayer–bilayer equilibrium of VA/PC mixtures and their miscibility were determined by measurements of collapse and spreading pressures. VA, PC, and VA/PC mixtures were dissolved in benzene as the spreading solvent. The solution was added with an Agala micrometer syringe onto the double-distilled water. After complete evaporation of the solvent, the surface pressures of the monolayers were measured by Whilhemy's method using a surface tensiometer (model CBVP-A3, Kyowa Kaimenkagaku Co., Ltd., Tokyo, Japan), and the surface pressure area per lipid molecule curve was obtained. The collapse pressures of the monolayer (surface pressures at the transition point from monolayer to bilayer or solid states) were determined from the inflection points on the curves. The spreading pressures of VA/PC mixtures at an air/water interface (surface pressures at the transition point from bilayer or solid states to monolayer) were obtained from a steady surface pressure value at 12–24 h after the addition of the lipid or the lipid mixture on water. Both the collapse and spreading pressures were determined at 25 °C. Details of the monolayer techniques were described elsewhere (Nakagaki et al., 1985).

3. Results and discussion

3.1. Stably dispersed particles of VA and PC mixtures

Fig. 1 shows the diameter of the dispersed particles as a function of VA mole fraction (X_{VA}). Separation of the dispersion to oil/water phases was not observed in the dispersions of VA and PC mixture in the range of $X_{VA} = 0$–0.7 at room temperature within 72 h after preparation. At $X_{VA} = 0.8$, the particle diameter was considerably larger at 180 nm, and the separation was observed at room temperature 72 h after preparation. At $X_{VA} = 0.9$, the particle diameter was 240 nm and the separation was observed at room temperature within 24 h after preparation. The chemical purity of VA was determined by HPLC and TLC method and no degradations were observed during the experiments.

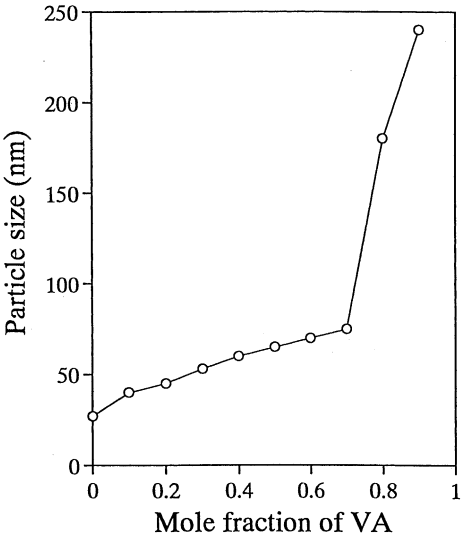

Fig. 1. Weight-averaged diameter of dispersed particles (just after preparation) represented as a function of X_{VA} in the mixture determined by DLS at 25 °C.

3.2. Aqueous space inside the dispersed particles

Fig. 2 shows the trapped volume of the particles per mole of PC at various X_{VA} values. The trapped

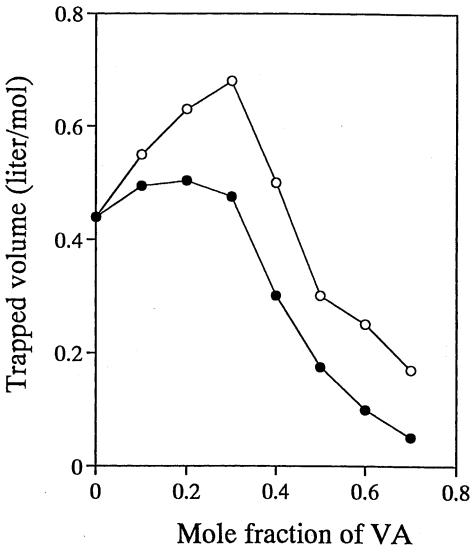

Fig. 2. Trapped aqueous volume inside the dispersed particles represented as a function of X_{VA} in the mixture. Volume of inner space of per mole of PC (○); volume of inner space of per total mole of the lipid (VA + PC) (●).

volumes of small unilamellar vesicles (diameter 20–50 nm), large unilamellar vesicles (200–1000 nm), and multilamellar vesicles (diameter 400–3000 nm) were estimated to be 0.2–0.5, 7–10, and 3–4 l mol^{-1}, respectively (Szoka and Papahadjopoulos, 1978). At $X_{VA} = 0$, small unilamellar PC vesicles (diameter 27 nm) had a trapped volume of 0.44 l mol^{-1}, which agrees with the reported value (Szoka and Papahadjopoulos, 1978). The trapped volume of the dispersed particles of VA/PC was the highest at $X_{VA} = 0.3$, and then decreased sharply above $X_{VA} = 0.4$. The trapped volume was also calculated on the basis of total moles of VA and PC, and is represented in Fig. 2. The drastic decrease in the trapped volume indicates that some structural change occurs in the dispersed particles as a result of addition of VA.

3.3. Fluorescence quenching

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 VA/PC particles, it only quenches the fluorescence of DSHA in the outer aqueous phase. In the modified Stern–Volmer plot, the $I_0[Q]/(I_0 - I)$ versus $[Q]$ plots (the I values had been corrected for dilution) were linear. Fig. 3 shows the ratio of the number of the molecules of the external to total (external plus internal) membrane (P) for VA/PC particles as a function of X_{VA}. PC liposomes (diameter 27 nm) that 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 and internal surfaces of small unilamellar vesicles (Huang, 1969; Huang and Mason, 1978). The P value for the VA/PC particles increased with increase in the X_{VA}. This result suggests that some structural changes occur in the dispersed particles by the addition of VA.

3.4. Collapse and spreading pressure of VA and PC mixtures

The nonolayer–bilayer equilibrium of VA/PC mixtures are estimated on the basis of the measurements of collapse and spreading pressures. The collapse pressure is considered as the transition surface pressure from the monolayer at the water surface to the bilayer, while spreading pressure is considered as the transition surface pressure form bilayer to monolayer (Handa et al., 1991) and has the same value as the collapse pressure. The collapse and spreading pressures of VA were consistent with each other (22.2 mN m^{-1}) and is good agreement with the reported value (23.0 mN m^{-1}, Loels and Shah, 1968). The collapse and spreading pressures of PC were also consistent with each other (47.0 mN m^{-1}), and the values agree with the reported collapse pressure of about 45.0 mN m^{-1} (Handa et al., 1991). The collapse and spreading pressures of a lipid generally have different values, and are dependent on the miscibility of the lipids in the monolayer and bulk phase (Defy et al., 1966).

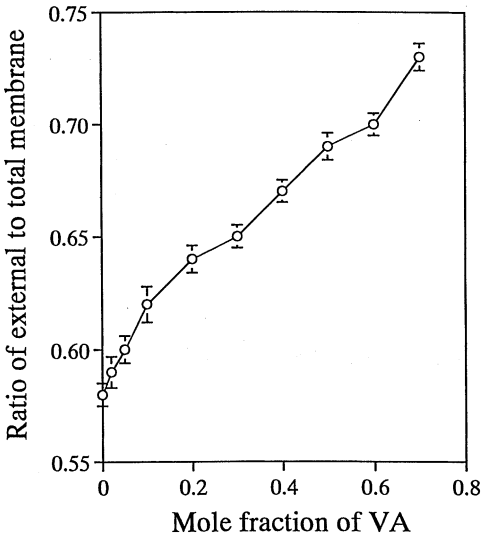

Fig. 3. Ratio of external to total (external plus internal) membrane in the lipid mixture determined by fluorescence quenching represented as a function of X_{VA} in the mixture. Each point represents the mean ± S.E.M. of three measurements.

The collapse and spreading pressures of VA/PC mixture at 25 °C were obtained as a function of X_{VA} and, therefore, provide a phase diagram for the monolayer (M)–PC bilayer (B)–VA solid (S) equilibrium, as shown in Fig. 4. The collapse pressure varies with X_{VA} in the mixed monolayer, whereas the spreading pressure was constant at 45.0 mN m^{-1} in the X_{VA} range of 0.03–0.97. On the basis of the surface phase rule (Defy et al., 1966), VA and PC were freely miscible in a mixed monolayer at an air/water interface (M),

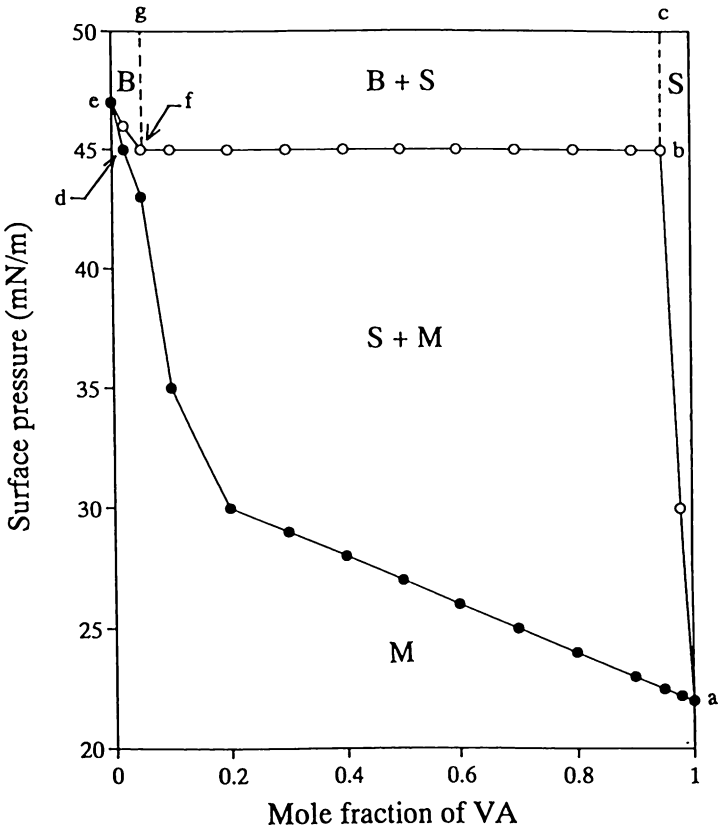

Fig. 4. Monolayer–bilayer equilibria of RP/PC mixture at 25 °C in the presence of water. Spreading pressure (○); collapse pressure (●). The solubilities of VA solid (*S*) in PC and PC in the RP solid (*S*) are evaluated from the inflection point for the spreading pressure, *f*, (VA mole fraction of approximately 0.03) and *b* (PC mole fraction of approximately 0.03), respectively.

but only partially miscible in the bulk phases, i.e. PC bilayers (*B*) and VA solids (*S*). The solubility of VA solid (*S*) in PC is evaluated from the inflectional point of spreading pressure, *f*, as the VA mole fraction of approximately 0.03. The solubility of PC in the VA solid (*S*) was evaluated from the inflection point for the spreading pressure, *b*, as the PC mole fraction of approximately 0.03. On the phase diagram in Fig. 4, a mixed monolayer exists in the region designed by *M*. Coexisting in the regions designated by $S + M$ and $B + S$ are VA solid and mixed monolayer and PC bilayers, and VA solids, respectively. On the horizontal line, *bf*, at surface pressure of 45.0 mN m^{-1}, the system consists of PC bilayers, *f*, which contain a limited amount (3%) of VA, and the VA solid phase, *b*, which contains about 3% PC. The mixed monolayer, *d*, which contain approximately 100% PC and has a surface pressure of 45.0 mN m^{-1}, is in equilibrium both with the bilayers, *f*, and the solid phase, *b*.

When the monolayer is formed on the surface of the VA-rich solid phase, *b*, the hydrophobic solid (emulsion particles), can be stably dispersed in water and coexists with the bilayers, *f* (liposomal vesicles).

3.5. Structural changes in the dispersed particles

The alterations in structure of the dispersed particles from the vesicular structure occur on the basis of the trapped volume and fluorescence quenching measurements. An increase in X_{VA} of the dispersed particles leads to a reduction in the fraction of PC, which participates in the formulation of the liposomal bilayers, and it is suggested that the PC monolayers take part in the formation and stabilization of dispersed particles

Table 1
Fraction of DPPC participating in the formation of vesicle bilayers (ξ_1) in VA/DPPC particles

Mole fraction of VA (X_{VA})	Trapped volume (v) (l mol^{-1} of DPPC)	ξ_1[a]	Ratio of external to total membrane (P) determined by fluorescence quenching	ξ_1[b]
0	0.44	1.0	0.58	1.0
0.1	0.55	–	0.62	0.89
0.2	0.63	–	0.64	0.83
0.3	0.68	–	0.65	0.78
0.4	0.50	–	0.67	0.71
0.5	0.30	0.68	0.69	0.62
0.6	0.25	0.56	0.70	0.54
0.7	0.17	0.39	0.73	0.36

[a] ξ_1 calculated by Eq. (2).
[b] ξ_1 calculated by Eq. (3).

in water. Handa et al. (Handa et al., 1991) reported that the fraction of PC that forms bilayer vesicles, ζ_1, can be calculated from the trapped volume, v, as follows:

$$\zeta_1 = \frac{v}{v_0} \tag{2}$$

here, v_0 is the trapped volume of small unilamellar vesicles ($v_0 = 0.44\,\mathrm{l\,mol^{-1}}$; see Table 1). The ζ_1 values calculated are presented in Table 1. The increased v values in the range of $X_{VA} = 0.1$–0.4 probably due to the increased size of the dispersed particles. The ζ_1 values larger than 1.0 in Table 1 do not necessarily show that all particles have a vesicular structure.

The fraction, ζ_1, is also calculated on the basis of the fluorescence quenching measurements (Fig. 3). The ζ_1 value is correlated with the ratio of external to total (external plus internal) membrane, P, in VA/PC particles (Handa et al., 1991).

$$\zeta_1 = \frac{1}{1-P_0}\left((1-P) - s\left(\frac{X_{VA}}{1-X_{VA}}\right)\right) \tag{3}$$

Here, P_0 is the ratio for the liposomal vesicles of PC and is 0.58; s is the solubility of PC in the separate solid phase of VA, equivalent to mole fraction of 0.03 as determined by spreading pressures (Fig. 4); $(1-P)$ is the fraction of PC that is inaccessible to the Cu^{2+} added to the outer aqueous phase of the dispersion; and $sX_{VA}/(1-X_{VA})$ is the fraction of PC solubilized in the separate VA phase.

As shown in Table 1, Eq. (3) gives ζ_1 values that are close to the values evaluated by the trapped volume method. A large percentage of PC molecules are found in structural formations other than bilayer vesicles and the RP separated from bilayers is stabilized by the PC monolayer as emulsion particles in aqueous media.

3.6. Formation of dispersion by lipid composition

VA can be classified as a neutral lipid and forms monolayer with and without phospholipid. Neutral lipids such as ubiquinone-10 (Handa et al., 1991), triglyceride (Handa et al., 1990; Hansrani et al., 1983), and α-tocopherol acetate (Asai and Watanabe, 1998) have limited solubility in phospholipid bilayer membranes, and form separate phases in aqueous media, which are stabilized by the closely packed phospholipid monolayer surrounding the phases (Handa et al., 1991; Miller and Small, 1983). Thus, the monolayer–bilayer equilibrium plays important roles in the structural formations of phospholipid-neutral mixtures in aqueous dispersions. Excess neutral lipid that separates from the PC bilayer membranes can be stably dispersed as small particles covered by PC monolayer.

When the PC content is less than the solubility in VA (PC mole fraction less than about 0.03; Fig. 4), the PC monolayer does not completely cover the hydrophobic VA particle surfaces. When $X_{VA} = 0.8$ or 0.9, probably due to the lack of the PC monolayer, the aggregation of the particles occurred and the particle size increased drastically ($\geqq$180 nm). Moreover, the separation into oil/water phases was observed after preparation and the dispersions were not stable. However, when the mole fraction of PC was higher, i.e. $X_{VA} = 0$–0.7, the PC monolayer covered the VA particles completely and stabilized the dispersion. When PC was excessive, the monolayer was in equilibrium with the PC bilayers (liposomes), and the particle surface had the maximum value: the spreading pressure of the bilayers. Therefore, the solubility between VA

and PC and the coexistence of emulsion and liposomal particles are critically important for the stabilization of the particles in water.

4. Conclusions

VA and PC were dispersed in water using sonication. The dispersions in VA mole fraction range of 0.1–0.7 were stable at room temperature for 3 days. A limited amount of VA was incorporated into PC bilayer membranes (approximately 3 mol%) and the excess VA separated from the PC bilayers was stabilized as emulsion particles by the PC surface monolayer. When the PC content was less than the solubility in VA ($X_{VA} \geqq 0.8$), PC monolayer did not completely cover the hydrophobic VA particle surfaces. In the case, the particle size increased drastically and the separation into oil/water occurred. The miscibility between VA and PC and the lipid composition were critically important for the stability of the dispersed particles (coexistence of emulsion particles (surface monolayer of PC + core of VA) with vesicular particles (bilayer)) of the lipid mixtures.

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