Formulation Research Laboratory, Kawashima, Eisai Co. Ltd., Gifu, Japan

# Structural differences in aqueous dispersions of α-tocopheryl acetate and phosphatidylcholine upon varying their molar fractions

Y. ASAI

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*Yasuyki Asai,PhD, Researcher, Formulation Research Laboratory, Kawashima, Eisai Co., Ltd., 1 Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 501–6195, Japan*
*y2-asai@hhc.eisai.co.jp*

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The purpose of this study was to investigate the structure of dispersed particles composed of α-tocopheryl acetate (TA) and phospholipid. TA 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 layer techniques. The dispersions in the TA mole fraction range of 0.1–0.7 were stable at room temperature for 3 days. A limited amount of TA was incorporated into PC bilayer membranes (approximately 5 mol%). The excess TA 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 TA (mole fraction of TA: more than 0.8), the PC monolayer did not completely cover the hydrophobic TA particle surfaces. In the case, the particle size increased drastically andseparation into oil and water occurred. The miscibility between TA 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 TA] with vesicular particles [bilayer]) of the lipid mixtures.

## 1. Introduction

α-Tocopherol is an indispensable lipid component of biological membranes. It has membrane-stabilizing properties due to its restriction of molecular mobility (Grams et al. 1972) and its ability to form complexes with unsaturated fatty acids (Erin et al. 1984). α-Tocopherol is an antioxidant which inhibits the peroxidation of membrane lipid (Niki 1987), and a deficiency of α-tocopherol in animal cells results in a change in the fluidity of the lipid membranes (Vajanamarhutue et al. 1979).

A number of physical techniques, including differential scanning calorimetry (DSC) (Massey et al. 1982), fluorescence spectroscopy (Schmidt et al. 1976), nuclear magnetic resonance (Perly et al. 1985, Wassall et al. 1986), and Fourier-transformed infrared spectroscopy (Villalain et al. 1986), have been used to investigate the interaction of α-tocopherol with phosphatidylcholine (PC). Some other lipids, such as diglyceride (Das and Rand 1986, Seddon 1990), monoglyceride (Nilsson et al. 1991), and menaquinone-4 (Handa et al. 1992), have appreciable solubility in PC bilayers. 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 a reversed cubic phase. α-Tocopherol has also been reported to induce a phase transition from the bilayer to hexagonal $H_{II}$ phase, but α-tocopheryl acetate (TA) does not induce this transition (Nakajima et al. 1990). It has been reported that the effect of TA on the fluidity and permeability of membranes is different from that of α-tocopherol (Villalain et al. 1986 Massay 2001).

In this study, in order to clarify the interaction between TA and phospholipid, we prepared dispersed particles of TA and soybean phosphatidylcholine (PC), (TA/PC particles) by sonication and characterized them to investigate the dispersal mechanism using several physicochemical techniques. The structure of TA/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 TA and PC were evaluated by surface monolayer techniques.

## 2. Investigations, results and discussion

Fig. 1 shows the mean diameter of the dispersed particles as a function of TA mole fraction ($X_{TA}$). Particle size distribution was single type. Separation of the dispersion to oil/water phases was not observed in the dispersions of TA and PC mixture in the range of $X_{TA} = 0–0.7$ at room temperature within 72 h after preparation. At $X_{TA} = 0.8$, the particle diameter was considerably larger at 160 nm, and the separation was observed at room temperature 72 h after preparation. At $X_{TA} = 0.9$, the particle diameter was 220 nm and the separation was observed at room temperature within 24 h after preparation

Fig. 2 shows the trapped volume of the particles per mol of PC at various $X_{TA}$ values. The trapped volumes of small unilamellar vesicles (diameter 20–50 nm), large unilamellar vesicles (200–1000 nm), and multilamellar vesicles (dia-

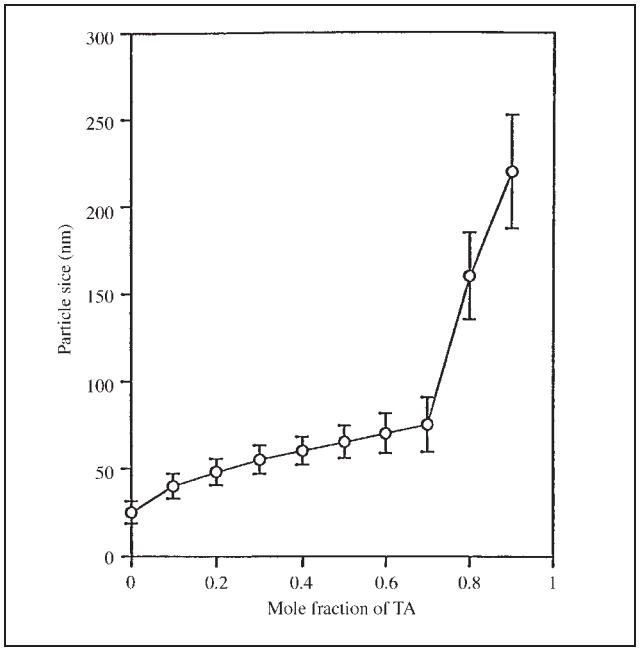


Fig. 1: Weight-averaged diameter of dispersed particles (just after preparation) represented as a function of $X_{TA}$ in the mixture determined by dynamic light scattering at 25 °C

meter 400–3000 nm) were estimated to be 0.2–0.5, 7–10, and 3–4 $L \cdot mol^{-1}$, respectively (Szoka and Papahadjopoulos 1978). At $X_{TA} = 0$, small unilamellar PC vesicles (diameter: 27 nm) had a trapped volume of 0.41 $L \cdot mol^{-1}$, which agrees with the reported value (Szoka and Papahadjopoulos 1978). The trapped volume of the dispersed particles of TA/PC was the highest at $X_{TA} = 0.3$, and then decreased sharply above $X_{TA} = 0.4$. The trapped volume was also calculated on the basis of total moles of TA and PC, and is presented 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 TA.

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 TA/PC particles, it only quenches the fluorescence of DSHA in the outer aqueous phase. In the modified Stern-Volmer plot, the $I_0 \cdot [Q]/(I_0 - I)$ vs [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 TA/PC particles as a function of $X_{TA}$. 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 TA/PC particles increased with increase in the $X_{TA}$. This result suggests that some structural changes occur in the dispersed particles by the addition of TA.

The monolayer-bilayer equilibrium of TA/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 the 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 TA were consistent with each other (22.2 mN/m). The collapse and spreading pressures of PC were also consistent with each other (47.0 mN/m), and the values agree with the reported collapse pressure of about 45.0 mN/m (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).

The collapse and spreading pressures of TA/PC mixture at 25 °C were obtained as a function of $X_{TA}$, and therefore, provide a phase diagram for the monolayer (M)-PC bi-

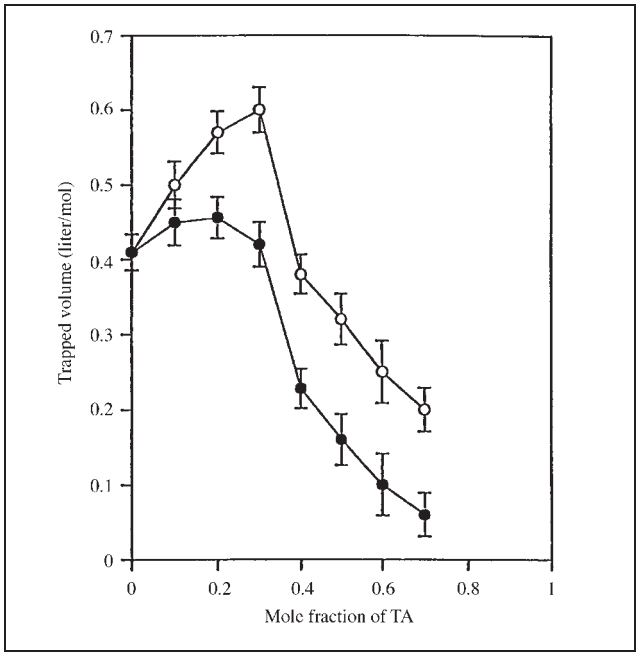


Fig. 2: Trapped aqueous volume inside of the dispersed particles represented as a function of $X_{TA}$ in the mixture. Volume of inner space of per mole of PC (○); volume of inner space of per total mole of the lipid (TA + PC) (●)

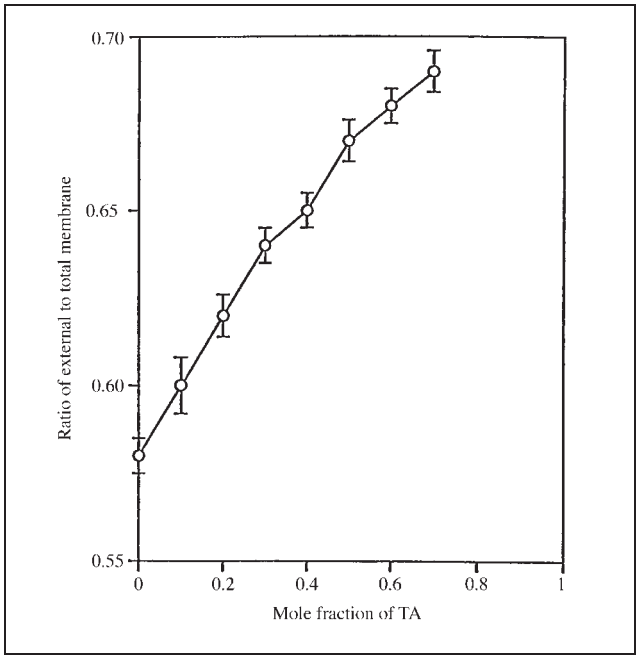


Fig. 3: Ratio of external to total (external plus internal) membrane in the lipid mixture determined by fluorescence quenching represented as a function of $X_{TA}$ in the mixture. Each point represents the mean ± S.D. of three measurements

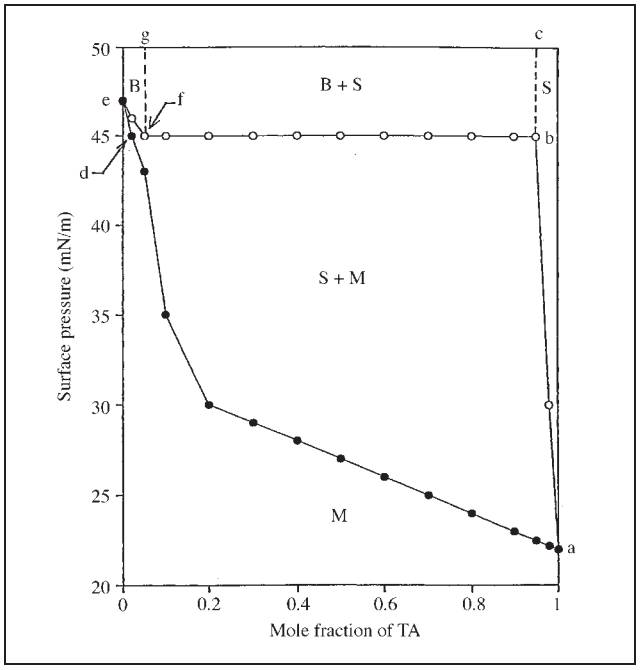


Fig. 4: Monolayer-bilayer equilibria of TA/PC mixture at 25 °C in the presence of water. Spreading pressure (○); collapse pressure (●). The solubilities of TA solid (S) in PC and PC in the TA solid (S) are evaluated from the inflection point for the spreading pressure, f, (TA mole fraction of approximately 0.05) and b (PC mole fraction of approximately 0.05), respectively

layer (B)-TA solid (S) equilibrium, as shown in Fig. 4. The collapse pressure varies with $X_{TA}$ in the mixed monolayer, wheres the spreading pressure was constant at 45.0 mN/m in the $X_{TA}$ range of 0.05–0.95. On the basis of the surface phase rule (Defy et al. 1966), TA and PC were freely miscible in a mixed monolayer at an air/water interface (M), but only partially miscible in the bulk phases, i.e., PC bilayers (B) and TA solids (S). The solubility of TA solid (S) in PC is evaluated from the inflectional point of spreading pressure, f, as the TA mole fraction of approximately 0.05. The solubility of PC in the TA solid (S) was evaluated from the inflection point for the spreading pressure, b, as the PC mole fraction of approximately 0.05. 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 TA solid and mixed monolayer and PC bilayers, and TA solids, respectively. On the horizontal line, bf, at surface pressure of 45.0 mN/m, the system consists of PC bilayers, f, which contain a limited amount (5%) of TA, and the TA solid phase, b, which contains about 5% PC. The mixed monolayer, d, which contain approximately 100% PC and has a surface pressure of 45.0 mN/m, is in equilibrium both with the bilayers, f, and the solid phase, b. When the monolayer is formed on the surface of the TA-rich solid phase, b, the hydrophobic solid (emulsion particles), can be stabely dispersed in water and coexists with the bilayers, f (liposomal vesicles).

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 $X_{TA}$ of the dispersed particles leads to a reduction in the fraction of PC, which participates in the formation of the liposomal bilayers, and it is suggested that the PC monolayers take part in the formation and stabilization of dispersed particles in water. Handa et al. (1991) reported that the fraction of PC that forms bilayer vesicles, $\xi_1$, can be calculated from the trapped volume, v, as follows:

$$\xi_1 = (v/v_0) \quad (1)$$

here, $v_0$ is the trapped volume of small unilamellar vesicles ($v_0 = 0.42 \cdot mol^{-1}$, see Table). The $\xi_1$ values calculated are presented in the Table. The increased v values in the range of $X_{TA} = 0.1–0.4$ probably due to the increased size of the dispersed particles. The $\xi_1$ values larger than 1.0 in the Table do not necessarily show that all particles have a vesicular structure.

The fraction, $\xi_1$, is also calculated on the basis of the fluorescence quenching measurements (Fig. 3). The $\xi_1$ value is correlated with the ratio of external to total (external plus internal) membrane, P, in TA/PC particles (Handa et al. 1991).

$$\xi_1 = [1/(1 - P_0)] \cdot [(1 - P) - s \cdot X_{TA}/(1 - X_{TA})] \quad (2)$$

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 TA, equivalent to mole fraction of 0.05 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 $s \cdot X_{TA}/(1-X_{TA})$ is the fraction of PC solubilized in the separate TA phase.

As shown in the Table, eq. (2) gives $\xi_1$ values that are close to the values evaluated by the trapped volume method. A large percentage of PC molecules is found in structural formations other than bilayer vesicles and the TA separated from bilayers is stabilized by the PC monolayer as emulsion particles in aqueous media.

TA can be classified as a neutral lipid and forms monolayer with and without phospholipid. Neutral lipids such as ubiquinone-10, and triglyceride (Handa et al. 1990; Hansrani et al. 1983) 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-

**Table: Fraction of PC participating in the formation of vesicle bilayers ($\xi_1$) in TA/PC particles**

| Mole fraction of TA ($X_{ta}$) | Trapped volume (v) ($L \cdot mol^{-1}$ of PC) | $\xi 1^*$ | Ration of external to total membrane (p) determined by fluorescence quenching | $\xi_1{}^{**}$ |
|---|---|---|---|---|
| 0 | 0.41 ± 0.024 | 1.0 | 0.58 ± 0.005 | 1.0 |
| 0.1 | 0.50 ± 0.031 | – | 0.60 ± 0.008 | 0.94 |
| 0.2 | 0.57 ± 0.028 | – | 0.62 ± 0.006 | 0.87 |
| 0.3 | 0.60 ± 0.036 | – | 0.64 ± 0.005 | 0.81 |
| 0.4 | 0.38 ± 0.026 | – | 0.65 ± 0.005 | 0.75 |
| 0.5 | 0.32 ± 0.034 | 0.78 | 0.67 ± 0.006 | 0.67 |
| 0.6 | 0.25 ± 0.041 | 0.59 | 0.68 ± 0.005 | 0.58 |
| 0.7 | 0.20 ± 0.029 | 0.49 | 0.69 ± 0.006 | 0.46 |

$\xi_1{}^*$ calculated by eq. (2); $\xi_1{}^{**}$ calculated by eq. (3).

bilayer equilibrium plays an important role in the structural formation 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 TA (PC mole fraction less than about 0.05, see Fig. 4), the PC monolayer does not completely cover the hydrophobic TA particle surfaces. When $X_{TA} = 0.8$ 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$160 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., $X_{TA} = 0–0.7$, the PC monolayer covered the TA 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 TA and PC and the coexistence of emulsion and liposomal particles are critically important for the stabilization of the particles in water.

## 3. Experimental

α-Tocopheryl acetate (TA) was purchased from Eisai Chemical Co., Ltd. (Ibaraki, Japan). 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) sulphate 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).

### *3.1. Preparation of dispersed particles*

TA and PC were dissolved in chloroform. After evaporation of the solvent, water was added to give a final combined concentration of TA and PC of 5 mM. The mixtures were sonicated for 30 min under a stream 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 nitrogen gas was filled into the empty head space.

### *3.2. Determination of particle size*

Dynamic light scattering (DLS) measurements of the sonicated dispersions of TA/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.

### *3.3. Determination of the trapped volume inside the dispersed particles*

A dried mixture of TA and PC was hydrated with a 70 mM calcein solution instead of water for the preparation of the dispersion. Entrapped 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 Bartlett (Bartlett 1959).

### *3.4. Fluorescence quenching*

Fluorescence quenching techniques were used to obtain the information on structural changes ratio of the number of the molecules of external to total (external plus internal) membrane in the TA/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. TA/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

$$I_0 \cdot [Q]/(I_0 - I) = (1/P) \cdot [Q] + 1/KP \quad (3)$$

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

### *3.5. Measurements of collapse and spreading pressures*

Monolayer-bilayer equilibrium of TA/PC mixtures and their miscibility were determined by measurements of collapse and spreading pressures. TA, PC and TA/PC mixtures were dissolved in benzene as the spreading solvent. The solution was added with an Agala micrometer syringe onto double-distilled water. After complete evaporation of the solvent, the surface pressures of the monolayers were measured by Wilhemy'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 TA/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 state surface pressure value 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).

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