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Understanding water-membrane interactions is a fundamental issue in biophysics since these interactions are at the basis of many key molecular processes occurring in membranes. The hydrogen-bond network of water molecules is highly dynamic and its dynamical structure influences membrane fluidity and proton transport across membranes. We investigate the dynamics of water hydrogen-bonds network in model membranes using dielectric relaxation spectroscopy in the TeraHertz range. This frequency interval is suitable for obtaining information on the collective low-energy modes of the hydrogen-bond network of water molecules. In this paper we review the technique and present some preliminary experimental results.

Keywords: bound water; confined water; dielectric relaxation; DOPC; lyotropic liquid crystals; phospholipids; THz time domain spectroscopy

INTRODUCTION

Studying the influence of hydration on lipid membrane functioning is a topical issue in biophysics. Hydration of lipid membranes affects the

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function of membrane proteins directly through water-protein interactions and indirectly through water-lipid interactions [1]. Moreover many membrane phenomena are known to be strongly influenced by water either bound to lipids head-groups or forming clusters of hydrogen-bond networks (HBN) within the membrane cavities. For instance, water hydration of membrane is strictly related to the fluidity of membrane that is of fundamental importance for phenomena like membrane fusion or drug transport [2]. However, many of the details of the role of water have remained poorly understood. Another interesting question arises when considering the anomalously high mobility of protons across membranes that has been explained by the formation of water hydrogen-bonds wires across the hydrocarbon region of membranes [3].

These observations and speculations point to a structural complexity of water interacting with membranes, which has prompted much effort in elucidating the dynamic water structure using various spectroscopic tools. Much information has been obtained by using techniques as NMR or time-resolved Mid-Infrared spectroscopy (MIR) [4]. NMR experiments may provide with information about the orientational order within the water-shell around lipid-headgroups and have helped in clarifying the difference between lateral and perpendicular translational diffusion rate of water at lipid-water interface [5]. On the other hand time-resolved MIR may provide with information on the lifetime of water stretching vibrations, thus probing locally the water hydrogen-bond strength [6]. However these techniques give little access to highly collective modes that lie in the far-infrared part of the radiation spectrum. For instance, in the case of pure water it has been shown that in the THz interval of frequencies there are specific spectral signatures of motions occurring on a large molecular scale length and implying a structural reorganization of the HBN [7].

With these considerations in mind we have applied THz Dielectric Relaxation Spectroscopy to the study of hydration in membrane-models made of stack of 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) lipid bilayers in order to investigate the influence of membrane confinement on the HBN low-energy modes. DOPC has been chosen since it is an unsaturated lipid generally used as a component of model membranes mimicking real ones [8].

We have studied the THz dielectric relaxation of these membranes by varying their level of hydration in order to investigate the possible heterogeneity of the HBN within the DOPC membranes (bulk-like water, water 'pools', etc.).

For these measurements we have used THz Time Domain Spectroscopy (THz TDS) [9]. THz TDS allows one to measure the complex

dielectric constant without using intermediate mathematical analysis based on Kramers-Kronig equations. In this paper we will review these experiments and will provide with a preliminary analysis of the data. The results are discussed within the framework of a two-times Debye model as explained in the following.

EXPERIMENT

THz TDS experiments were performed on membrane models made of stacked DOPC bilayers. This lipid displays a liquid-crystalline phase for relative humidity higher than 45%. DOPC was purchased from Avanti Polar Lipids Inc. Solutions of DOPC and pure chloroform were prepared at a concentration of about 15 g/L. Drops of these solutions were deposited on a fused quartz window, with the chloroform is let to evaporate. It is known that in this way stacks of lipid bilayers are formed by self-assembly [10]. Samples of few hundreds micron of thickness were readily realized by successive depositions. These samples have been then inserted in a variable-thickness cell of our design. The sealed cell was first exposed to a nitrogen flow to dry the lipid and was subsequently put in contact with a reservoir at controlled humidity.

THz TDS spectra in the frequency range 0.2–1.8 THz were measured by means of a spectrometer based on Time Domain THz Spectroscopy as schematically shown in Figure 1. A 130 fs laser pulse of 800 nm of wavelength is used for producing by means of Frequency Difference Generation a broad bandwidth single-cycle THz pulse. The field strength (rather than intensity) of the THz pulse transmitted through the sample is measured in the time domain by means of electro-optics sampling using a small part of the 130 fs pulse for probing at suitable time-delay the THz field impinging on the electro-optics crystal (details of this technique may be found in reference [11]). In addition, for each sample a linear mid-Infrared spectrum between 2000 and 4000 cm^{-1} has been recorded by using a standard two beams spectrometer. Some examples of these spectra in a reduced interval of wavenumbers are shown in Figure 2 where only the water contribution is displayed. For comparison a spectrum of pure water (black line) is included. An example of spectrum for a fully hydrated sample (93% RH) on a more extended range is shown in the inset. The spectral structure in the interval 2800–3000 cm^{-1} represent vibrational modes associated to DOPC.

In the THz TDS experiments, the presence of the sample leads to a reduction of the THz pulse amplitude and to a temporal shift of the THz trace. These two quantities contain information about the

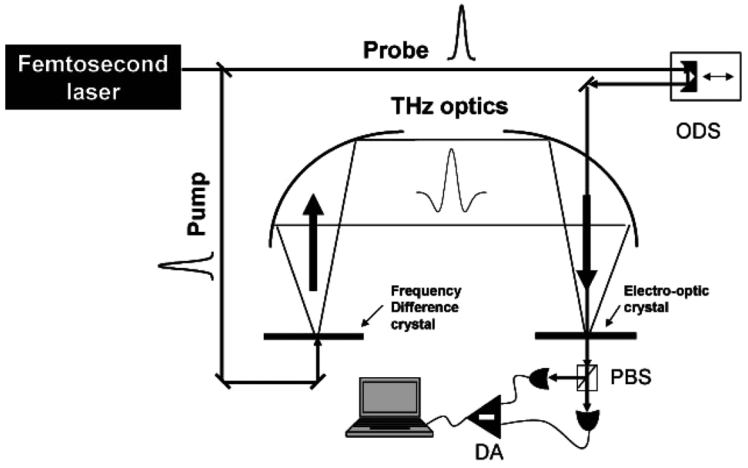

FIGURE 1 Experimental set-up for THz Time Domain Spectroscopy. ODS: optical delay stage; PBS: polarizing beam splitter; DA: differential amplifier.

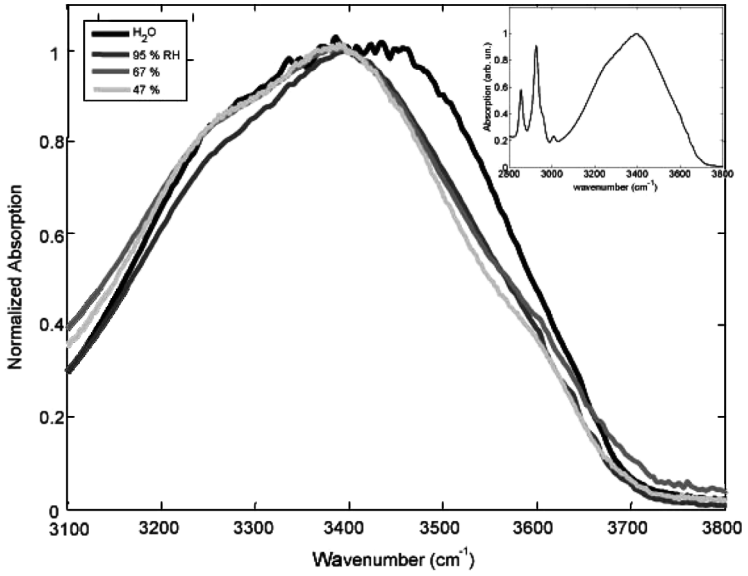

FIGURE 2 Infrared spectra for DOPC at different hydration levels over a reduced range of wavenumbers where only the water spectral contribution is shown (the values in the legend refer to relative humidity RH). Pure water spectrum (black lines) is included for comparison. Inset: extended spectrum for the 93% RH sample. The peaks in the interval 2800–3000 cm^{-1} are vibrational modes associated to DOPC.

absorption coefficient and refractive index of the sample, respectively. The analysis is most easily performed in the frequency domain. In principle, a comparison of the Fourier transformation of the THz temporal traces with and without the sample provides the frequency-dependent absorption coefficient and refractive index of the sample. The extraction of the complex dielectric function of the sample is somewhat complicated by the fact that the sample, in our case, is contained within a cell with windows that also affect the THz wave. In this case a better way to extract complex dielectric function is to single out the sample response from measurements at varying thicknesses of the sample. With this approach the reference is the sample itself and the contribution of the cell is directly taken into account. In Figure 3 we show the THz traces for a fully hydrated DOPC sample as a function of sample thickness.

The time-domain data may be converted to the frequency domain to obtain the frequency-dependent absorption coefficient and the refractive index using Eqs. (1) and (2) respectively:

$$\alpha(\nu) = \frac{\ln P(\nu, d) - \ln P(\nu, d + \delta d)}{\delta d} = k(\nu) \times 2\pi\nu, \tag{1}$$

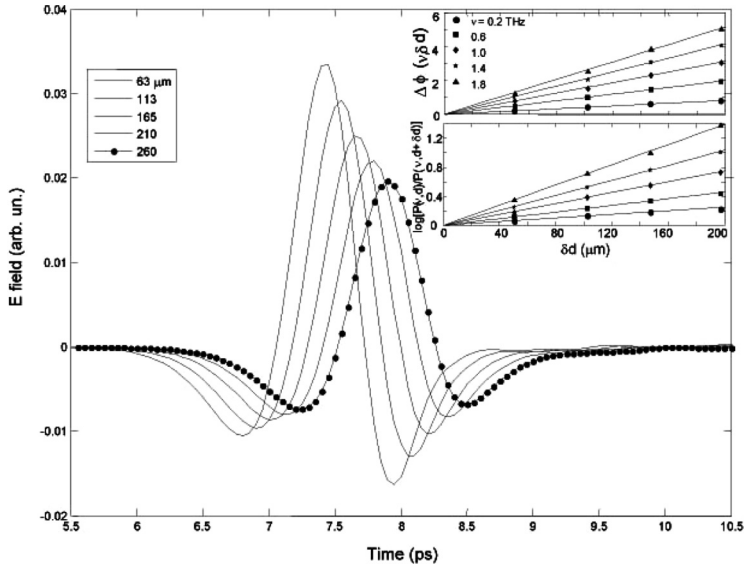

FIGURE 3 THz electric field as transmitted through fully hydrated (93% RH) DOPC sample at different thicknesses. Inset: power logarithm and phase of the same traces in the frequency domain as a function of the thickness variation (for clarity only few representative frequencies are displayed).

$$n(\nu) = \frac{\phi(\nu, d + \delta d) - \phi(\nu, d)}{2\pi\nu\delta d} \times c, \tag{2}$$

where P is the power spectrum and ϕ is the frequency-dependent phase of the transmitted THz pulse at frequency ν; d is the sample thickness and δd is the change in thickness between two measurements; c is the speed of light and k is the imaginary part of the complex refractive index ($\hat{n} = n + ik$).

As it is apparent from Eqs. (1) and (2), the phase and power depends linearly on δd at a given frequency. Therefore for each frequency a linear model can be used for fitting the experimental results in order to extract n and k as shown in the inset of Figure 3 for some selected frequencies. From the complex refractive index the complex dielectric response ($\hat{\varepsilon} = \varepsilon_r + i\varepsilon_i$) can be obtained by using the following relationships

$$\varepsilon_r(\nu) = n^2(\nu) - k^2(\nu), \tag{3}$$

$$\varepsilon_i(\nu) = 2n(\nu)k(\nu). \tag{4}$$

In Figure 4 the inferred dielectric functions are shown for different levels of hydration. We limit our attention to a range of relative

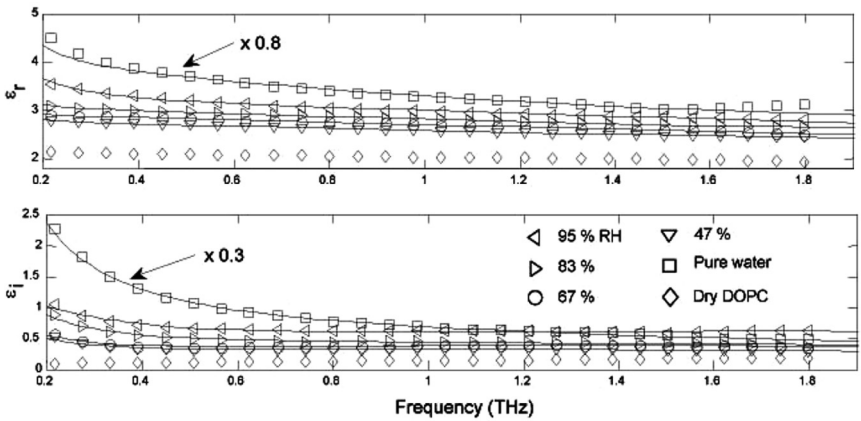

FIGURE 4 Dielectric relaxation function of DOPC at different hydration levels. Both real and imaginary parts are displayed. Solid lines are the results of fits based on a two-times Debye model as explained in the text. For comparison the dielectric relaxation function of pure water is included. Note that the real and imaginary parts of the latter have been multiplied by a factor 0.8 and 0.3, respectively.

humidity going from 45% to 100% as this range is the most interesting from a biological point of view. The response of pure water is shown for comparison. The solid lines in the figure refer to fits based on a Debye model as explained in the following section. Two things are worth of noting in Figure 4. First we observe that the dielectric response, both real and imaginary part, of water-DOPC mixture increases as a function of relative humidity. Second, the dielectric response of dry DOPC is quite dispersionless in the considered range. This is due to the fact that the dielectric response of DOPC is mainly due to the lipid polar heads, which contributes to the dielectric response outside our frequency window, in the MHz range [10]. The slight variation with frequency observed for dry DOPC is most likely due to the fact that it is never possible to obtain completely dry samples as our linear mid-infrared spectra display always a small water contribution even if the sample is exposed to nitrogen flow for few days. In any case, the absence of significant dispersion of the DOPC allows us in the following to separate the dielectric response of water in DOPC from that of DOPC itself.

DEBYE MODEL

Given the flat response of dry DOPC in the frequency range under study here, we assume that the frequency dependent contribution is given by water, while the DOPC contribution may be taken into account by a frequency-independent constant.

For pure water samples the double (or two-terms) Debye model has been shown to give almost satisfactory fits to the dielectric response in the 0.2–2 THz range [12]:

$$\hat{\varepsilon}(\nu) = \varepsilon_\infty + \frac{\varepsilon_s - \varepsilon_2}{1 + i2\pi\nu\tau_1} + \frac{\varepsilon_2 - \varepsilon_\infty}{1 + i2\pi\nu\tau_2} \tag{5}$$

where ε_∞ is the dielectric constant in the high frequency limit (optical dielectric constant), ε_2 is the intermediate dielectric constant, τ_1 is the first Debye relaxation time, and τ_2 is the second Debye relaxation time. ε_s is the contribution to the static (zero-frequency) dielectric constant of the system described by Eq. (5).

The most interesting parameter for discussing HBN dynamics is the Debye time τ_1. In pure water at room temperature τ_1 is about 8 ps [12]. There is still a large debate about the assignment of this time to specific modes in water. However its relationship with the collective reorganization of the hydrogen-bonds network has been generally accepted [7,13]. Given the limits of space for this report,

in the following, we will focus our attention on the results we get for τ_1 as a function of hydration level.

RESULTS AND DISCUSSION

Before analyzing the dielectric relaxation let us consider the IR absorption spectra shown in Figure 2. Compared to bulk water we observe a reduction of the spectral weight of the high frequency side of the stretching band. This side is generally assigned to water molecules with a 'solitary' character, i.e., water molecules that are weakly hydrogen-bonded. A similar behaviour has been observed for an analogous phospholipid by Righini et al. [6]. The authors in Ref. [6] interpret this behaviour as a signature of the presence of water molecules sparsely distributed in the proximity of the first layer bound to polar groups or in the hydrocarbon region. Therefore they conclude that the number of molecules involved in the hydrogen-bonds network is reduced and consequently the number of 'solitary' molecules. The reduction of the number of 'solitary' molecules leads to the depletion of the high frequency part of the spectrum.

The THz TDS data, analyzed using Eq. (5) to fit the dielectric data shown in Figure 4, reveal a decrease in τ_1 compared to bulk water. As in the case of bulk water, a single-time Debye model results in an extremely poor fit. We have let all five parameters (ε_∞, ε_2, ε_s, τ_1, and τ_2) free to vary in the fitting routine. The results for τ_1 are reported in Figure 5 as a function of the estimated number of water molecules per lipid. This number is calculated starting from the infrared linear spectra that give information about the relative concentration of water and DOPC (see inset of Fig. 2).

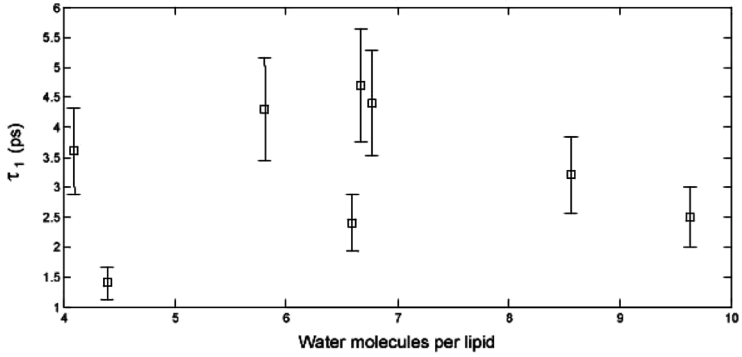

FIGURE 5 Debye time τ_1 vs the number of water molecules per lipid molecule.

Before providing with a tentative interpretation of the results shown in Figure 5 we wish to remark that this is a preliminary analysis of our data. The main weak point of this analysis is the limited interval of frequencies spanned in our measurements. This makes the use of our multi-parameter fits a bit delicate, although the requirement that both the real and imaginary part of the dielectric constant be fitted simultaneously over the entire frequency window greatly reduces the freedom in the choice of the parameters. A better confidence of the fit might be achieved by imposing some constraint on one of the fit parameters. This could be done, for instance, for ε_s by comparing our data with analogous measurements in the GHz range. This will be the subject of a future publication. With the above considerations in mind, let us consider the results of Figure 5. They show a dramatic reduction of the Debye time τ_1 as compared to the value of bulk water (about 8 ps at the same temperature of our experiments).

This result is in qualitative agreement with analogous dynamics observed in THz TDS experiments performed on inverse micelles [14]. As in Ref. [14] our results prove that THz TDS measurements are greatly sensitive to vibrational modes of the water hydrogen-bonds network that are highly collective in nature. In particular τ_1 is strongly related to the number of water molecules involved in the hydrogen-bonds network [15]. Our results therefore indicate that the confinement of water between the lipid bilayers strongly limits the extent to which collective motions can occur in membranes. This ends up in a 'blue' shift of this collective mode that, seen in the time domain, corresponds to a speeding up of the temporal dynamics. These results point to the presence of sparsely distributed water 'pools' in the membrane. This interpretation is in qualitative agreement with the results of our linear MIR measurements reported in Figure 2.

CONCLUSIONS

We have investigated the Dielectric Relaxation of water confined in phospholipids membranes by means of THz Time Domain Spectroscopy. We show that this technique is suitable for providing information about the dynamics of the hydrogen-bonds network of water molecules in membranes. We have analyzed our data based on a two-times Debye model. This preliminary analysis shows that there is a dramatic effect of this confinement resulting into a speeding up of the Debye time τ_1. We interpret this result with a suppression of the highly collective HBN mode. This is due to the fact that water is sparsely distributed in the membrane and around the first layer of

water molecules strongly bound to the lipid head groups. These results indicate that a precise measurement of τ_1 might give information about the spatial extension of HBN within membrane. In particular measurements at lower hydration levels might provide information about the extension of the hydrogen-bonds wires whose existence is assumed for explaining the anomalous large mobility of protons in membranes.

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