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Research paper

Formation and structure of stably dispersed particles composed of retinal with dipalmitoylphosphatidylcholine: coexistence of emulsion particles with bilayer vesicles

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Abstract

In order to develop an intravenous formulation of all-*trans*-retinal (vitamin A aldehyde, VAA) for the treatment of night blindness, VAA and dipalmitoylphosphatidylcholine (DPPC) were sonicated and the dispersions in the VAA mole fraction range of 0.1–0.7 were stable at room temperature for 3 days. In order to clarify the dispersal mechanism, the dispersed particles were characterized and the interaction between VAA and DPPC was investigated using several physicochemical techniques. Dynamic light scattering measurements showed that the diameter of the dispersed particles was 50–70 nm. A limited amount of VAA is incorporated into DPPC bilayer membranes (approximately 5 mole%). The trapped aqueous volume inside the particles was determined fluorometrically using the aqueous space marker calcein and the volume in the VAA/DPPC particles was decreased remarkably with the addition of VAA 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 VAA separated from the DPPC bilayers is stabilized as emulsion particles by the DPPC surface monolayer. The monolayer–bilayer equilibrium of VAA/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 VAA) with vesicular particles (bilayer) was critically important for the formation of the stably dispersed particles of the lipid mixture.

Keywords: Retinal; Vitamin A aldehyde; Dipalmitoylphosphatidylcholine; Dispersion; Structure; Monolayer–bilayer equilibrium

1. Introduction

All-*trans* retinal (vitamin A aldehyde, VAA, Fig. 1) is a major component in visual receptor membranes. A deficiency of VAA results in night blindness [1]. Owing to their polyenic structure, VAA is virtually insoluble in water and chemically labile, which makes their manipulation difficult. In order to overcome and to develop an injectable formulation of VAA for the treatment of the night blindness, we dispersed the VAA with dipalmitoylphosphatidylcholine (DPPC) using sonication. For the formation of the dispersion, it will be important to obtain the information on the interaction of VAA with DPPC. VAA is classified as a neutral lipid and the interaction of VAA with phospholipids have been reported. A number of physical techniques, including nuclear magnetic resonance [2], electron spin resonance and fluorescent spectroscopy [3] have been used to investigate the interaction of the VAA with phosphatidylcholine (PC). These studies suggests that VAA have limited solubility in the membrane (less than 1 mole%) and does not affect the fluidity.

Some neutral lipids, such as diglyceride [4], monoglyceride [5], menaquinone-4 [6], and α-tocopherol [7] have appreciable solubility in PC bilayers (>20 mole%) 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 [8,9], ubiquinone-10 [10], α-tocopherol acetate [11] and retinyl palmitate [12] have low solubilities in PC bilayers (<5 mole%) 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 mono-

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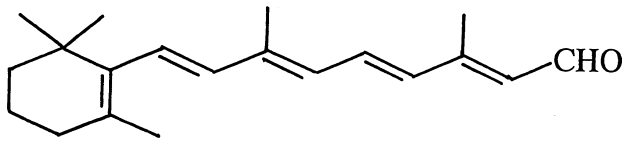

Fig. 1. Structure of all-*trans* retinal.

layers of the droplets are in equilibrium with the bilayer [9,10].

In this study, for the development of the injectable formulation of VAA, we dispersed the VAA with DPPC by sonication and characterized the dispersed particles using several physicochemical techniques. The structure of VAA/DPPC particles was determined by dynamic light scattering, fluorescence quenching and analysis of the trapped aqueous volume inside the particles. The miscibility and solubility of VAA and DPPC were evaluated by differential scanning calorimetry (DSC)and surface monolayer techniques.

2. Materials and methods

2.1. Materials

All-*trans*-retinal (vitamin A aldehyde, VAA) and L-α-dipalmitoylphosphatidyl -choline (DPPC) were purchased from Sigma Co., Ltd. (St. Louis, MO).

Copper (II) sulfate pentahydrate ($CuSO_4(5H_2O)$ was purchased from Wako Pure Industrial Ltd. (Osaka, Japan). Calcein (3,3′-bis[N,N-bis(carboxymethyl)aminomethyl]-fluorescein) were from Dojin. (Kumamoto, Japan). *N*-Dansylhexadecylamine (DSHA) was from Lambda Co., Ltd. (Graz, Austria).

2.2. Preparation of dispersed particles

VAA and DPPC were dissolved in chloroform. After evaporation of the solvent, water was added to give a final combined concentration of VAA and DPPC of 5 mM. The mixtures were sonicated for 30 min under a stream of nitrogen gas 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.

2.3. Determination of particle sizes

Dynamic light scattering (DLS) measurements of the sonicated dispersions of VAA and DPPC were performed with a DLS-7000 DL submicron-analyzer (Ohtsuka Electronics Co. Ltd., Osaka, Japan) at 25°C. The data were analyzed by the histogram method [13], and the weight averaged particle sizes were evaluated.

2.4. Determination of the trapped volume inside the dispersed particles

A dried mixture of VAA 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 [14] 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 [15].

2.5. Fluorescence quenching

Fluorescence quenching techniques were used to obtain information on structural changes (ratio of external to total (external plus internal) membrane) in the VAA/DPPC dispersed particles. Fluorescence quenching techniques have been previously described [16]. In this study, $CuSO_4$ was used as a quencher for the DSHA fluorescence embedded in the lipid particles. The VAA/DPPC dispersed particles containing 1 mole% 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 [17], 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.

2.6. Solubility of VAA in DPPC membrane and of DPPC in VAA

In order to determine the miscibility of VAA and DPPC for the formation of the dispersed particles, the solubility of VAA in DPPC membrane and of DPPC in VAA, differential scanning calorimetry (DSC) was performed using a Model DSC-100 (Seiko-Denshi Co. Ltd., Tokyo, Japan). The VAA/DPPC mixtures (total 1.5×10^{-6} mole) 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 to 70°C with constant heating rates of 2°C/min. All calorimetric data was obtained from samples during the heating phase.

2.7. Measurements of collapse and spreading pressures

VAA, DPPC and VA/DPPC mixtures were dissolved in benzene as the spreading solvent. The solution was added with an Agla micrometer syringe onto double-distilled

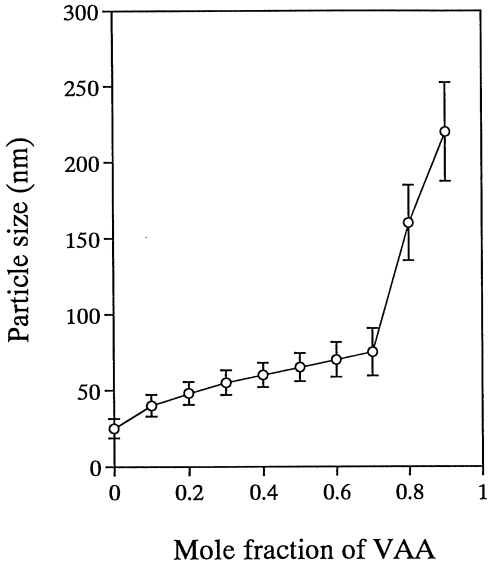

Fig. 2. Weight-averaged diameter of dispersed particles represented as a function of mole fraction of VAA (X_{VAA}) in the mixture determined by dynamic light scattering (DLS). Each point represents the mean diameter ± SEM of the measurement.

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 a surface pressures–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 VAA/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 [18,19].

3. Results

3.1. Size and stability of the dispersed particles from VAA and DPPC mixtures

Fig. 2 shows that the diameter of the dispersed particles obtained by these methods is represented as a function of the VAA mole fraction (X_{VAA}). Separation of the dispersion to oil/water phases was not observed in the dispersions of the VAA and DPPC mixture in the range of $X_{VAA} = 0$–0.7 within 72 h after preparation. At $X_{VAA} = 0.8$, the particle diameter was considerably larger at 160 nm, and separation was observed 72 h after preparation. At $X_{VAA} = 0.9$, the particle diameter was 220 nm and the separation was detected within 24 h after preparation.

3.2. Aqueous space inside the dispersed particles

Fig. 3 shows the trapped volume of the particles per mole of DPPC at various X_{VAA}. The trapped volumes of small unilamellar vesicles (diameter 20–50 nm), large unilamellar vesicles (diameters 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, respectively [20]. At $X_{VAA} = 0$, small unilamellar DPPC vesicles (diameter: 25 nm) had a trapped volume of 0.41 l/mol^{-1}, which agrees with the reported value. The trapped volume of the dispersed particles of the VAA/DPPC mixture was highest at $X_{VAA} = 0.3$, then decreased sharply above $X_{VAA} = 0.4$. The trapped volume was also calculated on the basis of total moles of VAA 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 VAA.

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 [21]. When the nonpenetrating fluorescence quencher $CuSO_4$ is added to VAA/DPPC dispersed particles, it only quenches the fluorescence of the DSHA in the outer aqueous phase. In the

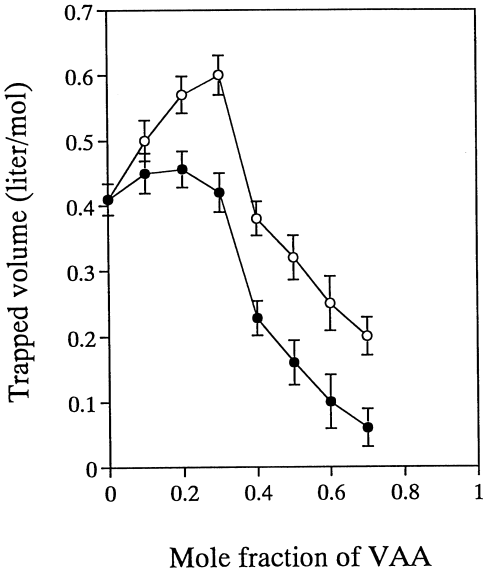

Fig. 3. Trapped aqueous volume inside of the dispersed particles represented as a function of mole fraction of VAA (X_{VAA}) in the mixture. Volume of inner space of per mole of DPPC (○- - - - ○), volume of inner space of per total mole of the lipid (VAA + DPPC) (●- - - - ●). Each point represents the mean ± SEM of the three measurements.

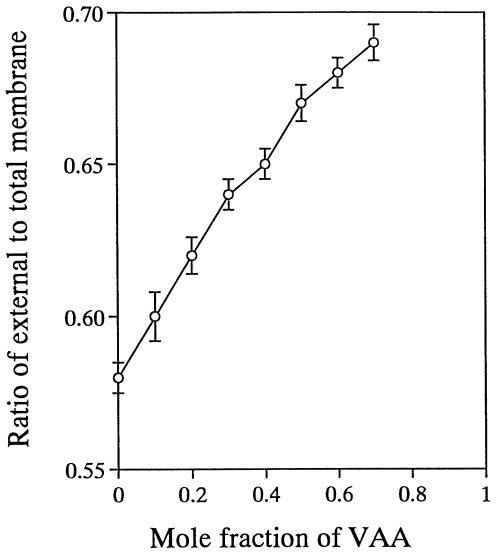

Fig. 4. Ratio of the external to total (external plus internal) membrane in the lipid mixture determined by fluorescence quenching represented as a function of the mole fraction of VAA (X_{VAA}) in the mixture. Each point represents the mean ± SEM of the three measurements.

modified Stern–Volmer plot, the $I_0([Q]/(I - I_0)$ vs. [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 VAA/DPPC dispersed particles as a function of X_{VAA}. The 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 [22,23]. The P value for the VAA/DPPC dispersed particles increased with increases in the X_{VAA}. These results suggest structural changes in the dispersed particles by the addition of VAA.

3.4. Solubility of VAA in DPPC membrane and of DPPC in VAA

Fig. 5 shows the solubilities of VAA in DPPC membrane and of DPPC in VAA were determined by differential scanning calorimetry (DSC). The addition of VAA decreased the phase transition temperature, and at X_{VAA} values higher than 0.05, the phase transition temperature was constant at 38°C (Fig. 4a). This indicates that the solubility of VAA in the DPPC membrane was equivalent to a mole fraction of 0.05. The phase transition enthalpy decreased with an increase in X_{VAA}, and the phase transition was abolished at $X_{VAA} = 0.95$ (Fig. 4b). This indicates that at $X_{VAA} = 0.95$, DPPC was completely incorporated in VAA and that the solubility of VAA in the DPPC membrane was equivalent to a mole fraction of 0.05.

3.5. Collapse and spreading pressures of VAA and DPPC mixtures

The monolayer–bilayer equilibrium of VAA/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 [10] and has the same value as

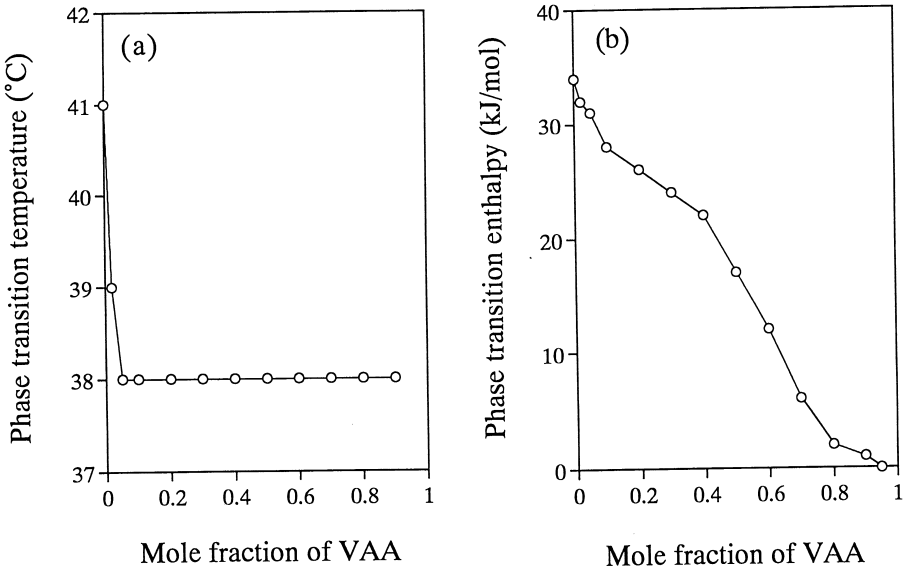

Fig. 5. (a) Phase transition temperature represented as a function of mole fraction of VAA (X_{VAA}) in the mixture determined by differential scanning calorimetry (DSC). (b) Phase transition enthalpy represented as a function of mole fraction of VAA (X_{VAA}) in the mixture determined by differential scanning calorimetry (DSC).

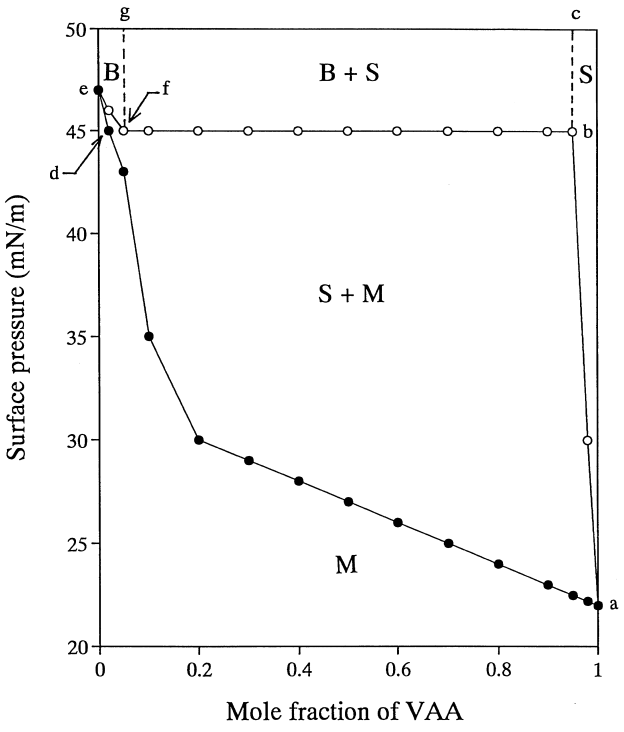

Fig. 6. Monolayer–bilayer equilibria of the VAA/DPPC mixture in the presence of water at 25°C. Spreading pressure (○- - - -○), collapse pressure (●- - - -●). The spreading pressure of the mixture is presented by the line *abfe*. The collapse pressure of the mixture is presented by the line *adg*. The stable dispersion containing an excessive amount of VAA is represented by the line *bfd* (surface pressure = 45.0 mN/m), where the emulsion particles (combination of monolayer, *d*, and VAA 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.

the collapse pressure. The collapse and spreading pressures of VAA 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 [10]. 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 [9,19].

The collapse and spreading pressures of the VAA/DPPC mixture were obtained as a function of X_{VAA}, and therefore, give a phase diagram for the monolayer (M) – DPPC bilayer (B) – VAA solids (S) equilibrium, as shown in Fig. 6 The collapse pressure varies with X_{VAA} in the mixed monolayer, while the spreading pressure was constant at 45.0 mN/m in the X_{VAA} range of 0.05–0.95. On the basis of the surface phase rule [19,24] it is found that VAA 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 VAA solids (S). The solubility of VAA solid (S) in DPPC is evaluated from the inflectional point of spreading pressure, *f*, as the VAA mole fraction of approximately 0.05. The solubility of DPPC in the VAA 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 VAA in DPPC bilayer membranes and of DPPC in VAA as determined 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 VAA solid and mixed monolayer and DPPC bilayers and VAA 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 VAA, and the VAA 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 VAA rich solid phase, *b*, the hydrophobic solid can be stably dispersed in water and coexists with the bilayers, *f* (liposomal vesicles).

4. Discussion

4.1. 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

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

Mole fraction of VAA (X_{VAA})	Trapped volume (v) (l/mole of DPPC]	ξ_1 [a]	Ratio of external to total membrane (p) determined by fluorescence quenching	ξ_1 [b]
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

[a] Calculated by Eq. (2).
[b] Calculated by Eq. (3).

basis of the trapped volume and fluorescence quenching measurements. An increase in X_{VAA} 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. [10] reported that the fraction of DPPC which forms bilayer vesicles, ξ_1, may be calculated from the trapped volume, v, as follows

$$\xi_1 = (v/v_0) \quad (2)$$

Here, v_0 is the trapped volume of small unilamellar vesicles ($v_0 = 0.41$ l/mol^{-1}, see Table 1). The ξ_1 values calculated are presented in Table 1. The increased values of v in the range of $X_{VAA} = 0.1$–0.4 are probably due to the increased size of the dispersed particles as a result of the increased X_{VAA}.

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 VAA/DPPC dispersed particles [10].

$$\xi_1 = [1/(1 - p_0)] \cdot [(1 - p) - s\cdot_{XVAA}/(1 - X_{VAA})] \quad (3)$$

Here, p_0 is the ratio of the liposomal vesicles of DPPC and is 0.58. Where s is the solubility of DPPC in the separate solid phase of VAA, equivalent to a mole fraction of 0.05 as determined by DSC (Fig. 5). $(1 - p)$ is the fraction of DPPC, which is inaccessible to the Cu^{2+} added to the outer aqueous phase of the dispersion. $s(X_{VAA}/(1 - X_{VAA})$ is the fraction of DPPC solubilized in the separate VAA phase.

As seen in Table 1, Eq. 3 gives 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 VAA separated from the bilayers is stabilized by the DPPC monolayer as emulsion particles in aqueous media.

4.2. Stability of dispersion and lipid composition

When the DPPC content is less than the solubility in VAA (DPPC mole fraction less than about 0.05, see Figs. 5 and 6), the DPPC monolayer does not completely cover the hydrophobic VA particle surfaces and separation into oil/water phases was observed after preparation. On the other hand, when the mole fraction of DPPC was higher, i.e. $X_{VAA} = 0$–0.7, the PC monolayer completely covers the VAA particles and stabilizes the dispersion. When DPPC is excessive, the monolayer is in equilibrium with the DPPC bilayers (liposomes), and surface pressure of the monolayer at the particle surface has the maximum value: the spreading pressure of the bilayer [9,10]. Therefore, the coexistence of emulsion and liposomal particles is critically important for the stabilization of the particles in water.

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