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Orientation studies of hydrated dipalmitoylphosphatidylcholine multibilayers by polarized FTIR-ATR spectroscopy

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Polarized Fourier-transform infrared-attenuated total reflection spectroscopy has been applied to explore the temperature-dependence of molecular orientations in multibilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) of various degrees of hydration. The order parameter of the hydrocarbon chain, evaluated from the dichroic ratios of the antisymmetric and symmetric CH_2 stretching bands, was drastically decreased at the main (or gel to liquid-crystalline phase) transition temperature (T_m) irrespective of the water content, suggesting that the hydrocarbon chain is in a disordered state as a result of chain-melting associated with an increase in the number of the *gauche* conformers. On the other hand, the dichroic ratios of the polar bands of hydrated DPPC assignable to the symmetric PO_2^- stretching and asymmetric $N^+(CH_3)_3$ stretching modes were increased mainly at the pretransition temperature (T_p), except for less hydrated case. The dichroic ratios of both the OH stretching and OH_2 bending bands of water showed the same temperature-dependence as those of the polar bands. These results indicate that the pretransition is ascribable mainly to the reorientation of the polar groups of the DPPC and bound water, while the main transition is due to the orientational disorder of the hydrocarbon chains. For less hydrated DPPC, the reorientation of the polar groups and water did not occur around T_p, but in the higher temperature region around T_m. This is in accord with the previously reported observation that the pretransition disappears for less hydrated DPPC. In this case, the polar groups and water may reorient following the reorientation of the hydrocarbon chains near T_m.

Introduction

The structure of hydrated phospholipid bilayers has been of great interest in the relation to that of the lipid matrix in biomembranes. In addition to X-ray and neutron diffraction [1–4], NMR [5,6], ESR [7], Raman [8–10], and differential scanning calorimetric (DSC) [1,3,11,12] studies, Fourier transform infrared (FTIR) spectroscopy of aqueous phospholipid dispersions has been developed extensively during the last decade [13–20]. In particular, the polymorphic phase behaviour of phospholipid membranes has been investigated successfully by this technique using frequencies, bandwidths, and other spectral parameters [14,15,18]. Holmgren et al. [17] have studied the FTIR dichroism of phospholipid lamellae in the liquid-crystalline phases. As far as we know, however, nothing has been reported so far about the temperature-dependence of FTIR dichroism of phospholipid membranes.

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Previously, we have applied polarized FTIR-attenuated total reflection (ATR) spectroscopy to the study of polypeptide-containing phospholipid multibilayer systems and demonstrated that this is a powerful tool for determining the molecular orientation of both the lipids and peptides [21]. Recently, we have also studied by FTIR-ATR spectroscopy the hydration of phospholipid films [22]. In the present work, we deal with the temperature-dependent FTIR-ATR spectra of fully and partially hydrated multibilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and discuss the orientations of the hydrocarbon chains and the individual polar groups of DPPC. Special attention is also paid to the orientation of the bound water.

Materials and Methods

L-DPPC from the Sigma Chemical Co. was used without further purification. Chloroform, used as a solvent, was a specially prepared reagent from Nacalai Tesque, Kyoto. Water was purified as described previously [21].

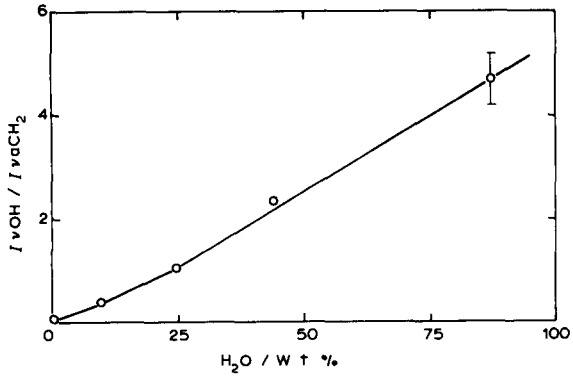

Fig. 1. Relation between the water content in the DPPC multibilayers and the intensity ratio $I_{\nu OH}/I_{\nu_a CH_2}$ in ATR spectra.

Dry multibilayer films of DPPC were prepared by uniformly spreading the chloroform solution (300–400 μl) of 11 mg · ml^{-1} on one face of a germanium ATR plate (52 × 18 × 2 mm) followed by the gradual evaporation of the solvent. The film thickness estimated from its total weight was about 3–4 μm. Then the germanium plate was assembled with a Teflon cell, which has two compartments, as reported previously [22]. The film-side compartment of the cell was connected to a jacket thermostated by a Neslab model RTE-4 refrigerated-bath circulator using a 1 : 1 mixture of ethylene glycol and water. The temperature of the sample was monitored by a copper-constantan thermocouple inserted into the film-side compartment. The accuracy of the temperature control and reading was within ±0.1°C.

The temperature-dependent spectroscopic study proceeded as follows: After the thermostated cell was mounted inside the sample chamber of the spectrophotometer, a known amount of water was carefully added to the film-side compartment (capacity 1.5 ml) to hydrate the DPPC films. The degree of hydration was determined with the aid of a calibration curve (Fig. 1), which gave the relation between the amount of water that had penetrated into the film and the intensity ratio of the OH stretching band of water to the antisymmetric CH_2 stretching band of DPPC. The calibration curve was obtained by recording the ATR spectra of weighed amount of DPPC and water on a germanium plate. Thus, the water contents (the weight of water in the film as a percentage of the total weight of the film plus water) were found to be 85 ± 5, 25 ± 2, and 10 ± 2 wt%, when 1.5, 8.0 · 10^{-3}, and 3.5 · 10^{-3} ml of water were added, respectively. In the latter two cases, spacers of the appropriate thickness were put into the film-side compartment to reduce its capacity. This procedure was necessary to keep the water in uniform contact with the whole film face, and also to prevent the removal of the film from the germanium surface. Prior to spectral measurements, the sample was incubated at 50°C for 0.5–1 h to complete the film hydration. Then the films were subjected to polarized FTIR-ATR measurements in the temperature range from 20 to 60°C. Each spectrum was measured after the sample was heated to a predetermined temperature and left for 5 min to reach thermal equilibrium. Spectra were recorded on a Nicolet 6000C FTIR spectrophotometer equipped with a mercury cadmium telluride detector. ATR measurements were accomplished using a Perkin-Elmer multiple-ATR attachment. The angle of incidence was 45° and the number of total reflection was 12 on the film side. To improve the polarization quality, two wire-grid polarizers were placed in parallel with each other after the ATR attachment. Three 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 accuracy of the frequency readings was better than ±0.1 cm^{-1}.

Dichroic ratio defined by $A_{\parallel}/A_{\perp}$ was calculated from the polarized ATR spectra. The absorbance (A) is obtained as the peak height of each absorption band. The base-line method was used to minimize the background artefact.

Results and Discussion

It has been known from DSC and X-ray diffraction studies [1] that approx. 30 wt% water is enough to complete the hydration of the DPPC films. Fully hydrated multibilayers of DPPC show characteristic transitions at 41.5 and approx. 34°C, which have been referred to as the main transition (the gel to liquid-crystalline phase transition) and the pretransition, respectively [11,12,15,18]. From DSC studies of partially-hydrated multibilayers of DPPC, Kodama et al. [11,12] have indicated that the main transition temperature (T_m) rises to 98°C on decreasing the water content to almost zero. The pretransition temperature (T_p) of the DPPC multibilayers is almost constant at water contents of more than 25 wt% and disappears at less than 17 wt% [11].

Fig. 2 illustrates the polarized FTIR-ATR spectra of fully hydrated DPPC multibilayers (with 85 wt% water) at 25°C (below T_p). Assignments of the major absorption bands are summarized in Table I. In the following sections we will discuss the molecular orientation of both the DPPC and bound water in the multibilayers from results obtained by polarized FTIR-ATR measurements.

Orientation of the hydrocarbon chains

Figs. 3a, b, and c show the polarized ATR spectra in the CH stretching region of fully hydrated DPPC at 25°C (below T_p), 38°C (between T_p and T_m), and 51°C (above T_m), respectively. Comparison between spectra a and b suggests that the dichroism of both the

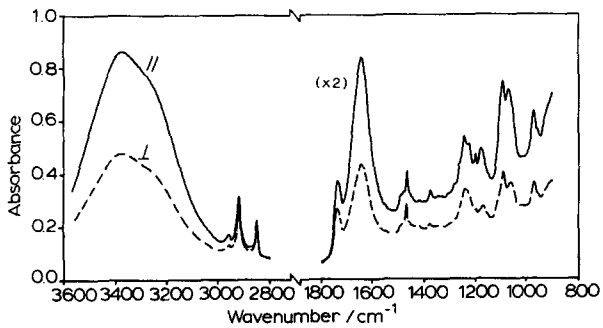

Fig. 2. Polarized FTIR-ATR spectra of fully hydrated DPPC multibilayers (with 85 wt% water) at 25 °C (below T_p). Solid and broken lines refer to the electric vector parallel and perpendicular to the plane of incidence, respectively.

antisymmetric and symmetric CH_2 stretching bands at 2918 and 2850 cm^{-1} is almost unaltered upon pretransition. Furthermore, no appreciable frequency change is observed for either band. However, the parallel dichroism for both bands is augmented in spectrum c, accompanied by a band shift to higher frequency. From the FTIR studies of fully hydrated DPPC, Mantsch et al. [14,15] have reported that the shifts to higher frequency of these two bands are prominent at T_m, but slight at T_p. The magnitude of the frequency shifts shown in Fig. 3 are quite consistent with those reported by Mantsch et al. [14,15]. A similar dichroic feature is observed for the CH_2 scissoring band at 1468 cm^{-1} (not shown here).

The temperature-dependence of the dichroic ratio of the symmetric CH_2 stretching band for the fully hydrated DPPC (with 85 wt% water) is shown by the broken line in Fig. 4A. Although the dichroic ratio in the lower temperature region is nearly equal to 1.0 and remains unaltered at T_p, it is sharply increased up to 1.4 at T_m. The same features are also found for both the antisymmetric CH_2 stretching and CH_2 scissoring bands (not shown here). These results suggest that the orientational changes of the hydrocarbon chains in DPPC occur at T_m.

TABLE I

Assignments of the major absorption peaks of hydrated DPPC in the gel phase below T_p

Wavenumber (cm^{-1})	Assignment
3380	OH stretching band of water
2957	asymmetric stretching band of terminal CH_3
2918	antisymmetric CH_2 stretching band
2874	symmetric stretching band of terminal CH_3
2850	symmetric CH_2 stretching band
1735	ester C=O stretching band
1640	OH_2 bending band of water
1468	CH_2 scissoring band
1377	symmetric bending band of terminal CH_3
1244–1224	antisymmetric PO_2^- stretching band [a]
1200	CH_2 wagging band of the *trans* conformer
1178–1168	antisymmetric C–O–C stretching band [b]
1090	symmetric PO_2^- stretching band
1068–1058	symmetric C–O–C stretching band [b]
970	asymmetric N^+-$(CH_3)_3$ stretching band

[a] Peaks are not resolved because of overlapping with the CH_2 wagging band.

[b] Frequencies of the peak maxima are different in *p*- and *s*-polarized spectra.

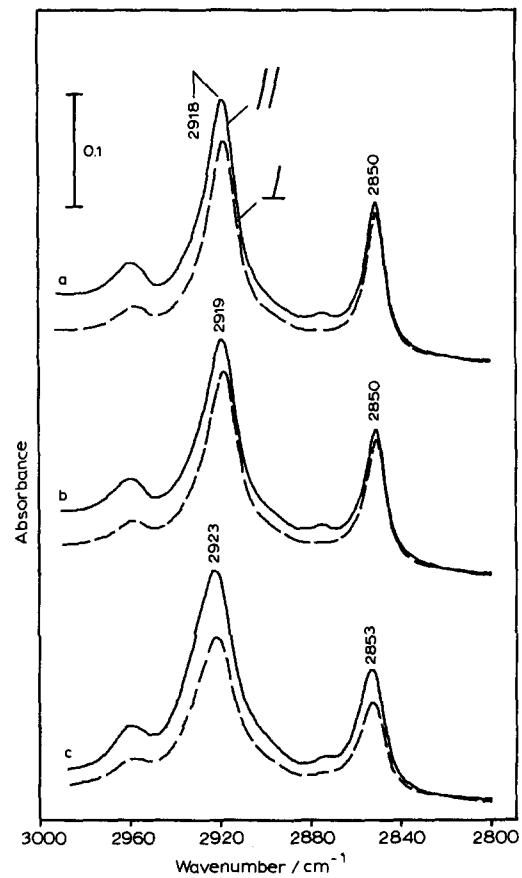

Fig. 3. Polarized FTIR-ATR spectra in the CH stretching region of the fully hydrated DPPC multibilayers (with 85 wt% water) at (a) 25 °C, (b) 38 °C, and (c) 51 °C. Solid and broken lines are the same as those in Fig. 2.

In order to evaluate the molecular orientation of DPPC, we used Flournoy and Schaffers' equation [23] on the assumption of uniaxial orientation of the hydrocarbon chains around the surface normal. This assumption was experimentally confirmed by the fact that no dichroism was found in the polarized transmission spectra. This is also reasonable, since the CH_2 scissoring band appears as a singlet at 1468 cm^{-1}, indicating that the hydrocarbon chains are predominantly packed in a hexagonal subcell lattice, where each hydrocarbon chain can rotate freely around its long axis oriented perpendicularly to the film surface. Therefore, the orientational order parameter of the hydrocarbon chain f is given by

$$f = -2(D-2)/(D+1.45) \quad (1)$$

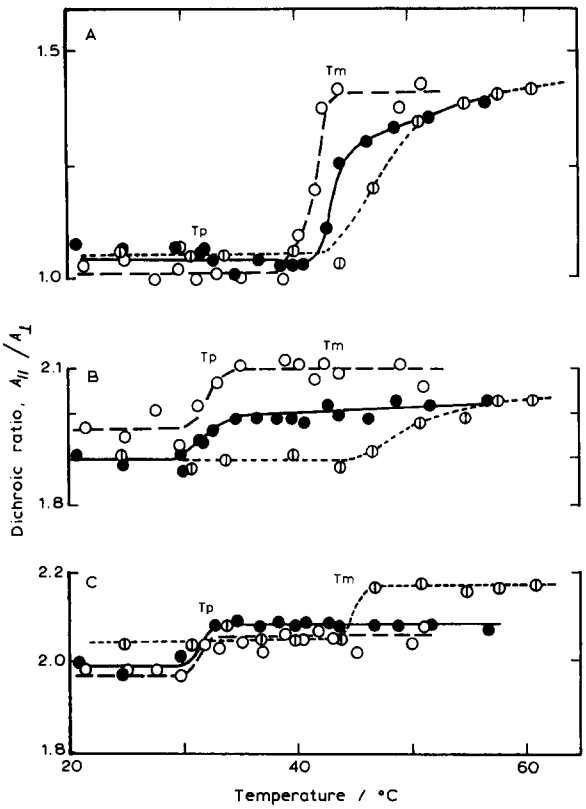

Fig. 4. Temperature dependence of the dichroic ratios of (A) the symmetric CH_2 stretching and (B) symmetric PO_2^- stretching bands of DPPC, and (C) the OH_2 bending band of water in multibilayers with 85 (○- - -○), 25 (●———●), and 10 wt% water (Ⓓ- - - -Ⓓ).

where D is the dichroic ratio ($A_{\parallel}/A_{\perp}$) of the antisymmetric and symmetric CH_2 stretching bands, the transition moments of which are considered to be freely rotated with the angle 90° around the chain axis [22].

Application of Eqn. 1 to the observed dichroic change for DPPC with 85 wt% water shown by the broken line in Fig. 4A indicates that the f value is 0.78 at lower temperature, remaining unaltered at T_p, but decreases to 0.40 at T_m. Since $f = 0$ ($D = 2.0$) and 1.0 ($D = 0.85$) imply the random and completely vertical orientation of the hydrocarbon chains, respectively [22], the sharp decrease in the f value at T_m suggests a phase-transition involving disorder of the hydrocarbon chains and the onset of molecular reorientation in the liquid-crystalline phase due to partial chain-melting associated with an increase in the number of *gauche* conformers. Lack of the reorientation of the hydrocarbon chains at T_p is consistent with the frequency invariance of the CH_2 stretching bands described above. Furthermore, the order parameter of the hydrocarbon chain in the gel phase (0.78) has nearly the same value as that obtained for the dry (solid) DPPC film in our previous study (0.72) [21]. This agreement shows that the hydrocarbon chains are mainly in the *trans*-zigzag conformation in the gel phase as in the solid state. These facts suggest little effect of hydration on the orientation of the hydrocarbon chains in the gel phase, and therefore suggest that there is a strong hydrophobic interaction between the adjacent hydrocarbon chains even in the fully hydrated gel state.

The f value of 0.40 in the liquid-crystalline phase implies that the hydrocarbon chains of DPPC are in a less ordered state than in the gel phase, but still maintain some degree of order of molecular orientation similar to the case of the fully hydrated DMPC [22].

For partially hydrated multibilayers, a temperature-dependence of the dichroism was also found for the CH_2 bands of DPPC. Solid and dotted lines in Fig. 4A indicate this for the symmetric CH_2 stretching band of DPPC with 25 and 10 wt% water, respectively. Similar to the case of fully hydrated DPPC, the dichroic ratios of the partially hydrated DPPCs are unaltered at T_p, but largely increased around T_m. It is apparent, however, that the changes are rather gradual and that they shift to higher temperatures for the less hydrated multibilayers (43 and 48°C for DPPC with 25 and 10 wt% water, respectively). The latter observations correspond well to the observed increase in T_m with decreasing the water content in the phase diagram [11]. The dichroic ratios in the gel and liquid-crystalline phases are almost independent of the degree of hydration within experimental error.

Orientation of the polar head groups

A characteristic temperature-dependence of the dichroic ratios also appears for the infrared bands due to the polar groups of DPPC. Fig. 4B shows those for the symmetric PO_2^- stretching band at 1090 cm^{-1} of the DPPC multibilayers with 85, 25 and 10 wt% water. In the cases of DPPC with 85 and 25 wt% water, the dichroic ratios of the band are apparently increased at T_p, but not at T_m. Similar temperature-dependence of the dichroism is also observed for the asymmetric $N^+(CH_3)_3$ stretching band of choline at 970 cm^{-1}. These facts indicate that both the phosphate and choline groups of DPPC are mainly reoriented at T_p.

On the other hand, the dichroic ratio of the symmetric PO_2^- stretching band of less hydrated DPPC with 10 wt% water does not change at T_p, and gradually increases around T_m (~ 50°C), as shown in Fig. 4B. The former result corresponds to the above-mentioned observation by DSC that the pretransition of DPPC disappears when the water content decreases to less than 17 wt% [11]. The latter result in Fig. 4B will be discussed in the next section in connection with the orientation of bound water.

Orientation of water

Fig. 4C shows the temperature-dependence of the dichroism for the OH_2 bending band (1640 cm^{-1}) of water in the DPPC multibilayers of various degrees of hydration. The dichroic ratios are increased at T_p for the multibilayers with 85 and 25 wt% water. For DPPC

with 10 wt% water, on the other hand, it is unaltered at T_p but increased in the higher temperature region around T_m. The same dichroic features are observed for the OH stretching band at 3380 cm^{-1}. It should be noted that these dichroic changes for the water bands are quite similar to those observed for the polar bands of DPPC shown in Fig. 4B. This result apparently indicates a strong cooperation between the polar groups of DPPC and the surrounding water. From these results we can conclude that for less hydrated DPPC with 10 wt% water, which does not show a T_p, the polar groups and bound water are reoriented at T_m following the reorientation of the hydrocarbon chains.

According to the ^{2}H-NMR study by Hauser et al. [24], there are three species of water in the hydrated DPPC multibilayers. They find that for DPPC with a water content of less than 20 wt% (10 water molecules per DPPC), all the water molecules are bound to DPPC; the water molecules within 20 and 35 wt% (10–20 water molecules per DPPC) are trapped between the adjacent lipid bilayers, and can be rapidly exchanged with bound water; and that the water molecules which exceed 35 wt% are free. These three different species of water have also been detected by the DSC study [25]. On the basis of this information, we can also have some insight into the orientation of the bound water in the hydrated DPPC multibilayers from the dichroism of the OH_2 bending band of water shown in Fig. 4C. For DPPC with 10 wt% water, the dichroic ratio of the band is almost equal to 2.2 above T_m, while for DPPCs with 25 and 85 wt% water, the ratios are 2.0 below T_p and 2.1 above T_p. This fact indicates that the bound water has a slight preference to orient with its symmetry axis directed perpendicularly to the film surface, while the trapped and free waters are in almost random orientation. Small changes in the dichroic ratio at T_p observed for the largely hydrated DPPC suggest that reorientation of bound water, which is present as a part of the total water, occurs at the pretransition. The result that the increase in the dichroic ratio at T_p is equally large for DPPC with 25 and 85 wt% water implies that the trapped water plays important role at the pretransition, as compared to the free water. This is consistent with the facts that the pretransition started to occur when the trapped water is incorporated into the adjacent lipid bilayers, and that T_p remains constant even in DPPC with large contents of free water [11].

Conclusions

The present study of FTIR-ATR dichroism of hydrated DPPC multibilayers provides useful information about the orientation of DPPC itself and of the bound water at the phase transitions of DPPC. The main transition of DPPC is ascribed to the orientational disorder of the hydrocarbon chains as a result of chain-melting associated with an increase in the number of the *gauche* conformers. This transition is independent of the degree of hydration. In contrast to this, the pretransition is associated with orientational changes of the polar groups of DPPC and the bound water. Reorientation of the polar groups and bound water depends upon the degree of hydration; it occurs at T_p for largely hydrated DPPC but at T_m for less hydrated DPPC which does not have the pretransition.

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