

## STUDY OF THE INTERACTION OF WATER WITH PHOSPHOLIPID BILAYERS BY FTIR SPECTROSCOPY

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**ABSTRACT.** The hydration of the dimyristoylphosphatidylcholine multi-bilayers has been studied by means of Fourier transform infrared spectroscopy. The structure of water confined in lipid membrane, under water-rich and water-poor conditions has been investigated by examining the OH stretching band region. We found that the structure of solvation water of lipid bilayer differs from bulk water. In addition, interaction of water molecules with carbonyl group and phosphate group of phosphatidylcholine have been observed through the downshift of the relative vibrational components representing hydrogen bonded groups. We infer that the disorder of the water network occurs especially in the interfacial region of phospholipids and it increases above the gel to liquid crystalline phase transition, giving rise to an increase of the absorption around  $3600\text{ cm}^{-1}$  which indicates the presence of weakly hydrogen-bonded water aggregates.

### 1. Introduction

Biological membranes play an important role in many cellular processes. A major component of biological membranes are the phospholipids. These have a polar head group and two hydrophobic hydrocarbon tails, which can differ in length and saturation. Due to their amphipathic character, phospholipids in aqueous medium have a tendency to aggregate spontaneously to ordered lamellar bilayers, in which the hydrophilic heads face the water at each surface of the bilayer, while the hydrophobic tails are shielded from the water in the interior. The study of the self-organized formation of lipid bilayers in presence of water represents a major challenge for the understanding the chemical and biological functions of cell membranes.

In the past few years, much efforts were focused on the incoherent thermal motions of phospholipid model membranes (D'Angelo *et al.* 2008; Wanderlingh *et al.* 2008; Trapp *et al.* 2010; Wanderlingh *et al.* 2010; Rifici *et al.* 2014; Wanderlingh *et al.* 2014; Rifici *et al.* 2016) and on the dynamics of water molecules at the interface with membrane (Swenson *et al.* 2008; Hishida and Tanaka 2011; Calero *et al.* 2016; Martelli *et al.* 2018). The latter studies are particularly important because both structure and functions of lipid membranes are critically affected by the hydration and by the structure and dynamics of

water molecules at the membrane surface. Furthermore, water is a key element in a variety of structural and functional roles that membranes play in living cells (Lee 2004). For example, coupling phenomena between membranes, such as adhesion, fusion, and stacking, depend on hydration forces deriving from the restructuring of water (Chaplin 2006; Ball 2008).

The interaction of phospholipids with bathing water is determined by the location of the water molecules with respect to the phospholipid ones. External water governs the aggregation of phospholipid molecules, due to the hydrophobic effect by which their apolar parts (the fatty chains) are shielded from a contact with water for minimizing the total free energy. Water molecules located in spaces between headgroups form intramolecular and intermolecular H-bonded cross-bridges between these headgroups. Water molecules accommodated in the free-volumes of the hydrocarbon region act on the conformation of fatty chains and contribute to increase the membrane permeability. But, in spite of the great importance of water-phospholipid interaction, the factors and the basic mechanisms that regulate this interaction are still poorly understood.

Very recently, by Fourier transform infrared (FTIR) spectroscopy (D'Angelo *et al.* 2017) and Molecular Dynamics simulation (Conti Nibali *et al.* 2014), we have analysed in detail the far-infrared spectral region of dimyristoylphosphatidylcholine (DMPC) to investigate the molecular subpicosecond motions. The spectroscopic study was performed by monitoring the dehydration of the bilayers and allowed us to reveal the existence of a rich variety of low frequency optical modes having strong similarities with the connectivity band in liquid bulk water.

In the present work we present a FTIR study of the interactions of water with phospholipids paying special attention to the formation of H-bonds between water and lipid molecules. The hydration is obtained by exposing the sample to an atmosphere of variable relative humidity (RH). In this way, the degree of hydration of the bilayers could be changed rather continuously between the anhydrous and fully hydrated state. The system investigated is a stack of phospholipid bilayers based on one of most studied electrically neutral phospholipids, i.e. 1,2-dimyristoyl-*sn* glycerol-3-phosphocholine (DMPC), with symmetric saturated fatty chains. DMPC-based lipid bilayer has a gel to liquid crystalline transition temperature of 297 K in its fully hydrated state. The liquid crystalline phase of this bilayer absorbs 25-30 water molecules per lipid (Hauser *et al.* 1981). The phase state is highly sensible to the presence of water (lyotropic phase behavior): when the water content is lowered below a threshold value corresponding to a number of *twelve water* molecules for polar head, the main transition temperature is shifted to higher values, the order among the hydrocarbon chains increases, so that the partially hydrated multibilayers are in gel phase at temperatures at which they would be in liquid crystalline phase if they were hydrated above the threshold value.

## 2. Experimental details

DMPC was purchased from Avanti Polar Lipids (Birmingham, AL, USA) and used without further purification. Phospholipids were dissolved in a solution of 2:1 chloroform/methanol and then dried. Fourier-transform infrared (FT-IR) spectra were taken at 25°C in attenuated total reflectance (ATR) mode, by using a single reflection horizontal

ATR accessory, having a diamond crystal fixed at incident angle of  $45^\circ$  (Platinum ATR, Bruker) mounted on a Vertex 80V FT-IR spectrometer (Bruker Vertex 80V). A background scan was recorded prior to the measurement and subtracted from the sample spectra. Each spectrum was averaged over 216 scans with a resolution of  $2\text{ cm}^{-1}$  and ATR corrected. The frequencies of peaks were determined from the center-of-gravity of the corresponding bands. Atmospheric moisture effects have been eliminated by performing measurements in an evacuated optics bench configuration. Aligned multilayers were obtained by spreading about  $70\ \mu\text{l}$  of a chloroform/methanol solution of phospholipids (concentration  $10\text{mg/ml}$ ) directly onto the ATR plate and evaporating the solvent. Subsequently a certain amount of water was added and then dried to form a stack of lipid bilayers. While evaporating the aqueous solvent, capillary forces flatten the membranes, which spontaneously form oriented ordered multilayer stack (Fringeli and Günthard 1981). The hydration degree of sample was regulated by adsorption via the gas phase corresponding to relative humidities of between 0 and 100% by changes of the RH of the ambient gas phase at fixed temperature by using saturated salt solutions. The saturated salt solutions, made up as a slushy mixture with distilled water and chemically pure salt, was enclosed in a sealed chamber placed in strict proximity of the sample. For the study nine salts are chosen (LiCl, CH<sub>3</sub>COOK, MgCl<sub>2</sub>, K<sub>2</sub>CO<sub>3</sub>, NH<sub>4</sub>NO<sub>3</sub>, NaCl, KCl, KNO<sub>3</sub>, K<sub>2</sub>SO<sub>4</sub>) so as to have a range of relative humidity of 11–98%. The values of equilibrium relative humidity at  $25^\circ\text{C}$  are given in Table 1.

TABLE 1. Selected saturated salt solutions and corresponding relative humidity at  $25^\circ\text{C}$ .

Salt	RH (%)
LiCl	11
CH <sub>3</sub> COOK	23
MgCl <sub>2</sub>	33
K <sub>2</sub> CO <sub>3</sub>	43
NH <sub>4</sub> NO <sub>3</sub>	64
NaCl	75
KCl	85
KNO <sub>3</sub>	95
K <sub>2</sub> SO <sub>4</sub>	98

### 3. Hydration behavior of phospholipids in the mid-infrared range: results and discussion

Figure 1 shows the infrared absorbance spectra of DMPC at various relative humidity levels in the full investigated frequency range. Increasing the hydration the spectra differ significantly in four distinct regions: above  $3000\text{ cm}^{-1}$ , between  $1700$  and  $1500\text{ cm}^{-1}$ , between  $1000$  and  $400\text{ cm}^{-1}$ , and below  $200\text{ cm}^{-1}$ . For liquid water these four frequency intervals correspond respectively to the regions where are observed the OH-stretching band, the OH-bending band, the librational band (centred at about  $675\text{ cm}^{-1}$ ) and the so-called connectivity band of water (centred at  $200\text{ cm}^{-1}$ ) consisting of the intermolecular OH–O stretching mode and of out-of-plane bending motion of the hydrogen bonded H atoms

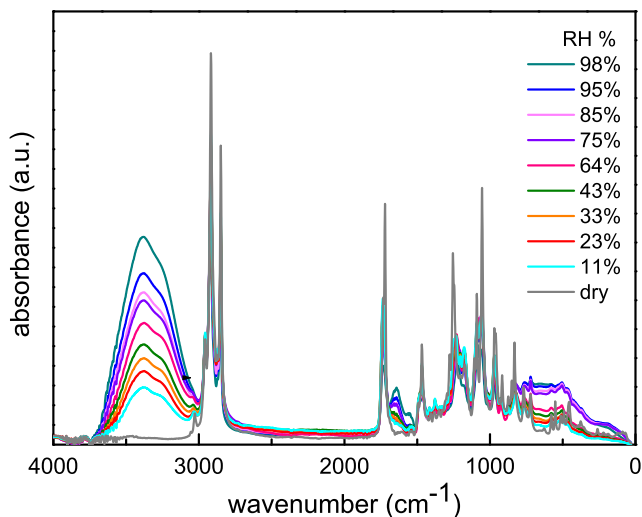


FIGURE 1. IR absorbance spectra of DMPC at different RH values.

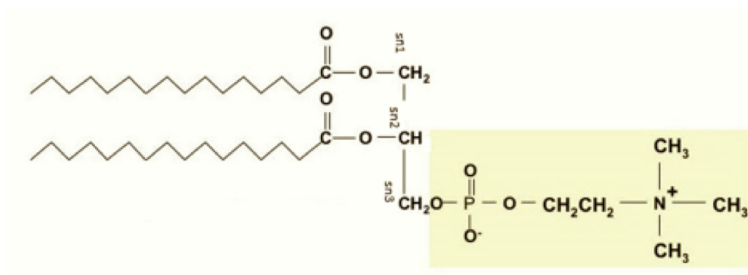


FIGURE 2. Schematic representation of DMPC molecule.

(Brubach *et al.* 2005). DMPC molecules have two proton-accepting groups that can interact with water to form hydrogen bonds: the phosphate group in the polar head and the ester carbonyl groups in the interfacial region (see Fig. 2). In the first stages of the hydration process, the water molecules interact with the phosphate group and, at a second step, with the carbonyl groups (Nagle and Wiener 1988). The formation of hydrogen bonds between these groups and water molecules can be monitored by observing the frequency shift of their stretching frequencies. It is expected that upon hydrogen-bonding these frequencies decrease as a consequence of the elongation of the chemical bonds in the relative functional groups (Lewis and McElhaneý 1998). Furthermore also the conformation of the acyl chains change with the hydration (Lewis and McElhaneý 1998).

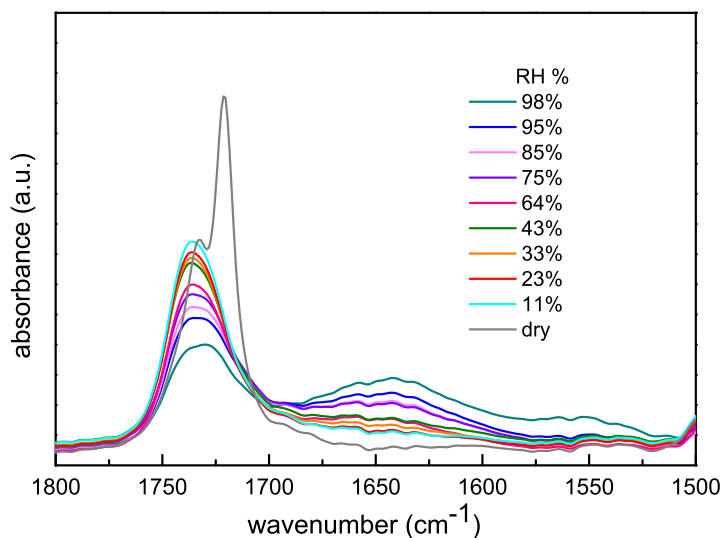


FIGURE 3. Absorption spectra of hydrated DMPC in the frequency range from 1800 to 1500  $\text{cm}^{-1}$  at different hydrations.

In order to better appreciate the hydration induced conformational changes in the bilayers and to investigate specific interactions between water and lipid molecules, we have analyzed the infrared spectra of DMPC separately in three regions. The first region extending from 1300 to 1000  $\text{cm}^{-1}$  is originated from molecular vibrations of the hydrophilic head group. The second region, which extends to about 1500 to 1300  $\text{cm}^{-1}$ , encompasses the bending vibrations of the methylene and methyl groups of the hydrophobic hydrocarbon chains, whereas the third region ranging from 1800 to 1500  $\text{cm}^{-1}$  includes the motions of the interfacial area. Figure 3 shows the infrared absorbance spectra of DMPC for the spectral region from 1800 to 1500  $\text{cm}^{-1}$ . This spectral range is dominated by the C=O stretching vibrations of the ester carbonyl group from the interfacial region, which give rise in the 1750-1700  $\text{cm}^{-1}$  region to at least two strong bands, reflecting the conformational non-equivalence of the two ester carbonyls. The band at 1742  $\text{cm}^{-1}$  is associated with the sn-1 C=O, while the corresponding band for the sn-2 C=O is found at 1725  $\text{cm}^{-1}$  (Lewis and McElhaney 1998). The frequency of these bands is sensitive to the glycerol moiety conformations and the arrangements of the acyl chains, but major changes occur upon hydrogen bonding by water. As shown in Fig. 3, these two bands are clearly visible in the dry sample whereas they merge into a single broad band for the hydrated samples. Moreover, this band moves toward lower frequencies, while its intensity decreases as the hydration increases. In particular as RH is increased above 75% the contour of this band reveals a two-component nature.

Hydration-induced changes in the contour and the frequency of this band are mainly a consequence of the change in the relative intensities of these two components, each of

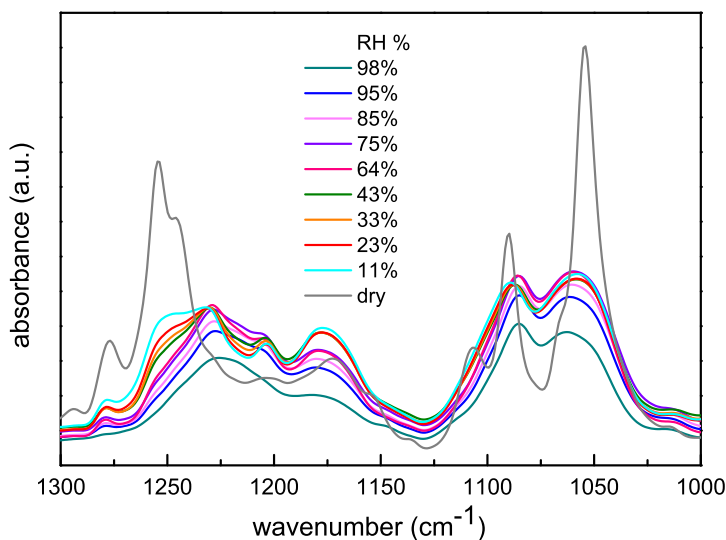


FIGURE 4. Absorption spectra of hydrated DMPC in the 1300 to 1000  $\text{cm}^{-1}$  spectral range.

which in turn consists of two underlying vibrational contributions of different intensity (Blume *et al.* 1982; Lewis *et al.* 1994). The higher frequency component is attributed to the non hydrogen bonded carbonyl group, whereas the lower frequency component band is associated to the hydrogen bonded carbonyl group. When lipids undergo the transition from the gel phase to the liquid phase, hydrogen bonding to both C=O groups increases, and this results in the increase of the relative intensity of the low-frequency component (Mantsch and McElhaneý 1991). Hence we suggest that the band shape change observed at RH above 75% indicates that, at this hydration degree, lipids have a sufficient water molecule content to pass into the liquid phase. This hypothesis is supported by the observation at RH values above 75% of a sharp increase (from 2850  $\text{cm}^{-1}$  to 2852  $\text{cm}^{-1}$ ) in frequency of the band corresponding to the symmetric  $\text{CH}_2$  stretching mode, and indicating the transition of the lipid bilayers from the liquid crystalline to the gel state (Mantsch and McElhaneý 1991; Lewis and McElhaneý 1998).

In the region below 1700  $\text{cm}^{-1}$  a band at about 1630  $\text{cm}^{-1}$  appears in all the spectra of hydrated samples, whose intensity increases on increasing the amount of water. It is due to the H-O-H bending vibration of water (Brubach *et al.* 2005). Concerning the region between 1300 and 1000  $\text{cm}^{-1}$  (see Fig. 4), it is dominated by the absorption originating from the phosphate group of the head groups. In the dry sample we observe an high band at 1255  $\text{cm}^{-1}$  with a shoulder at 1245  $\text{cm}^{-1}$ . As the hydration increases the band disappears; at the same time the shoulder becomes more prominent while moves from 1245  $\text{cm}^{-1}$  (dry) to 1223  $\text{cm}^{-1}$  (98% RH). We ascribe this vibration to the asymmetric stretching of  $\text{PO}_2$  (Mantsch and McElhaneý 1991; Lewis and McElhaneý 1998). The band that is

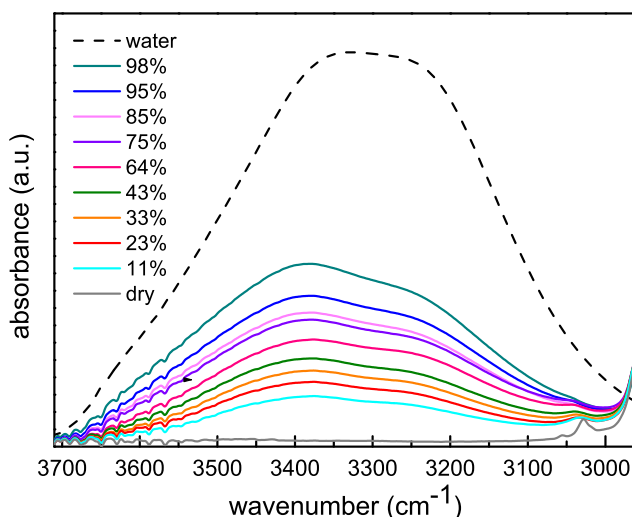


FIGURE 5. Absorption spectra of DMPC/water in the frequency region of O-H stretch vibration of H<sub>2</sub>O at different hydrations.

observed at  $1090\text{ cm}^{-1}$  in the dried sample and that shifts to  $1085\text{ cm}^{-1}$  in the sample with the highest water content is attributed to the PO<sub>2</sub> symmetric stretching (Mantsch and McElhaneý 1991; Lewis and McElhaneý 1998). The decrease of the wavenumber position of the PO<sub>2</sub> bands indicates the reduced mobility of the phosphate group, consequent to the formation of hydrogen bridges between phosphate oxygens in adjacent DMPC molecules. The strong absorbance at about  $1060\text{ cm}^{-1}$  originates from the vibration characteristic of the R-O-P-O-R' group (where R and R' represent the radical groups of the diesterified phosphate) (Goñi and Arrondo 1986).

Other water binding sites in phosphatidylcholines are the charged (CH<sub>3</sub>)<sub>3</sub>N<sup>+</sup> groups. The (CH<sub>3</sub>)<sub>3</sub> asymmetric stretching mode of the N(CH<sub>3</sub>)<sub>3</sub> group in PC is found at  $3050\text{ cm}^{-1}$ . We can note that the corresponding absorbance peak is clearly visible in the dried bilayers (see Fig. 5) and gradually disappears as the water content increases under the broad intense band appearing at higher wavenumbers. The  $\nu$ CN frequency is typically located at about  $970\text{ cm}^{-1}$ . We found that the  $\nu$ CN frequency in the fully hydrated sample ( $973\text{ cm}^{-1}$ ) is higher than that of the anhydrous sample ( $971\text{ cm}^{-1}$ ) (see Fig. 6). This finding indicates that the (CH<sub>3</sub>)<sub>3</sub>N<sup>+</sup> group is not hydrogen-bonded to water. This fact is not surprising because the (CH<sub>3</sub>)<sub>3</sub>N<sup>+</sup> trimethylammonium groups are hydrophobic in nature, thus they do not form strong interactions with the solvent, and, furthermore, promote formation of clathrates in the nearby water layers (Damodaran and Merz Jr. 1994).

The spectral range between  $3800$  and  $3000\text{ cm}^{-1}$  of the absorbance curves at all the RH values studied is shown and compared with that of pure bulk water in Fig. 5. This region is dominated by the broad intense absorption band of OH stretching vibrations of the sorbed

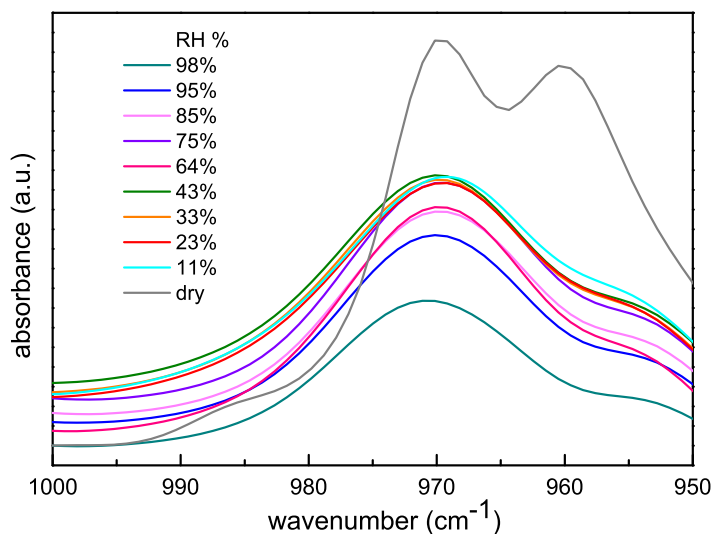


FIGURE 6. Absorption spectra of DMPC/water in the 1000-950  $\text{cm}^{-1}$  spectral range.

water. The total area of this band is observed to increase with increasing RH, corresponding to the uptake of water. In order to compare the shape of this band at the various hydration levels, the experimental data were normalized to the maximum intensity value and plotted in Fig. 7. We can clearly observe that the OH band shape of hydrated lipid systems strongly differs from that of pure bulk water. Furthermore its shape changes with the RH level. Both observations indicate that in the lipid system the normal hydrogen-bonded structure is strongly changed. We infer that the interactions of water molecules with lipid molecules as well as the confinement of water in membrane interfaces visibly affect the low-frequency and high-frequency sides of the OH stretching band. In pure bulk water this band is usually separated into three main components, whose assignment is controversial. In the present study we adopt the assignment of Brubach *et al.* (2005) and distinguish three types of water molecules coexisting in the bulk water: a) molecules connected tetrahedrally, forming extended water network, whose collective in-phase O-H stretching vibrations give rise to the low energy component at about  $3240 \text{ cm}^{-1}$ ; b) molecules of the so-called intermediate water: these water molecules are more weakly coupled in a distorted tetrahedral structure in which one hydrogen bond is lacking. The out of phase O-H stretching vibrations of these molecules give rise to the medium-energy component at about  $3420 \text{ cm}^{-1}$ ; c) molecules which do not form a rigid ordered network but are monomers or part of small isolated aggregates multimer water, corresponding to the highest energy component centered at  $3580 \text{ cm}^{-1}$ .

In lipid membranes, we observe that the high frequency shoulder near  $3600 \text{ cm}^{-1}$  increases with the RH level, indicating the increase of the relative abundance of the multimer water molecules. This result is in agreement with what has been reported by Binder (2007)



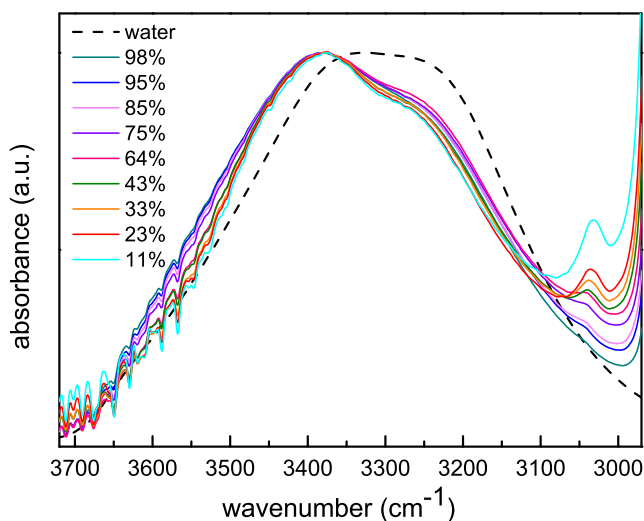


FIGURE 7. Normalized absorption spectra of DMPC/water in the frequency region of O-H stretch vibration of H<sub>2</sub>O at different hydrations.

for POPC. The increase of this shoulder reflects the formation of "free" water and, in lipid bilayer, it can be related to an increasing number of water molecules accommodated in the free-volumes of the hydrocarbon region. It is interesting to note that the increase of this high energy component of OH stretching band is more relevant at RH > 75%, that, as mentioned above, should correspond to a number of water molecules sufficient for switching the system from the gel phase to liquid crystalline phase. Also this finding is in accordance with the results found in POPC (Binder 2007), with the difference that in POPC the lyotropic phase transition is observed near RH = 44%. In the liquid crystalline phase the distances between DMPC molecules and the mobility of fatty chains markedly increase, and this situation allows for the formation of free volumes that are defects torsional states of dihedral angles in the packing of acyl chains. The increased free volume let more water molecules to penetrate deeper into the DMPC bilayer. The intermediate broad component at 3380 cm<sup>-1</sup> is the main contribution in our system. Because this component does not seem to change with the hydration level, we can hypothesize that it arises from the interlamellar water.

Finally the intensity of the component at the lowest energy appears to be strongly reduced compared to that of bulk water. This finding indicates the decrease of the water molecules connected tetrahedrally. Most interestingly, the intensity of this band progressively increases as RH increases up to 64%, whereas it begins to decrease when the RH is increased above a value of 75%. This finding, together with the related observation of the evident increase of the shoulder at 3600 cm<sup>-1</sup>, leads us to believe that in the first steps of water sorption, the water molecules are mainly incorporated in well-ordered network, and the connectivity of the network water increases. A further increase of hydration has the effect to induce

disorder in the water structure, increasing the fraction of multimer water. This can be explained by the fact that when the head groups reach a full hydrated state, the hydrocarbon chains experience both interchain and intrachain disorder and an increase of flexibility. This augmented mobility favors the formation of free volumes in proximity of the apolar regions, in which water molecules are caged (Träuble 1971).

#### 4. Conclusions

In conclusion, the interaction sites of phospholipids with the water molecules and the arrangement of water in lipid membranes were examined by ATR-FTIR spectroscopy while systematically changing the hydration level. The formation of hydrogen bonds between the phosphate group in the polar head and the ester carbonyl groups in the interfacial region and water molecules were monitored by observing the frequency shift of their stretching frequencies while changing the water content. It was found that the carbonyl band region changes with the hydration, the shape and the frequency of the revealed bands reflecting the lyotropic melting of the acyl chains. This finding indicates that the water absorption influences and involves not only the head and interface regions but also the hydrophobic core. We also found that the local hydrogen-bonding environment of hydration water in phospholipid membranes is strongly perturbed. The OH-stretching band shape of hydrated lipid systems strongly differs from that of pure bulk water and strongly depends on the RH level. The high- and the low-frequency tails of that band reveal respectively the existence of “multimer” and “tetrahedrally connected” water molecules. The observed variations of the intensity of these two spectral component with the degree of hydration lead us to conclude that the water molecules are mainly incorporated in well-ordered network in the first steps of water sorption. When the water content increases to a level near that required for the full hydrated state of head groups, disorder in the water structure is induced. This disorder is related to the more rapid increase of the high-frequency tail of the OH band. It is believed that it reflects the formation of free volumes in proximity of the aliphatic chain, in which water molecules are caged. Our results suggest that a careful analysis of the O–H stretching region as a function of hydration is an efficient tool to distinguish between the different arrangements of hydration water molecules and to unravel the structure of water confined in lipid bilayer.

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