Interaction of α-Tocopherol Acetate with Phosphatidylcholine and Their Formation of Small Dispersed Particles

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Stable aqueous dispersions of α-tocopherol acetate (α-TA) were obtained by cosonication with dipalmitoylphosphatidylcholine (DPPC) in the α-TA mole fraction range of 0.1—0.8. In order to clarify the dispersal mechanism, the dispersed particles were characterized and the interaction between α-TA and DPPC was investigated using several physicochemical techniques. Dynamic light scattering measurements showed that the diameter of the dispersed particles was 50—75 nm. The trapped aqueous volume inside the particles was determined fluorometrically using the aqueous space marker calcein. The trapped volume in the α-TA/DPPC particles decreased remarkably with the addition of α-TA into small unilamellar vesicles of DPPC. The decline in the fraction of vesicular particles was also confirmed by fluorescence quenching of *N*-dansylhexadecylamine in the DPPC membrane by the addition of the quencher $CuSO_4$. These results indicate that the excess α-TA separated from the DPPC bilayers is stabilized as emulsion particles by the DPPC surface monolayer. The monolayer–bilayer equilibrium of α-TA/DPPC mixtures was estimated by measurement of spreading and collapse pressures. The results showed that the coexistence of emulsion particles (surface monolayer of DPPC+core of α-TA) with vesicular particles (bilayer) was critically important for the formation of the stably dispersed particles of the lipid mixture.

Key words α-tocopherol acetate; dipalmitoylphosphatydylcholine; particle size; trapped volume; monolayer–bilayer equilibrium

α-Tocopherol is an indispensable lipid component of biological membranes. It has membrane-stabilizing properties due to its restriction of molecular mobility[1] and its ability to form complexes with unsaturated fatty acids.[2,3] α-Tocopherol is an antioxidant which inhibits the peroxidation of membrane lipids,[4] and a deficiency of α-tocopherol in animal cells results in a change in the fluidity of the lipid membranes.[5]

A number of physical techniques, including differential scanning calorimetry (DSC),[6] fluorescent spectroscopy,[7] nuclear magnetic resonance,[8,9] and fourier transform-infrared spectroscopy,[10] have been used to investigate the interaction of α-tocopherol with phosphatidylcholine (PC). Some other neutral lipids, such as diglyceride,[11,12] monoglyceride,[13] and menaquione-4,[14] 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 reversed cubic phase. α-Tocopherol has also been reported to induce a phase transition from the bilayer to a hexagonal H_{II} phase, but α-tocopherol acetate (α-TA) does not induce this transition.[15] It has also been reported that the effect of α-TA on the fluidity and permeability of membranes is different from that of α-tocopherol.[16]

In this study, in order to clarify the interaction between α-TA and PC, we prepared dispersed particles of α-TA and dipalmitoylphosphatidylcholine (DPPC) by cosonication and characterized them to investigate the dispersal mechanism using several physicochemical techniques. The structure of α-TA/DPPC particles was investigated by dynamic light scattering (DLS), fluorescence quenching and analysis of the trapped aqueous volume inside the particles. The miscibility and solubility of α-TA and DPPC were evaluated by DSC and surface monolayer techniques.

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Experimental

Materials α-TA was supplied by Eisai Co., Ltd. (Tokyo, Japan). L-α-DPPC, copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and calcein (3,3′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein) were purchased from Wako Chemical Co., Ltd. (Osaka, Japan). *N*-Dansylhexadecylamine (DSHA) was from Lambda Co., Ltd. (Graz, Austria).

Preparation of Dispersed Particle α-TA and DPPC were dissolved in chloroform. After evaporation of the solvent, water was added to give a final combined concentration of α-TA and DPPC 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.

Determination of Particle Sizes DLS measurements of the sonicated dispersions of a-TA and DPPC 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,[17] and the weight averaged particle sizes were evaluated.

Determination of the Trapped Volume inside the Dispersed Particles A dried mixture of α-TA and DPPC 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[18] after solubilization of the lipid particles by the addition of 10% Triton X-100, and the aqueous volume trapped per mole of DPPC was evaluated. The DPPC in the dispersion was assayed by the method of Bartlett.[19]

Fluorescence Quenching Fluorescence quenching techniques were used to obtain information on structural changes (ratio of external to total [external plus internal] membrane) in the α-TA/DPPC dispersed particles. Fluorescence quenching techniques have been previously described by Matsuzaki *et al.*[20] In this study, $CuSO_4$ was used as a quencher for the DSHA fluorescence embedded in the lipid particles. α-TA/DPPC 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,[21] one can estimate the exposed fraction of DSHA P, so that

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

where, I_0 is fluorescence intensity in the absence of the quencher, I the intensity afterquenching by Cu^{2+}, $[Q]$ the concentration of Cu^{2+} and K the

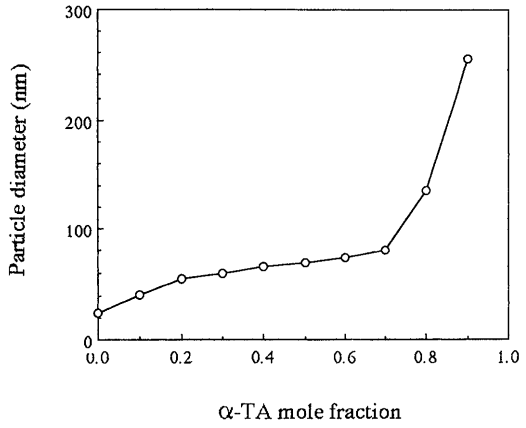

Fig. 1. Weight-Averaged Diameter of Dispersed Particles Represented as a Function of Mole Fraction of α-TA (X_{TA}) in the Mixture Determined by DLS

Stern–Volmer constant.

Solubility of α-TA in DPPC Membranes and of DPPC in α-TA In order to investigate the solubility of α-TA in DPPC membranes and of DPPC in α-TA, differential scanning calorimetry (DSC) was performed using a Model DSC-100 (Seiko-Denshi Co., Ltd., Tokyo, Japan). α-TA/DPPC mixtures (total 1.5×10^{-6} mol) in 40 μl of water were placed in a DSC pan and sealed. An equal volume of water was placed in the reference pan. Temperature scans were made from 10 °C to 70 °C with constant heating rates of 2 °C/min. All calorimetric data was obtained from samples during the heating phase.

Measurements of Collapse and Spreading Pressures α-TA, DPPC and a-TA/DPPC mixtures were dissolved in benzene as the spreading solvent. The solution was added with an Agla micrometer syringe onto 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 a surface pressurse–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 α-TA/DPPC 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 the surface pressure at 12—24 h after the addition of the lipid or lipid mixture on water. Both the collapse and spreading pressures were determined at 25 °C. Details of the monolayer techniques have been described elsewhere.[22,23]

Results

Stably Dispersed Particles of α-TA and DPPC Mixtures Figure 1 shows the diameter of the dispersed particles as a function of the α-TA mole fraction (X_{TA}). Figure 2 shows the DLS histograms of the particles of $X_{TA}=0.5$ and 0.8, and the weight-averaged diameters were 69.3 nm and 134.9 nm, respectively. Separation of the dispersion to oil/water phases was not observed in the dispersions of the α-TA and DPPC mixture in the range of $X_{TA}=0$—0.8 within 72 h after preparation. At $X_{TA}=0.9$, the particle diameter was considerably larger at 253 nm, and separation was observed 72 h after preparation. At $X_{TA}=0.95$, the particle diameter was 290 nm and the separation was detected within 24 h after preparation.

Aqueous Space inside the Dispersed Particles Figure 3 shows the trapped volume of the particles per mole of DPPC at various X_{TA}. 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 to 0.5, 7 to 10, and 3 to 4 liter·mole^{-1}, respectively.[24] At $X_{TA}=0$,

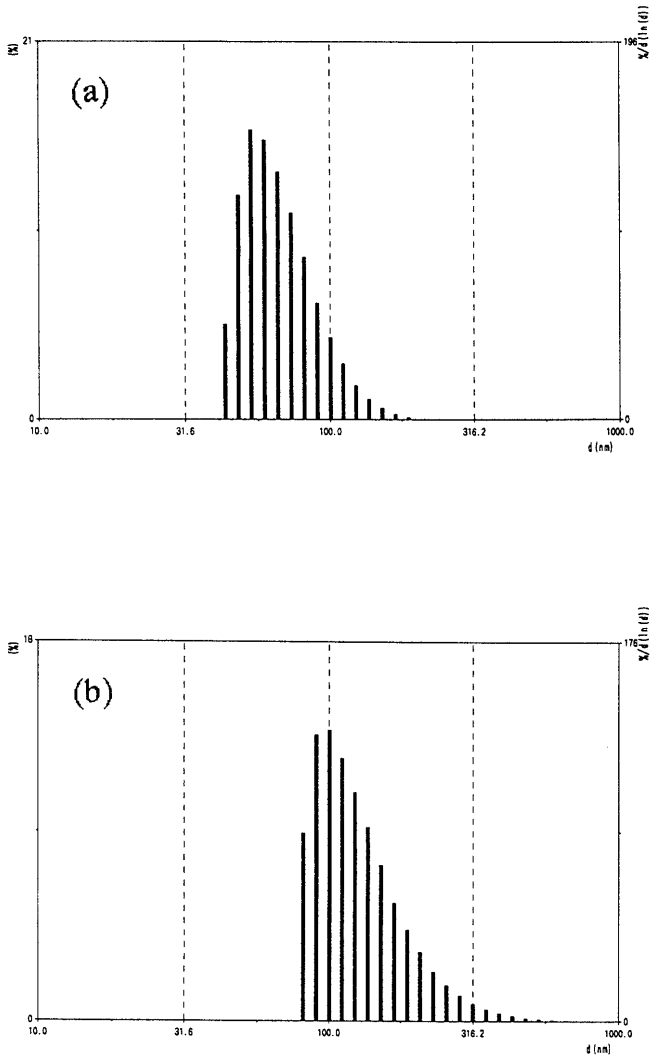

Fig. 2. Histograms of Weight-Averaged Particle Size Evaluated from DLS Data for the Dispersion α-TA/DPPC Mixture

Mole fraction of α-TA (X_{TA}) in the mixture: (a) $X_{TA}=0.5$, particle size: 69.3±28.5 nm (mean±SD), (b) $X_{TA}=0.8$, particle size: 134.9±59.2 nm (mean±S.D.).

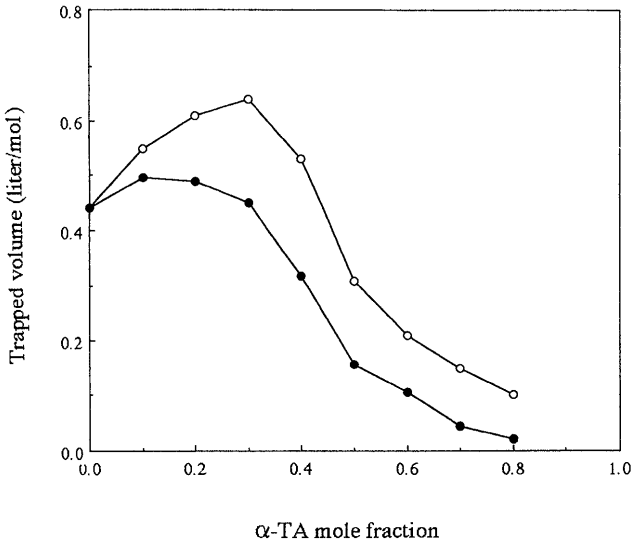

Fig. 3. Trapped Aqueous Volume Inside the Dispersed Particles Represented as a Function of the Mole Fraction of α-TA (X_{TA}) in the Mixture.

Volume of inner space per mole of DPPC (○—○), volume inner space of per total mole of (α-TA+DPPC) (●—●).

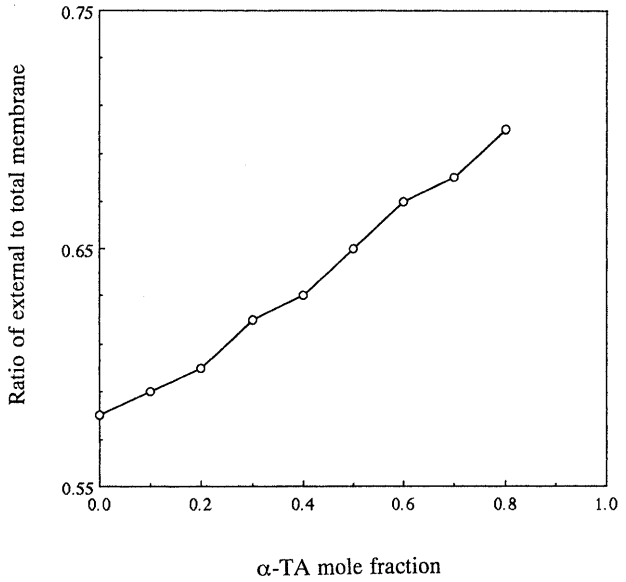

Fig. 4. Ratio of the External to Total (External Plus Internal) Membrane (p) in the Lipid Mixture Determined by Fluorescence Quenching Represented as a Function of the Mole Fraction of α-TA (X_{TA}) in the Mixture

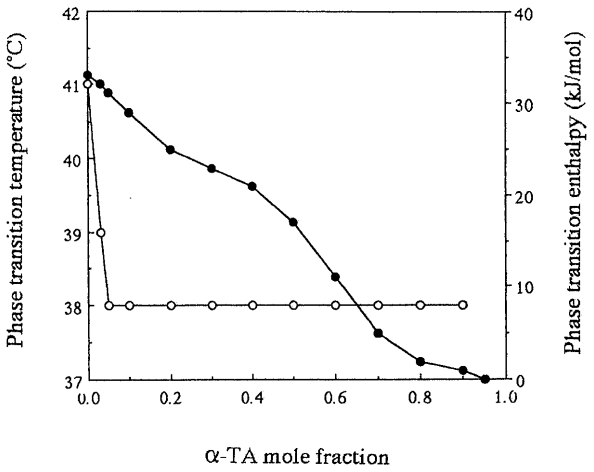

Fig. 5. Phase Transition Temperature and the Enthalpy Represented as a Function of the Mole Fraction of α-TA (X_{TA}) in the Mixture Determined by DSC

Phase transition temperature (○—○), phase transition enthalpy (●—●).

small unilamellar DPPC vesicles (diameter: 22.7 nm) had a trapped volume of 0.44 liter·mole^{-1}, which agrees with the reported value. The trapped volume of the dispersed particles of the α-TA/DPPC mixture was highest at $X_{TA}=0.3$, then decreased sharply above $X_{TA}=0.4$. The trapped volume was also calculated on the basis of total moles of α-TA and DPPC, and is represented in the same figure. The dramatic drop in the trapped volume indicates that some structural change occurs in the dispersed particles as a result of the addition of α-TA.

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.[25] When the nonpenetrating fluorescence quencher $CuSO_4$ is added to α-TA/DPPC dispersed particles, it only quenches the fluorescence of the DSHA in the outer aqueous phase. In the modified Stern–Volmer plot, the $I_0 \cdot [Q]/(I-I_0)$ *vs.* $[Q]$ plots (the I values had been corrected for dilution) were linear. Figure 4 shows the ratio of the external membrane to the total (external plus internal) (P) for a-TA/DPPC dispersed particles as a function of X_{TA}. DPPC 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.[26,27] The P value for the α-TA/DPPC dispersed particles increased with increases in the X_{TA}. These results suggest structural changes in the dispersed particles by the addition of α-TA.

Solubility of α-TA in DPPC Membranes and of DPPC in α-TA Figure 5 represents the solubility of α-TA in DPPC membranes determined by DSC. The addition of α-TA decreased the phase transition temperature, and at X_{TA} values higher than 0.05, the phase transition temperature was constant at 38 °C. This indicates that the solubility of α-TA in the DPPC membrane was equivalent to a mole fraction of 0.05. The phase transition enthalpy decreased with an increase in X_{TA}, and the phase transition was abolished at $X_{TA}=0.95$. This indicates that at $X_{TA}=0.95$, DPPC was completely incorporated in α-TA and that the solubility of α-TA in the DPPC membrane was equivalent to a mole fraction of 0.05.

Collapse and Spreading Pressures of α-TA and DPPC Mixtures The monolayer–bilayer equilibrium of α-TA/DPPC 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 from bilayer to monolayer,[22] and has the same value as the collapse pressure. The collapse and spreading pressures of α-TA were consistent with each other (18 mN/m), and the values agree with the reported collapse pressure of about 19 mN/m.[28] 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.[29] The collapse and spreading pressures of a lipid mixture generally have different values, and are dependent on the miscibility of the lipids in the monolayer and bulk phase.[23,30]

The collapse and spreading pressures of the α-TA/DPPC mixture were obtained as a function of X_{TA}, and therefore, give a phase diagram for the monolayer (M)–DPPC bilayer (B)–α-TA solids (S) equilibrium, as shown in Fig. 6. The collapse pressure varies with X_{TA} in the mixed monolayer, while 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,[23,31] it is found that α-TA and DPPC are freely miscible in a mixed monolayer at an air/water interface (M), but only partially miscible in the bulk phases, *i.e.* DPPC bilayers (B) and α-TA solids (S). The solubility of α-TA solid (S) in DPPC is evaluated from the inflectional point of spreading pressure, f, as the α-TA mole fraction of approximately 0.05. The solubility of DPPC in the α-TA solid (S) was evaluated from the inflection point for the spreading pressure, b, as the DPPC mole fraction of approximately 0.05. These findings agree with the limited solubility of α-TA in DPPC bilayer membranes and of DPPC in α-TA as de-

termined by DSC.

On the phase diagram in Fig. 6, a mixed monolayer exists in the region designated by M. Coexisting in the regions designated by S+M, and B+S are an α-TA solid and mixed monolayer and DPPC bilayers and α-TA solids, respectively. On the horizontal line, *bf*, at a surface pressure of 45.0 mN/m, the system consists of DPPC bilayers, *f*, which contain a limited amount (5%) of α-TA, and the α-TA solid phase, *b*, which contains about 5% DPPC. The mixed monolayer, *d*, which contains approximately 100% DPPC 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 α-TA rich solid phase, *b*, the hydrophobic solid can be stably dispersed in water and coexists with the bilayers, *f* (liposomal vesicles).

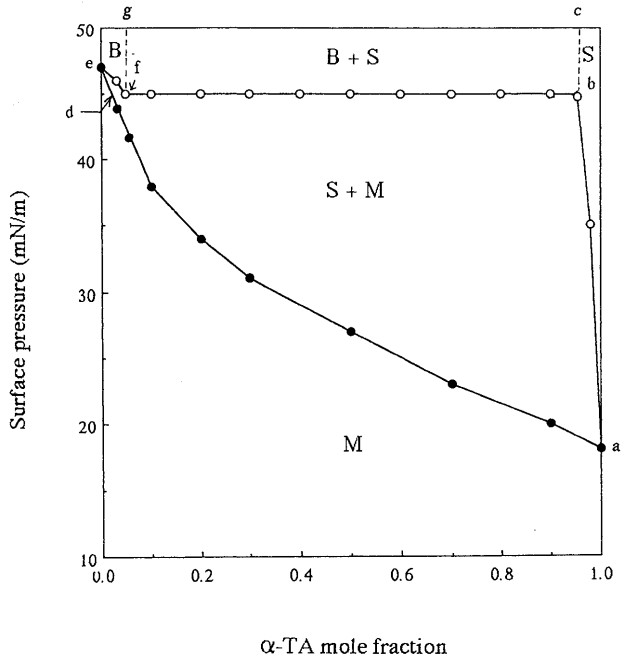

Fig. 6 Monolayer–Bilayer Equilibria of the α-TA/DPPC Mixture in the Presence of Water

Spreading pressure (○—○), collapse pressure (●—●). The spreading pressure of the mixture is presented by the line *abfe*. The collapse pressure of the mixture is presented by the line *adg*. The stable dispersion containing an excessive amount of α-TA is represented by the line *bfd* (surface pressure=45.0 mN/m), where the emulsion particles (combination of monolayer, *d*, and α-TA core, *b*) coexist with the bilayer, *f*. Without the bilayer, the monolayer has a lower surface pressure and a decreased effect on the stabilization of the emulsion.

Discussion

DPPC and a Neutral Lipid, α-TA α-TA can be classified as a neutral lipid[15] and forms monolayers with and without phospholipids.[28] Neutral lipids have limited solubility in phospholipid bilayer membranes,[32,33] and form separate phases in aqueous media, which are stabilized by the closely packed phospholipid monolayer surrounding the phases.[30,34] This kind of equilibrium has been observed in the dispersions composed of PC and ubiquinone-10[29] or triglyceride.[32,35] The monolayer–bilayer equilibrium, thus, plays an important role in the structural formation of phospholipid–neutral lipid mixtures in aqueous dispersions. Excess neutral lipid which separates from the PC bilayer membranes can be stably dispersed as small particles. On the other hand, neutral lipids, such as α-tocopherol,[15] diglyceride,[11,12] monoglyceride[13] and menaquinone-4[14] have appreciable solubility in phopholipid bilayers. The addition of these lipids to the bilayers changes the hydrophilic–lipophilic balance and induces a phase transition from a bilayer to a hexagonal H_{II} or reversed cubic phase.

Structural Changes in the Dispersed Particles It is presented that the alterations in the 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 DPPC which participates in the formation of the liposomal bilayers, and it is suggested that the DPPC monolayers take part in the formation and stabilization of dispersed particles in water. Handa *et al.*[29] reported that the fraction of DPPC which forms bilayer vesicles, ξ_1, may 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=0.44 liter·mole^{-1}, see in Table 1). The ξ_1 values calculated are presented in Table 1. The increased values of *v* in the range of X_{TA}=0.1—0.4 are probably due to the increased size of the dispersed particles as a result of the increased X_{TA}.

The fraction, ξ_1, is also calculated on the basis of the fluorescence quenching measurements (Fig. 4). The ξ_1 value is correlated with the ratio of external to total (external plus internal) membrane, *p*, in α-TA/DPPC dispersed particles.[29]

$$\xi_1=[1/(1-p_0)]\cdot[(1-p)-s\cdot X_{TA}/(1-X_{TA})] \quad (2)$$

Here, p_0 is the ratio of the liposomal vesicles of DPPC and is

Table I. Fraction of DPPC Participating in the Formation of Vesicle Bilayers (ξ_1)

α-TA mole fraction (X_{TA})	Trapped volume (*v*) [liter·mole^{-1} of PC]	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	—	0.59	0.96
0.2	0.61	—	0.60	0.92
0.3	0.64	—	0.62	0.85
0.4	0.53	—	0.63	0.80
0.5	0.31	0.70	0.65	0.71
0.6	0.21	0.47	0.67	0.61
0.7	0.15	0.34	0.68	0.48
0.8	0.10	0.23	0.70	0.23

a) Calculated by eq.1. *b)* Calculated by eq. 2.

0.58. s is the solubility of DPPC in the separate solid phase of α-TA, equivalent to a mole fraction of 0.05 as determined by DSC (Fig. 5) and spreading pressures (Fig. 6). $(1-p)$ is the fraction of DPPC, which is inaccessible to the Cu^{2+} added to the outer aqueous phase of the dispersion. $s \cdot X_{TA}/(1-X_{TA})$ is the fraction of DPPC solubilized in the separate α-TA phase.

As seen in Table 1, eq. 2 give ξ_1 values which are close to the values evaluated by the trapped volume method. A large percentage of DPPC molecules are found in structural formations other than bilayer vesicles, and the α-TA separated from the bilayers is stabilized by the DPPC monolayer as emulsion particles in aqueous media.

Stability of Dispersion and Lipid Composition When the DPPC content is less than the solubility in α-TA (DPPC mole fraction less than about 0.05, see Figs. 5 and 6), the DPPC monolayer does not completely cover the hydrophobic α-TA particle surfaces. When $X_{TA}=0.90$ or 0.95, separation into oil/water phases was observed after preparation, and the dispersions were not stable. However, when the mole fraction of DPPC was higher, *i.e.* $X_{TA}=0$—0.8, the PC monolayer completely covers the α-TA particles and stabilizes the dispersion. When DPPC is excessive, the monolayer is in equilibrium with the DPPC bilayers (liposomes), and the particle surface has a maximum value: the spreading pressure of the bilayers. Therefore, the coexistence of emulsion and liposomal particles are critically important for stabilization of the particles in water.

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