

Effect of hydration on the long-range order of lipid multilayers investigated by in situ time-resolved energy dispersive X-ray diffraction

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Received 20 April 2005; in final form 11 May 2005

Available online 9 June 2005

Abstract

In situ time-resolved energy dispersive X-ray diffraction (EDXD) was applied to investigate the hydration kinetics of oriented dioleoylphosphocoline (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) membranes. The long-range order in the investigated lipid multilayers has been found to vary as a function of hydration in a nonmonotonic way. In the first stage, water adsorption increased the long-range order along the normal to the lipid bilayer whereas, in the second one, a progressive loss of interbilayer coherence was observed. Possible molecular mechanisms underlying the experimental observations are discussed.

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1. Introduction

Water is essential to the structure and dynamics of all biologically relevant molecules. Among these, phospholipids usually bind considerable amounts of water at their lipid headgroup often establishing a liquid-crystalline lamellar arrangement, known as L_α phase, which is the structural basis of biological membranes. Since in the presence of water most lipids exist as aggregate structures, often bilayer in nature, some water interacting intimately with the surface should be considered as a part of the structure itself, the two separate structures being considered as one [1]. More in detail, the water interacting with lipids has been categorized as free water, trapped, weakly bound and tightly bound [2–4].

Nevertheless, lipid hydration is known to affect not only the molecular interactions in the headgroup region

but also the organization and motions of the acyl chains comprising the hydrocarbon core (HC) [5,6].

The utility of ultra-high-resolution ¹H magic-angle-spinning nuclear magnetic resonance (NMR) for determining the nature and extent of lipid hydration at the membrane/water interface has been recently demonstrated [7]. More recently [8], deuterium (²H) NMR studies have shown that the phosphatidylglycerol headgroup of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)] (POPG) undergoes changes of conformation and average orientation with respect to the membrane surface as a function of hydration.

Unfortunately, although a basic phenomenon in membrane biophysics, the exact physical-chemical principles driving the lipid hydration have not yet been understood in detail. Molecular dynamics (MD) studies have recently shown a strong correlation between the degree of hydration of lipid bilayers and the structure of interbilayer water. Indeed, in the headgroup region, water molecules are ordered by phosphate, choline and carbonyl groups. While waters form hydrogen bonds

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with phosphate and carbonyl groups, they do not hydrogen bond with the choline group but form a clathrate structure around the choline methyl group [9].

On the other hand, water associated with lipid headgroups and in contact with the interfacial region has been supposed to be the main determinant of the dipole potential of lipid membranes. In a MD study, Tobias [10] has shown that, as a result of the orienting effect of the negatively charged unesterized phosphate groups on water molecules, the dipoles of water molecules on the surface of fully hydrated lipid bilayers point toward the membrane surface. It means that tightly bound water molecules are ordered and polarized.

Further support to this hypothesis has been recently provided by Cheng et al. [11] who have demonstrated the ordered orientation of the hydration water at the surface of phospholipids bilayers by use of coherent anti-stokes raman scattering (CARS) microscopy. The authors provided the experimental evidence that water molecules close to the bilayer surface are strongly ordered with their dipoles aligned against the bilayer dipole, that the lipid polar headgroup affects the orientation of the water molecules and that the percentage of oriented molecules decreases as the interlamellar spacing increases. Previously, Hristova and White [12] reported a comprehensive study on the effect of hydration on the HC structure of dioleoylphosphocoline (DOPC) bilayers and observed a distinctive structural change upon completion of the DOPC hydration shell enforcing the general idea that the water in the first hydration shell of the lipid is definitely more structured than the bulk water. Given that the number of water molecules in the hydration shell can vary as a function of the phase state of the lipids, the type of lipid headgroup, the electric charge on the headgroup, the HC organization and the presence of *cis*-double bonds, in principle it should be possible to differentiate between structural changes of different lipids upon progressive hydration [13,14].

In this Letter, we report on the hydration kinetics of oriented DOPC, and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) membranes by means of in situ time-resolved energy dispersive X-ray diffraction (EDXD). In this study, we took specific advantage of using highly aligned lipid membranes which allow for more accurate diffraction analyses with respect to liposomic aqueous dispersions and are excellent model systems of biological membranes [15, and references therein].

This work is devoted to comparatively explore the effect of hydration on the structural features of two multilamellar lipid systems exhibiting different lipid headgroups and a number of systematic variations in relevant physical–chemical parameters [1]. In addition, the hydration behavior of DOTAP could be of some supplementary importance since this cationic lipid has been using as DNA complexing agent for gene delivery systems in therapy studies [16]. Adsorbed water may

play a major role in the effectiveness of lipid drug delivery systems where lipid–cell interactions are involved.

2. Materials and methods

2.1. Sample preparation

Multilayered stacks of highly aligned DOPC and DOTAP membranes were prepared depositing 0.5 mg of lipid onto the oriented surface of freshly cleaved $\langle 100 \rangle$ silicon wafers (area $\sim 1.5 \text{ cm}^2$) by evaporating from an isopropanol solution (10 mg/ml). The amount of lipid sample spread over an area of 1.5 cm^2 should result in ~ 1200 DOPC bilayers [17].

All the lipids were purchased from Avanti Polar Lipids in the lyophilized form and used without further purification. After drying under a vacuum over at least 12 h to remove any residual solvent, the samples were alternatively hydrated from a vapour saturated atmosphere, at $T = 293 \text{ K}$, in a sample chamber suitably designed to overcome the experimental inadequacy that had previously led to the vapour–pressure paradox [17].

2.1.1. Time-resolved EDXD experiments

X-ray diffraction experiments were carried out by using an EDXD apparatus elsewhere described [18]. The X-ray source is a standard Seifert tube operating at 50 kV and 40 mA whose Bremsstrahlung radiation is used whereas the detecting system is composed of an EG&G liquid-nitrogen-cooled ultrapure Ge solid-state detector. The diffractometer, equipped with step motors and a collimating system, operates in vertical θ/θ geometry and both the X-ray tube and the detector can rotate around their common center in which the lipid coated wafer is placed. The uncertainty associated to θ is $\Delta\theta = 0.001^\circ$ and it directly affects the uncertainty Δq associated to the transfer momentum q ($q = \cos t E \sin \theta$; $\cos t = 1.01354 \text{ \AA}^{-1} \text{ keV}^{-1}$). For kinetics experiments, the diffraction intensity was collected and automatically stored every 1 s. Biological samples are not damaged by EDXD experiments as elsewhere discussed [19].

3. Results and discussion

The EDXD pattern of multilayered DOPC film, collected at $t = 0$ ($T = 290 \text{ K}$, relative humidity RH $\sim 45\%$), is displayed in Fig. 1 as a function of momentum transfer q . Four orders of sharp Bragg peaks ($00l$) were recorded with elevated accuracy indicating a high degree of translational order along the normal to the lipid bilayer and a lamellar periodicity $d = 2\pi/q = 48.4 \text{ \AA}$. The EDXD pattern of DOTAP (data not reported) showed very similar features and a lamellar periodicity $d = 47.5 \text{ \AA}$. After closing the chamber windows the

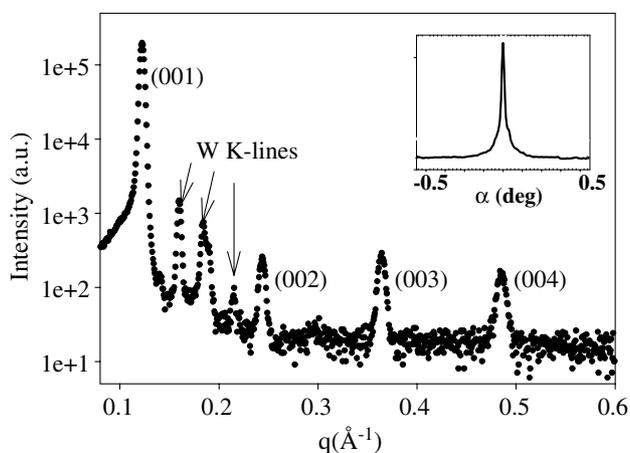


Fig. 1. EDXD pattern of pure DOPC shows four sharp Bragg reflections (00*l*) of a lamellar structure. The W K-lines of the incident radiation, indicated by arrows, are superimposed to the diffraction pattern. A representative rocking scan measured at the first-order Bragg peak proves the very good alignment of the DOPC membranes (mosaicity better than 0.1°).

water adsorption onto the lipid film immediately proceeds. A series of EDXD patterns were collected during the exposure of the lipid film to the water vapour and were automatically stored every 1 s. Time-resolved EDXD patterns representative of the temporal evolution of the first-order Bragg peak of DOPC as a function of increasing hydration are reported in Fig. 2. Upon hydration, adsorbed water molecules result in the continuous swelling of DOPC membranes. The increase of the repeat distance *d* is reflected in the change of the

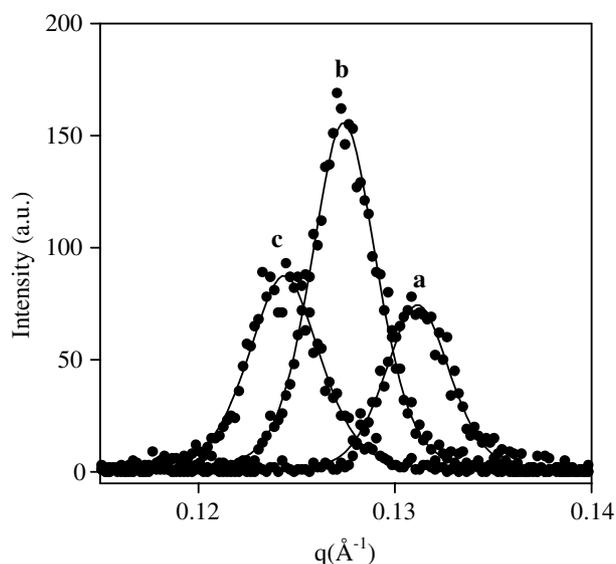


Fig. 2. Representative time-resolved EDXD patterns of DOPC collected upon hydration as a function of time. At *t* = 0 s: (a) the system is in a dry state at the equilibrium with the surrounding environment while at *t* = 174 s, (b) it is in the most ordered state. Finally, at *t* = 800 s the hydration process is complete.

diffraction Bragg peak position which moves to lower *q*-values as a function of time that is as a function of increasing hydration. Although impeded by the hydrophobic chains, water molecules permeate vertically through the lipid film even if the real situation is more complicated with respect to a simple diffusive phenomenon because of additional effects such as the presence of different adsorption coefficients and the lateral transport near defects [20,21]. It is clear that the information content of the EDXD patterns of Fig. 2 is not only restricted to the simple statement of the increasing *d* value, i.e., to the continuous swelling of lipid membranes displayed in Fig. 3. The individual data points were obtained by fitting the first-order Bragg peak of each single diffraction pattern by the following Pseudo-Voigt function (to clarify one data point every 50 s is shown):

$$I(q) = ac \frac{1}{1 + \left(\frac{q-q_0}{b}\right)^2} + a(1-c)e^{-\frac{1}{2}\left(\frac{q-q_0}{b}\right)^2},$$

where *a* and *c* are weight parameters, *b* is the full width at half maximum (FWHM) and *q*₀ is the peak position in the *q*-space.

On the other hand, by careful analysis of the EDXD patterns of Fig. 2, the hydration phenomenon appears to be quite attractive. In fact, while the lipid membranes swell monotonously as shown in Fig. 3, the lamellar signals show an unusual behavior which is common to both the investigated lipid samples. The intensity of the first-order Bragg peak of DOPC and DOTAP as a time function is shown in Fig. 4. As evident, it firstly increases, passes through a marked maximum and then falls off until a plateau is reached.

While there is certainly a consensus that *d* increases as a result of water adsorption, there could remain controversy in assigning the increase of the lamellar signal. Therefore, which is the reason of such an anomalous behavior? To answer this question one should bear in

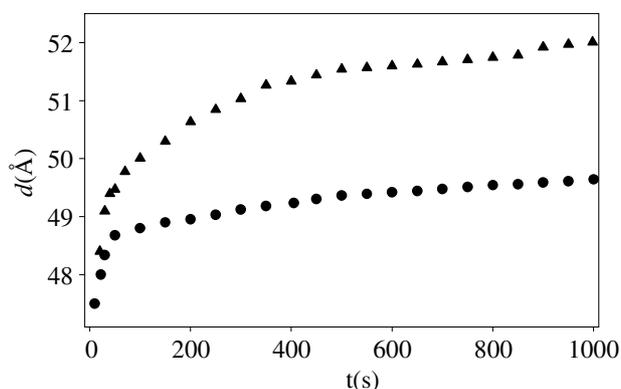


Fig. 3. Temporal evolution of the lamellar repeat distances *d* of DOPC (triangles) and DOTAP (circles) obtained by fitting a Pseudo-Voigt function to the first-order Bragg peak of the EDXD patterns.

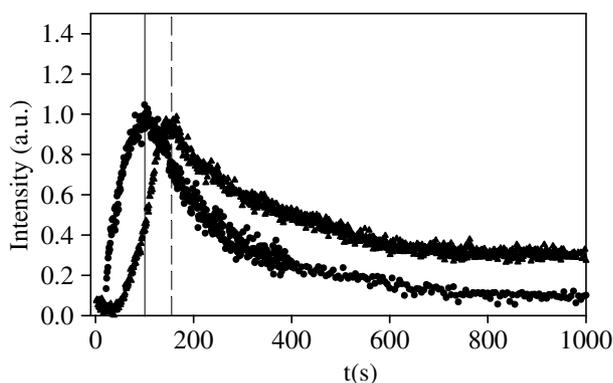


Fig. 4. Temporal evolution of the normalized first-order Bragg peak intensity of DOPC (triangles) and DOTAP (circles). Vertical lines (solid and dashed for DOTAP and DOPC, respectively) are simply guides to the eye separating two distinct hydration regimes: in the first one, water molecules promote a relevant gain of interbilayer spatial coherence whereas, in the second one, behave as bulk water promoting bilayer undulations and reducing the long-range order along the normal to the lipid bilayer.

mind which structural information are related to the scattered intensity of multilayered systems.

Specifically, the scattered intensity of a stack of layers is a quantitative measure of the order along the normal to the plane of the layers [22]. In the case of perfectly flat and equally spaced layers, the resultant Bragg peaks are independent of their order of equal intensity and width. However, in multilayered lipid films, small variations in the bilayer separations usually occur due to lattice defects, bending fluctuations and water-induced bilayer undulations which produce stacking disorder and force the crystalline long-range order to be lost. Thus, as a result of hydration, the resulting scattering distribution shows simultaneous loss in intensity and broadening of the Bragg peaks proportional to the square of the diffraction order [23]. Nevertheless, one can still observe Bragg-peak scattering due to the safeguarded quasi long-range order [24]. For that reason, the temporal evolution of the first-order Bragg peak intensity suggests that the long-range order along the normal to the DOPC and DOTAP lipid bilayer severely depends on the degree of hydration. Indeed, our EDXD data show that, at the lowest water content, the long-range order is minimum probably due to the presence of several lattice defects common in dehydrated structures. Upon hydration, two distinct hydration regimes can be unambiguously discriminated. In the first stage, the water binding to the polar lipid headgroups strongly increases the correlation length of the spatial domains within which the sample scatters coherently. Conversely, in the second stage, after the highest long-range order is reached (i.e., the maxima in the intensity profiles of Fig. 4), intermembrane coherence extends less far. What should be emphasized here is that the results of our swelling measurements provide experimental evidence that water

molecules interacting with lipids can strongly modulate the order of multilamellar lipid systems and that, more remarkably, not all the adsorbed water molecules behave the same. In principle, the first stage of the water adsorption could appear very puzzling since multilayers which incorporate a greater amount of water usually exhibit broader Bragg peaks [23]. On the other hand, for both the lipid samples there is an intermediate structure displaying sharper first-order Bragg peak than that of both the starting and the final conditions. This means that more layers contribute coherently to Bragg diffraction which leads to the notion of a better packing due to less bilayer fluctuations [24]. By this observation, we infer the existence of a strongly ordered intermediate structure at a specific level of hydration for each lipid species, i.e., at a specific interlamellar separation that is $d = 49.8$ and 48.5 Å for DOPC and DOTAP, respectively. Since membrane modulated biological function can depend strongly on small changes in the physical properties of the membrane such an intermediate structure may have, in principle, some biological relevance. Upon further hydration ($t > 1000$ s, data not reported), the d -spacing and the peak intensity indicate that the relaxation process is still going on but water adsorption only results in a continuous swelling of lipid membranes and in a monotonous decrease in peak intensity. All these observations show a correlation between reduced ordering and increased hydration which is sign of the penetration of free water molecules, i.e., bulk waters, between the interbilayer region. When these parameters do not vary anymore the relaxation process is completed. The total relaxation time was about 5000 s for DOPC whereas DOTAP was found to swell indefinitely. This is consistent with the general expectation that there is a net surface charge on DOTAP bilayers responsible of the about unlimited increase in d with increasing water content [25].

From a kinetic point of view, the central importance of long time scale relaxation processes in hydrating and ordering both pure lipid systems and lipid/DNA complexes has been recently highlighted ([26] and references therein).

Our findings are consistent with the main conclusions of the work of Hristova and White [12] who hypothesized a discrete structural change taking place when the DOPC first hydration shell becomes filled just at 12 water molecules per lipid ($d \sim 50$ Å). Nevertheless, since the observed break in the Bragg spacing curve as a function of adsorbed waters also coincided with a change in the method of hydration, there was a small possibility, discussed by the authors, that it was an experimental artefact due to the change of the hydration protocol. Conversely, in our EDXD experiments, only one sample is used and the water adsorption occurs from a water-saturated atmosphere. Our results show the existence of a most ordered structure

at $d \sim 50 \text{ \AA}$ just close to the completion of the filling of the DOPC hydration shell. Anyway, about the origin of the physicochemical mechanism underlying the observed phenomenon further hypotheses can be made. As mentioned above, water molecules close to the bilayer surface have been found to be very ordered and polarized [11]. The interaction between the lipid headgroups in the starting almost dry state is replaced by stronger water-headgroups H bonds between water and lipids. Thus, first adsorbed water molecules could progressively modulate the molecular arrangement of lipids with the headgroups locked into a more ordered and stable structure.

Alternatively, DOPC could undergo a structural rearrangement upon hydration. In a recent publication, Yang et al. [27] showed the observation of a rhombohedral phase in pure DOPC system at reduced level of hydration (RH < 45%). This structure, firstly reported by Luzzati [28,29] and interpreted as an intermediate phase between the lamellar and the hexagonal ones, seems only to appear on the dehydrated state of a lamellar phase. Thus, roughly speaking, the observed increase in intensity could be related to the bilayer electron density rearrangement following the rhombohedral to lamellar phase transition [30].

In principle, all these mechanisms could be responsible for the local gain of electron density observed in the first stage of the investigated hydration kinetics. By comparing the hydration behavior of DOPC and DOTAP, we argue that the number of water molecules interacting with the lipid headgroup is the chemical rationale governing the temporal evolution of the hydration process. Although both the lipids present a similar hydration behavior, dissimilarity in the relaxation times between DOPC and DOTAP are also evident the main difference being a longer time for DOPC to reach the ordered intermediate structure. At the molecular level, both DOPC and DOTAP contain choline groups as well as a glycerol backbone thus their strong water-binding behavior is not surprising [1]. Furthermore, the methylation of the headgroup N^+ , whose importance in the hydration process has been recently elucidated, is also the same. Although it is usually assumed that it is the choline, itself, in the headgroups which imparts the water binding character to the investigated lipids, this turns out not to be exclusively the case. On the contrary, DOPC does contain a phosphate entity in the headgroup and this could account for the DOTAP hydration kinetics appearing faster than that of DOPC. According to the interpretation that distinct structural changes occur upon completion of the first hydration shell, this effect could be rationalized by arguing that, due to the presence of a phosphate entity, the volume around the headgroup of DOPC available to water molecules is larger than that of DOTAP so that the time required to fill the hydration shell could be a bit larger.

4. Conclusions

Our structural results have shown that the long-range order in multilamellar lipid systems strictly depends on the hydration level but in a nonmonotonic way. First adsorbed water molecules promote the gain of spatial coherence along the normal to the lipid bilayer. At a distinct value of the interlamellar separation, a very locked packing can be inferred from the fact that more layers contribute coherently to Bragg diffraction and the sharpest Bragg peaks are detected in the diffraction pattern. After a maximum spatial coherence is reached, water molecules penetrate into the interbilayer region, behave as bulk water and produce second order disorder. According to the most accredited evidences reported in the literature, both theoretical and experimental, the initial gain and subsequent loss of long-range order could be interpreted in terms of the ordering effect of the highly polarized water molecules adsorbed close to the bilayer surface or, alternatively, to the completion of the lipid hydration shell. These findings confirm the existence of a correlation between the degree of hydration of lipid bilayers and the structure of interbilayer water as recently proposed [1,6–9]. Differences in the hydration behavior of DOPC and DOTAP have been ascribed to the presence of a phosphate entity in the DOPC headgroup which enlarges the volume accessible to water. Lastly, the present study have confirmed the ability of in situ time-resolved EDXD to provide a detailed evolution of the bilayer structure as a function of hydration in the temporal range of the seconds.

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