The Interaction of Menaquinone-4 with Phospholipid Membranes

Yasuyuki ASAI*,[a] and Sumio WATANABE[b]

Formulation Research Laboratory, Kawashima, Eisai Co., Ltd.,[a] Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 501–6195, Japan and Tsukuba Research Laboratories, Eisai Co., Ltd.,[b] Tokodai, Tsukuba, Ibaraki 300–2635, Japan.
Received January 5, 1999; accepted February 24, 1999

The effects of menaquinone-4 (MQ) on dipalmitoylphosphatidylcholine (DPPC) membranes were studied by surface monolayer and fluorescence techniques. DPPC and MQ have been proven to be freely miscible in the mixed monolayer at an air/water interface, and to be partially miscible in a bulk phase, *i.e.*, the bilayer and solid phase. There is an expanding interaction between MQ and DPPC in the MQ/DPPC mixed monolayers. The solubility of MQ in the DPPC is about 20 mol%, and the solubility of DPPC in MQ is about 10 mol%. The addition of MQ induced a proportional decrease in the phase transition temperature of DPPC membrane and a broadening of the temperature range of the transition.

Key words menaquinone-4; dipalmitoylphosphatidylcholine; monolayer–bilayer equilibrium; solubility; phase transition

Neutral lipids, such as triglyceride,[1,2] ubiquinone-10[3] and α-tocopherol acetate,[4] have low solubilities in the bilayers of phosphatidylcholine (PC) and form droplets separated from the bilayers in an aqueous medium. The droplets of a neutral lipid are covered with a PC monolayer and are stabilized as emulsion particles. The surface monolayers of the droplets are in equilibrium with the bilayers.[1,3]

Some other neutral lipids, such as diglyceride,[5,6] monoglyceride[7] and α-tocopherol,[8] have appreciable solubilities in bilayers of PC. The addition of such a neutral lipid to the bilayers changes the hydrophilic–lipophilic balance and induces a bilayer-to-reversed cubic phase transition.[5—8] It is difficult to disperse these nonbilayer phases of reversed topology stably as small particles in an aqueous medium.

A neutral lipid, menaquinone-4 (MQ) has vitamin activity in animals (vitamin K_2): the activation of a carboxylase induces the modification of prothrombin, blood clotting factor, and other proteins in plasma and tissues.[9—11] Although MQ is localized in membranes of microsomes, very little is known about its interaction with phospholipids and its localization and organization in the bilayer. In order to clarify the interaction of MQ with the phospholipid membrane, we prepared mixed monolayer films from MQ and dipalmitoylphosphatidylcholine (DPPC) and determined their behavior. In addition, the effect of MQ on the phase transition behavior of DPPC membrane was determined using a fluorescence polarization technique.

Experimental

Materials MQ was purchased from Eisai Chemical Co., Ltd. (Ibaraki, Japan). L-α-DPPC was purchased from Sigma Chemical Co., Ltd. (St. Louis, U.S.A.). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Determination of the Behavior of MQ in Mixed Monolayers with DPPC and Measurements of Collapse and Spreading Pressures MQ, DPPC and MQ/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 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 MQ/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.[12,13]

The Effect of MQ on the Phase Transition of DPPC Membrane The effect of MQ on the gel to liquid–crystalline phase transition of the DPPC membrane was determined using a fluorescence polarization technique (probe: DPH) as reported.[14] DPPC and MQ were dissolved in chloroform and then mixed at a suitable ratio. The solvents were evaporated under a stream of nitrogen gas at 70 °C. The lipid film was hydrated to give a total concentration of the total lipids of 1 mM with 4.25 mM phosphate–NaOH buffer (pH 7.3). The lipid dispersion was then sonicated with a probe-type sonicator (Tomy Seiko Co., Ltd., Tokyo, Japan) at 50 °C for 10 min. DPH was added at 1 mol% of total lipids. All fluorescence measurements were carried out using a Model F-4500 fluorescence spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan) equipped with a thermoregulated cell compartment, Atago Coolnics Model REX-C10 (Atago Co., Ltd., Tokyo, Japan). The degree of polarization (P) was calculated using the following equation:

$$P=(I_{VV}-C_f \cdot I_{VH}) / (I_{VV}+C_f \cdot I_{VH})$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analysis (second) polarizers, respectively. C_f ($=I_{HV}/I_{HH}$) is the grating correction factor.

Results and Discussion

Monolayer Properties of MQ and DPPC The behavior of MQ in mixed monolayers with DPPC was examined by determination of the properties of binary mixtures of MQ with DPPC spread on subphases of water. The surface pressure–area curves for mixed monolayers are presented in Fig. 1. MQ and DPPC have a limiting area of 46 and 58 Å^2 per molecule, respectively. Figure 2 shows the mean molecular area of mixed monolayers of MQ and DPPC mixtures obtained in the miscible region of the surface pressure isotherm (Fig. 1) at two different surface pressures, 5 and 8 mN/m. The upper surface pressure of MQ was 9 mN/m and the mean molecular areas for the mixtures have been taken from Fig. 1 at the given surface pressures. The broken line between the end points in the figure represents the additivity rule of monolayer areas.[15] It can be seen that at a surface pressure of both 5 and 8 mN/m, the molecules occupy a volume in the film that is larger than would be expected from the pure compounds alone. It is suggested that there is an expanding effect brought about by the interaction between the molecules of the film.

Monolayer–Bilayer Equilibrium of MQ/DPPC Mixtures The collapse and spreading pressures of the MQ/

* To whom correspondence should be addressed.

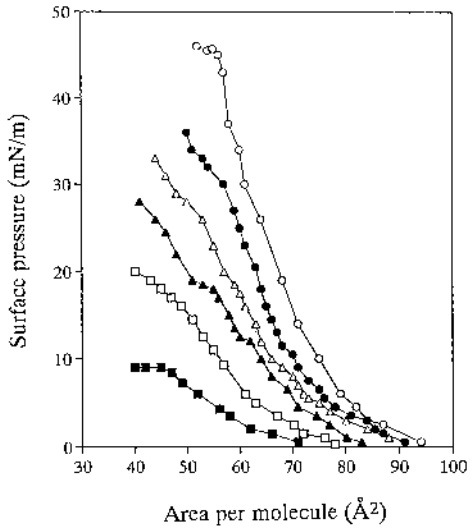

Fig. 1. Surface Pressure–Area Curves for Mixed Monolayers of MQ and DPPC at Different Molar Ratios at 25°C

X_{MQ}=0 (○), 0.2 (●), 0.33 (△), 0.5 (▲), 0.67 (□), 1.0 (■).

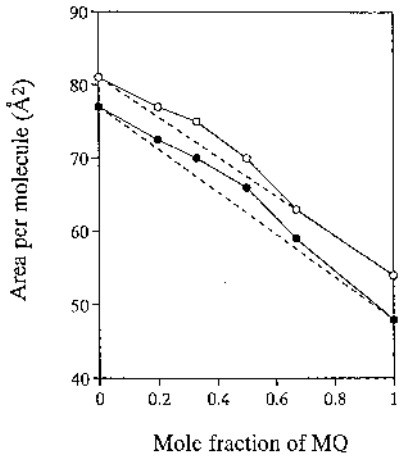

Fig. 2. Average Area per Molecule *versus* Molar Ratio of the Lipids for Mixed Monolayers of MQ and DPPC at 5 mN/m (○) and at 8 mN/m (●) at 25 °C

The broken lines represent the additivity rule.[15]

DPPC mixture were obtained as a function of the mole fraction of MQ (X_{MQ}), and therefore, gave a phase diagram for the monolayer (M)–DPPC bilayer (B)–MQ solids (S) equilibrium, as shown in Fig. 3. The collapse pressure of DPPC, which is the surface pressure at the transition from the monolayer to the bilayers, was 45.0 mN/m. The spreading pressure of DPPC, which is the surface pressure at the transition from bilayers to monolayer,[9] was identical to the collapse pressure. The collapse and spreading pressures of MQ, which are the surface pressures at the monolayer–solid equilibrium of this compound, were 9.0 mN/m. The collapse and spreading pressures of DPPC were also consistent with each other (45.0 mN/m), and the values agree with the reported collapse pressure of about 45.0 mN/m.[12] 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 (bilayer or solid).[16]

The collapse pressure varied with X_{MQ} in the mixed monolayer, while the spreading pressure remained constant at 42.0

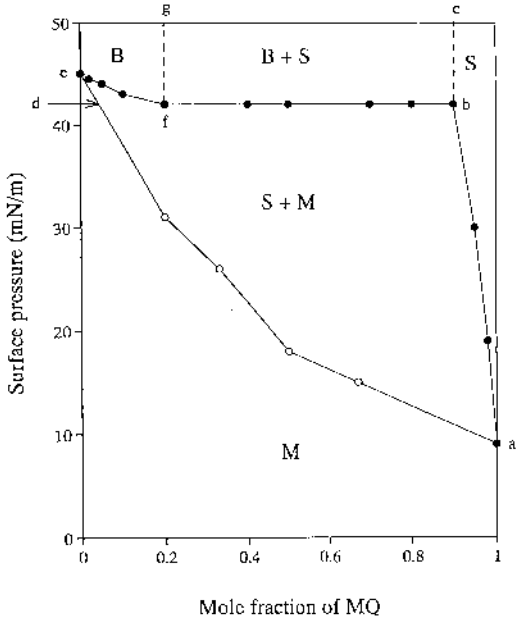

Fig. 3. Monolayer (M)–DPPC Bilayer (B)–MQ Solids (S) Equilibrium of the MQ/DPPC Mixture in the Presence of Water at 25 °C

Collapse pressure (○—○), spreading 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 solubility of MQ solid (S) in DPPC is evaluated from the inflection point for the spreading pressure, *f*, as the MQ mole fraction of approximately 0.2. The solubility of DPPC in the MQ solid (S) is evaluated from the inflection point for the spreading pressure, *b*, as the DPPC mole fraction of approximately 0.1. A mixed monolayer exists in the region designated by M. Coexisting in the regions designated by S+M, and B+S are an MQ solid and mixed monolayer and DPPC bilayers and MQ solids, respectively.

mN/m in the X_{MQ} range of 0.2—0.9 (the line *fb* in Fig. 3). On the basis of the surface phase rule,[13] it was found that DPPC and MQ are freely miscible in a mixed monolayer at an air/water interface (M), but only partially miscible in the bulk phase, *i.e.*, the DPPC bilayer (B) and MQ solid phase (S). The solubility of MQ solid (S) in the DPPC is evaluated from the inflectional point of spreading pressure, *f*, as a DPPC mole fraction of approximately 0.2. The solubility of DPPC in the MQ solid (S) was evaluated from the inflection point for the spreading pressure, *b*, as a mole fraction of approximately 0.1.

On the phase diagram in Fig. 3, a mixed monolayer exists in the region designed by M. Coexisting in the regions designated by S+M, and B+S are an MQ solid and mixed monolayer and DPPC bilayers and MQ solids, respectively. On the horizontal line, *bf*, at a surface pressure of 42.0 mN/m, the system consists of DPPC bilayers, *f*, which contain about 20% MQ, and the MQ solid phase, *b*, which contains about 10% DPPC. The mixed monolayer, *d*, which contains approximately 3% DPPC and has a surface pressure of 42.0 mN/m, is in equilibrium both with the bilayer, *f*, and the solid phase, *b*.

The Effect of MQ on the Phase Transition of DPPC In Fig. 4, the fluorescence polarization (*P*) of DPH incorporated into DPPC liposomes is plotted as a function of the temperature. The *P* values of DPH in DPPC liposomes decreased rapidly at 40—42 °C, which corresponded to the phase transition temperature determined by differential scanning calorimetry and X-ray diffraction.[17] An increase in the amount of MQ incorporated into DPPC liposomes lowered

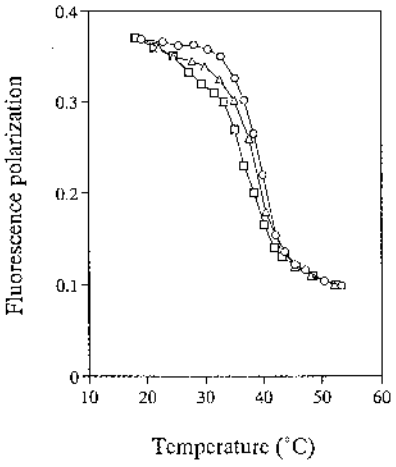

Fig. 4. Relationship between Incubation Temperature and Fluorescence Polarization Using DPH as a Function of the Lipid Mole Fraction of MQ (X_{MQ}) in the Lipid Mixtures

X_{MQ}=0 (○—○), 0.1 (△——△), 0.2 (□—□).

the transition temperatures with a decrease of P values. At X_{MQ}=0, 0.1 and 0.2, the phase transition temperatures were 41, 38 and 36 °C, respectively. An increase of X_{MQ} induced a proportional decrease of phase transition temperature and a broadening of the temperature range of the transition.

References and Notes

1) Tall A. R., Small D. M., *Adv. Lipid. Res*., **17**, 1—15 (1980).
2) Handa T., Saito H., Miyajima K., *Biochemistry*, **29**, 2884—2890 (1990).
3) Handa T., Asai Y., Miyajima K., Kawashima Y., Kayano M., Ida K., Ikeuchi T., *J. Colloid Interface Sci*., **143**, 205—213 (1991).
4) Asai Y., Watanabe S., *Chem. Pharm. Bull.*, **46**, 1785—1789 (1998).
5) Das S., Rand R. P., *Biochemistry*, **25**, 2882—2889 (1986).
6) Seddon J. M., *Biochemistry*, **29**, 7997—8002 (1990).
7) Nilsson A., Holmgren A., Lindblom G., *Biochemistry*, **30**, 2126—2133 (1991).
8) Nakajima K., Utsumi H., Kazama M., Hamada A., *Chem. Pharm. Bull.*, **38**, 1—4 (1990).
9) Johnson B. C., *Mol. Cell. Biochem*., **38**, 77—121 (1981).
10) Vermeer C., *Mol. Cell. Biochem*., **61**, 17—35 (1984).
11) Suttie J. W., *Annu. Rev. Biochem*., **54**, 459—477 (1985).
12) Handa T., Ichihashi C., Nakagaki M., *Prog. Colloid Poly. Sci*., **71**, 26—31 (1985).
13) Nakagaki M., Tomita K., Handa T., *Biochemistry*, **24**, 4619—4624 (1985).
14) Iwamoto K., Sunamoto J., Inoue K., Endo T., Nojima S., *Biochim. Biophys. Acta*, **691**, 44—51 (1982).
15) Gains G. L. "Insoluble Monolayers at Liquid-Gas Interface," Wiley-Interscience, New York, 1966.
16) Yamamoto I., Mazumi T., Asai Y., Handa T., Miyajima, K., *Colloid Poly. Sci.*, **272**, 598—603 (1994).
17) Ladbrooke B. D., Williams R. M., Chapman D., *Biochim. Biophys. Acta,* **150**, 333—340 (1968).