Formation of the Dispersed Particles Composed of Soybean Oil and Phosphatidylcholine

Yasuyuki ASAI

*Formulation Research Laboratory, Kawashima, Eisai Co., Ltd.
(1 Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 501-6195, JAPAN)*

Edited by K. Tajima, Kanagawa Univ., and accepted February 20, 2003 (received for review December 24, 2002)

Abstract: The purpose of this study was to investigate the dispersal mechanism of soybean oil (SO) into phospholipid as a fat emulsion. SO 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 SO mole fraction range of 0.1–0.7 were stable at room temperature for 3 days. A limited amount of SO was incorporated into PC bilayer membranes (approximately 5 mol%). The excess SO 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 SO (mole fraction of SO: more than 0.8), the PC monolayer did not completely cover the hydrophobic SO particle surfaces. In the case, the particle size increased drastically and the separation into oil/water occurred. Therefore, the solubility between SO and PC and the coexistence of emulsion and liposomal particles are critically important for the stabilization of the particles in water.

Key words: soybean oil, soybean phosphatidylcholine, miscibility, stability, monolayer-bilayer equilibrium

1 Introduction

Soybean oil have been used to incorporate lipophilic drug substances (1) and dispersed with lecithins (phosphatides) from egg yolk or soybean as emulsions for o/w emulsions. However, little attention has been given to the fundamental properties of submicromized emulsions stabilized with lecithins (phosphatides), especially for parental use. Schuberth and Wrethind (2) studied various emulsifying agents for fat emulsions, at a constant concentration of egg yolk phosphatides (1.2%), and Benita *et al.* (3) reported the effect of concentration of phosphatides and non-ionic surfactant such as polyoxyethylene-polyoxypropylene block copolymers (Pluronic F68) on the stability of emulsions prepared with egg yolk phosphatides. Further investigation of emulsions stabilized with lecithins will be necessary to

obtain information such as the mechanism of stabilization and formation of the emulsion and characterization of the emulsion particles.

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

*Correspondence to: Yasuyuki ASAI, Formulation Research Laboratory, Kawashima, Eisai Co., Ltd., 1 Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 501-6195, JAPAN
E-mail: y2-asai@hhc.eisai.co.jp

2 Experimental

2.1 Materials

Soybean oil (SO) was purchased from Sigma Co., Ltd. (St Louis, MO). Soybean phosphatidylcholine was purchased from Ajinomoto Co., Ltd. (Tokyo, Japan). Calcein (3,3'-bis[*N,N*-bis (carboxymethyl)aminomethyl]-fluorescein) was from Dojin. (Kumamoto, Japan). Copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was purchased from Wako Pure Industrial Ltd. (Osaka, Japan). *N*-Dansylhexadecylamine (DSHA) was from Lambda Co., Ltd. (Graz, Austria).

2.2 Preparation of Dispersed Particles

SO and PC were dissolved in chloroform. After evaporation of the solvent, water was added to give a final combined concentration of SO 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.

2.3 Determination of Particle Size

Dynamic light scattering (DLS) measurements of the sonicated dispersions of SO/PC particles were performed with a DLS-7000DL submicron analyzer (Oh-tsuka Electronics Co., Ltd., Osaka, Japan) at 25°C. The data were analyzed by the histogram method (4), and the weight averaged particle sizes were evaluated.

2.4 Determination of the Trapped Volume inside the Dispersed Particles

A dried mixture of SO 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 (5) 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 (6).

2.5 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 SO/PC dispersed particles. Fluorescence quenching techniques have been previously described (7). In this study, CuSO_4 was used as a quencher for the DSHA fluorescence embedded in the lipid particles. 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 Stern-Volmer equation (8), one can estimate the exposed fraction of DSHA, P , so that

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

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.

2.6 Measurements of Collapse and Spreading Pressures

Monolayer-bilayer equilibrium of SO/PC mixtures and their miscibility were determined by measurements of collapse and spreading pressures. SO, PC and SO/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 evaporation of the solvent, the surface pressures of the monolayers were measured by Whilhemmy'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 SO/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 hr 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 (9).

3 Results and Discussion

3.1 Dispersed Particles of SO and PC Mixtures

Figure 1 shows the mean diameter of the dispersed particles as a function of SO mole fraction (X_{SO}). Separation of the dispersion to oil/water phases was not observed in the dispersions of SO and PC mixture in the range of $X_{SO} = 0$ -0.7 at room temperature within 72 hr after preparation. At $X_{SO} = 0.8$, the particle diameter was considerably larger at 131 nm, and the separation was observed at room temperature 72 hr after preparation. At $X_{SO} = 0.9$, the particle diameter was 230 nm and the separation was observed at room temperature within 24 hr after preparation.

3.2 Aqueous Space inside the Dispersed Particles

Figure 2 shows the trapped volume of the particles per mole of PC at various X_{SO} values. The trapped 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 liter·mole⁻¹, respectively (10). At $X_{SO} = 0$, small unilamellar PC

vesicles (diameter: 27 nm) had a trapped volume of 0.44 liter·mole⁻¹, which agrees with the reported value (10). The trapped volume of the dispersed particles of SO/PC was the highest at $X_{SO} = 0.3$, and then decreased sharply above $X_{SO} = 0.4$. The trapped volume was also calculated on the basis of total moles of SO 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 SO.

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 (11). When the nonpenetrating fluorescence quencher CuSO₄ is added to SO/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. **Figure 3** shows the ratio of the number of the molecules of the external to total (external plus internal) membrane (P) for SO/PC particles as a function of X_{SO} . PC liposomes (diameter 25 nm) that served as a control

Fig. 1 Weight-Averaged Diameter of Dispersed Particles (just after preparation) Represented as a Function of X_{SO} in the Mixture Determined by Dynamic Light Scattering at 25°C.

Fig. 2 Trapped Aqueous Volume Inside of the Dispersed Particles Represented as a Function of X_{SO} in the Mixture. Volume of inner space of per mole of PC (○); volume of inner space of per total mole of the lipid (SO+PC) (●).

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 (12, 13). The P value for the SO/PC particles increased with increase in the X_{SO} . This result suggests that some structural changes occur in the dispersed particles by the addition of SO.

3.4 Collapse and Spreading Pressure of SO and PC Mixtures

The monolayer-bilayer equilibrium of SO/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 from bilayer to monolayer (14) and has the same value as the collapse pressure. The collapse and spreading pressures of SO were consistent with each other (13.0 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 (14). The collapse and spreading pressures of a lipid generally have

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_{SO} in the Mixture. Each point represents the mean $\pm$ SD (standard deviation) of three measurements.

different values, and are dependent on the miscibility of the lipids in the monolayer and bulk phase (15).

The collapse and spreading pressures of SO/PC mixture at 25°C were obtained as a function of X_{SO} , and therefore, provide a phase diagram for the monolayer (M)-PC bilayer (B)-SO solid (S) equilibrium, as shown in Fig. 4. The collapse pressure varies with X_{SO} in the mixed monolayer, whereas the spreading pressure was constant at 45.0 mN/m in the X_{SO} range of 0.05-0.95. On the basis of the surface phase rule (15), SO 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 SO solids (S). The solubility of SO solid (S) in PC is evaluated from the inflectional point of spreading pressure, f , as the SO

Fig. 4 Monolayer-Bilayer Equilibria of SO/PC Mixture at 25°C in the Presence of Water. Spreading pressure ($\circ$); collapse pressure ($\bullet$). The solubilities of SO solid (S) in PC and PC in the SO solid (S) are evaluated from the inflection point for the spreading pressure, f , (SO mole fraction of approximately 0.05) and b (PC mole fraction of approximately 0.05), respectively.

mole fraction of approximately 0.05. The solubility of PC in the SO 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 SO solid and mixed monolayer and PC bilayers, and SO 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 SO, and the SO 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 SO-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_{SO} 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 in water. Handa *et al.* (14) reported that the fraction of PC that forms bilayer vesicles, ξ_1 , can be calculated from the

trapped volume, *v*, as follows:

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

here, v_0 is the trapped volume of small unilamellar vesicles ($v_0=0.44$ liter·mole⁻¹, see **Table 1**). The ξ_1 values calculated are presented in **Table 1**. The increased *v* values in the range of $X_{SO}=0.1-0.4$ probably due to the increased size of the dispersed particles. The ξ_1 values larger than 1.0 in **Table 1** do not necessarily show that all particles have a vesicular structure.

The fraction, ξ_1 , is also calculated on the basis of the fluorescence quenching measurements (**Fig. 3**). The ξ_1 value is correlated with the ratio of external to total (external plus internal) membrane, *P*, in SO/PC particles (14).

$$\xi_1 = [1/(1-P_0)] \cdot [(1-P) - s \cdot X_{SO}/(1-X_{SO})] \quad \dots\dots\dots(3)$$

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 SO, 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 $s \cdot X_{SO}/(1-X_{SO})$ is the fraction of PC solubilized in the separate SO phase.

As shown in **Table 1**, Eq. (3) gives ξ_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 SO separated from bilayers is stabilized by the PC monolayer as emulsion particles in aqueous media.

Table 1 Fraction of DPPC Participating in the Formation of Vesicle Bilayers (ξ_1).

SO mole fraction (X_{SO})	Trapped volume (<i>v</i>) [liter·mole ⁻¹ of PC]	ξ_1^*	Ratio of outer total membrane(<i>p</i>) determined by fluorescence quenching	ξ_1^{**}
0	0.44	1.0	0.58	1.0
0.1	0.55	-	0.60	0.94
0.2	0.59	-	0.61	0.90
0.3	0.63	-	0.63	0.85
0.4	0.57	-	0.64	0.78
0.5	0.36	0.82	0.65	0.71
0.6	0.25	0.57	0.67	0.61
0.7	0.19	0.43	0.68	0.48
0.8	0.12	0.27	0.70	0.23

*calculated by eq.1; **calculated by eq.2.

3·6 Formation of Dispersion by Lipid Composition

SO can be classified as a neutral lipid and forms monolayer with and without phospholipid. Neutral lipids such as ubiquinone-10 (14), and triglyceride (16, 17) 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 (17, 18). Thus, the monolayer-bilayer equilibrium plays important roles in the structural formations of phospholipid-neutral lipid 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 SO (PC mole fraction less than about 0.05, see Fig. 4), the PC monolayer does not completely cover the hydrophobic SO particle surfaces. When $X_{SO} = 0.8$ or 0.9, probably due to the lack of the PC monolayer, the aggregation of the particles occurred and the particle size increased drastically (≥ 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., $X_{SO} = 0-0.7$, the PC monolayer covered the SO 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 SO and PC and the coexistence of emulsion and liposomal particles are critically important for the stabilization of the particles in water.

4 Conclusions

SO and PC were dispersed in water using sonication. The dispersions in SO mole fraction range of 0.1-0.7 were stable at room temperature for 3 days. A limited amount of SO was incorporated into PC bilayer membranes (approximately 5 mol%) and the excess SO 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 SO ($X_{SO} \geq 0.8$), PC monolayer did not completely cover the hydrophobic SO particle surfaces. In the case, the particle size increased drastically and the separation into oil/water

occurred. The miscibility between SO 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 SO] with vesicular particles [bilayer]) of the lipid mixtures.

References

1. S. LJUNBERG and R. JEPPSON, Intravenous Injection of Lipid Soluble Drugs, a Preliminary Report, *Acta Pharm. Suec.*, Vol. **54**, 435-440 (1970).
2. O. SCHBERTH and A. WRETHINED, Intravenous Infusion of Fat Emulsion, Phosphatides and Emulsifying Agents, *Acta Chiripida Scandinavica*, Vol. **278** (Supp), 3-21 (1961).
3. S. BENITA, D. FRIEDMAN and M. WEINSTOCK, Pharmacological Evaluation of an Injectable Prolonged Release Emulsion of Physostigmine in Rabbits, *J. Pharm. Pharmacol.*, Vol. **38**, 653-658 (1986).
4. E. GULARI, E. GULAR, Y. TSUNASIMA and E. CUE, Photon Correlation Spectroscopy of Particle Distributions, *J. Chem. Phys.*, Vol. **70**, 3965-3972 (1979).
5. T.M. ALLEN and L.G. CLELAND, Serum-Induced Leakage of Liposome Contents, *Biochim. Biophys. Acta*, Vol. **597**, 418-436 (1980).
6. G.R. BARTELETT, Phosphorus Assay in Column Chromatography, *J. Biol. Chem.*, Vol. **234**, 466-468 (1959).
7. K. MATSUZAKI, Y. TAKAISHI, T. FUJITA and K. MIYAJIMA, Hepelcin A, an α -aminoisobutyric Acid Containing Antibiotic Peptide, Induced Fusion of Egg Yolk L- α -phosphatidylcholine Small Unilamellar Vesicles, *Colloid and Polym. Sci.*, Vol. **269**, 604-611 (1991).
8. R.A. BADELEY, *Modern fluorescence spectroscopy*, Vol. **2**, (EL WEHRY, ed.), Plenum Press, New York, 112-119 (1976).
9. M. NAKAGAKI, K. TOMITA and T. HANDA, Interaction of Differently Oriented Lipids in Monolayer: Mixed Monolayers of 16-(9-anthroxyloxy) Palmitic Acid with Phosphatidylcholine and Cholesterol, *Biochemistry*, Vol. **24**, 4619-4624 (1985).
10. F.Jr. SZOKA and D. PAPAHAZIOPOULOS, Procedure for Preparation of Liposomes with Large Internal Aqueous Space and High Capture by Reverse-Phase Evaporation, *Proc. Natl. Acad. Sci. USA*, Vol. **75**, 4194-4198 (1978).
11. K. IWAMOTO and J. SUNAMOTO, Fluorescence Depolarization Studies on N-dansylhexadecylamine in Liposomal Bilayers, *Bull. Chem. Soc. Jpn.*, Vol. **54**, 399-403 (1981).
12. C.H. HUANG, Studies on Phosphatidylcholine Vesicles. Formation and Physical Characteristics, *Biochemistry*, Vol. **8**, 344-351 (1969).
13. C.H. HUANG and J.T. MASON, Geometric Packing Contains in Egg Phosphatidylcholine Vesicles, *Proc. Natl. Acad. Sci. USA*, Vol. **75**, 308-310 (1978).
14. T. HANDA, Y. ASAI, K. MIYAJIMA, Y. KAWASHIMA, M. KAYANO, K. IDA and T. IKEUCHI, Formation and Structure of Stably Dispersed Small Particles Composed of Phosphatidyl-

- choline and Ubiquinone-10: A pool of Ubiquinone-10 Separated from Lipid Bilayers, *J. Colloid Interface Sci.*, Vol. **143**, 205-213 (1991).
15. R. DEFY, I. PRIGOGINE, A. BELLMANS and D.E. EVERETT, *Surface Tension and Adsorption*, Longmans, Green, London, pp.71-84 (1966).
16. T. HANDA, H. SAITO, K. MIYAJIMA, Phospholipid Monolayers at the Triolein-Saline Interface: Production of Microemulsion Particles and Conversion of Monolayers to Bilayers, *Biochemistry*, Vol. **29**, 2884-2890 (1990).
17. P.K. HANSRANI, S.S. DAVIS and M. GROVES, The Preparation and Properties of the Sterile Intravenous Emulsions, *J. Parenter. Sci. Technol.*, Vol. **37**, 145-150 (1983).
18. K.M. MILLER and B.M. SMALL, Triolein-Cholesteryl Olate-Cholesterol-Lecithin Emulsions: Structural Models of Triglyceride-Rich Lipoproteins, *Biochemistry*, Vol. **22**, 443-451 (1983).
-