Micelle-Mediated Transport of Vitamin K_1 through Porous Membranes: Contribution of Phosphatidylcholine–Bile Salt Mixed Micelles

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Diffusion of vitamin K_1 solubilized by phosphatidylcholine-sodium deoxycholate mixed micelles through porous membranes having various pore characteristics was examined. The membranes include Nuclepore, Duragard and nitrocellulose membranes, which were intended to mimic the narrow channels in the vicinity of absorptive brush border. The diffusion coefficient of the mixed micelles was 4.6—5.5 × 10^{-7} cm^2/s, from which the hydrodynamic radius was calculated to be about 50 Å. The dependence of the diffusivity on pore size showed that the transport of the micelles is hindered by pores having a radius ratio of the diffusate to the pore of about 0.05 or larger.

Keywords self-diffusion; porous membrane; phosphatidylcholine; bile salt; mixed micelle; transport; vitamin K_1

While the major route of administration of liposomal drugs is parenteral, attempts have been made to use liposomes as drug carriers for oral administration.[1-3] However, the role of liposomes in the absorption remains a subject of controversy.[4-6] In the intestinal tract, it is questionable whether the physical integrity of liposomes is maintained in such a severe environment, because the vesicles normally encounter physiological surfactants such as bile salts (BSs) which tend to disintegrate the lipid bilayer.[7,8] BSs form mixed micelles with phosphatidylcholine (PC) under normal physiological conditions, contributing to the solubilization of ingested fat, cholesterol and fat-soluble compounds.[9,10] It is therefore very likely that drug-loaded liposomes are damaged more or less by BSs in the intestine. For the disintegration of drug-loaded liposomes, the following sequence of events might be generally involved: (1) surfactant binding and disintegration of the liposomes, (2) solubilization of the drug by the resulting mixed micelles, and (3) micelle-mediated transport to the absorptive epithelium.

Vitamin K_1 (VK_1), which is required for the synthesis of the vitamin-dependent clotting factors, is water-insoluble. The oral administration of the vitamin would therefore be subject to the above stages when the vitamin is given as a liposomally associated form.[11] We have studied the disintegration rate of liposomes by BSs and the solubilization of VK_1 by PC–BS mixed micelles[12,13] and the data indicated an enhanced recovery of blood coagulation after the oral administration of liposomal VK_1.[11]

Transport of PC–BS mixed micelles in the vicinity of the absorptive epithelium is also an important factor for absorption of such water-insoluble drugs as VK_1.

The purpose of the present paper is to describe how the mixed micelles affect the vitamin transport across porous membranes with different pore sizes which mimic the narrow channels in the vicinity of absorptive brush border.

Experimental

Materials Purified egg PC (Coatsome NC-10, lot #70128, 70303, Nichiyu Liposome Co., Tokyo) was used as received. Sodium deoxycholate (SDOC, Sigma, St. Louis, MO) was chromatographically pure and was used without purification. VK_1 was supplied by Eisai Co. (Tokyo) and was used as received. *p*-Aminobenzoic acid (PABA) was purchased from Wako Pure Chem. Industries and recrystallized from ethanol. Methanol, ethanol, isopropanol and *n*-hexane were of high performance liquid chromatography (HPLC) quality.

Membranes Polycarbonate membranes (Nuclepore, Nuclepore Co., Pleasanton, CA) with different pore sizes (pore size 0.05 μm, thickness 5 μm; 0.2, 10; 0.4, 10; 0.6, 10; 0.8, 10) were used. Nitrocellulose membranes (Toyo Roshi, Tokyo) with different pore sizes (pore size 0.1 μm, thickness 110 μm, porosity 0.65; 0.2, 133, 0.73; 0.3, 140, 0.75; 0.45, 145, 0.78; 0.65, 150, 0.79; 0.8, 150, 0.80) were used. Polypropylene microporous membranes (Duragard 3501 (hydrophilic), pore size 0.04 × 0.4 μm, thickness 25 μm; Duragard 3401 (hydrophilic), 0.02 × 0.2, 25, Polyplastics Co., Tokyo) were also used.

Preparation of Mixed Micelles Known amounts of PC stock solution (*n*-hexane–ethanol, 98:2), SDOC stock solution (methanol) and VK_1 stock solution (ethanol) were taken into a 500 ml round-bottomed flask and mixed. The organic solvents were removed by a rotary evaporator. The mixture was first gently hydrated by adding an aliquot of phosphate buffer (0.05 M PBS, pH 8.0, ionic strength 0.2) to give a transparent solution and then the total volume was adjusted to give a desired concentration of the mixed micelles (50 mM). The molar ratio of PC and SDOC was always maintained at unity.[13] The concentration of VK_1 solubilized by the mixed micelles used for the diffusion study was 13.0—15.0 mM.

Diffusion Cell Figure 1 shows the diffusion cell, which is basically of the same type as that used by Roy *et al.*[14] It was entirely fabricated from acrylic resin material. The membrane was fixed between the circular faces of the donor and receptor compartments, coupled with a Teflon O ring. The membrane was immersed in distilled water or mixed micelle solution for several hours before being used in a diffusion experiment. The effective surface area of the membrane exposed was 6.16 cm^2. The lower compartment acted as the reservoir of vitamin-loaded mixed micelle solution (11.7 ml), and the upper compartment acted as a sink (20 ml). The solution was first introduced through the port of the lower compartment with the

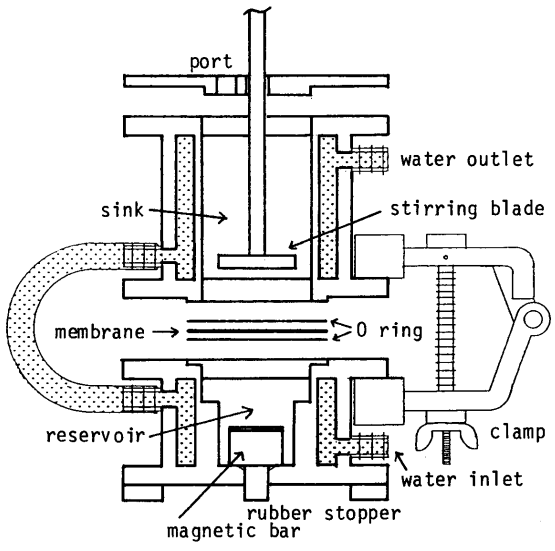

Fig. 1. A Diffusion Cell

cell upside-down and the port was closed with a rubber stopper after carefully removing bubbles. Both compartment were double-jacketed for temperature control (25°C) and were equipped with a magnetic bar (receptor) and a stirring blade (donor). An aliquot (25 μl) was taken periodically from the upper compartment. Because the lower compartment was closed during the experiment, bulk flow would be avoided. Adsorption of solubilized VK_1 was negligible for all membranes used.

When a membrane of exposed area S and thickness h is mounted and the concentrations of the donor (volume of V_1) and the receptor (volume of V_2) are C_1 and C_2, respectively, provided that the total amount of a diffusate in the system, M_t, is $C_1V_1+C_2V_2$, we have the following equations:

$$\ln\left[1-\frac{(V_1+V_2)C_2}{M_t}\right]=-\frac{(V_1+V_2)SP}{V_1V_2}t \quad (1)$$

$$P=\frac{Df}{h} \quad (2)$$

where P is the permeability coefficient, D is the diffusion coefficient, and f is the fractional coefficient of membrane available for diffusate passage.

Ultraviolet (UV) Assay of PABA Samples (25 μl) were withdrawn for UV assay (277 nm) from the upper cell before stirring and at appropriate intervals thereafter. An equivalent volume of the buffer was returned so that the volume remained constant.

HPLC Assay of VK_1 Each sample (25 μl) was collected in a 10 ml stoppered glass test tube containing 0.375 ml of water and 0.6 ml of isopropanol, and VK_1 was extracted with *n*-hexane (5 ml) for 30 min. After centrifugation at 3000 rpm for 30 min, 2 ml of the organic phase was transferred to a 10 ml glass tube and evaporated to dryness, and immediately thereafter 1—5 ml of isopropanol was added. The volume of the sample injected was always 50 μl. The chromatographic system used was a Shimadzu LC-6A apparatus equipped with an SPD-6A UV detector, a reversed-phase column (Nucleosil-C_{18}, 5 μm, 15 cm × 4.6 mm i.d.) and a Shimadzu C-RIA reporting integrator. The mobile phase was methanol (100%) at a flow rate 1 ml/min. The column eluate was monitored at 254 nm with a sensitivity of 0.001 a.u.f.s.; chart speed, 1.0 cm/min. The minimum detectable quantity of VK_1 was 5 ng.

Table I. The Permeability Coefficients of PABA through Nuclepore and Duragard Membranes and the f Values

Nominal pore diameter[a] (μm)	P_{PABA}[b] (× 10^4, cm/s)	Nominal thickness[a] (× 10^4, cm)	f[c]
0.8	4.29	10	0.051
0.6	3.77	10	0.045
0.4	4.37	10	0.052
0.2	3.81	10	0.045
0.1	3.36	5	0.020
0.05	2.49	5	0.015
0.04 × 0.4[d]	2.16	25	0.064
0.02 × 0.2[d]	1.30	25	0.039

a) Supplied by the manufacturer. *b*) $P_{PABA}=Df/h$. *c*) The diffusion coefficient of PABA in water at 25°C is 8.4×10^{-6} cm²/s.[15] *d*) Duragard membranes, slit dimension.

Table II. The Permeability Coefficients of PABA through Nitrocellulose Membranes and the f Values

Nominal pore diameter[a] (μm)	P_{PABA}[a] (× 10^4, cm/s)	Nominal thickness[a] (× 10^2, cm)	Nominal porosity[a] (%)	f[c]
0.8	2.53	1.50	80	0.45
0.65	1.70	1.50	79	0.30
0.45	1.62	1.45	78	0.28
0.3	1.62	1.40	75	0.27
0.2	1.52	1.33	73	0.24
0.1	1.71	1.10	65	0.22

a) Supplied by the manufacturer. *b*) $P_{PABA}=Df/h$. *c*) The diffusion coefficient of PABA in water at 25°C is 8.4×10^{-6} cm²/s.[15]

Results and Discussion

Characterization of Membranes and a Diffusion Cell To characterize the diffusional system with membranes having different pore characteristics, a diffusion study across the membranes was carried out using PABA as a standard diffusate, the molecular size of which is sufficiently small compared with the pore sizes of the membranes used. As the diffusion coefficient of PABA in water at 25°C is 8.4×10^{-6} cm²/s,[15] the f value can be calculated.

Tables I and II show the permeability coefficients, P_{PABA}, through Nuclepore and Duragard membranes and nitrocellulose membranes, respectively, and their f values in addition to the nominal parameters of the membranes supplied by the manufacturers.

Porous membranes are generally characterized in terms of three quantities, pore radius, pore length or thickness, and pore density. The f value reflects the pore density (ε) and the tortuosity (τ) of the membranes. Since the Nuclepore membranes (polycarbonate track-etch membranes) have pores that are essentially uniform circular cylinders, pore length is nearly equal to membrane thickness. The deviation of pore alignments from the normal falls within 30°, the average pore length being assumed to exceed membrane thickness only by 6.8%,[16] namely the tortuosity = 1.068. Assuming that $f=\varepsilon/\tau$, the ε values ranged from 0.048 to 0.056 for the membranes with pore sizes 0.2—0.8 μm (diameter) and 0.016 for the membrane with 0.05 μm pore size.

The Duragard membrane has slit-type pores which are reported to be relatively straight.[17] The f values of the two kind membranes were 0.064 and 0.039, respectively, which are comparable to those of the Nuclepore membranes.

In contrast, nitrocellulose membranes are of more than 100 μm in thickness and have ants' nest-like channels. The f values are much larger than the others. As the nominal porosity is given, the τ values may be calculated, ranging from 2 to 3. These values seem to fall in a reasonable range for such membranes.

Diffusion Studies of VK_1-Loaded Mixed Micelles Effective BS concentrations for mixed micelle formation with PC have been described elsewhere for various BSs.[13,18-20] The size and shape of the mixed micelles depend on the ratio of BS to PC in the micelle when the lipid is solubilized by BS.[19,21] The total ratio of the components in the dispersion, however, might not be equal to the ratio of the mixed micelle since some BS could exist as monomers. The monomer concentration is referred to as intermicellar concentration (IMC) and is different from the critical micelle concentration (CMC) determined for the BS alone.[21,22]

It is well known that dilution of mixed micelles beyond a critical concentration results in release of BS molecules from the mixed micelles, eventually leading to formation of vesicles.[19,20] It is therefore not always appropriate to use a diffusional system in which the concentration gradient across membranes is established with regard to the mixed micelles, particularly when the initial concentration in the receptor remains zero or much lower than that in the reservoir.

VK_1 is water-insoluble and is well solubilized by PC–BS mixed micelles.[13] Therefore, the diffusional study of the

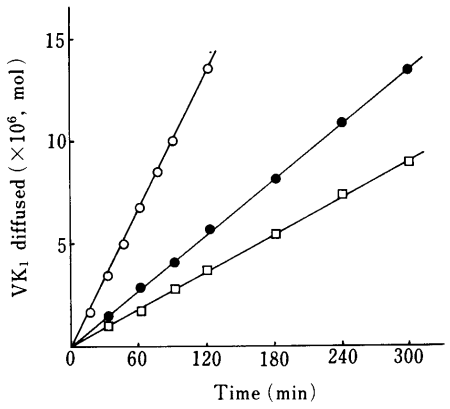

Fig. 2. The Amount of VK_1 Diffused through the Porous Membranes with Time

(○) Nuclepore membrane (pore size, 0.2 μm; VK_1 initial concn., 13.2 mM); (●) nitrocellulose membrane (0.2 μm, 14.5 mM); (□) Duragard membrane (0.04 × 0.4 μm; 14.9 mM). Temperature: 25 °C.

TABLE III. The Permeability Coefficient Ratio ($R = P_{mc}/P_{PABA}$) for Nucleopore Membranes and Duragard Membranes with Various Pore Sizes

Nominal pore diameter[a] (μm)	P_{mc} (× 10^5, cm/s)	P_{PABA} (× 10^4, cm/s)	R (P_{mc}/P_{PABA})
0.8	2.82	4.29	0.066
0.6	2.41	3.77	0.064
0.4	3.09	4.37	0.070
0.2	2.34	3.81	0.062
0.1	1.40	3.36	0.042
0.05	0.827	2.49	0.033
0.04 × 0.4[b]	0.544	2.16	0.025
0.02 × 0.2[b]	—[c]	1.30	—

a) Supplied by the manufacturer. *b*) Duragard membrane, slit dimension. *c*) Extremely slow.

TABLE IV. The Permeability Coefficient Ratio ($R = P_{mc}/P_{PABA}$) for Nitrocellulose Membranes and Various Pore Sizes

Nominal pore diameter[a] (μm)	P_{mc} (× 10^5, cm/s)	P_{PABA} (× 10^4, cm/s)	R (P_{mc}/P_{PABA})
0.8	1.47	2.53	0.058
0.65	0.918	1.70	0.054
0.45	0.842	1.62	0.052
0.3	0.907	1.62	0.056
0.2	0.851	1.52	0.056
0.1	0.564	1.71	0.033

a) Supplied by the manufacturer.

vitamin was conducted against corresponding vitamin-free mixed micelle solution. The concentration changes of the vitamin with time imply that the vitamin is transferred by the self-diffusion of the mixed micelles across membranes.

Figure 2 shows typical diffusional rates of VK_1 loaded in the mixed micelles through Nuclepore (pore size, 0.2 μm), Duragard (0.04 × 0.4, slit size) and nitrocellulose (pore size, 0.2 μm) membranes. No lag time was observed since pores in the membrane were allowed to be occupied by the VK_1-loaded mixed micelle solution in the initial experimental setup.

The relationship between concentration change of VK_1 and time basically follows Eq. 1. The permeability coefficient, P_{mc}, was calculated from the initial linear portions. Tables III and IV show the ratio of P_{mc} to P_{PABA}, R, for the Nucleopore membranes and nitrocellulose membranes with various pore sizes, respectively, in addition to the results with the Duragard membranes (Table III).

The R value would reflect the ratio of diffusion coefficients of mixed micelles and a standard substance (PABA), for the same apparatus and membrane.[23] It was noted that the R values for the Nuclepore membranes remain almost constant (average = 0.066) down to the 0.2 μm pore size and decrease at the 0.05 μm pore size. These results indicate that hindered diffusional transfer of the mixed micelles begins to occur in the channels of less than 0.2 μm pore size. The Duragard membranes, which have oblong channels as indicated, showed resistance to micelle transfer, which is largely limited by the shorter dimension of the channels.

Nitrocellulose membranes are much thicker and consequently have higher tortuosity of channels than Nuclepore membranes. The R values of the membranes were found to be comparable to those of the Nuclepore membranes (the average = 0.055) and the lower limit of constancy also follows suit.

From these results, the apparent diffusion coefficient of the mixed micelles was calculated to be $4.6—5.5 \times 10^{-7}$ cm^2/s, based on the average R values independent of the pore sizes of both Nuclepore and nitrocellulose membranes and the diffusion coefficient of PABA. The dependence of the micelle diffusion coefficient on micelle concentration, which may lead to the diffusion coefficient at infinite dilution, was not followed since changes of the molar ratio (PC/BS) of the mixed micelles are possible with decreasing concentration and consequently changes of the loading capacity of the vitamin may occur.

The obtained diffusion coefficient might tentatively be assigned to the 50 mM mixed micelles. If this magnitude could be taken as approximating to the diffusion coefficient of the mixed micelles, the familiar Stokes–Einstein equation could be applied to obtain the hydrodynamic radius:

$$D_{mc} = \frac{kT}{6\pi\mu r} \tag{3}$$

where k is Boltzman's constant, T is temperature, μ is the viscosity of the continuous phase, and r is the hydrodynamic radius. When the D_{mc} values is taken as 5.0×10^{-7} cm^2/s, the radius is given as 49 Å. This result is comparable to the hydrodynamic radius of PC–taurocholate mixed micelles (1 : 1 molar ratio) at a similar concentration (5 g/dl).[24]

As described earlier, the VK_1-loaded mixed micelles start experiencing hindrance to their diffusion in pores of 0.2 μm or less in diameter. The reduction of the diffusivity is frequently expressed as a function of the radius ratio of the diffusate and the pore size. If the critical pore diameter is assumed to be 0.2 μm, the ratio is obtained as 0.051. This implies that the pore size is about 20 times larger than the size of the diffusate in diameter.

For a cylindrical pore such as those of Nuclepore membranes used in this study, Faxen's formula gives

$$\frac{D_p}{D_\infty} = 1 - 2.04\lambda + 2.09\lambda^3 - 0.59\lambda^5 \tag{4}$$

where D_p and D_∞ are the diffusion coefficients in the pore and in bulk solution, respectively.[25] $\lambda = r_d/r_p$ where r_d and r_p are the radii of the diffusate and the pore. When $\lambda = 0.051$, $D_p/D_\infty = 0.90$, which seems to agree reasonably well with the theoretical prediction according to Eq. 4.

Although the complexity of the absorptive epithelium in the intestine may lead to difficulty in physical modeling, the geometric or physical figures of villi and microvilli can be clearly visualized by electron micrography, and reveal spaces of 30—150 μm for villi with a depth of 1—1.5 mm and 0.01—0.1 μm for microvilli with a depth of about 1 μm.[26] Accordingly, the diffusional process in the vicinity of the absorptive cell surface would be a primary process depending on the physical dimensions of the mixed micelles approaching the site of absorption. It is apparent that the VK_1-loaded micelles can readily reach the bottom of villi without diffusional hindrance, even without taking account of waving motions of the villi. Channels of microvilli may offer some resistance to diffusion of the mixed micelles, which is however likely to be compensated for by the short length of the channels.

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