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Influence of gramicidin on the dynamics of DMPC studied by incoherent elastic neutron scattering

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Abstract

By using the fixed energy window method in incoherent elastic neutron scattering, molecular motions in the 150 ps timescale in highly oriented multilayers of 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) membranes in excess of water (D_2O) have been studied as a function of temperature, in the range from 27 to 325 K. The same system in partially deuterated form and with the addition of a pore-forming peptide (gramicidin) has also been investigated. By proper orientation of the membrane plane with respect to the scattering wavevector Q, information on in plane and out of plane motions of lipid membranes have been derived. Two relevant dynamical transitions were observed at $T = 297$ K and at $T = 270$ K. The former is related to the structural main transition from gel to liquid phase of the phospholipid bilayer, while the latter is related to a transition of the aqueous solvent. The inclusion of gramicidin shifts the main transition down to 294 K and the second transition up to 276 K. In both cases the observed dynamical transitions show an enhanced mobility in the direction normal to the membrane plane.

1. Introduction

Cell membrane is one of the most important constituents of living organisms: it provides a partially permeable barrier consisting mostly of lipids and proteins that regulate the flux of material in and out of the cell [1]. Membrane lipids are amphiphilic molecules with a hydrophobic tail and a hydrophilic head group. Due to this feature, in aqueous environment they self-assemble in the form of continuous bilayer structures with the heads interacting with the water molecules on the surfaces and the tails interacting with each other in the interior. The bilayers are therefore surrounded by water and their structure acts as a barrier to water soluble molecules and provides a framework for incorporating membrane proteins which perform many important physiological functions for the cell activity.

The interactions between proteins, peptides and membrane lipids are of fundamental importance to the structure and function of all biological membranes. Indeed, the secondary structure and the activity of many integral transmembrane proteins are modulated by the physical properties of the host lipid bilayer [2–4]. Because of the complexity of the real cell membrane, the properties of this system are studied in biomimetic model systems mainly realized by phospholipids which form lamellar structures with characteristics very similar to the ones of natural membranes. Phospholipids bilayers exhibit lyotropic liquid crystalline properties and present a variety of phases as temperature is changed [5]. Going from low to high temperatures, they pass from a crystal/subgel phase, with highly ordered tails and head groups, to a gel phase (L_β) which shows little disorder in the tail region and a number of different head group conformations; then a fluid phase (L_α) is approached which shows a liquid-like disorder throughout the whole system. In this phase the bilayer is a two-dimensional fluid, meaning that the phospholipids are free to move in the plane of the bilayer. The hydrocarbon chains are highly disordered and

therefore the transition to the (L_α) phase is regarded as the melting of the bilayer with a characteristic temperature T_m.

Just below T_m, a ripple phase (P_β) is found in which the lamellae structure is deformed by periodic modulation. The transition from gel to ripple phase is referred to as pre-transition and the transition to liquid phase is called main transition.

The pre- and main transition temperatures decrease with increasing head group hydration and unsaturation of the acyl chains. Pure phospholipid bilayers show very sharp phase transitions taking place over a temperature range of 0.8–1.5 °C. However, this temperature range is broadened in the presence of cholesterol, small molecules and oligo- or polypeptides interacting with the bilayer. These molecules also influence the structure of the low temperature phases of the bilayer.

A knowledge of the behaviour of lipid bilayers is therefore of great importance for understanding the functioning of a cell. Moreover there is a novel and growing interest in membrane structure and dynamics due to the possibility of making pores on the surface [6]. Pore-forming proteins and peptides comprise a class of molecules ranging from small cationic peptides to large ion channel proteins having the ability to create a pathway for molecules that cannot normally cross lipid bilayers. From a biotechnological point of view the ability to create a selective and regulated pathway into a cell is a task of great interest for biotherapeutic and biosensor applications. Transport and delivery systems, antibiotics, biosensors, cell permeabilization and killing molecules are some of the potential applications.

In this paper we report an incoherent elastic neutron scattering study on the interactions between a model membrane and a pore-forming peptide, in conditions of excess of water (D_2O). Multibilayer membranes of phospholipids have been the object of several investigations using neutron scattering, mostly from a structural point of view [7–9] but also from a dynamical one [10–12].

The membrane system is realized by highly oriented, hydrated phospholipid bilayer stacks of DMPC (1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine) in the hydrogenated form and in the partly deuterated form DMPC-d54, where 54 out of 72 hydrogens (in the acyl chain) are substituted by deuteria. The peptide inserted into the membrane is gramicidin D, a well known pore-forming 15-residue oligopeptide showing antimicrobial activity [13]. DMPC exhibits clear transitions between gel L_β, rippled gel P_β and liquid crystalline L_α phases in water when the lipid sample is pure [14] and it has been widely used as a biomimetic membrane [15] also in neutron scattering studies [8, 10]. The pre-transition for a pure DMPC occurs at 14 °C, while the main transition is found at 24 °C [16].

The membranes used in this experiment were supported on mica but a comparison of the same system supported on silicon was also performed, in a limited range of temperatures.

2. Experimental details

2.1. Sample preparation

DMPC and DMPC-d54 have been purchased from Avanti Polar Lipids; gramicidin (bacillus brevis) was purchased from Fluka and consists of a mixture of A, B and C species. Aligned multilayers of DMPC membrane and 40:1 lipid/peptide DMPC with gramicidin (DMPC/G) are obtained following the preparation suggested by Hallock *et al* [17], using as supporting substrate both Si and mica plates. Each substrate was covered with about 1.5 mg of lipids per cm^2. Following the cited procedure, the lipids and peptides were dissolved in an excess of 2:1 $CHCl_3/CH_3OH$ (chloroform/methanol). The lipid–peptide solution was dried and then for each mg of lipids it was redissolved in 50 μl 2:1 $CHCl_3/CH_3OH$ containing a 1:1 molar ratio of naphthalene to lipid–peptide. The solution was then spread and dried on both the faces of the substrate plates. To remove the naphthalene and any residual organic solvent, the samples were vacuum dried overnight. The samples were indirectly hydrated at 40 °C in 96% relative humidity using a saturated potassium sulfate D_2O solution for 12 days, after which 28 mole of D_2O per mole of lipid–peptide were added. The plates were stacked and equilibrated at 4 °C for 12 additional days. The alignment was then verified by ^{31}P-NMR chemical shift [18] and resulted in more than 70% of aligned lipids.

High quality mica: V-1 grade Muscovite, was purchased from SPI Chem., in rectangular sheets of size 45×25 mm^2 and thickness of 150 μm. The samples were prepared by stacking six plates spaced by 100 μm aluminium spacers on the edges for a total surface of about 100 cm^2, excluding the outer faces. The final sample thickness was about 2.5 mm. Silicon wafer was kindly provided from ST-microelectronics, shaped in a rectangular sheet of 43×25 mm^2 and thickness of 250 μm. The sample preparation was similar to that for mica but only five plates were used to achieve the same final thickness. All the samples were prepared in order to have an overall transmission of about 92%.

2.2. Spectrometer

The IN13 backscattering spectrometer at the Institut Laue Langevin facility was used to perform incoherent elastic neutron scattering scans, with a transferred momentum ranging from $Q = 0.4$ to 4.7 $Å^{-1}$, corresponding to an explored spatial region ranging from 30 to 2 Å. The instrumental resolution is of $E = 8$ μeV (full width at half maximum), which matches the energy width of dynamical processes with a decay time of 150 ps. Consequently only dynamics faster than that value can be ascertained. The explored temperatures range from 27 to 325 K with a precision of ± 1 K and an acquisition time of about one hour per temperature. In the region of the morphologic transitions, experimental data have been acquired with a step of 5 °C. At high temperatures the absence of hysteresis effect was checked by performing a few up and down temperature cycles. By using the software package ELASCAN available at ILL, the data were corrected to take account of incident flux, cell scattering, self-shielding and detector response, and normalized to the sample at the lowest temperature $T = 27$ K.

Considering the rather high exchange momentum, nearly all of the coherent scattering can be regarded as effectively incoherent. To a good approximation, therefore, the behaviour

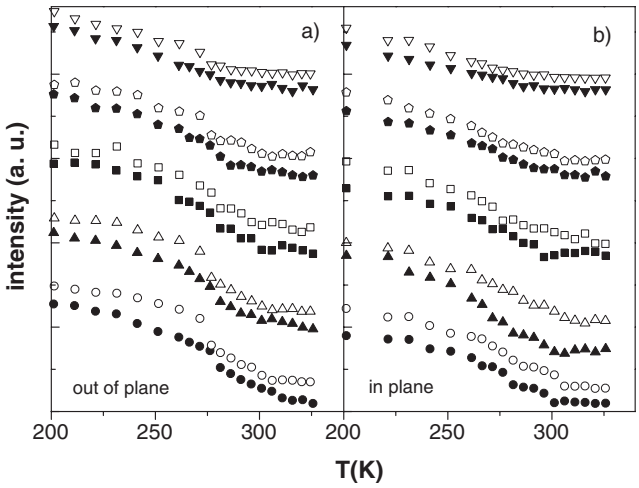

Figure 1. Temperature dependence of FEW scans for the most representative Q values for pure DMPC (empty symbols) and DMPC/G (full symbols) for (a) the out of plane and (b) in plane configurations: triangle-down for $Q = 4.14$ Å^{-1}, pentagon $Q = 2.65$ Å^{-1}, square for $Q = 2.06$ Å^{-1}, triangle-up for $Q = 1.77$ Å^{-1} and circle for $Q = 1.47$ Å^{-1}. The curves are arbitrarily shifted along the intensity axis for greater clarity.

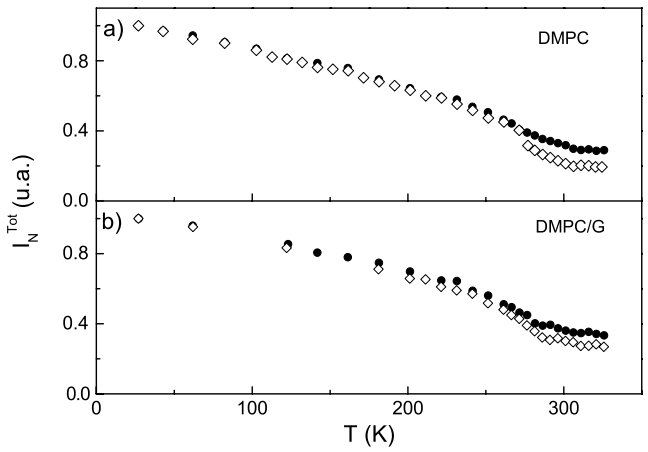

Figure 2. Temperature dependence of FEW scans integrated over all the Q-range and normalized to the lowest temperature for both in plane (full symbols) and out of plane (empty symbols) components of DMPC (a) and DMPC/G (b).

of the intensities integrated over a fixed energy window centred at zero energy (fixed energy windows (FEW) method) will reflect the single-particle dynamics of the proton and deuteron populations in the samples. For each measured sample the scattered intensity was collected at two different orientations: 45° and 135° with respect to the incident beam in order to maximize the contribution of the out of plane and in plane dynamics respectively.

3. Experimental results

In figure 1 FEW scans for some detectors in the temperature range of 200–325 K for DMPC and DMPC/G are shown. The two different experimental arrangements, corresponding to in plane and out of plane, are reported in the right (b) and the left (a) sides of the figure 1, respectively. Although the IN13 spectrometer covers an extended Q range from 0.4 to 4.7 Å^{-1} by means of 32 detectors, we have expressly chosen to plot those detectors with Q values below 2.27 Å^{-1} that are particularly significant for the correlation length of samples investigated, taking care of summing neighbouring detectors to reduce statistical uncertainty. Moreover, FEW scans at higher values of Q have also been shown to evidence the dynamics on smaller correlation lengths.

A distinct dip at $T = 297$ K appears in the in plane component at $Q = 1.47$ Å^{-1}. The inclusion of gramicidin leads to a shift of this transition from 297 to 294 K. Analogously with findings of Rheinstädter *et al* [10] the dip at 297 K is strongly reduced (or absent) in the out of plane components (see figure 1(a)), revealing an anisotropic scattering. Another relevant drop in the temperature dependence of the neutron intensity of both in plane and out of plane components of DMPC is observed at $T = 270$ K, although it is more evident and sharp in the out of plane setup. Its presence does not show a notable dependence on a particular Q value in the full investigated range. The addition of gramicidin shifts the related transition up to $T = 276$ K.

In figure 2(a) the temperature dependence of FEW scans integrated over all Q-ranges and normalized to the intensity at the lowest temperature (I_N^{Tot}) for both in and out of plane components of DMPC is reported in order to highlight anisotropic effects. A similar plot was drawn for the DMPC/G sample and is shown in figure 2(b). This representation offers a general view of molecular mobilities of the investigated lipids. A decrease in the intensity measured in the FEW scans corresponds to a higher facility to move upon unfreezing and vice versa. We observe that in both investigated samples the decrease in the intensity in the out of plane orientation is more enhanced than in plane one. The differences are more prominent above 270 K.

In figures 3(a) and (b) the I_N^{Tot} of DMPC, DMPC/G, DMPC-d54/G in the temperature range affected by the transitions ($T > 150$ K) in both orientations are compared in order to evidence the effect of gramicidin and of isotopic substitution on membrane dynamics. We can observe that the dynamics of the membrane is hindered by the inclusion of gramicidin and that this hindrance is more enhanced in the out of plane direction. It has been observed that the influence of gramicidin on the in plane dynamics is already evident even below the freezing at 270 K. A measurement performed on DMPC-d54/G sample in the in plane configuration (shown in figure 3(a)) has revealed a FEW intensity further increased.

In this experiment we have chosen to use mica as the substrate for the membrane mainly because it is much more suitable for the ^{31}P-NMR chemical shift measurements that we had to perform to check the correct membrane alignment. Mica is also easier to manipulate and it is widely used as a substrate for phospholipids and other biological compounds. Although the measured signal of the bare mica sample holder was found to be higher than the one from Si by a factor of eight, it is still small in comparison with the signal of our samples and it does

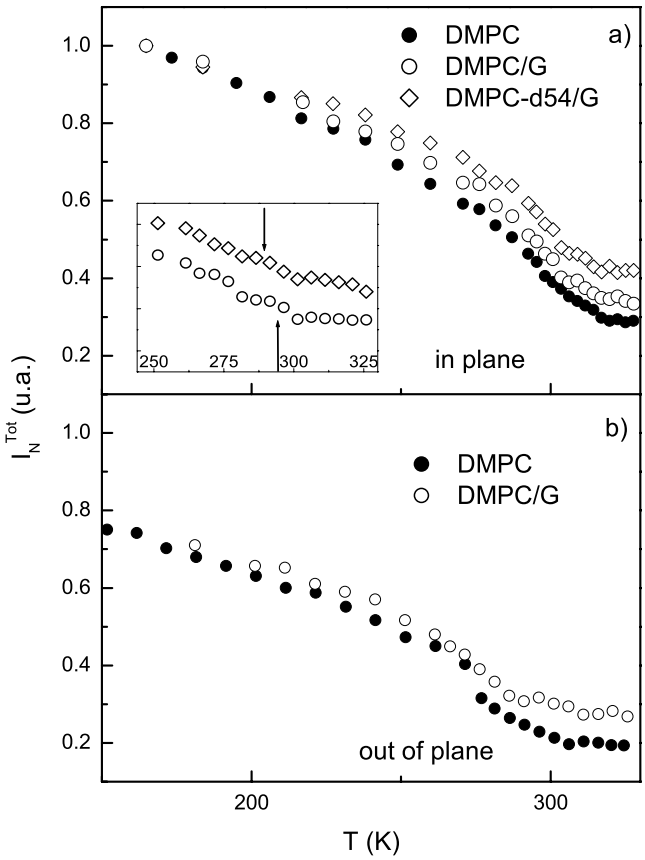

Figure 3. Comparison among the temperature dependence of FEW scans of DMPC (full circle), DMPC/G (empty circle) and DMPC-d54/G (empty diamond) for both in plane (a) and out of plane (b) components. In the inset of panel (a) scans at $Q = 1.47$ Å^{-1} of DMPC/G and DMPC-d54/G, arbitrarily shifted along the intensity axis to make the comparison easier, are shown.

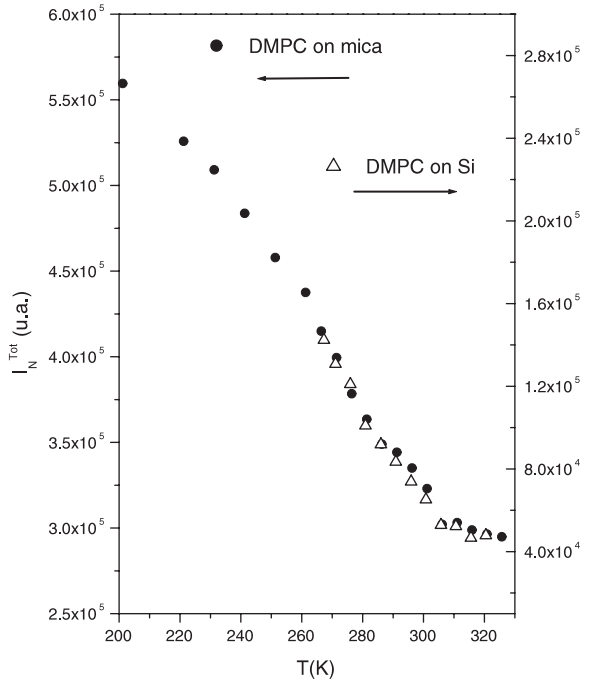

Figure 4. Comparison of the temperature dependence of FEW scans integrated over all Q-range of DMPC bilayers supported on mica (full circle) and on silicon (empty triangle).

not seem to have any influence on the signal coming from the membrane. This is illustrated in figure 4 where the intensity, integrated over all the detectors, is shown for both DMPC on mica and on Si. In the common temperature region a good overlap of the two spectra is observed.

4. Discussion

As referred to before, sharp drops in the FEW intensity as a function of the sample temperature indicate the onset of an increased molecular mobility, which can be related to relaxations or transitions toward a different structural phase of the sample. In the present work the transition temperatures have been evaluated by the intersection between the slopes of the region before the transition and of the front of the unfreezing curve, as commonly used in differential scanning calorimetry [19]. More detailed information about the transitions has been obtained by analysing the FEW behaviour at different Q values, based on the common expectation that every single transition involves specific length scales in the sample.

The sharp decrease in the FEW scans observed at $Q = 1.47$ Å^{-1} for pure DMPC (see figure 1(b)) indicates a first order transition at $T_m = 297$ K, which can be ascribed to the melting of the acyl chains. As a matter of fact, the increase in the molecular mobility takes place at the same temperature where the transition from the rippled gel to the lamellar liquid crystalline phase occurs in DMPC [16]. Moreover, the Q value of 1.47 Å^{-1} is very close to the value of the correlation peak of lipid acyl chains in DMPC ($Q = 1.40$ Å^{-1}) [20], showing that the length scale directly involved in the transition is the interchain distance between nearest neighbouring lipids. Furthermore, lipid bilayers show a quasi-bidimensional liquid structure [20]. Then, the difference revealed in the in and out of plane setups describes the expected high anisotropy of the chain dynamics.

Very recently, in an analogous neutron experiment, the melting of the acyl chains has been revealed in DMPC-d54 at $T = 293$ K, in good agreement with the expected value for the deuterated membrane [10]. Differently from Rheinstädter's findings, the transition we observe appears broader. This has to be ascribed to the lower instrumental resolution of the IN13 spectrometer that implies a smaller sensitivity for revealing dynamic transitions. Moreover, the temperature step we used (5 °C) smoothes steep changes in the FEW intensity, favouring apparent broadening of transitions.

The addition of gramicidin in DMPC changes the mobility of the host phospholipid bilayer, shifting the location of the high temperature transition up to 294 K (see figure 1(b)). Changes in the decrease of the scattered intensity with temperature in the range 270 K $< T <$ 300 K can also be observed. It is known that the presence of gramicidin in lipid bilayers influences their thermotropic phase behaviour [21, 22]. The revealed small shifting of 3 °C of

the high temperature transition is in agreement with recent measurements, showing that the disruptions of lipid packing promoted by small amounts of gramicidin produce modest decreases in the temperature (∼3 °C) of the main phase transitions of DMPC [21, 22]. In the same papers it was also shown that the increase in the peptide concentration (corresponding to a lipid/peptide ratio below 50:1) favours the appearance of a two-component main phase transition, with a more cooperative, lower temperature endotherm superimposed over a less cooperative, higher temperature endotherm. This finding could explain the observed changes in the scattered intensity below 297 K. Indeed, in our sample there is a higher peptide content (40:1 lipid/peptide) and so a clear two-component main phase transition is expected, with consequent changing mobilities over an extended temperature region.

The second transition that we observe at $T_f = 270$ K is similar to the transition noticed at 271 K in the deuterated DMPC [10]. These authors, on the basis of the observed highly isotropic character and the high sensibility to the correlation lengths of heavy bulk water ($Q = 2$ Å^{-1}) and the ice Bragg peaks (Q values of 1.605, 1.706 and 1.817 Å^{-1}), attributed the transition to mobility of water molecules in the layer between the membrane stacks. It was supposed that, upon cooling, mobile bulk-like water in the centre of the water layer may come out from the membranes and condense as polycrystalline ice outside the lamellar structure. Differently from Rheinstädter *et al* we observe a high anisotropy in the scattering, the mobility in the out of plane direction being predominant over the in plane one at temperatures above 250 K (see figure 2(a)). Moreover, this transition can be seen at almost all of the Q values explored, including the value of $Q = 1.47$ Å^{-1}, where it appears very evident. These differences can be explained by considering that in our sample only the hydration water is deuterated: consequently the predominant part of the scattered intensity is due to the hydrogen atoms of the acyl chains (that are nonexchangeable and much more than those on the polar heads) rather than to the aqueous solvent environment. Hence we can speculate that the transition at 270 K reflects not only some transitions taking place in the hydration water, but also an increase of mobilities of the membrane. Both the unfreezing of aqueous solvent and contingent increase of hydration shells following from the migration of solvent into the membrane stacks of lamellae assure more flexibility to the bilayers, favouring a more accentuated dynamics of the lipid chains.

The inclusion of gramicidin shifts the beginning of low temperature transition from 270 to 276 K (see figure 1(a)). Moreover, it imposes more severe restrictions on the out of plane dynamics (see figure 3(a)). It is known that the insertion of gramicidin into the bilayer is accompanied by dehydration of the lipid head groups surrounding the peptide [23]. As a consequence, changes in the interlamellar spacing and large mechanical stresses are expected in the bilayer stacks. Moreover, hydration forces arise when lamellar surfaces are brought to separation $\leqslant 10$ Å. It can be reasonably supposed that the presence of peptide is correlated with the presence of both hydration forces and mechanical stresses of the host bilayers, though a convincing explanation for the observed increased temperature requires further investigations and auxiliary information by different techniques. The restrictions of the membrane mobility imposed by gramicidin are more easily understandable. In fact, it is well documented that already at a few degrees below the main transition the gramicidin becomes immobile [24]. Gramicidin incorporated into DMPC interacts primarily with the polar head group and glycerol-backbone region of the phospholipid molecules by electrostatic and hydrogen-bonding interactions and secondly with hydrocarbon chains by hydrophobic and van der Waals's interactions. As a consequence of these interactions the contiguous lipids are expected to have hampered dynamics and the whole membrane mobility will slow down. Moreover, the effects of gramicidin on lipid acyl chain motion in the liquid crystalline phase were derived from NMR experiments [25, 26]. It was found that gramicidin suppresses fast motions with correlation times shorter than 0.1 ns. The increase in the FEW intensity we found above the main transition in DMPC/G confirms this finding. All the changes in the freezing dynamics we observed in the system composed of phospholipids and gramicidin involve greatly the acyl chains. The measurement performed in the DMPC-d54/G sample in the in plane setup gives clear evidence of the dependence of the system mobility on the hydrocarbon chains. The deuteration of the lipid acyl chains masks their mobility, consequently the increase of the scattered intensity observed in the deuterated sample (see figure 3(a)) shows that a relevant part of the measured signal is due to the contribution of acyl chains. In the inset of the figure is shown the detail of the FEW scans at $Q = 1.47$ Å^{-1} for DMPC/G and DMPC-d54/G samples in the region of the main transition. In the deuterated sample a lowering of the transition temperature from 294 K to 289 K is observed, in agreement with what is expected in a deuterated lipid [27].

5. Conclusions

By analysing the temperature dependence of FEW scans of pure DMPC and DMPC/G membranes, information about the molecular mobilities in these systems have been derived. We have found that acyl chain motions are strongly involved in the main transition from ripple gel to liquid crystalline phase. We also observed a second transition at temperatures close to the freezing of pure heavy water that we have ascribed to a sudden increase of the membrane flexibility. A strong anisotropy has been found for the motions involved in both transitions: the out of plane dynamics is strongly enhanced in comparison with the in plane one and involves mainly the acyl chains. The presence of gramicidin strongly hampers molecular motions of the lipids and causes the lowering of the main transition temperature and the raising of the transition temperature correlated to the hydration water.

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