Langmuir

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pubs.acs.org/Langmuir

Water State and Carbonyl Distribution Populations in Confined Regions of Lipid Bilayers Observed by FTIR Spectroscopy

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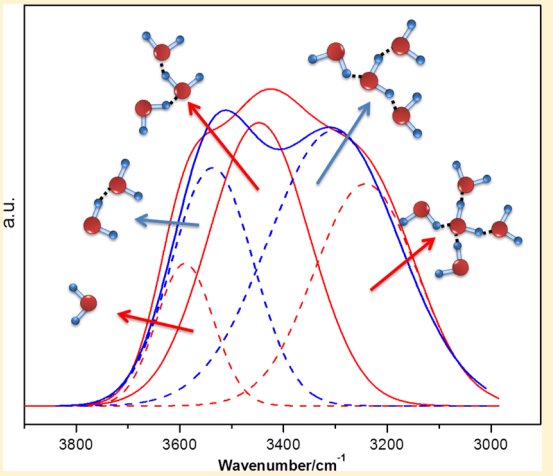
ABSTRACT: It has been suggested that water in confined regions presents different properties than bulk water, mainly because of the changes in water population species that may be induced by the adjacent walls of different polarities in terms of hydrogen bond formation. In this context, it would be expected that lipids in the gel and the fluid states should offer different templates for water organization. The presence of water pockets or defects in lipid bilayers has been proposed to explain the insertion of charged and polar peptides and amino acids in membranes. In this work, we provide direct evidence by means of FTIR spectroscopy that water band profiles are changed whether lipids are in the solid state, in the gel state after heating and cooling across the phase transition, or in the fluid state. The different bands found in each case were assigned to different H-bonded water populations in agreement with the exposure of carbonyl groups.



INTRODUCTION

The comprehension of the role of water in biological systems has regained increasing interest in the last few years because it has been recognized that it has a profound effect on the activity of enzymes, the stability of biological structures such as DNA, and the structure and function of membranes.[1−3] Specifically, it is not possible to have a complete picture of protein membrane interaction without considering the role of interfacial water in the thermodynamic process of minimizing the free energy of the interactions.

Because thermodynamics is related to order−disorder and energy transitions, the formation of structures as a result of hydrogen bonds between water molecules themselves and with lipids and proteins is important to understanding the behavior of water near the lipid surfaces. In this regard, it is known that the properties of water adjacent to different wall materials strongly differ from those of bulk water becauses of its interaction with hydrophilic−hydrophobic groups.[4,5] Furthermore, structural and dynamic properties of the water of hydration and their influence on the dynamic behavior of biomembranes, as studied by neutron scattering techniques,[6] revealed the strong interaction of the first hydration layer with the membrane surface and a reduced self-diffusion of aqueous solvent parallel to the membrane surface. It is concluded that protein/lipid complex are strongly affected by the amount of solvent interacting with the lipids and the membrane proteins. In particular, the lipids and their ability to attract solvent molecules play important roles in the hydration-induced flexibility of biomembranes. Moreover, it has been argued that H-bond networks play an essential role in recognition and binding. In short, it is now accepted that the properties of these confined regions of water are due to the influence of the adjacent wall of the materials.[4,5,7]

In the case of lipid membranes, some water molecules are likely to be confined between hydrocarbon chains, mainly at the first carbon atoms.[5] Water facing apolar regions would be organized in a different H-bond array than that bound to polar groups such as CO and PO, and also it would be different from the H-bond coordination in pure water.

Thus, it is of interest to explore the water properties in terms of H-bond formation in the function of the distribution of membrane polar groups exposed to water. The membrane surface properties have been examined via phospholipid headgroup IR absorption mainly by the carbonyl and phosphate band displacement due to hydration and dehydration and have been correlated with the water bands reflecting the water state.

In the presence of lipids, a 3411 cm^{-1} peak and the shoulder at 3250 cm^{-1} were ascribed to the O−H stretching mode of water molecules in an effective H-bonded structure (network water) whereas the weak high-frequency shoulder near 3600 cm^{-1} was assigned to water in a structure of distorted (weakened) hydrogen bonds, named multimer water, to describe the monomer of water molecules without H-bond formation. Hence, water forms transient microdomains of two possible states: low density network water of high connectivity and higher density multimer water of lower connectivity.[8−11]

Received: November 5, 2012
Revised: December 22, 2012

   dx.doi.org/10.1021/la304390r | *Langmuir* XXXX, XXX, XXX−XXX

According to these authors, thermotropic and lyotropic measurements are interpreted in terms of the significantly larger fraction of multimer water adsorbed by the liquid-crystalline phase in comparison to the gel state. Thus, lipid melting would give rise to the transfer of water from the network state to the multimeric state in considerable amounts.[11,12] Low-connectivity domains would prevail in the fluid phase, and those of high connectivity would prevail in the gel state.

Changes in water stretching bands are relatively small, indicating a flexible HB network as proposed for bulk liquid. In cationic lipids, a broad central peak at 3411 cm^{-1} has been reported to be coincident with that found in phospholipids in the gel state.[12,13]

The frequency band corresponding to the phosphate groups in phosphatidylcholines does not vary when lipids go through the gel–liquid phase transition.[13,14] This indicates that the hydration shell around the phosphates is altered neither by the phase change nor by the temperature. In contrast, it has been recognized that water may penetrate the lipid bilayer to a plane located in the region of the carbonyl groups.[14,15] Under this condition, the carbonyl band has been shown to vary with curvature, osmotic stress, and lipid phase state. Apparently, these groups are sensitive to small changes in the bilayer interfaces in relation to their exposure to water. In this regard, we have shown previously that the intensity and band centers are displaced if the lipids are either in a planar gel or in a corrugated phase, that is, in spontaneous or induced curvatures.[16,17]

To determine the correlation of the potential H-bond group formation with the possible changes in the adjacent water structure, we have correlated the shift of bound and unbound carbonyl populations at the gel–fluid transition temperature with the evolution of the water band distribution in saturated lipids.

This analysis may give a more complete picture of the H bond networks at the interphase in relation to the membrane surface topology.

METHODOLOGY

Materials. 1,2-Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL), and its purity was checked by thin layer chromatography using a chloroform/methanol/water mixture. A single spot was found; therefore, it was used without further purification (>99% pure).

FTIR Spectroscopic Measurements of Water Bands. Samples for infrared spectroscopy were prepared by hydrating 3–5 mg of the dry lipid with the addition of 50–70 μL of H_2O at pH 7.4 followed by vigorous vortex mixing at temperatures well above the gel/liquid-crystalline phase transition of the lipid. After hydration, lipid dispersions were stabilized at 18 °C and squeezed between the windows of a sample cell equipped with a spacer of appropriate thickness (CaF_2 windows). Once the sample was mounted in the instrument holder, its temperature was controlled by an external, computer-controlled circulating water bath. Spectra were registered before and after full lipid hydration and at temperatures below and above the lipid phase transition (i.e., 18 and 25 °C, respectively).

For the monitoring of the carbonyl group bands, a similar process was followed by replacing water with D_2O.

The spectra were recorded with an FTIR Nicolet TM 380 spectrophotometer provided with a DTGS detector and a KBr beam splitter. A total of 320 scans were recorded for hydrated samples with 2 cm^{-1} resolution. A number of different samples (no less than three) were processed to obtain a standard deviation below the resolution of the equipment.

The full hydration of the lipids was monitored by the saturation of the peak of the $\nu_{asym}(PO_2^-)$ band, which shows an absorption at 1240 cm^{-1} in the solid state and shifts to 1230 cm^{-1} when saturated with water.[13,14]

In the spectral regions of interest, the observed absorption bands are usually the result of the summation of broad overlapping components. In such cases, Fourier deconvolution was used to determine the frequencies of the component bands accurately (band narrowing factors: 1.6–2.2), followed by curve fitting to obtain band widths and intensities. Each band was simulated by a Gaussian–Lorentzian function for which best-fit estimates of band shape were achieved with an ~70% Gaussian contribution.

FTIR spectroscopy is especially suitable for identifying H bonds between interfacial water molecules and phospholipid headgroups. Generally speaking, the formation of an H-bonded complex between two atoms A and B, A–H···B, leads to a weakening of the A–H bond and therefor a downshift of the frequency of the A–H stretching vibration by a few tens of cm^{-1}. The width in frequency of the $\nu_{1,3}(OH)$ band reflects the distribution in strength of the H bonds between the phospholipid and interfacial water molecules.

The OH stretching band of water is centered at around 3380–3405 cm^{-1} and shifts to higher frequencies when solutes promote nonlinear hydrogen bonding and to lower frequencies when stronger H bonding is promoted by the increase in the number of linear H bonds.[17,18]

Thus, IR measurements provide a semiquantitative picture of the water arrangement around solutes dissolved in it.

RESULTS AND DISCUSSION

The FTIR spectra for pure water show a broad band in the 3330–3400 cm^{-1} region and a lower band at around 1700 cm^{-1} (Figure 1, blue line). This last band overlaps with that corresponding to the stretching frequency of the carbonyl

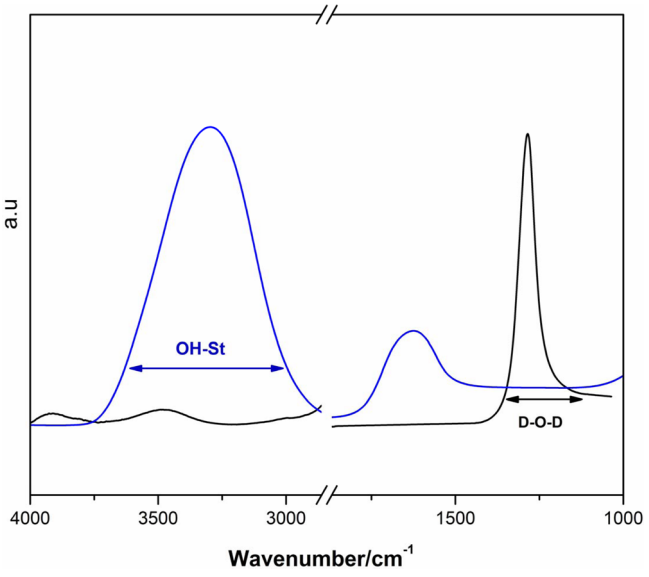


Figure 1. Pure water (blue line) and deuterated water spectra (black line).

dx.doi.org/10.1021/la304390r | Langmuir XXXX, XXX, XXX–XXX

groups in phospholipids. To inspect the behavior of carbonyl groups of lipids in different states, they must be dispersed in deuterated water. In this case, the water band at 1700 cm^{-1} is displaced to lower frequencies and the region of carbonyl groups can be analyzed without interference (Figure 1, black line).

The comparison and analysis of the bands at 3330−3400 cm^{-1} in water and that at 1740 cm^{-1} for lipids in D_2O allows the correlation of the behavior of water bands in comparison to the carbonyl group populations in different hydration states.

In Figure 2A, the broad band centered at 3411 cm^{-1} indicates a wide distribution of H bonds in the presence of solid lipids (i.e., lipids without swelling as described in Materials and Methods for the preparation of liposomes), similar to that reported when NaCl is dissolved in it. This is interpreted as a consequence of the fact that before the process of swelling the lipid lattice distorts the H-bond network, promoting fewer bound water molecules. Under this condition, the PO stretching band is around 1240 cm^{-1}, denoting that lipids have not been hydrated.

In contrast, when lipids are swollen by heating to above the phase transition and then cooled to 18 °C (gel state), two shoulders, one at 3242.3 cm^{-1} and another at 3589.3 cm^{-1}, can be seen in the deconvoluted spectra in addition to a central band at 3450.3 cm^{-1} (Figure 2B). This change follows the evolution of water in the process of full hydration of lipids, a classical method employed to prepare multilamellar liposomes. In this process of hydration, the PO band shifts and saturates at 1230 cm^{-1} (Table 1), as reported.[13,14]

The lateral water bands at lower and higher frequencies denote the presence of bound and unbound water populations, respectively, when lipids are swollen in water and maintained in the gel state at 18 °C. Urea, a well-known water disrupting agent, shifts the water band to lower frequencies at 5 M, denoting strong hydrogen bond formation[18,19]

The central band at 3450.3 cm^{-1} can probably be assigned to the band observed in pure water that has been shifted to higher frequencies by the lipids as a result of the higher exposure and concomitant hydration of the phosphate groups. The shift to higher frequencies when lipids are swollen and then maintained in the gel state accounts for the water species influenced by the exposed phosphates, not the phosphate band stretching that appears at 1230 cm^{-1} (Table 1). This central band was reported for lipids in the gel state and for cationic lipids.[9,19,9,20]

The central band observed at 3450.3 cm^{-1} for lipids in the gel state disappears above the phase-transition temperature, and the lateral ones are now more intense and centered at 3298.2 cm^{-1} and 3554.6 cm^{-1} (Figure 2C).

The comparison of water bands in the gel (18 °C) with those in the fluid (25 °C) state denotes that, in the first case, swollen lipids absorb some of the water molecules. In the fluid state, the central peak, ascribed to phosphate hydration in the gel state, is not observed. The peak at 3298.2 cm^{-1} in the fluid state is congruent with the strengthening of H bonds between water molecules. This is consistent with the reinforcement of H bonds between water molecules in the presence of nonpolar residues such as the CH_2 groups exposed to water. This is compatible with the formation of water clustering in between the lipid acyl chains when the bilayer is in the liquid-crystalline state. The peak at 3554.6 cm^{-1} can be assigned to less H-bonded water molecules because of its comparatively higher peak frequency and is compatible with the low connectivity states reported elsewhere.[11,12]

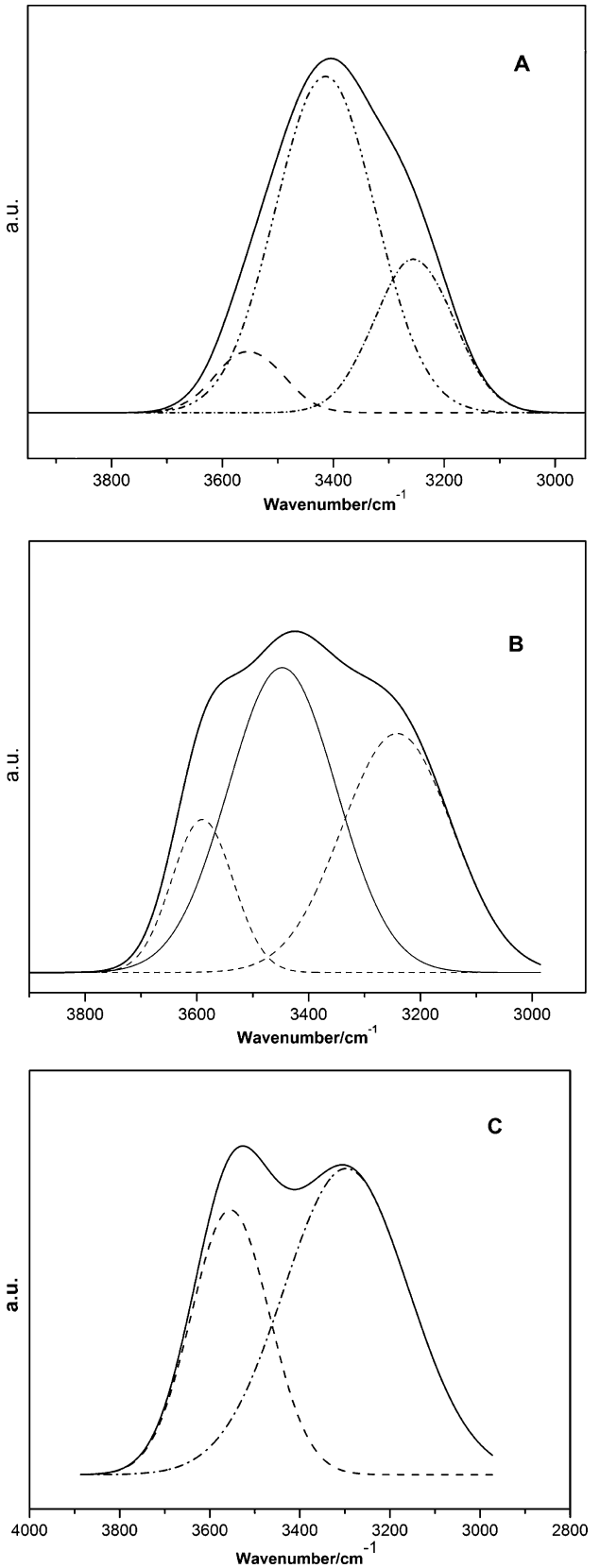


Figure 2. (A) Water band in the presence of solid DMPC before swelling. (B) Water bands in the presence of DMPC after heating and cooling and then after cooling to the gel state (18 °C). (C) Water bands in the presence of DMPC in the fluid state (25 °C). Solid lines correspond to spectra obtained experimentally. Dashed and dotted lines are deconvolutions showing the band components.

dx.doi.org/10.1021/la304390r | *Langmuir* XXXX, XXX, XXX−XXX

Table 1. Carbonyl and Phosphate Bands for DMPC in the Gel and Fluid States[a]

	DMPC gel state (18 °C)	DMPC fluid state (25 °C)
$\tilde{\nu}_p$/cm^{-1} st C=O unbounded	1742.0 (0.2)	1735.6 (0.3)
$\tilde{\nu}_p$/cm^{-1} st C=O bounded	1725.9 (0.2)	1719.8 (0.3)
$\tilde{\nu}$p/cm^{-1} st antisym PO_2^-	1229.5 (0.2)	1230.0 (0.2)
$\tilde{\nu}$p/cm^{-1} st sym PO_2^-	1086.3 (0.2)	1087.4 (0.2)

[a]Standard deviations are reported in parentheses.

Water deeply penetrates the headgroup region, interacting with phosphate and carbonyl groups, in a region that is 5−10 Å from the headgroup surface.[14,15] However, only carbonyl groups appear to be affected at the phase transition (Table 1).

In the spectra of lipids in D_2O, an unbound CO band is found at 1742 cm^{-1} in the gel state and shifts to lower frequencies in the fluid state (Figure 3 and Table 1). The same

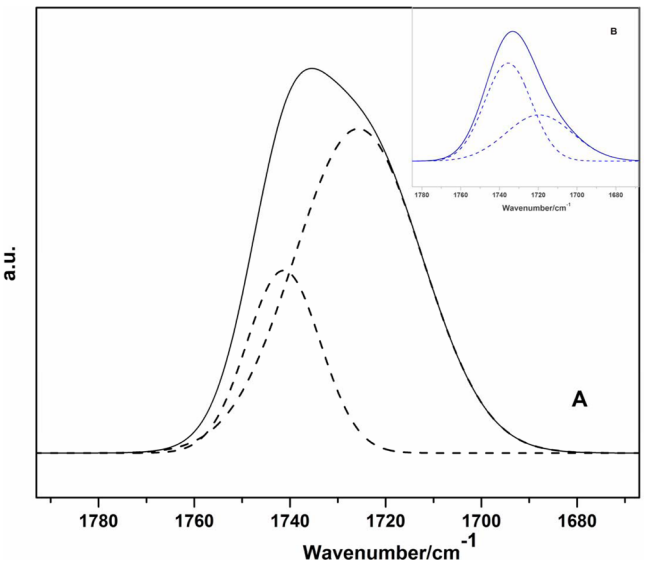


Figure 3. Carbonyl group bands of DMPC in (A) the gel and (B) the liquid-crystalline state.

is observed for the bound CO population (Table 1). The splitting of the CO band could be attributed to two populations of carbonyl groups exposed to a more and a less polar environment, for example, as a result of different distances from the phosphate group. This means that both CO populations form stronger hydrogen bonds, most probably with water, when the lipids are in the fluid state because the relative population of bound to unbound CO groups is much higher in the gel than in the fluid state. That is, parallel to the increase in the exposure of both populations to water, the distribution of bound (hydrated) to unbound (less hydrated) carbonyl groups is not even and differs according to the lipid-phase state. This is an interesting point indicating that the changes at the phase transition, sensed by following the CO distribution, correspond to water penetration.

The shift to lower frequencies in both populations is in correlation with the disappearance of the central band at 3450.3 cm^{-1} at the expense of the bands at 3298.2 and 3554.6 cm^{-1} denoting a rearrangement of the water structure. A possibility is that the shift to lower frequencies of the 3589.3 cm^{-1} peak observed in the gel state would be due to a strengthening of water structure in an icelike network induced by the exposed carbonyl groups.

In conclusion, both carbonyl populations form stronger H bonds affecting the water populations, meaning that CO and water conformations are linked. When CO molecules are less exposed, water bands are centered at 3242.3 and 3589.3, and when CO molecules are more exposed, those bands are centered at 3298.2 and 3554.6 cm^{-1}.

How is water at interfaces different from that in the bulk?

Different water species have been postulated to be present in pure water.[20,21] The presence of lipids can promote some of these species, as a detriment to others depending on the lipid phase state. Thus, the total number of water bands found under the different conditions of the lipids in contact with it can be summarized in five water populations (Figure 4). Assuming that

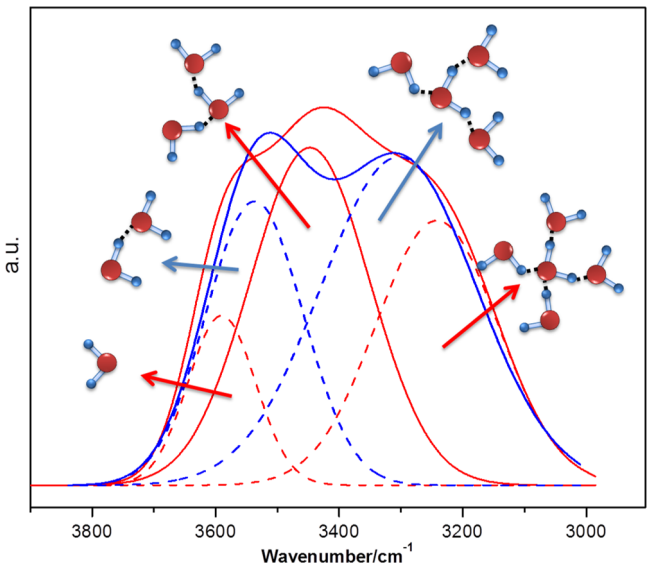


Figure 4. Hypothetical distribution of water species and the corresponding water bands in the gel (red curve) and the liquid-crystalline (blue line) states as derived from deconvolution. From left to right: monomers (0H bonds), dimers (1H bond), trimers (2H bonds), tetramers (3H bonds), and pentamers (4H bonds).

the values of frequency are given by the strength of the H bonds between them and that this is given by its coordination, those populations are four H-bonded (3242. cm^{-1}), 3H bonded (3298 cm^{-1}), 2H bonded (3450.3 cm^{-1}), 1H bonded (3554.6 cm^{-1}), and 0H bonded (3589.3 cm^{-1}). In terms of water species, they correspond to pentamers (a tetrahedrally coordinated centered molecule), tetramers, trimers, dimers, and monomers, respectively. In the gel state, the 4H/2H/0H populations predominate, and in the fluid state , only the 3H and 1H populations are present.

The amount of water sequestered by lipids varies according to the methods employed in its determination. It has been reported that the gel phase of DMPC has 12 to 13 water molecules.[21,22] Likewise, the fluid state is correlated with 18−20 water molecules[22,23] or with 27 water molecules/lipid.[22,23,24]

However, the structural and dynamic properties of the water of hydration and their effects on the dynamic behavior of biomembranes have been ascribed to the first hydration layer that involves at most one to two water layers from the lipid surface.[6,24,25]

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A high core of hydration given by 14 water molecules was derived from reverse micelle studies,[25,26] and 6 of them are condensed on each phosphate, which is not altered by the phase transition[13,14] (Table 1).

The 4H, 2H, and 0H populations, associated with the three bands in the gel state, correspond to the organization of water molecules in the outer shell of the condensed groups, which is consistent with the six water molecules in the hydration shell of the phosphates.[13,14] In the fluid state, the carbonyl groups would bind an additional four molecules as proton acceptors, and another two molecules can interact with the CO double bond, accounting for the total of 8, which amounts to the 14 water molecules reported for micelles.[25,26] This point is further discussed below.

Carbonyls are proton acceptors. Thus, when these are exposed to water a tetrahedrically coordinated water molecule can give a proton to an exposed carbonyl free electron pair, thus displacing the 4H population to a 3H population, which would explain the frequency shift from 3242.3 to 3298.2 cm^{-1}.

The gel−fluid transition implies that there is also a transition from the 2H and 0H populations to the 1H population. This appears as a dismutation, 2H → 1H and 0H → 1H (i.e., deprotonation is concomitant with protonation), suggesting that the 1H population is more stable than the other two in the presence of exposed CO molecules. This may account for the donation of a proton to the CO double bond in which sterically only one proton can be accommodated. This interaction would be rather weak in comparison to a neat CO−HO interaction.

The difference in the frequency of the transition from 4H to 3H is 56 cm^{-1} (blue shift), and that for the 2H to 1H transition is 104.3 cm^{-1} (blue shift). This suggests that when a pentamer (i.e., a tetrahydrically coordinated molecule) breaks an H bond in the water structure the energy involved is lower than that corresponding to the breaking of an H bond in a trimer. It might be possible that in the first case the H bond broken with water is rebuilt with the exposed carbonyl and thus the frequency shift is lower than expected. The high shift in frequency from trimer to dimer would account for the breaking of H bonds without the possibility of reforming.

However, the transition from 0H (monomer) to 1H (dimer) is 34.7 cm^{-1} (red shift). A red shift was ascribed to the water dangling of OH bonds in the hydration shells around dissolved nonpolar hydrocarbons observed in aqueous solutions of alcohols and found at air−water interfaces.[26,27] Thus, it is reasonable that the presence of fluid lipids promotes a water structure similar to that found in hydrophobic interphases.

It is noteworthy that the 50 cm^{-1} frequency shift similar to the 60 to 70 cm^{-1} difference between free OH and 1H-bonded OH groups in low-temperature benzene−water clusters[27,28] was ascribed to the formation of π−H bonds in liquid water. According to these results, the double bond of carbonyl groups might admit water molecules in addition to those bound to the free electron pairs on the oxygen. These molecules would complete the 14 water molecules as described above.[25,26]

Distributions of the different kinds of hydrogen-bonded molecules seem to be similar to those in the bulk liquid, although the relative populations of the molecules with different numbers of hydrogen bonds vary through the interface according to the carbonyl exposure. However, this means that the confinement effect is weakly dependent on the details of the interactions with the lipid surface.

It can be concluded that when carbonyl groups are exposed at least part of the water is organized in different H-bonding coordination water species that may confer different surface free energies to the membrane.

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Notes

The authors declare no competing financial interest.

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