Dielectric Spectroscopy as a Sensor of Membrane Headgroup Mobility and Hydration

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ABSTRACT Dielectric spectroscopy is based on the response of the permanent dipoles to a driving electric field. The phospholipid membrane systems of dimyristoylphosphatidylcholine and dioleoylphosphatidylcholine can be prepared as samples of multilamellar liposomes with a well known amount of interlamellar water. For optimal resolution in dielectric spectroscopy one has to design the experimental set-up so that the direction of the permanent headgroup dipole moment is mostly parallel to the field vector of the external radio frequency (rf) electric field in this layered system. A newly developed coaxial probe technique makes it possible to sweep the measuring frequency between 1 and 1000 MHz in the temperature range 286–323 K. The response yields both the dispersion (ϵ') and the absorption part (ϵ'') of the complex dielectric permittivity, which are attributed to the rotational diffusions of the zwitterionic phosphatidylcholine headgroup and the hydration water, respectively. Although the contributions of the headgroup and the hydration dipole moments to the dielectric relaxation are found to be situated close together, we succeeded in separating them. In the language of the Debye description, we propose to assign the lower frequency portion of the signal response to the relaxation contributed by the headgroups. The respective relaxation frequency is a discrete value in the range of 15–100 MHz and it shows normal temperature dependence. The contribution of the hydration water molecules exhibits a similar behavior in the range of 100–500 MHz but with the attributed relaxation frequency as the center of an asymmetric distribution of frequencies in analogy to simulation models known from the literature. Activation energies are derived for each of these relaxation processes from the Arrhenius plots of the temperature-dependent relaxation frequencies.

INTRODUCTION

As an approach, the electrical response of a system of dipoles to an incident electromagnetic wave could be used for the investigation of the system dynamics. The portion of the signal that is contributed by the permanent dipoles is named dielectric relaxation and was first described by Debye (1929). Its typical frequencies are in the radio frequency (rf) range.

Investigations of aqueous colloidal solutions of predominantly phospholipid vesicles showed a dispersion peak around 80 MHz that is due to the rotational diffusion of the zwitterionic headgroups of the phospholipid molecules (Pottel et al., 1984). According to the shape of the dispersion/absorption curves around 80 MHz, Pottel et al. concluded that there should be an additional underlying process. They then assumed that this previously unrecognized portion close to the relaxation time of the headgroup was caused by motionally perturbed water (Kaatze et al., 1984) that behaved differently from the bulk water.

In another application of dielectric spectroscopy to layered systems of lipid membranes, the thermotropic phase transition of highly hydrated 1,2-dimyristoyl-*sn*-glycerophosphatidylcholine (DMPC) was monitored for a single frequency wave (Enders and Nimtz, 1984). Although still in the rf range (4–12GHz), those experiments had been carried out far apart from the relaxation frequencies of the involved dipoles. The effect of the thermotropic phase transition of DMPC was clearly resolvable in the dielectric signals. The resulting curves could even be extrapolated to draw conclusions concerning the relaxation times and their temperature dependence in terms of activation energies.

However, for a direct determination of the dielectric relaxation times, one should conduct measurements directly in the frequency range where the respective dispersion and absorption processes occur. A new method was therefore developed, which now allows one to measure ϵ' and ϵ'' based on 201 single values in the frequency range from 1 MHz up to 1000 MHz (Kohlsmann, 1995). Some special requirements had to be fulfilled to conduct successful experiments. For maximal signal amplitude, the electric rf field vector should oscillate parallel to the responding dipoles, i.e., almost in the planes of the membrane surfaces under test. The field should in principle be totally confined to the sample to minimize ghost effects and reflections from the interfaces. Generally, the highly anisotropic character of the systems under investigation requires that their dielectric response has to be treated with $\epsilon = \epsilon' + \epsilon''$ as a tensor. However, it will be useful to adopt the system to the scalar formulation of the Debye equations. It will be seen later that we succeeded in satisfying these conditions by a proper experimental set-up.

Two principal kinds of permanent molecular dipoles are present in systems of fully hydrated lipid membranes in excess water. These are, first, the headgroup dipoles that are

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oriented at an angle of approximately 75° with respect to the bilayer normal (Essmann et al., 1995); they contribute a significant dipole component in the planes of the membrane surfaces under test. Then, water itself consists of polar molecules the orientation of which will be largely influenced around the lipid interface. Hydration may thus be envisaged as the formation of more or less fixed shells of bound water the extensions of which can be expressed in terms of pair correlation functions. The oscillatory character of the corresponding orientation polarization will be smoothed off in the liquid crystalline (L_α) phase resulting in a steadily falling polarization profile (Essmann et al., 1995).

We were not interested in observing the well known relaxation of the free water molecules in this study. Water itself, which may be regarded as a pair of OH dipoles that share the oxygen, probably is the most mobile permanent dipole. Its relaxation frequency of 18 GHz thus reveals the upper limit for dielectric measurements (Franks, 1973). However, the water molecules adjacent to a biological membrane (up to a distance of 6 Å at least) are different from those in the bulk water in that they can be envisaged as being bound in several shells (Murthy and Worthington, 1991) with a decreasing correlation strength (Hauser, 1975). The relaxation of these water molecules should show up in the frequency range between the slow headgroup dipoles and the mobile bulk water molecules (Pottel et al., 1984). This bound water can be described, in agreement with the results of computer simulations (Frischleder and Peinel, 1982), by an asymmetric distribution of water molecules with distinct relaxation times for any such shell. As an approach, we model this distribution and the corresponding surface potential (Gawrisch et al., 1992) by a continuously falling exponential decay (Cevc and Marsh, 1987; Volke et al., 1994). We thus choose an asymmetric distribution of relaxation times to be folded with the Debye equations (Böttcher and Bordewijk, 1973; Reichle, 1996). This corresponds to an altered course of ϵ' and ϵ'' as a function of frequency in contrast to the behavior found with a single frequency value; both the dispersion and the absorption curves of the bound water contribution will be more flattened, and the absorption curve will lose its symmetry. The validity of this approach will be demonstrated by separating the signals into two components each of which can be attributed to the respective dipole systems. This way, the effect of introducing weighed relaxation times to represent the response of the hydration water dipoles in the frequency range of 100–500 MHz becomes clearly visible in the experimental results.

In the following section, we will describe the preparation method of the sample layers as well as the experimental set-up for taking and evaluating the data of the dielectric dispersion and absorption from a rf electromagnetic wave that is applied from a tiny coaxial probe and reflected within the responding sample surfaces.

As the first results we will present the temperature dependence of dielectric dispersion and absorption from two systems of hydrated lipid membranes, namely DMPC and 1,2-dioleoyl-*sn*-glycero-phosphatidylcholine (DOPC) membranes, in the form of logarithmically plotted functions of the measured relaxation frequencies versus the reciprocal temperature. These Arrhenius plots will yield an activation energy (Glasstone et al., 1941) for each of the relaxation processes.

MATERIALS AND METHODS

Sample preparation

Preparation started by depositing a sample of dried lipid like a pile of pancakes. Under hydration, this stack would take up water and swell (Helfrich, 1978; Harbich and Helfrich, 1984), thus forming elongated multimembrane structures. The aim was to produce many membranes running more or less parallel with the glass support surface and thus exposing their headgroup dipoles under suitable angles.

DMPC and DOPC of high purity (>99%) were used as purchased (Avanti Polar Lipids, Alabaster, AL). Water was of Seradest (Millipore, Bedford, MA) quality and chloroform was of spectroscopic grade. A weighed amount of 10 mg of lipid was dissolved in 500 μl of chloroform and then applied in small amounts (~5 μl) onto a carefully cleaned glass support. The glass support was kept on a blotting paper in a petri dish and at a temperature of 323.2 K. Most of the solvent evaporated very quickly from the 5-μl volume and a lipid spot of ~2-3 mm in diameter was obtained. Adding spot onto spot formed a pile of almost dry lipid. After all material was applied, the dish was loosely covered to keep off any dirt and kept for 8–10 h at 323.2 K under reduced pressure (~1.5 kPa) to completely remove the chloroform and to obtain pure lipid in a dry stack. A drop (250 μl) of distilled water was applied onto the blotting paper below the glass support. The petri dish was then closed and the sample was exposed to the warm water vapor (again at 323.2 K) for another 4 h so that lipid membranes could form all through the sample and could slowly take up water by swelling. Final weighing gave ~50% (by weight) water content for DMPC and ~56% for DOPC. The glassy looking sample was finally brought, at elevated temperature (303 K at least), into whole-area contact with the measuring coaxial probe. To assure good contact, the probe was lowered and lifted several times while being monitored with an eyepiece. If the probe was continuously in touch with the sample, our measurements showed that the mean orientation of the membranes was sufficient for measuring good signals. Finally, the sample was enveloped with a drop of exterior water to shield it from dirt during the measurements and to prevent effects that might be due to dehydration.

Techniques and evaluation

Both microscopy (light and electron) and x-ray scattering were used to check the macroscopic and the microscopic state of the samples. These experiments are not the focus of this paper; the technical details are given elsewhere (Klösgen and Helfrich, 1993; Hartung et al., 1994).

The experimental set-up for dielectric spectroscopy is schematically drawn in Fig. 1. It mainly consists of a conventional Hewlett-Packard network analyzer (HP 8752C) with a built-in synthesized source and an additional miniaturized coaxial probe. The inner and outer conductor of the coaxial probe form the capacity of the terminating complex impedance. The network analyzer is programmed to scan the chosen frequency range of 1–1000 MHz in 201 logarithmically equidistant steps. Both the amplitude and the phase of the reflected wave are measured by splitting the signal and by comparing it in a receiver to the unreflected wave. The effect of physical parameters of the dielectric dispersion (ϵ') and of the dielectric absorption (ϵ'') is regarded as a contribution to the terminating complex impedance of the coaxial probe (Stuchly, 1980). Changes both in amplitude and phase of the reflected wave can thus be attributed to the respective values of ϵ' and ϵ''. To obtain qualitative results, the experiment has to start with a calibration procedure (Marsland and Evans, 1987) for ϵ' and ϵ''

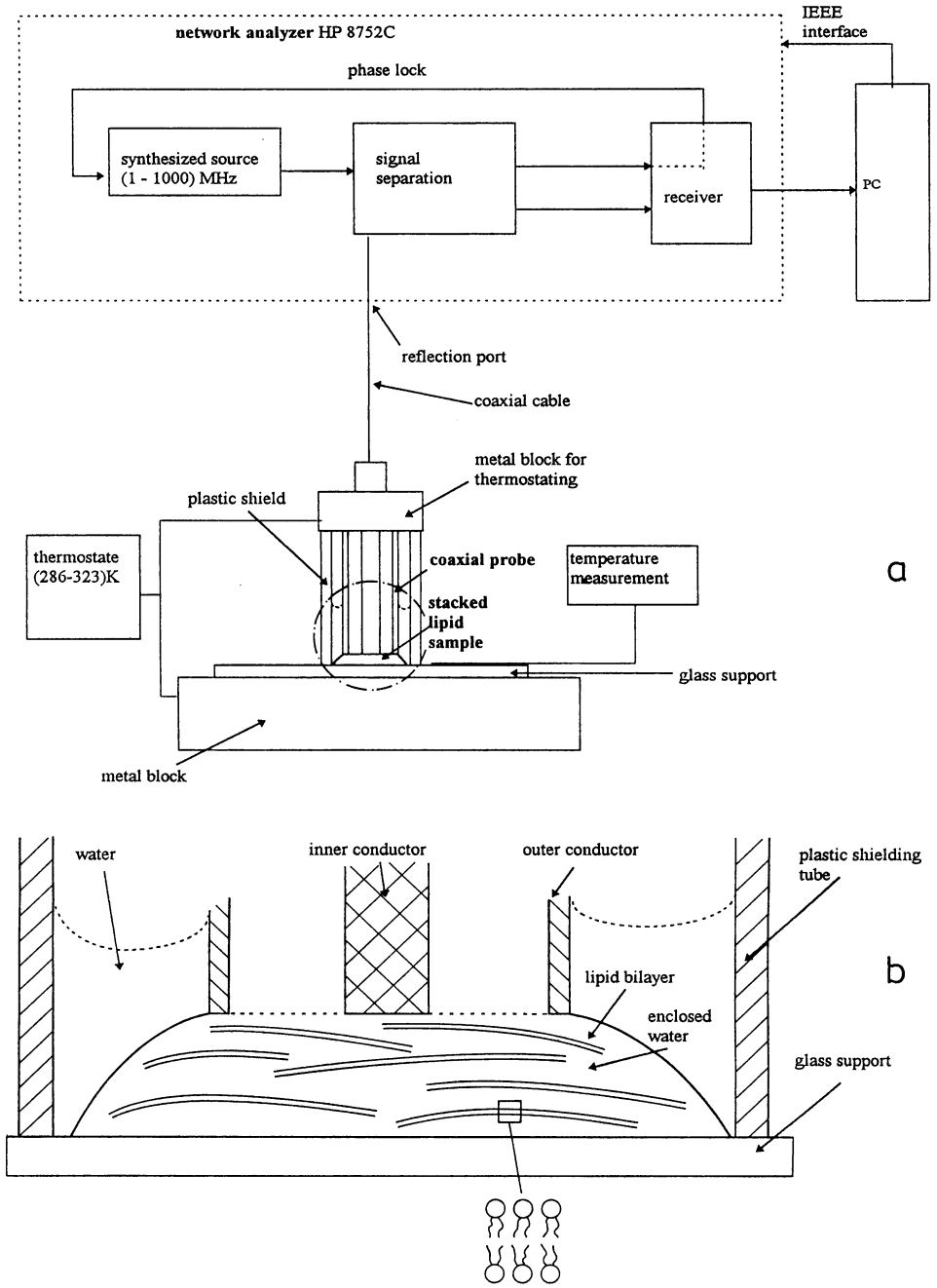

FIGURE 1 Scheme of the experimental set-up to obtain the dielectric data of stacked lipid layers by use of the coaxial microprobe. (*a*) Main features of the HP network analyzer. The coaxial cable connected to the reflection port is designed as a microprobe (Ø = 3 mm). It is directly contacted to the stacked lipid sample. (*b*) Mechanical details as the enlarged inset of *a*.

using two liquids of different polarity (methanol and bidistilled water) with well known temperature-dependent relaxation frequencies and air as the third standard with a constant value of $\epsilon' = 1$. Possible effects of a temperature gradient over the probe have to be avoided to keep the sample temperatures known and constant at ± 0.1 K. This is guaranteed by a metal block of high heat capacity that sits slightly above and below the contact plane and so fixes the preset temperature.

The analyzer takes data during an overall measuring time of 20 s per run, averaging sequentially over five values of amplitude and of phase. These mean amplitudes and phases provide direct input for laboratory-made software that was developed to calculate the frequency-dependent 201 values for ϵ' and ϵ'' as expressed by the two Debye equations. We then obtain the dispersion and the absorption curves. For our system containing two distinct dipole components with the respective relaxation frequencies f_1 and f_2 of the two distinguishable ensembles of permanent dipoles, the Debye equations can be written as follows (Debye, 1929):

$$\epsilon' = \epsilon_\infty + \Delta\epsilon_1 \frac{1}{1 + (f/f_1)^2} + \Delta\epsilon_2 \frac{1}{1 + (f/f_2)^2} \qquad (1)$$

and

$$\epsilon'' = \Delta\epsilon_1 \frac{(f/f_1)}{1 + (f/f_1)^2} + \Delta\epsilon_2 \frac{(f/f_2)}{1 + (f/f_2)^2}. \qquad (2)$$

The quantities herein are the effective dielectric dispersion ϵ' and the dielectric absorption ϵ'' of a composed system with two components, with f as the frequency of the perturbing electric field and f_1 and f_2 as the temperature-dependent relaxation frequencies of the respective compo

nents. The quantity $\Delta\epsilon_1$ represents the contribution of the first component to the static dielectric polarization, whereas $\Delta\epsilon_2$ is assigned to the second one. The induced polarization at very high frequencies is a slowly falling function tending to a value that corresponds to the optical refractive index. Its contribution is contained in ϵ_∞ (Debye, 1929). The rf power level is adjusted sufficiently low so as not to evoke thermal effects. The geometries of the inner and the outer conductors of the coaxial probes are designed to concentrate approximately 98% of the field energy into the sample (Schnupp, 1995), thus giving an optimal signal-to-noise-ratio.

RESULTS

The values of ϵ' and ϵ'' were measured in systems of water-saturated DMPC and DOPC membranes over the whole frequency range (1–1000 MHz) and at 24 different temperatures between 286 and 323 K, both heating and cooling the sample. Slight differences between the up and down temperature runs could be attributed to hysteresis effects.

1,4-Butanediol was used as a test sample for our newly developed coaxial probe technique. A plot of originally measured values of ϵ' and ϵ'' as a function of the rf frequency applied is given in Fig. 2 *a* for a temperature of 298.2 K. The upper line represents the dielectric dispersion of the relaxing dipoles. It is almost constant at low frequencies where it approaches the limiting value of the static polarization. The noisy appearance of the curves in the range of smaller frequencies (less than 10 MHz) is of no physical significance. It is caused by the fact that the exact measurement of the typically small phase shift, originating from the low capacity contributed by our microprobe, is experimentally difficult for the HP network analyzer at large wavelengths. A larger coaxial probe with its higher capacity would increase the phase shift and improve the measurements below 10 MHz, but the sample geometry restricts the coaxial probe diameter to 3 mm. On the other hand we are not interested in the frequency range less than 10 MHz, because the dielectric permittivity is constant in this range (see Fig. 2 *b*). In the case of a logarithmically plotted axis, the relaxation function of a system of uniformly relaxing dipoles is symmetric. This is evident in Fig. 2 *a*. The curve was fitted to obtain the Debye parameters as given in Eqs. 1 and 2. They are $\Delta\epsilon_0 = 26.7 \pm 0.3$, $\epsilon_\infty = 6.1 \pm 0.2$, and $f_0 = 237.4 \pm 4.5$ MHz, and the fitted relaxation frequency coincides with literature values (Böttcher and Bordewijk, 1973).

Fig. 2 *b* gives an illustration of the more complicated relaxation behavior of oriented lipid samples. It shows the experimental results of a DMPC membrane sample at 308.5 K. In contrast to the results of the 1,4-butanediol measurements, the shape of the DMPC dispersion and absorption curve is obviously asymmetric. We will demonstrate that its asymmetry can be explained as the result of two overlapping processes of rotational diffusion. In the case of hydrated lipid membranes, these are performed by the zwitterionic phosphatidylcholine headgroup and the bound water.

The distinct parameters of the Debye equations for such systems of two dipole components (Eq. 1) were obtained

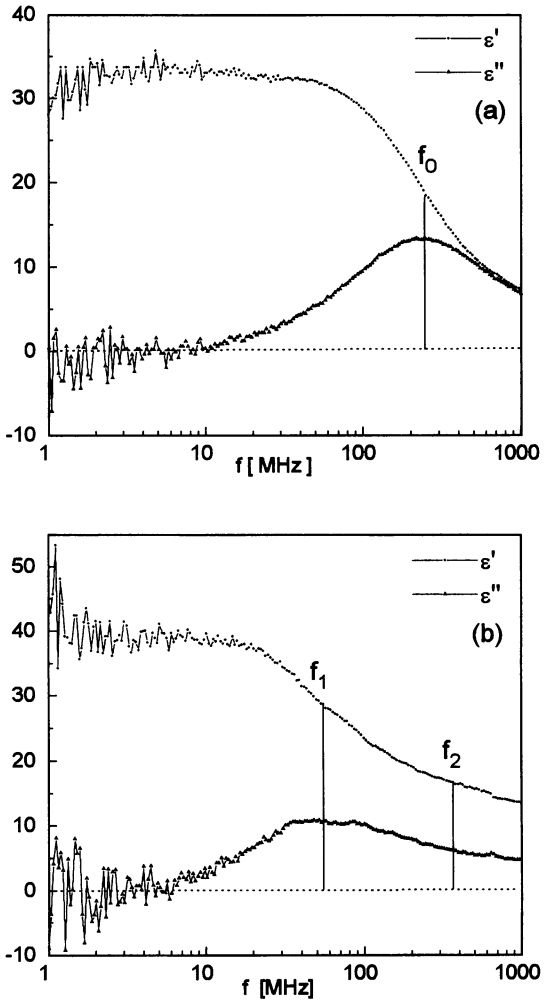

FIGURE 2 Examples for original data obtained in our application of dielectric spectroscopy with a microprobe. (*a*) This figure exhibits the complex permittivity of the reference sample (1,4-butanediol) as a function of the applied rf frequency of 1–1000 MHz. The upper line designates the real contribution and the lower one the imaginary contribution to ϵ. The experimental values are in agreement with the literature values. (*b*) The real (ϵ') and the imaginary part (ϵ'') of the dielectric relaxation of a DMPC stack as a function of the applied rf frequency 1–1000 MHz at T = 308.5 ± 0.1 K. The dispersion frequency f_1 can be attributed to the diffuse thermal rotational motion of the phosphatidylcholine headgroup whereas the dispersion contribution that is characterized by the relaxation frequency f_2 corresponds to the bound water.

from the measurements by a proper fit. Especially, the two characteristic relaxation frequencies f_1 and f_2 were extracted for any component: a distinct one for the headgroup contribution and another one as the center of a weighed distribution of dipoles that is characteristic for the system of relaxing dipoles of the bound water. It will be seen later that the description of the bound water dipoles by an asymmetric weighing function will fit the measured curves much better than one single relaxation frequency can do. The dispersion (see Fig. 2 *b*) slowly falls off in the range where the contributing dipoles exhibit their respective closely neighboring relaxations. The corresponding broad absorption peak is plotted in the lower line.

The relaxation of dipoles depends on temperature; for any temperature, we obtained a whole set of parameters for the dielectric relaxation. These temperature-dependent results of the fully hydrated membrane stacks of DOPC and of DMPC are presented all together in the Arrhenius plots of Fig. 3, *a* and *b*. The two relaxation frequencies for the hydration water molecules and for the headgroups are logarithmically plotted against the inverse of temperature. An activation energy may be evaluated from the slope. It is representative for the temperature-dependent motion of the respective dipoles in the external rf field. The activation energy values for the two distinguishably relaxing types of dipoles that were extracted from Fig. 3, *a* and *b* for the DOPC and DMPC systems are compiled in Table 1.

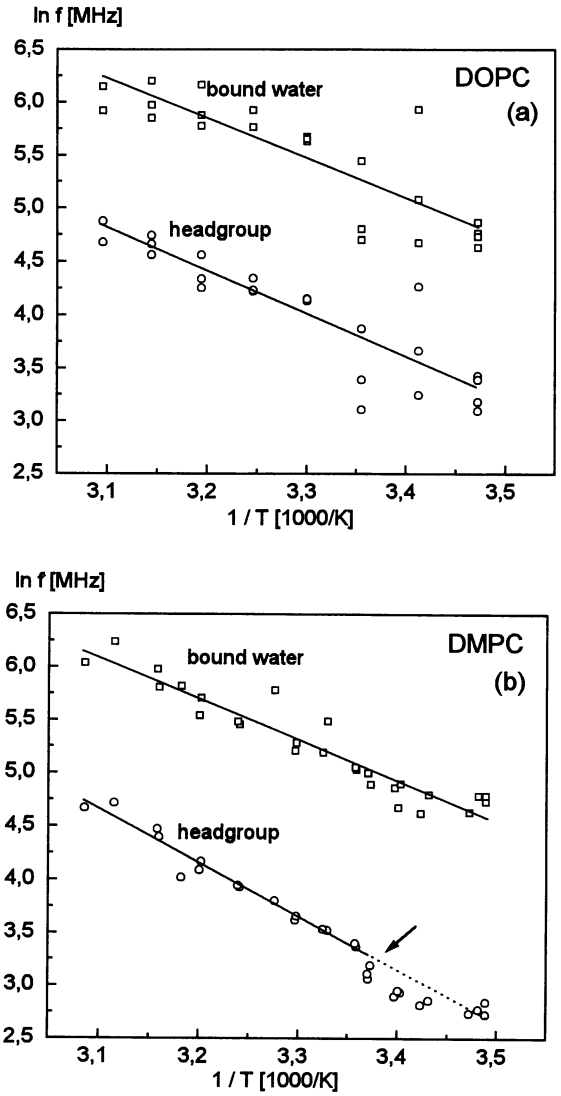

FIGURE 3 Arrhenius plots of measurements made with hydrated membrane stacks. (*a*) A plot of the two relaxation frequencies f_1 and f_2 as a function of inverse temperature for the DOPC stack experiments. The curves were obtained by fitting each measurement with two discrete relaxation frequency values f_1 and f_2 for any chosen temperature (288–323 K). Two activation energies, one as a characteristic of the relaxation of the headgroups and the other one as a characteristic of the water, can be extracted from the slopes. (*b*) The same relation is given as the Arrhenius plot for the DMPC stack measurements. Again, two relaxation frequencies are obtained to represent the system answer to the exciting field. As a difference, the headgroup answer exhibits a small but well distinguishable jump (see arrow) at the main transition temperature of 296.6 K.

DISCUSSION

The lipid samples were too dense to be directly controlled for their optical axiality by light microscopy. However, we knew from similar preparations of less lipid that preoriented multilamellar layers of lipid membranes can be formed by a proper preparation (Hartung et al., 1994). The more homogeneous the layers of dry lipid are before water uptake, the more extended regions of membranes that are oriented approximately parallel to the glass support show up as uniformly looking dark areas in the field of view. The borders would then either be decorated by single membranes that were swelling apart or form (paucilamellar) vesicles, or they would exhibit myelin-like structures, often as growing multilamellar tubes. Such samples were investigated by x-ray diffraction (Hartung et al., 1994). After less than 0.5 h of swelling, the samples exhibited the known and almost constant repeat distances of approximately 60 Å (DMPC) and 64 Å (DOPC). The high water content of 50% (DMPC) and 56% (DOPC) measured gravimetrically in our samples prepared for the dielectric spectroscopy seems to contradict these values. However, this value includes not only the hydration water layers close to the lipid headgroups that appear as a part of the lamellar ordered structure in the diffraction patterns but also a large amount of water that is present as well in a bulk phase and in water caverns. This could in principle be demonstrated by electron microscopy (see Fig. 4). Actually, the thick samples with extended structures that we examined in our spectroscopy could not be prepared for electron microscopy in an undistorted or in an unsorted way. We therefore looked into a special portion of the sample; a small amount was taken and further diluted and finally frozen to be observed by cryo-transmission electron microscopy. Thus, only pictures of the smaller objects could be taken, which consist of multilamellar liposomes that resemble onions. Large volumes of water are contained between these stacked multilamellar vesicles (Fig. 4) and within them. This portion of water is not visible in our small-angle x-ray diffraction patterns, but it contributes to the total water amount as weighed.

The formation of large oriented multilamellar domains was an important condition for getting reproducible and unambiguous results in our dielectric measurements. We performed a test with a preparation of 1,2-dioleoyl-*sn*-glycero-phosphatidyloline (DOPE) samples. DOPE is known to be in a hexagonal phase under our experimental conditions, which we verified by x-ray diffraction. In contrast to the large multilayers of DMPC and DOPC lying more or less parallel to the support surface, the permanent dipoles of the hexagonal phase of DOPE were insufficiently oriented with

TABLE 1 Activation energies as determined from the slopes of the Arrhenius plots of Fig. 3, *a* and *b*

	DOPC	DMPC
Headgroup	$\Delta E = 33.5 \pm 3.8$ kJ/mol	$\Delta E = 41.8 \pm 2.1$ kJ/mol
Bound water	$\Delta E = 30.1 \pm 4.2$ kJ/mol	$\Delta E = 31.8 \pm 3.8$ kJ/mol

respect to the rf field that is applied by our microprobe. The results of dielectric experiments were, as expected, poor. The previously good orientation (and signal amplitude) was also destroyed when we separated sample and probe several times. This was also true for samples of DMPC that were prepared by vortexing weighed amounts of water into dry lipid powder. Here, the presence of spherical liposomes (onions) is dominant and, as a consequence, the response of the system to the applied rf wave is rather uncorrelated. This undesirable effect can easily be avoided by proper preparation.

All experimental curves were analyzed for the distinct contributions of the two components (headgroups and bound water) to the total relaxation. For any temperature, we thus obtained two different relaxation frequencies. The temperature dependence of the relaxation frequencies is presented in logarithmic plots of frequencies versus inverse temperature, such as in Fig. 3. When the two curves obtained with DMPC membranes (see, e.g., Fig. 3 *b*) were analyzed, we recognized that the course of relaxation frequency over temperature was not steadily falling for both types of dipoles. Instead, we found a small jump in the value of the temperature-dependent relaxation frequencies for the lower frequency component (persumably contributed by the headgroups) whereas nothing comparable was visible for the more mobile one (attributed to bound water). This small frequency increase was observed exactly at 296.6 K (corresponding to an abscissa value of 3.372×10^{-3} K^{-1}) where the pretransition of completely hydrated DMPC membranes from the L_α phase into the more rigid $P_{\beta'}$ phase takes place (Taylor and Morris, 1995; Caffrey et al., 1991).

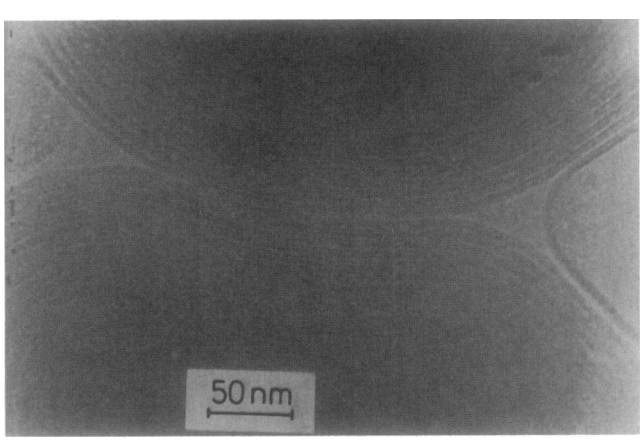

FIGURE 4 Electron micrograph (cryo-TEM), of a population of onion-like paucilamellar lipid vesicles (DOPC in 1 mM NaCl; for details of the method see Klösgen, 1993).

We take this as a confirmation that the lower frequency relaxation has to be attributed to the rotation of the headgroups. Their mobility will be restricted when the chains are packed closer within the $P_{\beta'}$-gel phase. The small jump of the headgroup relaxation frequency at the transition temperature may be explained as an indirect effect caused by the increased flexibility of the molten lipid chains. This will also increase the headgroup area per molecule and the free volume for the headgroup rotation. In the solid phase, the molecules are situated closer to each other as the chains of the lipids condensed into the $P_{\beta'}$ phase. The rotation of the dipoles will thus be cut down or at least restricted, resulting in a longer relaxation time that is characteristic for the gel phase.

The activation energy that we took from the slopes of curves such as in Fig. 3 *b* for inverse temperature values smaller than 3.372×10^{-3} K^{-1} therefore has to be ascribed to the motion of the DMPC headgroups of the fluid phase only. No activation energy was evaluated for the headgroup rotation of the gel phase as the determination of relaxation frequencies for values lower than 10 MHz, which is the range of the low temperature phase ($P_{\beta'}$), was difficult (see Fig. 2 *b*) because of the already mentioned poor accuracy of the measured ϵ' and ϵ'' values in this frequency range. An analysis of the low frequency range to yield an activation energy also for the $P_{\beta'}$ phase was thus not reasonable.

The phase transition itself as well as the change of structure in the hydrophobic region of the membrane (which is mostly an effect of the interaction of the lipid tails) may be more easily accessed by calorimetric (Caffrey et al., 1991), P-NMR (Dufourc et al., 1992), or x-ray diffraction methods (Caffrey, 1989; Smith et al., 1990). Application of ^{2}H-NMR (Ulrich et al., 1990, 1994; Gawrisch et al., 1992; Volke et al., 1994) may yield information about both the chain dynamics and the state of the bound water. In dielectric spectroscopy there is no direct response of the apolar tails as only the polar headgroups can interact with the applied rf wave. However, the indirect effect of the transition into the $P_{\beta'}$ phase is small but clearly visible in our experiments.

The higher value measured for the headgroup activation energy of DMPC (41.8 kJ/mol) as compared with the value obtained for DOPC (33.5 kJ/mol) can also be interpreted as a result of different steric hindrance caused by the different conformation of the chains involved.

The upper curves of Fig. 3, *a* and *b* correspond to the response of the hydration water. This portion of water differs from the bulk water. The dielectric relaxation frequency of bulk water can be observed at 18 GHz, whereas the relaxation of the bound water is shifted to lower frequencies. The respective temperature-dependent values lie in the frequency range of 100–500 MHz. This is also reflected in the higher activation energy of the bound water as compared with the literature value (~21 kJ/mol) of pure water (Franks, 1973). The two values evaluated for the hydration water from the DOPC measurements (30.1 ± 4.2 kJ/mol) and also from the DMPC experiments (31.8 ± 3.8

kJ/mol) are significantly higher and agree with each other within the experimental error, both as expected.

The response of the hydration water that is hidden in the total dielectric response can be further evaluated to obtain more information about the dipole density and the respective mobility distribution. As an approach, we separated the two contributions from the headgroup and water dipoles from each other by subtraction of the headgroup contribution. For example, the measurements of fully hydrated DMPC membranes at 308.5 K were analyzed by application of Eq. 1 to get two distinct contributions that will sum up to give the total relaxation of the system. These are f_1 = 50.9 ± 3.5 MHz and $\Delta\epsilon_1$ = 20.2 ± 1.2 for the headgroup rotation as well as f_2 = 345.4 ± 114 MHz and $\Delta\epsilon_2$ = 6.7 ± 1.0 for the contribution of the hydration water. The high frequency domain then follows, starting from ϵ_∞ = 13.1 ± 0.9. The quantity $\Delta\epsilon_2$ as the static dielectric polarization of the water dispersion has a value of approximately one-third of the headgroup value $\Delta\epsilon_1$.

According to Monte Carlo simulations (Frischleder and Peinel, 1982), we describe the hydration water by a series of shells that each include some bound water molecules, the number of which falls off with increasing distance from the membrane. This asymmetric density distribution of water molecules corresponds to an exponentially falling surface potential. The dielectric relaxation of the hydration water may then be approximated by choice of a single relaxation time for each of these water shells. Instead, we introduced, as an approach, a weighing function G with a continuously falling exponential decay for the relaxation frequencies of the hydration water. For symmetric distribution functions, the center of gravity would always coincide with the relaxation frequency as given by the first Debye equation (Eq. 1). This is generally not true for any asymmetric distribution function. One important attribute of the weighing function G was thus the demand that its center of gravity should be invariant to the half-width of the underlying asymmetric distribution. This center of gravity was defined to be identical to the relaxation frequency f_2 as obtained from an unweighed Debye equation. We posed this condition to get a discrete value that we could handle and discuss in terms of activation energies resulting from Arrhenius plots (see Fig. 3, *a* and *b*). On the other hand, we could transform the previously used single-component relaxation function, characterized by a singular frequency, into the more representative hydration water formulation with its exponentially decaying frequency distribution. As a result, we get a much better agreement between the theoretically calculated and the experimentally measured course of the dispersion data. This will be discussed below. A detailed derivation of the distribution function and the respective description of the water response by the resultant relaxation broadening is given in the Appendix.

With this choice of evaluation for the experimental data, we obtain a consistent description for the relaxation of fully hydrated lipid membranes (see above). As an example shown in Fig. 5 *a*, we replotted the data of Fig. 2 *a*. The

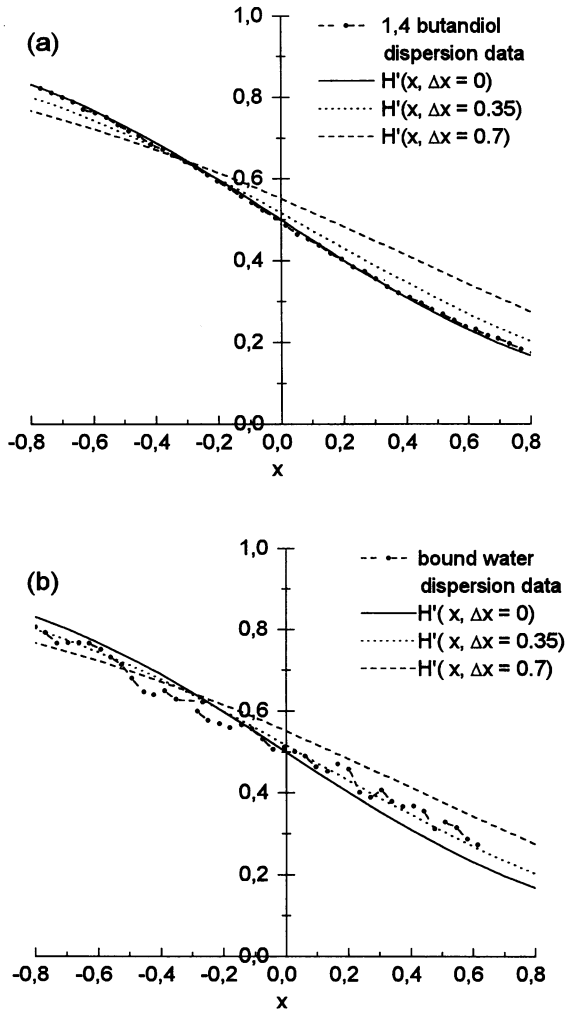

FIGURE 5 (*a*) The dispersion of the reference sample (1,4-butanediol) is redrawn in the formulation of the newly introduced dispersion function $H'(x,\Delta x)$; see Eq. 10. The abscissa designates the logarithmic frequency divided by the fitted relaxation frequency f_0 of the reference sample ($x = \ln(f/f_0)$). The functional decrease of H′ as calculated from the Debye equation (Eq. 1) for a system of relaxing identical dipoles appears as the straight line. This case is equivalent to a distribution function $G(x,x_0,\Delta x)$ with the half-width $\Delta x = 0$. Corresponding curves for two more values of a half-width Δx different from zero are also drawn, namely for $\Delta x = 0.35$ and for $\Delta x = 0.70$. They are less steep. The experimental data coincide with the expected description of $H'(x, \Delta x)$ with a half-width $\Delta x = 0$ of the underlying distribution. (*b*) The same is shown for the case of bound water. As an example, the dispersion was drawn from the measurements of fully hydrated DMPC membranes at 308.5 K. The abscissa value zero refers to the center of gravity of the bound water relaxation frequency (f_2 = 345.4 MHz). The ordinate represents the dielectric dispersion function of the water. The experimental results vary within the range $\Delta x = 0$ (lower line) and $\Delta x = 0.7$ (upper line) of $H'(x, \Delta x)$; see Eq. 10. We conclude that $\Delta x = 0.35$ (middle line) might be a reasonable mean value.

dispersion of the reference sample (1,4-butanediol) is now described by the newly introduced dispersion function $H'(x, \Delta x)$ as a function of the linearized frequency x, both as given in the Appendix. In fact, this is no more than a weighed presentation of Eq. 1. The butanediol data will prove to be rather illusive in the context of the presumed

distribution function $G(\Delta x)$ because their dispersion is governed by one singular relaxation frequency f_0 that, in terms of a frequency distribution, corresponds to $\Delta x = 0$.

For the abscissa, the newly introduced logarithmic variable x will be zero for the relaxation frequency f_0 of 1,4-butanediol and extend to approximately ± 0.8 in the experimentally applied rf range. The ordinate represents the dielectric butanediol dispersion as given by the dispersion function $H'(x, \Delta x)$. The theoretical course of H' as a function of x has been numerically calculated and drawn for three different distribution half-widths Δx as parameters. All experimental data nearly coincide with the expected best description of $H'(x, \Delta x) = 0$. This is also a proof of the low experimental error. The small systematic deviation of the experimental values to the theoretical ones is caused by fact that there is always a Gaussian distribution underlying any experimentally determined value.

In the case of dispersion of the hydration water as extracted from the measurements of highly hydrated stacks of DMPC or DOPC, the course of our experimental data in the same representation completely deviates from the corresponding $H'(x, \Delta x) = 0$. This is obvious from Fig. 5 *b* where the experimental data (taken from a measurement of DMPC at 308.5 K) scatter around the dispersion function $H'(x, \Delta x) = 0.35$. As proposed before (Enders and Nimtz, 1984), the idea of a uniformly relaxing ensemble of water dipoles is too simple.

According to these results, we propose the following interpretation for the total dielectric relaxation of our system. Our results can be completely explained if we assume two independent processes with two relaxing dipolar components. One constituent contributes to the signal by a unique response of the identical dipoles. This is the low-frequency contribution of the rotating headgroups. The other constituent is not as uniform. It consists of the hydration water molecules that are more or less bound by the membrane interface. This contribution has to be described by choice of a broadened distribution of relaxation frequencies. The exponential decay of the effective membrane surface potential is reflected in the asymmetric form of the underlying distribution (Eq. 9). The extent of asymmetry is owed to the half-width Δx. A reasonable mean half-width $\overline{\Delta x}$ could be derived to be approximately $\overline{\Delta x} = 0.35$ (see Fig. 5 *b*). This value corresponds, in the example encountered, to an additional frequency spread of approximately 120 MHz in addition to the normal wide frequency spread of 1200 MHz, calculated for a single dielectric relaxation at $f_2 = 345.4$ MHz.

The hydration water molecules may thus not be envisaged as being located in fixed shells of closely connected and similar neighbors. In that case, they would rather exhibit a few distinct relaxation frequencies that could be attributed to their well defined distinct states. We tried that, too, but the superposition of several distinct relaxation functions was not as satisfying. At least, one should necessarily end up with a reasonable model about the water adjacent to membranes. Moreover, the form of the measured values $\epsilon' = \epsilon'(f)$ reflects a different behavior and suggests as a straightforward model a continuous distribution of dipoles. In terms of the Debye description, the system of hydration water dipoles has then to be accounted for by an asymmetric distribution function that is similar to others that had been derived from Monte Carlo simulations (Frischleder and Peinel, 1982). In such simulations, the stacked system of membranes and water was modeled by a surface potential that is changing as the number of water molecules per shell changes as a function of their distance from the headgroup dipole.

CONCLUSION

The dielectric properties in biological materials are accessible by a low cost and easily transportable small device. A miniaturized probe allows one to take up signals from very narrow regions. We tested the method in the extremely reduced biological model system of stacked and highly hydrated lipid membranes. Relaxation curves were obtained for both absorption and dispersion. The measured signals could be analyzed and conclusions could be drawn about the underlying fundamental physical mechanisms. In the frequency range of 1–1000 MHz of the rf waves applied, we observed two distinguishable relaxation phenomena. One was attributed to the diffuse thermal rotational motion of the headgroups around axes approximately perpendicular to their permanent electric dipole moment. The second relaxation was interpreted to be due to water trapped around the membrane. This hydration water is hindered from a free rotation. The respective relaxation frequency was found to be much smaller than the one related to the rotation of free bulk water. Moreover, the relaxation of the hydration water did not represent a unique system of identical dipoles. It rather had to be attributed to a system of different dipoles that could be described by a continuous asymmetric distribution.

An interpretation using modified Debye's equations thus revealed different and distinguishable contributions of the headgroup and bound water molecules with either one distinct relaxation frequency or with a weighed distribution of frequencies according to the shell-like structure of the hydration water that is caused by the exponentially decaying surface potential. Distinct activation energies could be extracted and attributed from the temperature dependence of both relaxation processes.

Comparing the DMPC headgroup activation energy with the DOPC headgroup activation energy, we conclude that the smaller value for the DOPC sample is due to the less steric hindrance of the DOPC headgroup in the fluid phase. As expected, the values determined for bound water, from the experiments with both DOPC and DMPC, are identical within the experimental error. The quantity $\Delta\epsilon_2$ as the static dielectric polarization of the bound water dispersion has a value of approximately one-third of the headgroup value $\Delta\epsilon_1$.

APPENDIX

Derivation of an asymmetric weighing function G

The distribution G has to describe the contribution of the bound hydration water dipoles to the total dielectric dispersion in a stack of fully hydrated membranes. Representing the effect of an exponential decay of the membrane surface potential for the adjacent water, it shall itself be an asymmetric distribution. The center of gravity of the distribution has to coincide with the value of the relaxation frequency f_2 as obtained from the first Debye equation (dispersion, Eq. 1). A weighing function G that fulfills these conditions may be found as follows:

The second term in the Debye equation for the dispersion

$$\epsilon' = \epsilon_\infty + \Delta\epsilon_1 \frac{1}{1 + (f/f_1)^2} + \Delta\epsilon_2 \frac{1}{1 + (f/f_2)^2} \quad (1)$$

may be rewritten as the function

$$F'(x_2) = \frac{\epsilon' - \epsilon_\infty}{\Delta\epsilon_2} = \frac{1}{1 + e^{2x_2}} \quad (3)$$

when we introduce the substitution $\ln(f/f_2) = x_2$ to get a description with a linearized frequency axis.

We assume a relaxation broadening that we assign to the different shells of the hydration water. The dielectric response of this bound water is caused by an exponential decay of the membrane surface potential. This can be expressed by an asymmetric distribution of relaxation frequencies represented by an exponentially decaying weighing function G:

$$G = G_0\, e^{-\alpha x_2} \quad \text{with } x_2 \geq x^*, \quad (4)$$

where x^* is the minimal frequency of the distribution, x_2 is the physical variable with its proportionality constant α, and x is the free running parameter in the weighing function. We then have to find three conditions determine the constants G_0, α, and x^* in the weighing function. First, G has to be normalized to 1.

We thus find

$$G = \alpha e^{-\alpha(x_2 - x^*)} \quad (5)$$

Second, the exponentially decaying distribution function may be characterized by a half-width Δx. We then obtain by definition a connection between the minimal frequency x^* and the half-width Δx:

$$G(x^*) = 2G(x^* + \Delta x),$$

which yields

$$\alpha = \frac{\ln 2}{\Delta x} \quad (6)$$

The center of gravity of the weighing function should be invariant to changes in Δx:

$$\int_{x_2 = x^*}^{\infty} (x_2 - x) e^{-(\ln 2/\Delta x)\cdot x_2} dx_2 = 0 \quad (7)$$

The minimal frequency of the distribution is thus given as

$$x^* = x - \frac{\Delta x}{\ln 2}. \quad (8)$$

The latter condition allows us to use the Debye equations, and it ensures that one obtains the same distinct relaxation frequency to represent the hydration water molecules as without describing them by a weighed distribution.

Thus, we obtain the weighing function G in the following form:

$$G(x, x_2, \Delta x) = \frac{\ln 2}{e\Delta x} \cdot e^{+(\ln 2/\Delta x)x} e^{-(\ln 2/\Delta x)x_2}, \quad x_2 \geq x - \frac{\Delta x}{\ln 2}. \quad (9)$$

The relaxation of the bound water molecules may now be described (Reichle, 1996) by a dispersion function $H'(x, \Delta x)$ that we obtain when we fold the single component relaxation function $F'(x_2)$ (see Eq. 3) with the asymmetric frequency distribution function $G(x, x_2, \Delta x)$:

$$H'(x, \Delta x) = \frac{\ln 2\, e^{(\ln 2 x/\Delta x)}}{e\Delta x} \cdot \int_{x_2 = x - (\Delta x/\ln 2)}^{\infty} \frac{1}{1 + e^{2x_2}} e^{-(\ln 2 x_2/\Delta x)}\, dx_2 \quad (10)$$

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