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Fourier transform infrared-attenuated total reflection spectroscopy of hydration of dimyristoylphosphatidylcholine multibilayers

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The effect of hydration on the structure and molecular orientation of multibilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), cast on a germanium plate, was studied by means of polarized Fourier transform infrared (FT-IR)-attenuated total reflection spectroscopy. Compared with the dry state, the antisymmetric and symmetric CH_2 stretching bands of fully hydrated DMPC in the liquid-crystalline state were shifted to the higher frequency side, indicating the increase in the number of the *gauche* conformers. However, the dichroism of these bands revealed that the hydrocarbon chains of DMPC were still ordered and titled. The absorption bands of the glycerol ester, phosphoryl, and choline groups were broadened upon hydration, suggesting the activation of the librational or torsional motion. Furthermore, the dichroism of the polar head group bands of DMPC indicated that these groups retained a slight orientation even in the fully hydrated and fluid multibilayers.

Introduction

The structure of phospholipid bilayers has been of great interest as a basis to elucidate the functions of biomembranes. There have been considerable numbers of studies in the bilayer structure including the conformation and molecular packing of phospholipids by means of various techniques such as differential scanning calorimetry (DSC) [1,2], X-ray diffraction [1–4], electron microscopy [3], Raman [5] and infrared spectroscopy [6–9]. Akutsu et al. [10,11] and Fringeli [12] have independently applied the polarized infrared method to the study of the molecular orientation of phospholipids in the dry (or anhydrous) state. However, the molecular orientation of hydrated films has not been extensively investigated because of the strong infrared absorption of water. In order to overcome this difficulty, we applied the attenuated total reflection technique combined with the Fourier transform infrared (FT-IR) spectroscopy. To make in situ measurements of band frequency shifts induced by DMPC hydration, the cell previously described in Ref. 13 was used. Based on the observed frequency shifts and infrared dichroism, the structure and molecular orientation in the hydrated films were discussed in comparison with those in the dry ones. The sam-

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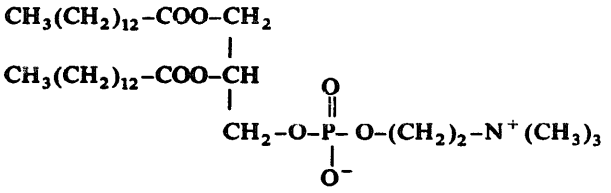
ple studied here was 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)



which is a very popular biomembrane phospholipid.

Materials and Methods

DMPC was purchased from Sigma Chemical Co. and used without further purification. Chloroform used as a spreading solvent was a specially prepared reagent (UVS-26) of Nakarai Chemicals Co. Ltd., Japan. Water was purified as described previously [14].

Cast dry films of DMPC were prepared by uniformly spreading the stock solution of 6 mg · ml^{-1} on one face of a germanium attenuated total reflection plate (52 × 18 × 2 mm) under streaming dry nitrogen. The film thickness was in the range from 1.5 to 4.5 μm. The germanium plate supporting the dry DMPC film was then assembled with the Teflon cell shown in Fig. 1 [13]. The cell consisted of two compartments, the film side of the germanium plate facing the one compartment. Temperature of the germanium plate was monitored by a copper-constantan thermocouple inserted through *c* or *d* into the second compartment (Fig. 1). The in situ spectroscopic study of DMPC hydration proceeded as follows:

(a) Cell, thermocouple, and germanium plate with overlaid cast dry film of DMPC were assembled and mounted inside the sample chamber of the spectrophotometer. The first spectrum was recorded.

(b) Without removing the cell, water was gently filled into the film side compartment through *a* or *b* (Fig. 1) to initiate DMPC film hydration. Within 1–2 min a second spectrum was recorded.

(c) Further spectra were recorded after step (b): 5 min, 20 min, 110 min, 280 min, and 22 h.

In this in situ hydration study, irreproducibility in sample preparation and repositioning of the sample were avoided. The temperature slowly

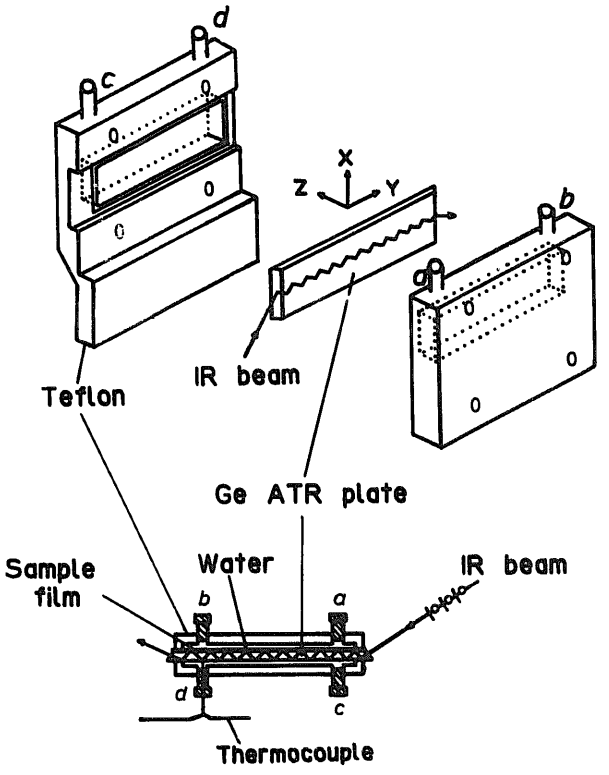


Fig. 1. Experimental set-up for infrared attenuated total reflection measurements of hydrated films.

drifted from 28 to 29.5 °C during the infrared measurements, but it was well above the gel to liquid-crystalline phase transition temperature of hydrated DMPC (24 °C).

The small (5 cm^3) capacity of the compartment prevented removal of the DMPC film from the germanium plate when water was introduced into the compartment. The FT-IR spectrophotometer was a Nicolet model 6000C. The sample was subjected to polarized infrared attenuated total reflection measurements. The detail of the spectral measurements was described elsewhere [14]. Five hundred interferograms collected with the maximum optical retardation of 0.25 cm were accumulated to yield spectra of high *S/N* ratio with a resolution of 4 cm^{-1}. The frequency reading was accurate to within ± 0.1 cm^{-1}.

Results and Discussion

Infrared attenuated total reflection spectra of dry and hydrated multibilayers of DMPC

Fig. 2a shows polarized infrared attenuated total reflection spectra of multibilayers of dry DMPC.

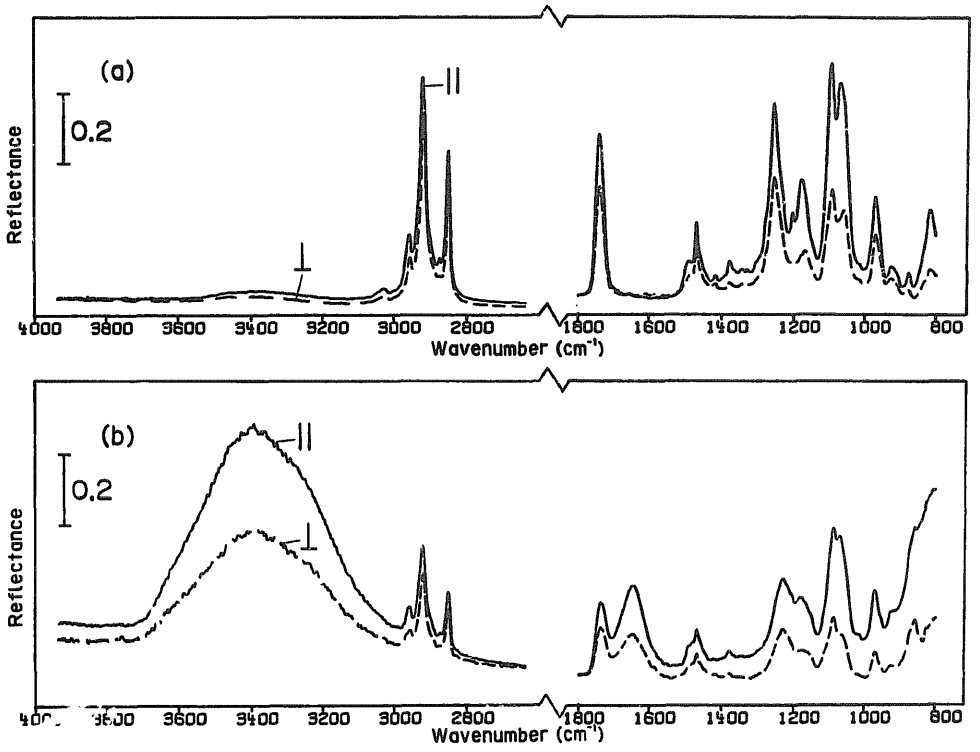


Fig. 2. Polarized infrared attenuated total reflection spectra of the (a) dry and (b) hydrated films of DMPC measured at 29 °C. Solid and broken lines refer to the electric vector parallel and perpendicular to the plane of incidence, respectively.

TABLE I

ASSIGNMENT AND DICHROIC RATIO OF MAJOR ABSORPTION BANDS FOR DRY AND HYDRATED FILMS OF DMPC

Dry film		Hydrated film		Assignment
Wavenumber (cm^{-1})	Dichroic ratio, r [a]	Wavenumber (cm^{-1})	Dichroic ratio, r [a]	
		3396	1.9±0.1	OH stretching band of water
3032	∥ [b]	– [c]		asymmetric CH_3 stretching band of N^+-$(CH_3)_3$
2957	1.8±0.1	2958	1.6±0.2	asymmetric stretching band of terminal CH_3
2918	1.1±0.0	2922	1.3±0.1	antisymmetric CH_2 stretching band
2874	>5	2875	∥ [b]	symmetric stretching band of terminal CH_3
2850	1.1±0.1	2853	1.3±0.1	symmetric CH_2 stretching band
1738	1.3±0.1	1735	1.4±0.1	C=O stretching band
		1647	1.9±0.1	OH bending band of water
1468	1.1±0.1	1468	1.3±0.1	CH_2 scissoring band
1378	2.0±0.3	1379	1.9±0.4	symmetric bending band of terminal CH_3
1254	1.3±0.2	1231	1.4±0.1	antisymmetric PO_2^- stretching band
1204	∥ [b]	– [d]		CH_2 wagging band of *trans* conformer
1176	2.7±0.2	1180	2.3±0.3	antisymmetric C-O-C stretching band
1093	1.9±0.3	1088	1.8±0.1	symmetric PO_2^- stretching band
1064	2.5±0.2	1069	2.4±0.2	symmetric C-O-C stretching band
970	1.4±0.1	970	1.9±0.1	asymmetric N^+-$(CH_3)_3$ stretching band

[a] $\Delta A_{\parallel}/\Delta A_{\perp}$.
[b] Parallel dichroism.
[c] Peak not resolved.
[d] Peak disappeared.

The frequencies and assignments of the major bands were summarized in Table I, being in good agreement with those previously reported [14,15].

The hydration of DMPC multibilayers occurred in less than 5 min (spectra not shown) after the introduction of water. Fig. 2b shows the polarized spectra of multibilayers of hydrated DMPC recorded after 22 h. The observed bands were also listed in Table I. New bands around 3400 and 1650 cm^{-1} are assigned to the OH stretching and bending vibrations of water, respectively. Since the multibilayer films were much thicker than the penetration depth of the evanescent wave (0.2–0.8 μm) of the attenuated total reflection measurements, these bands were not due to bulk water but to that which penetrated into the hydrophilic part of the DMPC multibilayers. The decrease in the intensity of the lipid bands indicates the swelling of the dry films on hydration. For example, after 5 min the intensity of the CH stretching bands of hydrated DMPC was about 53% (spectrum not shown) of that of the dry one and, as seen in Figs. 2a and 2b, about 44% after 22 h. The last result suggests that, provided the absorption coefficients of the dry and hydrated DMPC are equal, the film thickness has more than doubled on hydration. The intensity of the water bands indicates a water content of 40 wt% in the hydrated films, as compared with the peak intensity of the known amount of water (not shown). According to Ref. 1, 40 wt% of water is enough to complete the hydration of DMPC. The fast rate of lipid films hydration is in striking contrast to the slow ones reported for peptides in lipid bilayers [14,16,17] and for dry protein films [18].

Effect of hydration on the spectral features of DMPC films

Comparing with dry films, there happened noticeable changes in the spectral features, especially in band frequencies and widths of hydrated films. We will describe those for the hydrocarbon and polar group bands of DMPC separately.

Hydrocarbon bands. Two intense bands of dry DMPC at 2918 and 2850 cm^{-1} assigned to the antisymmetric and symmetric CH_2 stretching vibrations, respectively, were shifted to the higher frequency side (2922 and 2853 cm^{-1}) in the hydrated state as shown in Table I. It has been reported that the higher frequency shift of the CH_2 stretching bands is ascribed to the melting of the hydrocarbon chains accompanied by the introduction of the ***gauche*** conformers [8,19–22]. At the same time, the weak CH_2 wagging band of the all-***trans*** conformers at 1204 cm^{-1} in dry DMPC disappeared in the hydrated state. It is considered from these facts that the hydrocarbon chains of hydrated DMPC are in the liquid-crystalline state. The CH_2 scissoring band at 1468 cm^{-1} did not change in frequency upon hydration but broadened.

Polar group bands. The most remarkable change of the polar bands upon hydration was observed for the antisymmetric PO_2^- stretching mode. The sharp band at 1254 cm^{-1} was largely broadened and shifted to 1231 cm^{-1}, presumably due to the hydrogen bonding between phosphate group and water [22–24]. Other bands due to the symmetric PO_2^- stretching vibration (1093 cm^{-1}), the antisymmetric and symmetric C-O-C stretching vibrations (1176 and 1064 cm^{-1}), and the ester C=O stretching vibration (1738 cm^{-1}) are also shifted and broadened upon hydration. These facts reflect the changes in the environment and the motion of the respective polar groups in hydrated DMPC. The interaction between water and DMPC molecules causes the change in spatial packing of the polar groups of DMPC. On the other hand, the choline band at 970 cm^{-1} due to the asymmetric N^+-$(CH_3)_3$ stretching vibration did not change the position.

Effect of hydration on the molecular orientation

The dichroic ratio defined by

$$r = \Delta A_{\parallel} / \Delta A_{\perp} \qquad (1)$$

was evaluated for each band from the polarized infrared attenuated total reflection spectra as previously stated [14]. Here ΔA is the change in reflectance due to the presence of the film. The r values obtained are also given in Table I for both dry and hydrated DMPC. It is seen from this table that the dichroic ratios of the CH_2 stretching and scissoring bands are slightly increased upon hydration (from 1.1 to 1.3). The most prominent change of this value is seen for the choline band at

970 cm^{-1} (from 1.4 to 1.9). These changes in dichroism suggest the effect of hydration on the orientation of the hydrocarbon chains and the polar head groups of DMPC.

In order to characterize the molecular orientation of hydrated DMPC films, the equation Flournoy and Schaffers [25] proposed for films optically thicker than the penetration depth was applied to the polarized attenuated total reflection spectra of the present study. By using proper values of 4.00 and 1.44 for the refractive indices of germanium and film, respectively, and assuming the uniaxial orientation of the particular transition moment (TM) with respect to the surface normal (z axis), the orientational order parameter (F) of the transition moment can be generally related to the dichroic ratio (r) as follows [14]

$$F=\frac{r-2.00}{r+1.45} \tag{2}$$

Here, we assumed the model of the uniaxial orientation of the hydrocarbon chain as shown in Fig. 3. The transition moments of the antisymmetric and symmetric CH_2 stretching vibrations of the hydrocarbon chains are freely rotated with the angle 90° around the chain axes. Further, the hydrocarbon chain axes are uniformly oriented with an angle * α around the surface normal. Then, the expression of $F(\alpha)$ for transition moments of the CH_2 stretching and scissoring vibrations is given by

$$F(\alpha)=\frac{3\cos^2 90°-1}{2}\cdot\frac{3\cos^2\alpha-1}{2}=-\frac{1}{2}\cdot\frac{3\cos^2\alpha-1}{2} \tag{3}$$

On the other hand, the transition moment of the particular vibration of each polar group which constitutes the complex 'DMPC head group' is uniaxially oriented with an angle * β around the surface normal as also shown in Fig. 3. Then the orientational order parameter of the transition moment ($F(\beta)$) is expressed by

$$F(\beta)=\tfrac{1}{2}(3\cos^2\beta-1) \tag{4}$$

* Generally, the angles α and β have a distribution. Here, α and β mean the angles α_M and β_M which satisfy $\overline{\cos\alpha}=\cos\alpha_M$ and $\overline{\cos\beta}=\cos\beta_M$, respectively.

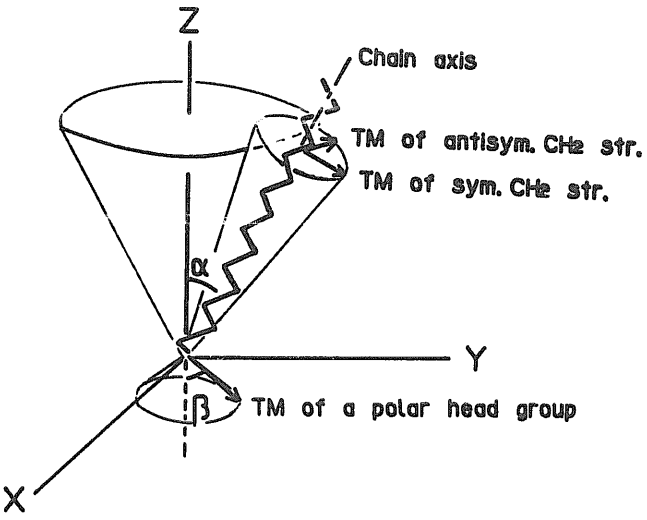


Fig. 3. Schematic representation of the uniaxial orientation of the hydrocarbon chain and of a polar head group of the DMPC molecule.

From Eqns. 2, 3, and 4, it is apparent that (i) in the case of complete vertical orientation where $\alpha=\beta=0°$, $F(\alpha)=-1/2$ and $F(\beta)=1$, (ii) in the case of complete horizontal orientation where $\alpha=\beta=90°$, $F(\alpha)=1/4$ and $F(\beta)=-1/2$, and (iii) in the case of random orientation where $\cos^2\alpha=\cos^2\beta=1/3$, or in the case of the particular orientation with $\alpha=\beta=54.7°$, $F(\alpha)=F(\beta)=0$ and then r becomes 2.00. Thus, it should be noted that in the present attenuated total reflection measurements $r=2.00$ implies random orientation, while in the ordinary transmission measurements no dichroism ($r=1.00$) implies random orientation.

In the present study, we discuss the orientation of water and DMPC semi-quantitatively on the basis of the above discussions.

Water. As shown in Table I, $r=1.9$ was obtained from both the OH stretching and bending bands. This suggests that the water molecules are almost randomly oriented in the DMPC bilayers. If a large part of water interacts with lipid molecules, noticeable orientation of water will be observed. However, it is not the case. Thus almost all of the water molecules seem to be free in the lipid bilayers.

Hydrocarbon chain of DMPC. Since $r=1.1$ was obtained for the CH_2 scissoring and antisymmetric and symmetric stretching bands of dry films (Table I), we have $\alpha\simeq 26°$ from Eqns. 2 and 3.

This result is in substantial agreement with the corresponding value (29–33°) of thin-dry films of dipalmitoylphosphatidylcholine (DPPC) reported by Fringeli [12]. Upon hydration, the dichroic ratios (r values) of these CH_2 scissoring and stretching bands increased to 1.3, resulting in $\alpha \simeq 35°$. This value means that the hydrocarbon chains of DMPC display some order and tilt even in the liquid-crystalline phase. The increase in the α value on hydration may be ascribed to a small increase in the number of ***gauche*** conformers. Thus it is concluded that the hydrophobic interaction between adjacent hydrocarbon chains plays an important role in the retention of the bilayer structure in the liquid-crystalline DMPC.

The spectra shown in Fig. 2b indicate that our film is in the liquid-crystalline smectic C state, where the hydrocarbon chains are fluid, uniaxially oriented and tilted [26]. This orientation of DMPC hydrocarbon chains is not contradictory to the generally accepted 'fluid mosaic model' of biomembrane structures in a liquid-crystalline state [27]. It is rather reasonable to consider 'incomplete fluidity' or 'partial rigidity' of the hydrocarbon chains, which is essential to maintain biomembrane functions like the stabilization of enzyme proteins and ion channels even in the liquid-crystalline state.

Polar head group of DMPC. For each absorption band of the polar head groups, the orientational order parameter ($F(\beta)$) and the tilt angle (β) of the transition moment are estimated from the r value in Table I by using Eqns. 2 and 4. The results for the dry and hydrated films are summarized in Table II. It is shown that in the dry films the transition moments of the C=O stretching and antisymmetric PO_2^- stretching bands are tilted by about 65° with respect to the surface normal. These values are compatible with the results (64°) of dry films of DPPC reported by Akutsu et al. [11] and those (63–67°) of DMPC by Nabedryk et al. [15].

It is also apparent that the β values of the bands of the polar groups are almost the same before and after hydration except for the asymmetric N^+-$(CH_3)_3$ stretching band. The persistence of the head group orientation in the liquid-crystalline phase L_α has also been shown by the neutron diffraction studies of fully hydrated DPPC [28]. However, there are infrared frequency shifts (especially remarkable for the antisymmetric PO_2^- stretching band) and the band broadening of these bands as stated before. The frequency shift reflects environmental changes of these groups, and the band broadening is the result of the activation of the librational or torsional motion of the polar head groups [29]. Therefore, the negligible change in the β values upon hydration reveals that although average orientation angles of the polar groups are almost unaltered, their distribution ranges are extended. It should be noted that, as in the case of the hydrocarbon chains, the polar head groups of DMPC are not completely disordered and remain slightly oriented even in the fully hydrated state. This result suggests that the electrostatic interaction between the polar groups of DMPC is still important for the retention of the bilayer structure in the hydrated state.

TABLE II

CHANGE IN THE ORIENTATION OF THE POLAR HEAD GROUPS FOR DRY AND HYDRATED FILMS OF DMPC

		$F(\beta)$		β	
		dry	hydrated	dry	hydrated
C=O str.		−0.25	−0.21	65°	64°
Antisym.	C-O-C str.	0.17	0.08	48°	52°
Sym.	C-O-C str.	0.13	0.10	50°	51°
Antisym.	PO_2^- str.	−0.26	−0.21	66°	64°
Sym.	PO_2^- str.	−0.04	−0.06	56°	57°
Asym.	N^+-$(CH_3)_3$ str.	−0.21	−0.03	64°	56°

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