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SHORT COMMUNICATION

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Formation and stability of dispersed particles composed of vitamin K_1, soybean oil and phosphatidylcholine

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Abstract The purpose of this study was to develop an intravenous formulation composed of vitamin K_1 (VK) for the treatment of blood coagulation with warfarin-induced hypoprothrombinaemia. VK was dispersed using sonication with soybean phosphatidylcholine (PC) and the dispersion mechanism was evaluated by characterizing the dispersed particles with dynamic light scattering, fluorescence spectroscopy and surface monolayer techniques. VK has an appreciable solubility in PC bilayers (approximately 20 mol%). Within the VK molar fraction of 0.1–0.9, the size of the dispersed particles increased at room temperature within 3 months. By addition of soybean oil (SO) to VK (molar ratio of VK:SO = 1:1), the solubility of the VK/SO mixtures in PC bilayers was decreased (approximately 5 mol%). The size of the aqueous dispersions at molar fractions of 0.1–0.7 was 50–70 nm and did not change for 3 months at room temperature. The solubility of the VK and VK/SO in PC bilayers is crucially important in the production of the stable aqueous dispersions of VK particles.

Key words Vitamin K_1 · Phosphatidylcholine · Soybean oil · Solubility · Stability

Introduction

Vitamin K_1 (VK) has been found in plants as a component of electron transport chains of chloroplasts participating in the photoreduction steps of the photosynthetic process [1]. VK is known to have "vitamin K activity" when fed to vitamin K deficient animals [2]. "Vitamin K activity" in animals is thought to be mainly the activation of a carboxylase which gives modification of protrombin, blood clotting factor and other proteins in plasma and tissues [3].

It was reported that VK associated with liposomes, administered orally, enhanced the recovery of blood coagulation in rabbits with warfarin-induced hypoprothrombinaemia [4]. In addition, VK was solubilized by bile salts (sodium deoxycholate, sodium cholate and their corresponding glycine conjugates) as mixed micelles for oral administration [5]. However, very little attention has been given to VK as an intravenous formulation. In this study, we focused on the preparation of injectable formulation of VK for the treatment of the previously mentioned diseases. Owing to its polyenic structure, VK is virtually insoluble in water and is chemically labile, which makes its manipulation difficult. In order to overcome these problems, we suspended VK in soybean oil (SO) and dispersed it with soybean phosphatidylcholine (PC) using sonication. For the formation of the dispersion for parental use, characterization of the physicochemical properties of the dispersed particle, such as the particle size, the structure and the physical stability should be clarified. The size should be smaller than that of the sterilization filter (0.22 μm) and should not increase during storage for a long period.

In this study, in order to clarify the interaction between VK and PC, we prepared dispersed particles of VK and PC by sonication and investigated the dispersion mechanism using several physicochemical techniques. In addition, SO was added to the VK and PC

mixture to improve the dispersiblity of VK as in the case of fat emulsions for intravenous administration [6]. The size and structure of the VK/PC and VK/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 VK/PC and VK/SO/PC were evaluated by surface monolayer techniques.

Experimental

Materials

VK and SO were purchased from Sigma Chemicals Co. (St Louis, Mo., USA). PC was purchased from Ajinomoto Co. (Tokyo, Japan). The fluorophore lipid *N*-(5-dimetylaminonaphtyalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphethanolamine trimetylammonium salt (Dansyl-DHPE) was from Molecular Probes (Eugene, Ore., USA). Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and calcein (3,3′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Methods

Preparation of the dispersed particles

VK and PC or VK, SO and PC were dissolved in chloroform and mixed. After evaporation of the solvent, water was added to give a final concentration of 5 mM of total lipids. Dispersed particles composed of VK and PC at various VK mole fractions ($X_{VK} = VK/[VK + PC]$) and particles composed of VK, SO and PC at various VK/SO mole fractions ($X_M = [VK + SO]/[VK + PC + SO]$) were obtained by sonication for 30 min under a stream of nitrogen gas at 50 °C. A model UD-200 probe-type sonicator (Tomy Seiko Co., Tokyo, Japan) was used at a power setting of 100 W. After cooling to room temperature, 5 ml of the dispersion was dispensed into the glass ampoules filled with nitrogen gas and the ampoules were sealed. The ampoules were stored at room temperature for 3 months.

Determination of particle size

The sizes of the VK/PC particles or VK/SO/PC particles stored at room temperature for 0, 1 and 3 months were measured with a DLS-7000DL submicron analyzer (Ohtsuka Electronics Co., Osaka, Japan) at 25 °C. The data were analyzed by the histogram method [7], and the weight-average particle sizes were evaluated.

Determination of the trapped volume inside the dispersed particles

In order to obtain information on the structural changes of VK/PC and VK/SO/PC particles as a function of X_{VK} and X_M, the volume trapped inside the particles was determined using the aqueous space marker calcein. Dried mixtures of VK and PC or VK, SO and PC (total 25 μM) were dispersed in a 5 ml of 70 mM calcein solution (pH 7.0) instead of water for the preparation of the dispersion. Untrapped calcein was removed by gel filtration (Sephadex G-50, buffer 5 mM tris(hydroxymethyl)aminomethane-HCl, 150 mM NaCl, pH 7.0). The volume of the calcein solution trapped in the dispersed particles was determined fluorometrically [8] after dissolution of the lipid particles after addition of 10% Triton X-100, and the aqueous volume trapped per mole of PC was evaluated. The PC in the dispersion was measured as described before [9].

Measurements of spreading pressure

In order to evaluate the miscibility of VK, SO and PC in the bulk phase, the spreading pressure of the lipid mixtures was measured. The VK, SO and PC were dissolved in chloroform and mixed. After evaporation of the solvent, the dried lipid mixtures were added on the surface of distilled water in a tensiometer (model CBVP-A3, Kyowa Kaimenkagaku Co., Tokyo, Japan). The spreading pressure of the lipid mixtures at the air/water interface was the steady-state value of surface pressure at 6–8 h after addition of the lipid or the lipid mixtures on the water surface at 25 °C. Details of the monolayer techniques were described elsewhere [10, 11].

Fluorescence quenching

The fluorescence quenching technique [12] was used to obtain information on structural changes [ratio of the number of PC molecules which exists in external and total (external plus internal) membrane] in the VK/SO/PC dispersed particles. $CuSO_4$ was used as a quencher for the Dansyl-DHPE fluorescence embedded in the lipid particles. The VK/SO/PC dispersed particles containing 1 mol% of Dansyl-DHPE were titrated with small aliquots of 1 M $CuSO_4$. The fluorescence intensity, I, at 515 nm (with excitation at 335 nm) was measured as a function of the Cu^{2+} concentration [Q]. Assuming that only the fluorescence of the Cu^{2+} accessible Dansyl-DHPE is quenched according to the Stern–Volmer equation [13], one can estimate the exposed fraction of Dansyl-DHPE, P, so

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

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.

Results and discussion

Size and stability of the dispersed particles

Figure 1 shows that the diameter of the dispersed particles was a function of X_{VK}, (Fig. 1a, for VK/PC particles) and X_M (Fig. 1b, for VK/SO/PC particles). Immediately after preparation, up to $X_{VK} = 0.7$, the particle size of VK/PC dispersions was almost 40 nm and at $X_{VK} = 0.8$ and 0.9, the size was increased to 80 and 150 nm, respectively. At $X_{VK} = 0.3$–0.9, the size was increased during storage at room temperature for 3 months. Up to $X_M = 0.7$, the size of the particles in the VK/SO/PC mixture varied between 50 and 70 nm. Separation of the dispersion into an air and a water phase or an increase in the size was not observed for VK/SO/PC at $X_M < 0.7$ within 3 months at room temperature; however, at $X_M = 0.8$, the particle diameter became considerably larger (160 nm), and phase separation was observed 72 h after preparation. At $X_M = 0.9$, the particle diameter was 220 nm and the separation was detected within 24 h after preparation.

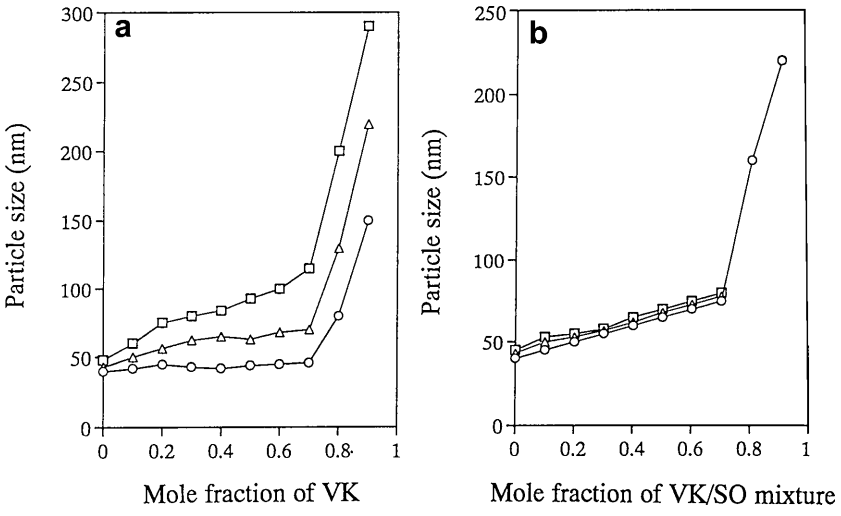


Fig. 1 **a** Weight-average diameter of dispersed vitamin K_1 (*VK*)/phosphatidylcholine (*PC*) particles as a function of X_{VK} (= VK/[VK + PC]). Dynamic light scattering (*DLS*) at 25 °C: immediately after preparation (O); after storage at room temperature for 1 month (△); after storage at room temperature for 3 months (□). **b** Weight-average diameter of dispersed VK/soybean oil (*SO*)/PC particles as a function of X_M (= [VK + SO]/[VK + PC + SO]). DLS at 25 °C: immetiately after preparation (○), after storage at room temperature for 1 month (△), after storage at room temperature for 3 months (□)

Water inside the dispersed particles

The volume of water trapped within the particles (per mole of PC) at various X_{VK} and X_M, determined immediately after preparation, is shown in Fig. 2. Small unilamellar vesicles (diameter: 20–50 nm), large unilamellar vesicles (diameter: 200–1000 nm) and multilamellar vesicles (diameter: 400–3000 nm) trapped amounts between 0.2 and 0.5, 7 and 10, and 3 and 4 $lmol^{-1}$, respectively [14]. At $X_{VK} = 0$, small unilamellar PC vesicles (diameter: 40 nm) trapped 0.45 $lmol^{-1}$, which agrees with the reported value. The trapped volume of water of the dispersed particles of VK/PC mixtures was approximately 0.5–0.6 $lmol^{-1}$ and nearly constant for $X_{VK} = 0$–0.7 (particle size: 40–50 nm). These data indicate that the particles were small unilamellar vesicles. At $X_{VK} = 0.8$ and 0.9, the particle (diameters: 80 and 150 nm, respectively) trapped a volume of 0.9 and 1.6 $lmol^{-1}$, respectively. These values indicate some structural changes of the vesicles of the particles.

The trapped volume of the dispersed particles of the VK/SO/PC mixture was highest at $X_M = 0.3$ (trapped volume: 0.6 $lmol^{-1}$), then it decreased sharply above $X_M = 0.4$. The dramatic drop in the trapped volume indicates structural changes caused by the addition of SO.

Miscibility of VK, SO and PC

The spreading pressures of VK/SO, VK/PC and VK/SO/PC at 25 °C are shown in Fig. 3. The spreading pressures of VK and SO were 13.4 and 20.0 mNm^{-1}, respectively (Fig. 3a) and varied linearly with the mole

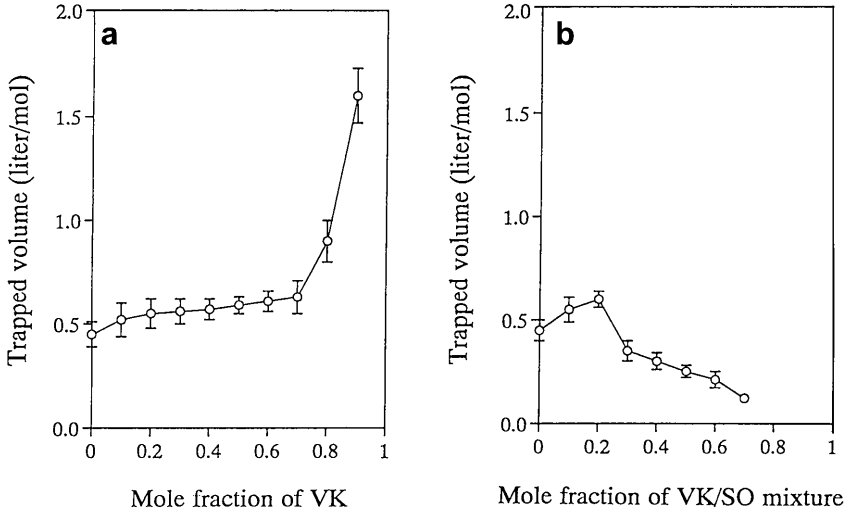


Fig. 2 **a** Trapped volume of water inside the dispersed particles as a function of X_{VK}. Each point represents the average of three samples. **b** Trapped volume of water inside the dispersed particles as a function of X_M

fraction of SO. The spreading pressure of a lipid mixture depends on the miscibility of the lipids in the bulk phases [11]. On the basis of the surface phase rule [11, 15], it was concluded that VK and SO were miscible in the bulk phases; therefore, the mixing ratio of VK and SO was chosen to be 1:1 in this study. The spreading pressures of VK and PC mixtures are shown in Fig. 3b. The solubility of VK in PC is evaluated from the inflection, *a*, at a VK molar fraction of about 0.2. The solubility of PC in VK was evaluated from the inflection point, *b*, at a VK molar fraction of about 0.8.

The spreading pressures of hydrated PC (lamellar bilayers of PC) and the VK/SO mixture (molar ratio of 1:1) were 45.3 and 17.2 mNm^{-1}, respectively (Fig. 3c). The spreading pressure of the VK and SO mixture varied with the molar fraction of VK. VK/SO and PC mixtures showed a decreasing spreading pressure up to $X_M = 0.05$, which remained constant at 43 mNm^{-1} at $X_M = 0.05$–0.95. These results suggest that VK/SO and PC were partially miscible in the bulk phases. The solubility of VK/SO in PC is evaluated from the inflection point of the spreading pressure, *a*, at a VK/SO molar fraction of about 0.05. The solubility of PC in VK/SO was evaluated from the inflection point for the spreading pressure, *b*, at a PC molar fraction of about 0.05. The monolayer–bilayer phase equilibrium for SO and PC was reported [16] and SO and PC were practically immiscible in the bulk phases. The solubilities of SO in PC and PC in SO were both 5 mol% [16]. Probably, the addition of SO to VK influences the miscibility of VK with PC and prevents VK from the formation of the hexagonal phase.

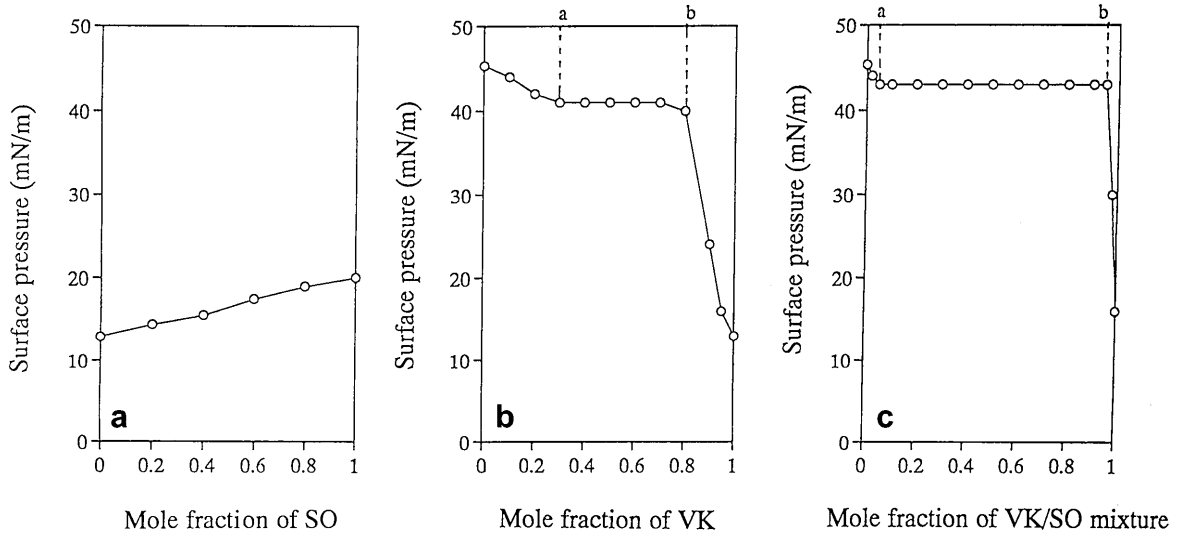
Fig. 3 **a** Spreading pressure of VK and SO mixtures on water at 25 °C. VK and SO are miscible in the bulk phase. **b** Spreading pressures of the VK and PC mixture on water at 25 °C. **c** Spreading pressure of the VK/SO and PC mixture on water at 25 °C

Fluorescence quenching

The fluorescence characteristics of Dansyl-DHPE are sensitive to the microenvironment around the probe, and the Dansyl fluorophore is located in the vicinity of the glycerol backbone of the lipid bilayers [17]. When the nonpenetrating fluorescence quencher $CuSO_4$ is added to dispersed VK/SO/PC particles, it only quenches the fluorescence of Dansyl-DHPE in contact with the outer aqueous phase. The modified Stern–Volmer plot, $I_0[Q]/(I - I_0)$ versus $[Q]$ (the I values were corrected for dilution), was linear. The ratio of the number of PC molecules of the external membrane to the total (external plus internal) (P) for dispersed VK/SO/PC particles is shown as a function of X_M at 25 °C in Fig. 4. At $X_M = 0$, P was 0.58. This agreed with the molar ratio of the number of PC molecules on the external membrane and the total (external plus internal) surface of small unilamellar vesicles [12, 18, 19]. The P value for the VK/SO/PC particles increased with X_M. These results suggest that an increase in X_M reduces the fraction of PC which participates in the formation of the liposomal bilayers.

Stability of the dispersed particles in an aqueous medium

VK can be classified as a neutral lipid and forms monolayers with and without phospholipid. A neutral lipid with limited solubility in PC bilayers (below 5 mol%) forms separate droplets of the excess neutral lipid. The droplets are covered with PC monolayers in equilibrium with the bilayers and form an emulsion. This distribution was observed in dispersions composed of PC and SO [16], trioleine [20] and α-tocopherol acetate [21]. Neutral lipids, such as diglyceride [22, 23],



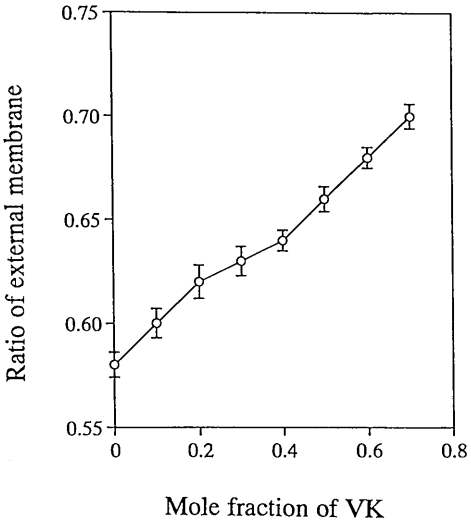


Fig. 4 Ratio of the number of PC molecules in the external to the total (external plus internal) membrane in the VK/SO/PC mixture determined by fluorescence quenching [probe: *N*-(5-dimetylaminonaphtyalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphethanolmine trimetylammonium salt, quencher: Cu^{2+}, at 25 °C] as a function of X_M. Each point represents the average of three samples

monoglyceride [24], menaquinone-4 [25] and α-tocopherol [26], have a high solubility (above 20 mol%) in PC bilayers. The monolayer in equilibrium with the separate phase, containing a low fraction of PC, does not stabilize the lipid phase in the form of small droplets (forming emulsions) in an aqueous medium [20, 21]. For $X_{VK} = 0.1$–0.9 the size of the VK/PC particles increased at room temperature within 3 months. These results are caused by the formation of the reversed phase (hexagonal H_{II} or cubic) and aggregation of the dispersed particles.

When SO was added to VK (molar ratio of 1:1), we obtained small particles (diameter: 50–70 nm) at $X_M = 0$–0.7, the oil/water phase separation was not observed and the size did not change at room temperature within 3 months. As mentioned earlier, SO has a limited solubility in the PC bilayer (5 mol%) and the addition of SO to VK changes the solubility of the neutral lipid mixture in the PC bilayers (5 mol%, Fig. 3). The VK/SO droplets are covered with PC monolayers in equilibrium with the bilayers and form an emulsion. When the PC content is less than the solubility in VK/SO (PC molar fraction less than about 0.05, Fig. 3), the PC monolayer does not completely cover the hydrophobic surface of the VK/SO particles. When $X_M = 0.8$ or 0.9, probably owing to the lack of PC monolayers, the particles aggregate and the particle size increased drastically (150 nm or greater). Moreover, separation into an oil and a water phase was observed after preparation; the dispersions were unstable. Therefore, the solubility of VK and SO in PC is crucially important for the stabilization of the particles in water.

Conclusions

VK has appreciable solubility in PC bilayers (approximately 20 mol%). Within $X_{VK} = 0.1$–0.9, the size of the dispersed particles increased at room temperature within 3 months. When SO was added to VK (molar ratio VK:SO = 1:1), the solubility of VK and SO mixtures in PC decreased (approximately 5 mol%). The particle size in the aqueous dispersions at $X_M = 0.1$–0.7 was 50–70 nm and did not change within 3 months at room temperature. The solubility of the neutral lipid in PC bilayer is probably critically important for the production of stable dispersions in aqueous media.

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