Effect of hydration on the structure of oriented lipid membranes investigated by *in situ* time-resolved energy dispersive x-ray diffraction

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In situ time-resolved energy dispersive x-ray diffraction (EDXD) was applied to investigate the effect of hydration on the structure of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-oriented membranes. The measurements allowed a very high density time sampling of the evolution of the structural properties of the DOTAP bilayer such as the lamellar *d*-spacing, the membrane thickness, and the size of the interbilayer water region. Time-resolved EDXD has been found to provide important information on the role played by free water molecules on the structure and fluidity of lipid bilayer. © *2005 American Institute of Physics*. [DOI: 10.1063/1.1952583]

Lipid hydration and its effect on the structure of lipid bilayers is a fundamental problem in membrane biophysics.[1,2] Water adsorption affects interactions in the headgroup region at a molecular level and results in the development of interbilayer interactions known as hydration forces.[3] The water interacting with lipids has been classified as tightly bound, weakly bound, trapped and free water and, as a result of interaction with water molecules, major changes can occur in both the structural and dynamic properties of lipids.[4] Furthermore, it has been recently shown that water molecules close to the bilayer surface are strongly ordered with their dipoles aligned against the bilayer dipole and that the percentage of oriented waters monotonously decreases as the interlamellar *d*-spacing increases.[5]

Since lipid bilayers are the supporting matrix of cell membranes, a second major goal is to measure their structural parameters quantitatively in the biologically relevant liquid-crystalline L_α phase at different levels of hydration. The headgroup-headgroup distance d_{HH} gives fundamental information about the fluidity and the elastic properties of lipid bilayers, whereas obtaining the water spacing between adjacent bilayers d_W is essential for evaluating interactions between membranes.[6] Insights into the correlation between the degree of hydration of lipid bilayers and the exact nature of interbilayer water could help towards answering the recurrent question about the relation between the structure of membrane surface water and lipid headgroups.[7]

In this letter, we demonstrate the feasibility of using the energy dispersive x-ray diffraction (EDXD) method as a means of monitoring temporal changes in the bilayer structure upon hydration. EDXD has recently emerged as a powerful tool to investigate the kinetic variation of the lipid bilayer structure induced by the change of physical and chemical parameters.[8] The proposed experimental setup is amenable to perform *in situ* hydration kinetics allowing to acquire accurate time-resolved images of the intrinsically low-resolution lipid bilayer structure. In particular, we report on the hydration kinetics of oriented 1,2-dioleoyl-3-trimethylammonium-propane-chloride (DOTAP-Cl) multibilayers system. In this study, we took specific advantage of using oriented lipid membranes, which strongly reduce acquisition times with respect to liposomic aqueous dispersions.[9] Oriented samples safeguard spatial information and allow for more accurate diffraction analyses.[10]

Although there is an extensive literature on the structure of neutral lipids, the present knowledge of the structural properties of charged lipids is still poor and needs to be implemented particularly due to the involvement of charged headgroups in cell activity.[11]

DOTAP was purchased by Avanti Polar Lipids in the lyophilized form and used without further purification. Oriented DOTAP membranes were prepared depositing 1 mg of lipid onto a flat freshly cleaved silicon wafer by evaporating from an isopropanol solution. After drying under vacuum for one day, the sample was hydrated from a vapor-saturated atmosphere, at $T=300$ K, in a sample chamber suitably designed to overcome the experimental inadequacy that had previously led to the vapor-pressure paradox.[12] A diffraction angle $\theta=1.06°$ was fixed that allowed to cover simultaneously an overall region of the reciprocal space $0.092<q<1.1$ Å^{-1} [$q=\text{const}(E)\sin\theta$; const$=1.014$ Å^{-1} keV^{-1}].

A series of time-dependent EDXD patterns were collected during the exposure of the lipid film to the water vapor, were automatically stored every 100 s, and are reported in Fig. 1 (250 patterns). As evident, the measurements allowed a very high density time sampling of the temporal evolution of the DOTAP bilayer structure. At $t=0$, the first four diffraction orders (00*l*) of a lamellar structure with a *d*-spacing $d=2\pi l/q=47.9$ Å were measurable with high accuracy, while occasional higher orders could be observed but were not accurately measured. Immediately after closing the chamber windows, the lamellar order of the L_α phase is maximum.

Upon hydration, the water adsorption results in a spontaneous and continuous swelling of the DOTAP membranes. The increase of the repeat distance is reflected in the change of the diffraction Bragg peaks positions which move to lower *q*-values as a function of time, that is, as a function of increasing hydration. As evident from Fig. 1, the lamellar signal changes remarkably in that the intensities of the diffraction orders continuously decrease. To the sake of clarity, the

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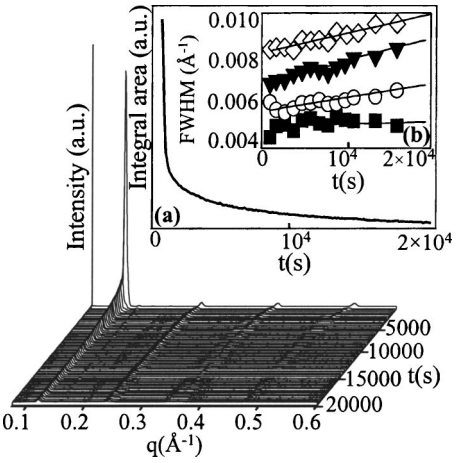

FIG. 1. Time-resolved EDXD patterns of DOTAP multibilayer system upon hydration. Water molecules are incorporated between lipid bilayers and promote loss of spatial coherence. Increasing lattice disorder leads to a progressive lowering and broadening of diffraction peaks, as confirmed by the temporal evolution of the integral area of the first-order Bragg peak (panel a) and by the FWHM of all the diffraction orders as a time function. The continuous lines are a guides to the eyes to show the general trends (panel b).

temporal evolution of the first-order Bragg peak intensity is reported in panel A, whereas the temporal evolution of the full width at half-maximum (FWHM) of all the diffraction orders is displayed in panel B. The onset of diffraction intensity decrease is directly interpretable in terms of order loss promoted by free water molecules that penetrate the multilayer system and reduce the scattering correlation length. Since FWHMs increase with increasing transfer momentum q, disorder of the second kind is indicated.[13] Interestingly, panel B also shows that FWHMs increase as a function of hydration, confirming that the intermembrane coherence progressively extends less far. We also observe that intensities from higher orders of diffraction gradually degrade.

All these observations consistently show a correlation between reduced ordering and increased hydration, which is consistent with the penetration of free water molecules between the interbilayer region.

With the aim to provide direct and quantitative evidence that such order loss could be entirely ascribed to the disordering effect caused by the adsorption of free water molecules, electron density profiles along the normal to DOTAP bilayers were calculated from the time-resolved EDXD patterns of Fig. 1, and some representative profiles are shown in Fig. 2. The electron density profile ($\Delta\rho$) along the normal to

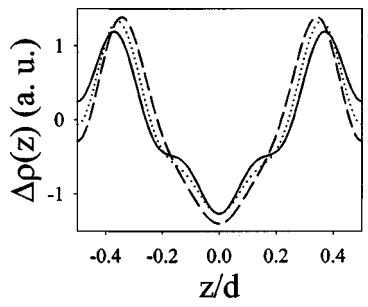

FIG. 2. Representative electron density profiles calculated from the time-resolved EDXD patterns collected at $t=300$ s (solid line), $t=1000$ s (dotted line), and $t=15000$ s (dashed line).

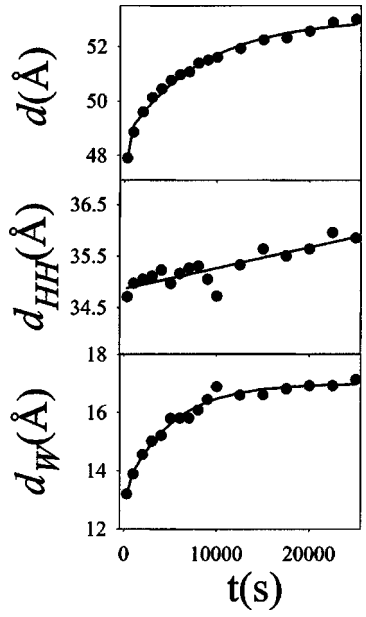

FIG. 3. Temporal evolution of the lamellar repeat distances obtained by the electron density profiles calculated applying Eq. (1). Representative values are depicted. The swelling of the DOTAP membranes is due essentially to the expansion of the water layer d_W between opposing bilayers. In addition, as a result of the greater mobility of the DOTAP molecules, the membrane thickness d_{HH} increases slightly. The continuous lines are the best fits to the data obtained applying Eq. (2): d; Eq. (3): d_W; and a linear regression: d_{HH}.

the bilayers (z) was calculated as a Fourier sum of cosine terms as

$$\Delta\rho = \frac{\rho(z) - \langle\rho\rangle}{[\langle\rho^2(z)\rangle - \langle\rho\rangle^2]^{1/2}} = \sum_{l=1}^{N} F_l \cos\left(2\pi l \frac{z}{d}\right), \tag{1}$$

where $\rho(z)$ is the electron density, $\langle\rho\rangle$ its average value, N is the highest order of observed reflections, F_l is the form factor for the $(00l)$ reflection, and d is the thickness of the repeating unit.

The phase problem was solved using the approach first suggested by Luzzati.[14] Due to intrinsic fluctuations in hydrated fluid bilayers, x-ray data from multilamellar arrays can yield electron density profiles only along the normal to the bilayers, giving a measure of the bilayer thickness d_{HH} and the size of the water region d_W. The electron-denser regions (i.e., the two maxima in the electron density profiles of Fig. 2) represent the DOTAP headgroups, while the pronounced central minimum corresponds to the terminal methyl groups of the opposing acyl chains. The distance between the maxima provides a sensible estimation of the membrane thickness d_{HH}.[6] The lamellar repeat distance d can be regarded as the sum of the membrane thickness d_{HH} and the water layer thickness d_W. Thus, the interbilayer water thickness is defined as $d_W = d - d_{HH}$. Accordingly, from the calculated electron density profiles, accurate information about the temporal evolution of the lamellar repeat distances can be retrieved. Figure 3 shows the results for the changes in the d-spacing, bilayer thickness, and water layer thickness as a function of hydration. While the bilayer thickness increases linearly, both the d-spacing and the water layer thickness are well fitted by the following double exponential models:

$$d(t) = d_1(1 - e^{t/\tau_1}) + d_2(1 - e^{t/\tau_2}), \tag{2}$$

$$d_w(t) = d_{w_1}(1 - e^{t/\tau_{w_1}}) + d_{w_2}(1 - e^{t/\tau_{w_2}}), \quad (3)$$

which prove the existence of two relaxation processes: a faster ($\tau_1 \sim 74$ s; $\tau_{w_1} \sim 79$ s) and a slower process on the time scale of a few thousands of seconds ($\tau_2 \sim 8165$ s; $\tau_{w_2} \sim 5080$ s).

The decomposition of the *d*-spacing into bilayer and interbilayer water thickness reveals that the discussed order loss is not only caused by an uptake of water. Indeed, concomitantly with the *d*-spacing swelling, the bilayer responds to the predominant water layer expansion by a simultaneous enlargement in membrane thickness as each single DOTAP molecule requires more space due to the increasing of motional freedom at higher levels of hydration.

Therefore, our structural findings demonstrate that the swelling of the DOTAP membranes depend not only on the adsorption of free water molecules by DOTAP multilayer but also, significantly, on the development of pronounced bilayers undulations, reflecting a more fluid lipid bilayer as hydration proceeds. Such fluctuating whole-body motions of the bilayers introduce lattice disorder and cause additional broadening of the Gaussian distributions that describe the thermal motion of the quasimolecolar fragments representing the bilayer nature of lipid membranes.

These findings confirm the existence of a correlation between the degree of hydration of lipid bilayers and the structure of interbilayer water, which modulates the fluidity of membranes as recently proposed.[7]

On the whole, the present study demonstrates the ability of *in situ* time-resolved EDXD to provide a quantitative and refined description of the dynamic evolution of the bilayer structure and fluidity as a function of hydration. Experiments carried out on samples at lower and higher levels of hydration than those of the present study are currently in progress and are expected to elucidate in detail how the lipid hydration is related to the structure and fluidity of the bilayers.

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