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A new experimental setup for the study of lipid hydration by energy dispersive X-ray diffraction

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Abstract

A new hydration cell has been constructed that allows to fully hydrate aligned lipid multibilayers as well as to study lipid hydration by using in situ time-resolved energy dispersive X-ray diffraction. The cell has been used to investigate the evolution of the lamellar *d*-spacing of dioleoylphosphocoline oriented membranes as a function of both relative humidity (RH) and time. The extreme sensitivity of *d* to RH was shown and two hydration regimes were detected.

1. Introduction

Lipid bilayers have been much studied during the last decades as model systems of biological membranes. Major focuses of such studies are the structural characterization of lipid bilayers, lipid hydration and the interaction between bilayers [1–5]. Some of the importance of lipid hydration comes from the postulated 'hydration force' arising from the water associated with the lipid headgroups [6 and references therein]. For scattering purposes, two distinct measurement conditions exist: liposomes and aligned multibilayers (Fig. 1). Liposomes consist of concentric multilamellar vesicles of bilayers with an average repeat spacing of about 60 Å arising from the sum of the membrane thickness plus a water layer. Aligned multibilayers are solid-supported multilamellar arrays of lipid bilayers oriented with their normals aligned along one axis perpendicular to the solid support.

In the case of diffraction, liposomes can be regarded as a set of independent stacks of parallel bilayers isotropically distributed in space. As a result, such powder-like samples diffract weakly and higher-order diffraction peaks are not usually observable. For the purpose of investigating structure, there are therefore advantages to studying oriented samples. In particular, aligned samples allow for the clear differentiation between in-plane (organization of acyl chains) and out-of-plane (lamellar *d*-spacing) correlations.

Nevertheless, there has been concern in studying aligned systems due to many reports of samples hydrated from water vapor exhibiting *d*-spacings much less than their equivalent liposomes immersed in water [7,8]. Since the chemical potential of water vapor in equilibrium with bulk water is the same, a question spontaneously arose about the physical reasons underlying such an anomalous discrepancy: why should the *d*-spacing be different? As such the so-called 'vapor pressure paradox' appeared to be well established [9] and theories were proposed to explain the paradox [10,11]. The 'vapor pressure paradox' persisted until recently, when Katsaras [12,13] demonstrated that the paradox was an experimental artifact resulting from large temperature gradients inside hydration chambers. Technical deficiencies present in previous cells impeded to achieve relative humidity (RH) better than 0.99 until Katsaras [14] designed a sample cell capable of RH = 1 suitable for X-ray diffraction of aligned lipid multibilayers. Taking advantage of this, oriented systems at 100% RH exhibited, for the first time, the same physical characteristics as

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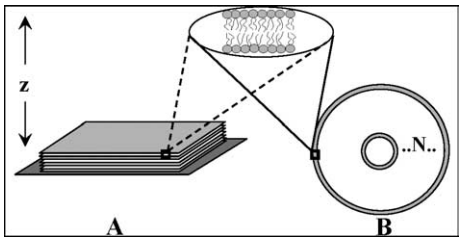

Fig. 1. Two types of samples for X-ray diffraction: (A) solid-supported oriented samples with the normals to the lipid bilayers aligned along one direction (z-direction); (B) unoriented powder-like liposomes made of N concentric lipid vesicles. In the zoom, the lipid bilayer is shown. Despite different geometries, physical properties of A and B samples are the same [12].

liposomes in solution [12–14]. Nowadays, achieving full hydration means obtaining the same d-spacings of liposomes immersed in liquid water [12].

In addition to structural studies, time-dependent hydration phenomena are emerging as important issues in many fields such as food and drug research. Furthermore, the uptake of water by lipid films is of special interest for using lipid films in membrane applications [15]. Unfortunately, despite several hypotheses, there is no straightforward interpretation of lipid hydration kinetics so far [16]. In this Letter, we present an experimental setup that allows for in situ time-resolved energy dispersive X-ray diffraction (EDXD) experiments with a precise control of RH and temperature. Although the basic principles are not new, experimental setup presented here allows to collect diffraction patterns both as a function of RH and time. It has been applied to study the hydration of solid-supported dioleoylphosphocoline (DOPC) oriented membranes. Because of its high sensitivity and the time resolution of the order of seconds, the method is expected to provide new insights into both the structural and kinetic aspects of lipid hydration.

2. Experimental setup

2.1. Hydration cell

The sample chamber is essential for studying fully hydrated oriented samples with X-rays [12]. Fundamental inspiration to designing the hydration cell arose from the outstanding research of Katsaras [12–14] and the Nagle's group [17–19]. The experimental setup was arranged according to the following principles: achieving full hydration, minimization of temperature gradients, having a water's effective evaporation area and a 'vapor volume' suitable to perform both structural and kinetic studies by EDXD. The sample cell shown in Fig. 2 is the final result of several previous attempts. It was entirely constructed from aluminum to minimize temperature gradients [13]. Water's evaporation area could vary between 0.5 and $\sim 10\,\mathrm{cm}^2$ while 'vapor volume' could be adjusted between 0.5 and $\sim 40\,\mathrm{cm}^3$. It has one entrance and one exit mylar windows whose surface (2.5×0.6 cm) was minimized in order to avoid water vapor condensation [19]. Oriented

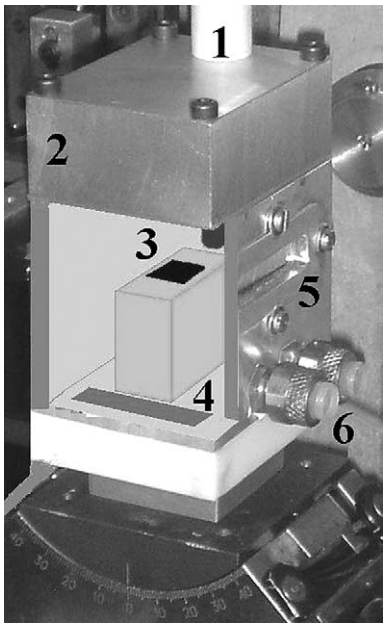

Fig. 2. Photograph of the hydration chamber: humidity controller (1); removable top (2); solid-supported lipid film (3); water reservoir on the bottom of the cell (4); mylar entrance window (5); refrigerator water circulator (6). The hydration chamber is mounted on a rotation motor that allows to align the membranes respect to the incident X-ray beam.

samples can be hydrated from water vapor in equilibrium with a reservoir of bulk water in the bottom of the sample cell. Attention is paid to use pure water because contaminants are known to lower the water vapor pressure [19]. RH is monitored by a humidity controller (Hygropalm, Rotronic ag, Germany). It has been recently shown that small temperature gradients (>0.008 °C) between the sample and the water vapor inside the cell are necessary to achieve full hydration [19]. Hydration shortfalls are usually overcome by mounting the sample on a Peltier element that cools the sample relative to the temperature of the water vapor [19] and raises the effective RH at the sample.

As explained by Chu et al. [19], this cooling procedure is potentially dangerous the main concern being condensation of liquid water at the solid/air interface of the sample thus reducing the intensity of diffraction remarkably. In the case of EDXD experiments, this drawback may be really dramatic. Indeed, the longer the wavelengths of the polychromatic incident X-ray radiation, the higher the absorption. In principle, EDXD patterns could become unusable.

The experimental approach presented here is quite different. The temperature of the cell, the sample and the water reservoir in the bottom of the cell are exactly the same (T_c) and are controlled via a refrigerator water circulator with accuracy of ±0.1 °C. Conversely, the temperature of the water vapor (T_v) is regulated by a system of lamps, the main effect being a slight increase of T_v with respect to T_c ($\Delta T = T_v - T_c \sim 0.1$ °C). Calling $P_v(T_v)$ the vapor pressure exerted by the gaseous particles trapped inside the cell and $P_{sat}(T_c)$ the saturated water pressure at T_c the RH measured by the humidity controller is $(\mathrm{RH})_{cell} =$

$P_v(T_v)/P_{sat}(T_v)$, whereas one can define the relative humidity at the sample as $(RH)_{sample} = P_v(T_v)/P_{sat}(T_c)$. So, heating the temperature of the water vapor with respect to T_c raises P_v and the effective RH at the sample.

2.2. Samples preparation

Multilayered stacks of highly aligned DOPC membranes were prepared by depositing 0.5 mg of lipid onto the oriented surface of freshly cleaved ⟨1 0 0⟩ silicon wafers (area ~1.5 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 [12]. DOPC was 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 was transferred to the sample chamber.

2.3. EDXD experiments

X-ray diffraction experiments were carried out by using an EDXD apparatus elsewhere described [20]. 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 $\Delta\theta = 0.001°$ and it directly affects the uncertainty Δq associated to the transfer momentum q ($q = \cos t \times E \times \sin\theta$; $\cos t = 1.01354$ Å^{-1} keV^{-1}). In contrast to traditional angular dispersive X-ray diffraction, energy dispersive X-ray diffraction (EDXD) permits the simultaneous acquisition of the spectrum points in the investigated region of the reciprocal q-space. This peculiar characteristic of the EDXD technique allows to perform kinetic studies. Biological samples are not damaged by EDXD experiments as elsewhere discussed [21].

3. Results and discussion

The purpose of our experiments was fully hydrating oriented lipid mulibilayers as well as measuring the lamellar d-spacing both as a function of RH and time. To this end, we examined oriented DOPC multibilayers hydrated through vapor over the hydration range of 0.5–1 RH at constant temperature $T = 27$ °C. In this region of the phase diagram, DOPC membranes are in the liquid-crystalline L_α phase where the hydrocarbonic chains are melted [22].

Accordingly, the EDXD pattern of multilayered DOPC film collected at RH = 0.5 (not reported) exhibited five orders of sharp Bragg peaks (0 0 l) indicating a high degree of translational order along the normal to the lipid bilayer and a lamellar periodicity $d = 2\pi/q = 42.2$ Å.

Closing the removable top, water molecules continued to break away from the water reservoir on the bottom of the cell and started to fill the water vapor's volume. As a result, P_v, i.e. RH monotonously increased. Upon hydration, both the diffraction intensity and the RH were registered and automatically stored every 1 s. Fig. 3 shows RH as a function of time. Intriguingly, we were able to monitor changes in RH as a function of time that was well fitted by the following function:

$$RH = RH_0 + \frac{a}{(1+\frac{t}{\tau})^b}, \tag{1}$$

where RH_0 is $RH(t=0)$, τ is the time constant and a and b are fitting parameters ($a = 0.51$; $b = -0.77$). For $t = \tau = 162$ s $RH(t=\tau) = 0.73$. At the equilibrium, the water activity of sorbed water equaled that of the vapor atmosphere in the sample chamber, i.e. the number of molecules leaving the liquid surface was exactly balanced by the number of molecules rejoining it. The change in RH initiated the adsorption of water onto the sample and the lipid did spontaneously swell. Fig. 4a shows the Bragg d-spacing of DOPC bilayers as a function of RH (one data point each 0.001 of RH). X-ray studies usually report d as function of osmotic pressure (P_{osm}) [19] which is related to RH by the following definition:

$$P_{osm} = -(kT/V_w)\ln(RH), \tag{2}$$

where T is the temperature, k is the Boltzmann constant and $V_w = 30$ Å^3 is the volume of a water molecule. Different hydration levels are usually produced using the technique of applying osmotic pressure by adding the immiscible polymer polyvinylpyrrolidone (PVP) to bulk water. By varying concentration of PVP the P_{osm} can be changed according to the procedure of McIntosh and Simon [23]. P_{osm} values are then turned into RH by applying Eq. (2). Nevertheless, experimental uncertainties can be large essentially due to the difficulty of preparing small samples with precise polymer concentrations. We claim the relevance of the results of Fig. 4 because a single sample was used and the RH was not calculated but measured directly. At each RH value, a diffraction pattern was collected and a lamellar d-spacing was calculated from the

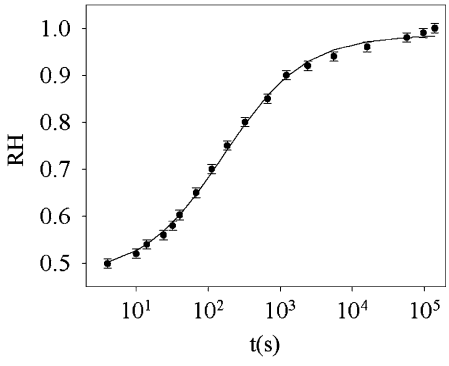

Fig. 3. Temporal evolution of measured RH. Solid line is the best fit to the data obtained applying Eq. (1).

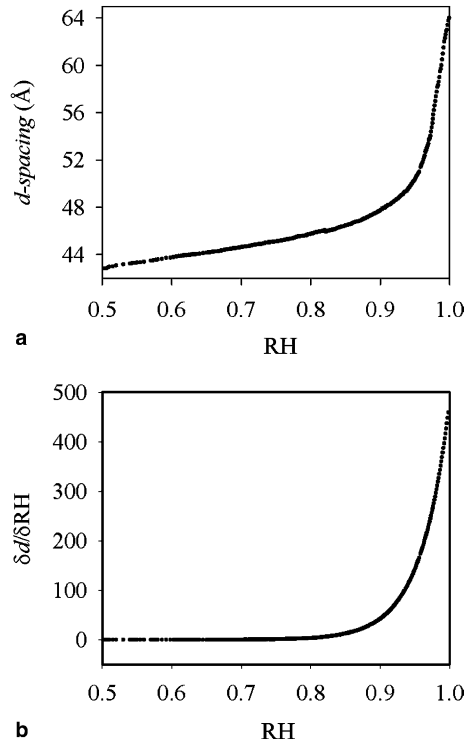

Fig. 4. Evolution of the lamellar d-spacing as a function of RH (a) and its first derivative, $d' = \delta d/\delta \mathrm{RH}$ (b), that allows to detect two distinct hydration regimes. In the first one ($0.5 < \mathrm{RH} < 0.9$), d linearly increases and the d' is nearly constant. In the second one ($\mathrm{RH} > 0.9$), an abrupt increase of d' can be observed. This profile shows the extreme sensitivity of d to RH in the humidity range ($0.9 < \mathrm{RH} < 0.1$).

position of the first-order Bragg peak (q_{001}) by applying the Bragg's law ($d = 2\pi/q_{001}$). The extreme sensitivity of d to RH provided by the method was evident (Fig. 4a). The hydration behavior was better elucidated by the first derivative $d' = \delta d/\delta \mathrm{RH}$ (Fig. 4b). Two hydration regimes were distinguished. In the first range ($0.5 < \mathrm{RH} < 0.9$), the increment of d, i.e. its first derivative, was essentially constant whereas in the second one ($\mathrm{RH} > 0.9$) a marked increase could be observed. At $(\mathrm{RH})_{\mathrm{cell}} = 0.990$ the calculated lamellar spacing was $d \sim 58.5$ Å whereas, at $(\mathrm{RH})_{\mathrm{cell}} = 0.999$, the DOPC membranes were fully hydrated as revealed by obtaining a lamellar spacing $d = 63.5$ Å [24].

The findings of Fig. 4 are in excellent agreement with those recently reported by Jendrasiak and Smith [1] who studied the hydration of a variety of phospholipids. In their work, the number of adsorbed water molecules per lipid molecule was estimated gravimetrically. According to our structural findings, the adsorption isotherm of DOPC showed two hydration regimes: in the first one the number of adsorbed water molecules increases linearly as a function of RH (up to $\mathrm{RH} \sim 0.85$) whereas, in the second one, an exponential increase occurs. Thus, the amount of water available to the lipid determines in large measure the hydration process.

The number of water molecules per lipid n_{w} at full hydration can be estimated using [25]

$$n_{\mathrm{w}} = [(Ad/2) - V_{\mathrm{L}}]/V_{\mathrm{w}}, \tag{3}$$

where A is the area/DOPC molecule, d is the lamellar spacing, V_{L} is the volume/DOPC molecule and V_{w} is the volume of a water molecule ($\sim$30 Å^3). By inserting the values reported by Tristram-Nagle et al. [25] ($A = 72.2$ Å^2; $V_{\mathrm{L}} = 1303$ Å^3) into Eq. (3), $n_{\mathrm{w}} = 33$ was calculated. Although this is a rough estimation, it is in good agreement with that reported by Tristram-Nagle et al. [25], $n_{\mathrm{w}} = 32.5$.

Thus, the designed hydration cell allowed us to obtain full hydration. This finding confirms the general expectation that very small humidity gradients ($\Delta\mathrm{RH} \sim 0.001$) are responsible for aligned multibilayers exhibiting smaller d-spacings. Furthermore, it means that $(\mathrm{RH})_{\mathrm{sample}} = P_{\mathrm{v}}(T_{\mathrm{v}})/P_{\mathrm{sat}}(T_{\mathrm{c}}) = 1$, when $(\mathrm{RH})_{\mathrm{cell}} = P_{\mathrm{v}}(T_{\mathrm{v}})/P_{\mathrm{sat}}(T_{\mathrm{v}}) = 0.999$. When $(\mathrm{RH})_{\mathrm{cell}} = 1$ water condensation at the solid/air interface of the lipid film occurred and higher-order diffraction peaks were removed from the EDXD pattern. Recently, Katsaras [12] proposed to monitor water condensation at the solid/air interface of multilayered lipid films through the intensity of the Bragg reflections. As the sample was hydrating, he observed that the intensity of the first-order Bragg peak increased until the lamellar d-spacing reached its limiting value. At full hydration, decreased Bragg intensity was indicative of water condensation on the sample. Although we agree that the intensity of first-order Bragg peak could provide useful information, its evolution as a function of hydration seemed to be more complicated. We have recently reported on the hydration kinetics of oriented DOPC membranes [26]. We observed that the intensity of the first-order Bragg peak firstly increased, passed through a maximum and then fell off until a plateau was reached. Nevertheless, the maximum in the intensity was not obtained at $\mathrm{RH} = 1$, as Katsaras observed, but a reduced level of hydration ($\mathrm{RH} \sim 0.7$). Upon further hydration ($0.7 < \mathrm{RH} < 1$), water adsorption resulted in a continuous swelling of lipid membranes and in a monotonous decrease in peak intensity. Thus, we believe that the decrease in Bragg intensity cannot be assumed as an indicator of when condensate forms. Once the full d-spacing was achieved, signs of water condensation were visually observed through the mylar windows. At that time, disappearance of higher-order Bragg reflections together with simultaneous increase of inter-peaks background in the diffraction pattern were detected. Both these facts were due to the scattering of excess liquid water condensed at the lipid/air interface and were considered indicative of water condensation.

In Fig. 5, the lamellar repeat distance as a function of time is shown. The d-spacing was well fitted by the following double exponential model:

$$d(t) = d_0 + d_1 \times (1 - \mathrm{e}^{t/\tau_1}) + d_2 \times (1 - \mathrm{e}^{t/\tau_2}), \tag{4}$$

where d_0 is the starting d-spacing at $t = 0$ ($\mathrm{RH} = 0.5$). Two relaxation processes were detected: one faster ($\tau_1 = 728$ s) which produces a slight increase in d-spacing ($d_1 = 4.4$ Å) and a slower process ($\tau_2 = 75796$ s) responsible for the ma-

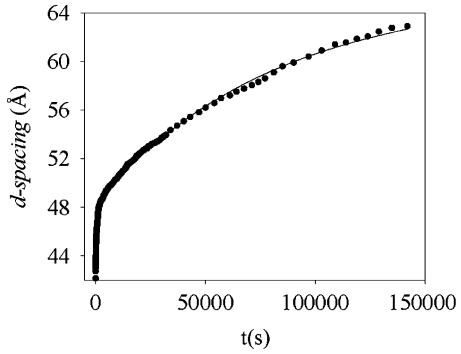

Fig. 5. Temporal evolution of the lamellar d-spacing. Solid line is the best fit to the data obtained applying Eq. (4).

jor part of the lipid swelling ($d_2 \sim 17$ Å). Equilibration times for obtaining final d-spacing of full hydration took up to 150 000 s. Here, we emphasize that adjusting the water's evaporation area and the 'vapor volume' allowed us to shorten this time remarkably.

4. Conclusion

Although the basic principles are not new [12–14], we claim that the experimental approach presented here is superior in three respects: (i) achieving full hydration, i.e. $(\mathrm{RH})_{\mathrm{sample}} = 1$, without using potentially dangerous Peltier currents; (ii) achieving full hydration when $(\mathrm{RH})_{\mathrm{cell}} = 0.999$ thus avoiding water condensation at the interface of lipid multilayer; (iii) a precise control of RH inside the cell together with the unique characteristics of in situ time-resolved EDXD allows to obtain simultaneous structural and kinetic information on lipid hydration.

Although biologically relevant full hydration is the most common measurement condition, lipid bilayers at reduced levels of hydration are of biophysical interest for investigating interactions between bilayers, phenomena of anomalous swelling and the role of the first layer of water molecules on the structure of lipid membranes. To this end, collecting high-resolution EDXD patterns at distinct hydration levels (i.e. at distinct RH) could provide new insights into the relation between hydration and structural changes of lipid bilayers at the molecular level.

Nowadays, accurate differences of relative humidities from 1 are difficult to measure by commercial humidity meters when RH is close to 1. Thus, when the relation between d-spacing and RH will be known accurately, lipid bilayer itself may be used as a very precise humidity meter.

References

[1] G.L. Jendrasiak, R.L. Smith, Cell. Mol. Biol. Lett. 5 (1) (2000) 35.
[2] J.N. Israelachvili, H. Wennerstrom, J. Phys. Chem. 96 (1992) 520.
[3] J.N. Israelachvili, H. Wennerstrom, Nature (Lond.) 379 (1996) 219.
[4] H. Wennerstrom, E. Sparr, Pure Appl. Chem. 75 (2003) 905.
[5] K. Hristova, S.H. White, Biophys. J. 74 (1998) 2419.
[6] S. Leikin, V.A. Parsegian, D.C. Rau, R.P. Rand, Annu. Rev. Phys. Chem. 44 (1993) 369.
[7] E.B. Sirota, G.S. Smith, C.R. Safinya, R.J. Plano, N.A. Clark, Science 242 (1998) 1406.
[8] J. Torbet, M.H.F. Wilkins, J. Theor. Biol. 62 (1976) 447.
[9] R.P. Rand, V.A. Parsegian, Biochim. Biophys. Acta 988 (1989) 351.
[10] R. Podgornik, V.A. Parsegian, Biophys. J. 72 (1997) 942.
[11] V.A. Parsegian, R. Podgornik, Colloid. Surf. A 129–130 (1997) 345.
[12] J. Katsaras, Biophys. J. 75 (1998) 2157.
[13] J. Katsaras, M.J. Watson, Rev. Sci. Instrum. 71 (4) (2000) 1737.
[14] J.F. Nagle, J. Katsaras, Phys. Rev. E 59 (6) (1999) 7018.
[15] H. Binder, B. Kohlstrunk, H.H. Heerklotz, Chem. Phys. Lett. 304 (1999) 329.
[16] H. Binder, B. Kohlstrunk, W. Pohle, J. Phys. Chem. B 104 (2000) 12049.
[17] Y. Lyatskaya, Y. Liu, S. Tristram-Nagle, J. Katsaras, J.F. Nagle, Phys. Rev. E 63 (2000) 011907.
[18] S. Tristram-Nagle, J.F. Nagle, Chem. Phys. Lipids 127 (2004) 3.
[19] N. Chu, N. Kučerka, Y. Liu, S. Tristram-Nagle, J.F. Nagle, Phys. Rev. E 71 (2005) 041904.
[20] R. Caminiti, V. Rossi Alberini, Int. Rev. Phys. Chem. 18 (2) (1999) 263.
[21] G. Caracciolo, G. Amiconi, L. Bencivenni, G. Boumis, R. Caminiti, E. Finocchiaro, B. Maras, C. Paolinelli, A. Congiu Castellano, Eur. Biophys. J. 30 (2001) 163.
[22] L. Yang, L. Ding, H.W. Huang, Biochemistry 42 (22) (2003) 6631.
[23] T.J. McIntosh, S.A. Simon, Biochemistry 32 (1993) 8374.
[24] G. Caracciolo, C. Sadun, R. Caminiti, M. Pisani, P. Bruni, O. Francescangeli, Chem. Phys. Lett. 397 (2004) 138.
[25] S. Tristram-Nagle, H.I. Petrache, J.F. Nagle, Biophys. J. 75 (1998) 917.
[26] R. Caminiti, G. Caracciolo, M. Pisani, P. Bruni, Chem. Phys. Lett. 409 (2005) 331.