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COMMUNICATION

Interaction of Ubiquinone-10 with Dipalmitoylphosphatidylcholine and Their Formation of Small Dispersed Particles

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ABSTRACT

Stable aqueous dispersions of ubiquinone-10 (UQ) were obtained by cosonication with dipalmitoylphosphatidylcholine (DPPC) in the UQ mole fraction range 0.1–0.7. To clarify the dispersal mechanism, the dispersed particles were characterized, and the interaction between UQ and DPPC was investigated using several physicochemical techniques. Dynamic light scattering (DLS) measurements showed that the diameter of the dispersed particles was 50–70 nm. A limited amount of UQ was incorporated into DPPC bilayer membranes (approximately 5 mol%). The trapped aqueous volume inside the particles was determined fluorometrically using the aqueous space marker calcein, and the volume in the UQ/DPPC particles decreased remarkably with the addition of UQ 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 UQ separated from the DPPC bilayers is stabilized as emulsion particles by the DPPC surface monolayer.*
Key Words: *Dipalmitoylphosphatidylcholine; Dispersion; Particle; Structure; Ubiquinone-10.*

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INTRODUCTION

Ubiquinone-10 (UQ) is a well-known constituent of the mitochondrial inner membrane (1,2), in which it is believed to function as a mobile carrier of electrons and protons in the mitochondrial electron transfer chain. The mobility, location, and association in phospholipid membrane have been investigated on the basis of ^{1}H-NMR (nuclear magnetic resonance) spectroscopy (3–7), differential scanning calorimetry (DSC), and X-ray diffraction measurements (8). There seems to be general agreement that a limited amount of UQ is incorporated into phospholipid bilayer membranes (less than 10 mol%), and that an appreciable fraction forms a separate phase located outside the bilayers (4,6,8).

In this study, to clarify the interaction between UQ and PC (phosphatidylcholine), we prepared the dispersed particles composed of UQ and dipalmitoylphosphatidylcholine (DPPC) by cosonication and characterized them to investigate the dispersal mechanism using several physicochemical techniques. The structure of UQ/DPPC particles was determined by dynamic light scattering (DLS), fluorescence quenching, and analysis of the trapped aqueous volume inside the particles. The miscibility and solubility of UQ and DPPC were evaluated by DSC. On the basis of these studies, some indication of the interaction between UQ and DPPC and the mechanism of formation of the dispersed particles and their stability was obtained.

EXPERIMENTAL

Materials

UQ was supplied by Eisai Company, Limited (Tokyo, Japan). L-α-Dipalmitoylphosphatidylcholine (DPPC) and copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were purchased from Wako Pure Chemical Industrial, Limited (Osaka, Japan). *N*-Dansylhexadecylamine (DSHA) was from Lambda Company, Limited (Graz, Austria). Calcein (3,3′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-fluorescein) was from Dojin (Kumamoto, Japan).

Methods

Preparation of Dispersed Particles

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

Determination of Particle Sizes

DLS measurements of the sonicated dispersions of UQ 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 (9), and the weight-averaged particle sizes were evaluated.

Solubility of UQ in DPPC Membrane and of DPPC in UQ

To determine the miscibility of UQ and DPPC for the formation of the dispersed particles and the solubility of UQ in DPPC membrane and of DPPC in UQ, DSC was performed using a model DSC-100 (Seiko-Denshi Co., Ltd., Tokyo, Japan). UQ/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°C to 70°C with constant heating rates of 2°C/min. All calorimetric data were obtained from samples during the heating phase.

Determination of the Trapped Volume Inside the Dispersed Particles

A dried mixture of UQ 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 (10) 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 (11).

Fluorescence Quenching

Fluorescence quenching techniques were used to obtain information on structural changes (ratio of external to total [external plus internal] membrane) in the UQ/DPPC dispersed particles. Fluorescence quenching techniques have been described previously (12). In this study, $CuSO_4$ was used as a quencher for the DSHA fluorescence embedded in the lipid particles. UQ/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 (13), one can estimate the exposed fraction of DSHA P, so that

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

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

RESULTS

Size and Stability of the Dispersed Particles from the Mixtures

Figure 1 shows that the diameter of the dispersed particles obtained by these methods is represented as a function of the UQ mole fraction X_{UQ}. Separation of the dispersion to oil/water phases was not observed in the dispersions of the UQ and DPPC mixture in the range $X_{UQ} = 0$–0.7 within 72 hr after preparation. At $X_{UQ} = 0.8$, the particle diameter was considerably larger at 165 nm, and separation was observed 72 hr after preparation. At $X_{UQ} = 0.9$, the particle diameter was 220 nm, and the separation was detected within 24 hr after preparation.

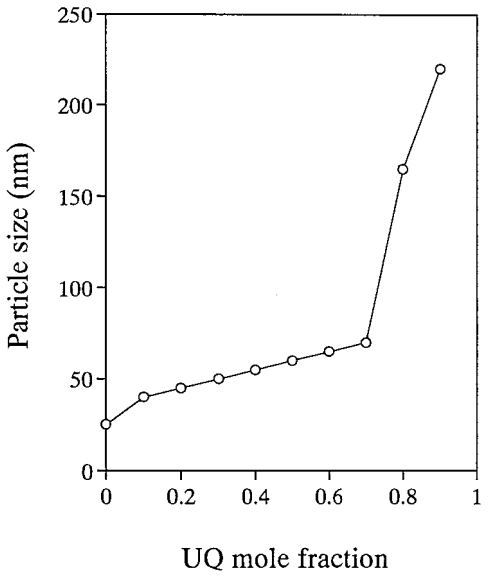

Figure 1. Weight-averaged diameter of dispersed particles represented as a function of mole fraction of UQ (X_{UQ}) in the mixture determined by DLS.

Solubility of Ubiquinone-10 in Dipalmitoylphosphatidylcholine Membrane and of Dipalmitoylphosphatidylcholine in Ubiquinone-10

Figure 2 shows the solubilities of UQ in DPPC membrane and of DPPC in UQ determined by DSC. The addition of UQ decreased the phase transition temperature, and at X_{UQ} values higher than 0.05, the phase transition temperature was constant at 38°C. This indicates that the solubility of UQ in the DPPC membrane was equivalent to a mole fraction of 0. 05. The phase transition enthalpy decreased with an increase in X_{UQ}, and the phase transition was abolished at $X_{UQ} = 0.95$. This indicates that, at $X_{UQ} = 0.95$, DPPC was completely incorporated in UQ, and that the solubility of UQ in the DPPC membrane was equivalent to a mole fraction of 0. 05.

Aqueous Space Inside the Dispersed Particles

Figure 3 shows the trapped volume of the particles per mole of DPPC at various X_{UQ}. 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 $L \cdot mol^{-1}$, respectively (14). At $X_{UQ} = 0$, small unilamellar DPPC vesicles (diameter 27 nm) had a trapped volume of 0.44 $L \cdot mol^{-1}$, which agrees with the reported value. The trapped volume of the dispersed particles of the UQ/DPPC mixture was highest at $X_{UQ} = 0.3$, then decreased sharply above $X_{UQ} = 0.4$. The trapped volume was also calculated on the basis of total moles of UQ 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 UQ.

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 (15). When the nonpenetrating fluorescence quencher $CuSO_4$ is added to UQ/DPPC dispersed particles, it only quenches the fluorescence of the DSHA in the outer aqueous phase. In the modified Stern-Volmer plot, the plots of $I_o \cdot [Q]/(I - I_o)$ versus $[Q]$ (the I values were corrected for dilution) were linear. Figure 4 shows the ratio of the external membrane

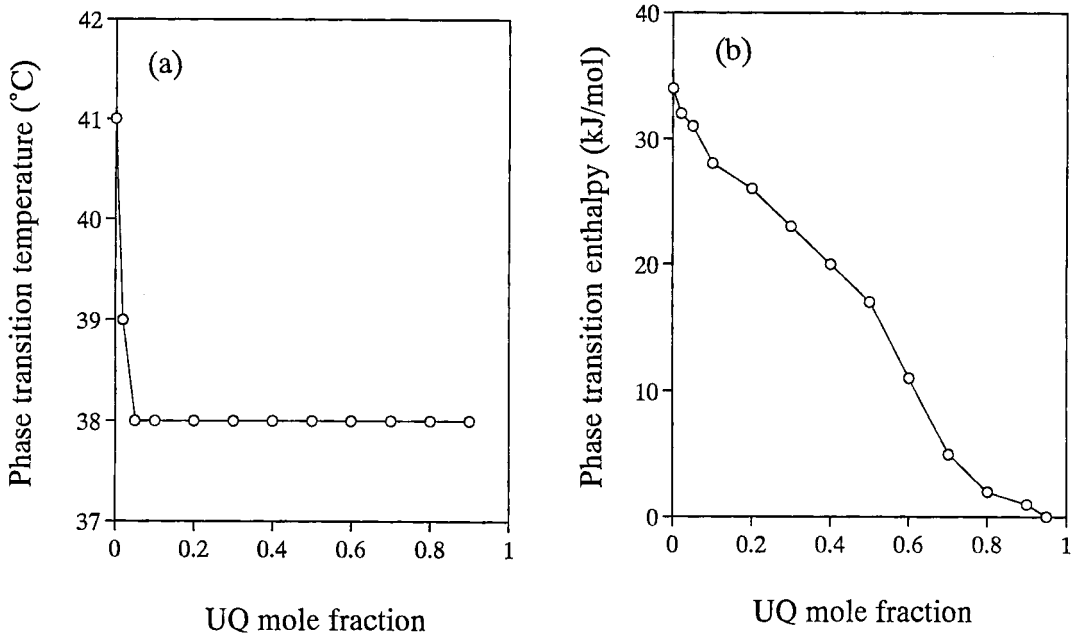

Figure 2. (a) Phase transition temperature represented as a function of mole fraction of UQ (X_{UQ}) in the mixture determined by DSC. (b) Phase transition enthalpy represented as a function of mole fraction of UQ (X_{UQ}) in the mixture determined by DSC.

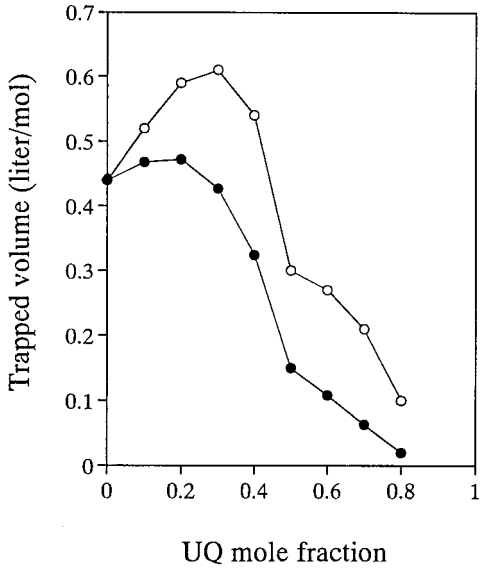

Figure 3. Trapped aqueous volume inside of the dispersed particles represented as a function of mole fraction of UQ (X_{UQ}) in the mixture: ○ volume of inner space per mole of DPPC; ● volume of inner space per total mole of the lipid (UQ + DPPC).

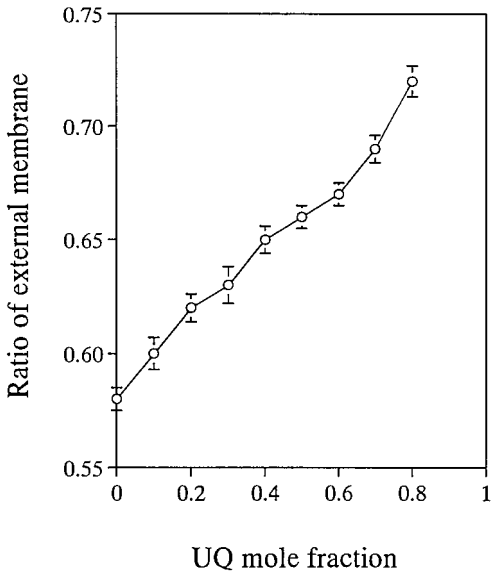

Figure 4. Ratio of the external to total (external plus internal) membrane in the lipid mixture determined by fluorescence quenching represented as a function of the mole fraction of UQ (X_{UQ}) in the mixture.

to the total (external plus internal) (P) for UQ/DPPC dispersed particles as a function of X_{UQ}. 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 (16,17). The P value for the UQ/DPPC dispersed particles increased with increases in the X_{UQ}. These results suggest structural changes in the dispersed particles by the addition of UQ.

DISCUSSION

Neutral Lipid Ubiquinone-10

UQ can be classified as a neutral lipid and forms a monolayer with and without phospholipid (18,19). Neutral lipids have limited solubility in phospholipid bilayer membranes (20,21) and form separate phases in aqueous media, which are stabilized by the closely packed phospholipid monolayer surrounding the phases (22,23). This kind of equilibrium has been observed in the dispersions composed of phosphatidylcholine and α-tocopherol acetate (24) or triglyceride (20,25). The monolayer-bilayer equilibrium thus plays an important role in the structural formation 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. On the other hand, neutral lipids such as diglyceride (26,27), monoglyceride (28), menaquinone-4 (29), and α-tocopherol (30) have appreciable solubility in phospholipid 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_{UQ} of the dispersed particles leads to a reduction in the fraction of DPPC that 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. We (24) reported that the fraction of DPPC that forms bilayer vesicles ξ_1 may be calculated from the trapped volume v as follows:

$$\xi_1 = (v/v_0) \tag{2}$$

Here, v_0 is the trapped volume of small unilamellar vesicles ($v_0 = 0.44$ L · mol^{-1}; see Table 1). The ξ_1 values calculated are presented in Table 1. The increased values of v in the range UQ = 0.1–0.4 are probably due to the increased size of the dispersed particles as a result of the increased X_{UQ}.

It is also presented that the alterations in the structure of the dispersed particles from the vesicular structure occur on the basis of the fluorescence quenching measure-

Table 1

Fraction of Dipalmitoylphosphatidylcholine (DPPC) Participating in the Formation of Vesicle Bilayers ξ_1

UQ Mole Fraction (X_{UQ})	Trapped Volume v (L · mol^{-1} of DPPC)	ξ_1[a]	Ratio of Exernal to Total Membrane (p) Determined by Fluoresence Quenching	ξ_1[b]
0	0.44	1.0	0.58	1.0
0.1	0.52	—	0.60	0.94
0.2	0.59	—	0.62	0.87
0.3	0.61	—	0.63	0.83
0.4	0.54	—	0.65	0.75
0.5	0.30	0.68	0.66	0.69
0.6	0.27	0.61	0.67	0.61
0.7	0.21	0.48	0.69	0.46
0.8	0.10	0.23	0.72	0.19

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

ments. An increase in X_{UQ} of the dispersed particles leads to a reduction in the fraction of DPPC that participates in the formation of the liposomal bilayers. It is suggested that the DPPC monolayers easily take part in the formation and stabilization of dispersed particles of emulsion structure in water . The fraction of DPPC forming bilayer vesicles ξ_1 is calculated on the basis of the fluorescence quenching measurements (Fig. 4) and is correlated with the ratio of external to total (external plus internal) membrane p in UQ/DPPC dispersed particles (24).

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

Here, p_0 is the ratio of the liposomal vesicles of DPPC and is 0.58, s is the solubility of DPPC in the separate solid phase of UQ, equivalent to a mole fraction of 0.05 as determined by DSC (Fig. 2). $(1 - p)$ is the fraction of DPPC that is inaccessible to the Cu^{2+} added to the outer aqueous phase of the dispersion, and $s \cdot X_{UQ}/(1 - X_{UQ})$ is the fraction of DPPC solubilized in the separate UQ phase. As seen in Table 1, the ξ_1 values show a decline in the fraction of vesicular particles in the X_{UQ} range 0–0. 7. In the preparation of the particles at 70°C, the hydrophobic liquid droplets of UQ separated from the bilayers are stabilized by the DPPC monolayer as emulsion particles in an aqueous medium (24).

Stability of Dispersion and Lipid Composition

When the DPPC content is less than the solubility in UQ (DPPC mole fraction less than about 0.05; see Fig. 2), the DPPC monolayer does not completely cover the hydrophobic UQ particle surfaces. When $X_{UQ} = 0.8$ or 0.9, separation into oil/water phases was observed after preparation because of the probable nonhomogeneity in the mixtures at room temperature (UQ is in a solid state), and the dispersions were not stable. On the other hand, when the mole fraction of DPPC was higher (i.e., $X_{UQ} =$ 0–0.7), the PC monolayer completely covered the UQ particles and stabilized the dispersion. When DPPC was excessive, the monolayer was in equilibrium with the PC bilayers (liposomes), and the surface pressure of the monolayer at the particle surface had the maximum value (24). Therefore, the coexistence of emulsion and liposomal particles was critically important for the stabilization of the particles in water.

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