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Towards high-resolution ^{1}H-NMR in biological membranes: magic angle spinning of bicelles

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Abstract

Proton line narrowing in biomembranes spun at the magic angle, for spinning speeds greater than 7 kHz, was investigated in two ways: increasing the field strength from 200 to 800 MHz and changing the membrane fluidity. The resolution that one can obtain on natural lipid membranes under the form of liposomes is 0.019 ppm at 800 MHz. On the other hand, spinning bicelles (disk-like model membranes made of synthetic long and short chain lipids) at the magic angle decreases the line width by an additional factor of 3 provided the bicelle is subjected to large orientational disorder. This leads to proton line widths of the order of 6 Hz at 500 MHz. The conjunction of high field, magic angle spinning and use of bicelle membranes should prove to be useful to solve membrane protein structure in a membrane environment. © 2002 Published by Elsevier Science B.V.

Keywords: Bicelle; MAS NMR; Proton line width in membrane; Membrane protein

1. Introduction

Protein structure isn't everything but it sure helps in understanding their function. So far, there are only two techniques that are able to depict the structure at the atomic resolution: solution NMR on one side and X-rays and neutron diffraction on the other. Both techniques became classical tools with soluble proteins. Nonetheless, it has also to be recognized that among the hundred thousand proteins that are coming after the unveiling of various genomes, including ours, 30–40% are supposed to be embedded or associated to biological membranes. Unfortunately, only a dozen such proteins have seen their structure solved to atomic resolution. This is essentially due to the intrinsic hydrophobicity/amphiphilicity borne by these proteins. It renders their crystallization difficult for studies by diffraction methods and their solubility poor at millimolar concentrations in biologically relevant media for solution NMR studies. In addition, the question is still opened about the relevance of structures obtained in a non-membranous environment. There is therefore a demand for techniques capable to bring structure in the membrane. Solid state wide line NMR has shown its potential for topology information after the pioneering works of Griffin, Cross and Opella [1–3]. Orientation of helices or fragments of proteins with respect to the membrane surface have been obtained. On the other hand, protein–protein interaction has been obtained in the membrane by high-resolution solid state NMR, using MAS techniques [4]. All the above experiments rely on specific protein labeling that may be tedious and expensive. As proposed by Davis et al. [5], proton NMR would be easier because one can use all the multidimensional NMR experiments that are used in solution studies. Recent examples of MAS of bicelles can be found in the literature [6–8]. There are however some difficulties inherent with this approach. First, the very large homogeneous dipolar proton interaction that is present in membrane media is difficult to cancel by magic angle spinning rates of tens of kHz. Second, the lipid membrane must be totally deuterated to only observe the protein protons. Whereas the second point is now being solved the first one is the object of the present contribution. In other words, can MAS of membranes bring sufficient line narrowing to observe proton resonances of a few hertz? The problem was attacked in trying to answer three questions: (i) What is the influence of field strength on the proton line width under magic angle

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spinning conditions? (ii) What is the influence of spinning speed on proton line width? (iii) What is the role of internal membrane dynamics in line narrowing processes?

We have chosen to work with pure lipid membranes both natural (egg phosphatidylcholine-water liposomes) and artificial (water-dispersed bicelles) for facility reasons. We will therefore look at proton line narrowing conditions for lipid protons. The results that are obtained with lipid resonances will nonetheless have to be validated with proteins embedded in a deuterated membrane. Bicelles are composed of a binary mixture of long chain (C14–C18) and short chain (C6–C8) phospholipids or bile salt analogues. Under certain conditions, the mixture of these two lipids was shown to form disk-like aggregates in aqueous solution. Because bicelle solutions are lyotropic liquid crystal systems, the application of magnetic field greater than about one tesla leads these disk-like assemblies to align with their bilayer normal perpendicular to the magnetic field, thus displaying nematic discotic order [9,10].

As it will be shown herein, both the combination of MAS at high field and reasonable speed rate and use of membranes under the form of bicelles offers the possibility to line narrow the resonances to few hertz that are the requirements for solving 3D structure. An example of such a resolution will be given for the transmembrane segment, neu_{tm35}, of the tyrosine kinase receptor, *neu*, embedded in perdeuterated bicelles.

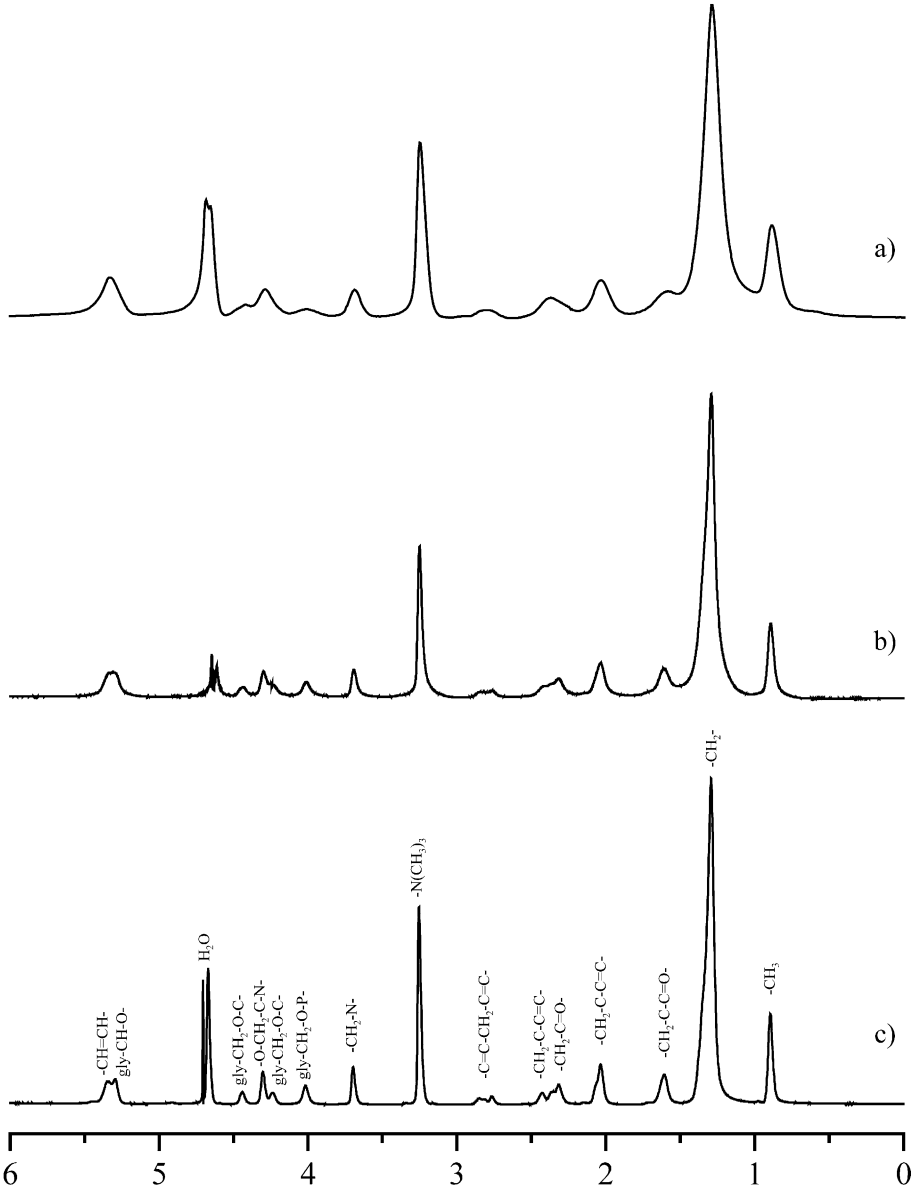

Fig. 1. ^{1}H-NMR spectra of egg phosphatidylcholine model membranes recorded at (a) 200, (b) 500 and (c) 800 MHz. All spectra are recorded under magic angle spinning conditions (10 kHz). Resonance assignment was performed with COSY experiments and using literature data. Acquisition conditions are described in Materials and methods.

1.1. Theoretical background

The theory for solid state magnetic and electric interactions that allows to describe the average spin Hamiltonian in a biological fluid membrane where fast (nanosecond time scale) axial rotation takes place has been already developed [5,11,12]. We will only outline here what is needed for the discussion.

When dealing with proton NMR in a fluid membrane, the direct spin–spin interaction dominates all other internal interactions and the spin Hamiltonian for an ensemble of interacting spins is written to first-order as:

$$\langle \mathscr{H} \rangle = -\sum_k \omega_{0k} I_{zk} - \sum_{k<1} \hbar \frac{\gamma_k \gamma_k}{2 r_{kl}^3} (3\cos^2 \beta_{\mathrm{NL}} - 1) \times [3 I_{zk} I_{zl} - \boldsymbol{I}_k \boldsymbol{I}_l] S_{kl}^{\mathrm{HH}} \quad (1)$$

where $\omega_{0k} = \gamma_k B_0 (1 - \sigma_k^{zz})$ is the Larmor frequency of nucleus k with the σ_k^{zz} the z component of the shielding tensor, r_{kl} the distance between nuclei k and l, β_{NL} the angle between the normal to the bilayer and the laboratory axis, and S_{kl}^{HH} the order parameter of the proton pair kl ($S_{kl}^{\mathrm{HH}} = \langle 3\ \cos^2 \beta_{\mathrm{PN}} - 1 \rangle / 2$). β_{PN} is the angle between the principal axis system and the bilayer normal. All the other symbols have their usual meaning. When using deuterium-labeled compounds to record deuterium NMR spectra, the electric quadrupolar interaction then dominates all other internal interactions and the Hamiltonian may be written to first-order as:

$$\langle \mathscr{H} \rangle = -\sum_k \omega_{0k} I_{zk} - \sum_{k<1} \omega_{Qk} \frac{1}{2} (3\cos^2 \beta_{\mathrm{NL}} - 1) \times [3 I_{zk}^2 - \boldsymbol{I}_k^2] S_k^{\mathrm{CD}} \quad (2)$$

Here, ω_{Qk} is the static quadrupolar coupling constant and S_k^{CD} the order parameter of the C–D bond ($S_k^{\mathrm{CD}} = \langle 3\ \cos^2 \beta_{\mathrm{PN}} - 1 \rangle / 2$). Under fast magic angle sample spinning, all angle-dependent terms in the above equations average to zero to yield the well-known Hamiltonian of solution state:

$$\langle \mathscr{H}_{\mathrm{iso}} \rangle = -\sum_k \omega_{0k}^{\mathrm{iso}} I_{zk} + \sum_{k<1} 2\pi J_{kl} I_{zk} I_{zl} \quad (3)$$

where $\omega_{0k}^{\mathrm{iso}} = \gamma_k B_0 (1 - \sigma_k^{\mathrm{iso}})$ is the isotropic chemical shift and J_{kl} the coupling constant for indirect spin–spin interaction. Eq. (3) is obtained for spinning speeds that are greater than the residual solid state dipolar or quadrupolar interactions. The direct homogeneous spin–spin interaction may be of the order of 10 kHz for bilayer membranes [13]. When performing MAS ^{1}H-NMR of membranes, one is expected to have to spin to rates that are much greater than 10 kHz due to the homogeneous character of the direct spin–spin interaction. The residual quadrupolar interaction may range from 100 to 20 kHz depending on membrane composition and temperature. Because of the intrinsic inhomogeneous character of the quadrupolar interaction, spinning rates of the order of the residual interaction would be enough to average it to zero. Unfortunately, the current state of the art in MAS probe design is limited to ca. 30 kHz, which may be insufficient to do so.

2. Materials and methods

2.1. Chemicals

Egg PC and synthetic DMPC, DCPC and {*sn*-2-$^2H_{27}$}-DMPC were purchased from Avanti Polar Lipids (Birmingham, AL, USA) and used without further purification. Perdeuterated {$^2H_{72}$} DMPC was synthesized according to Ref. [14]. D_2O and deuterium-depleted water were obtained from Eurisotop (St. Aubin, France). Possible hydrolysis of lipids was checked after completion of NMR experiments by thin layer chromatography. When more than 5% of

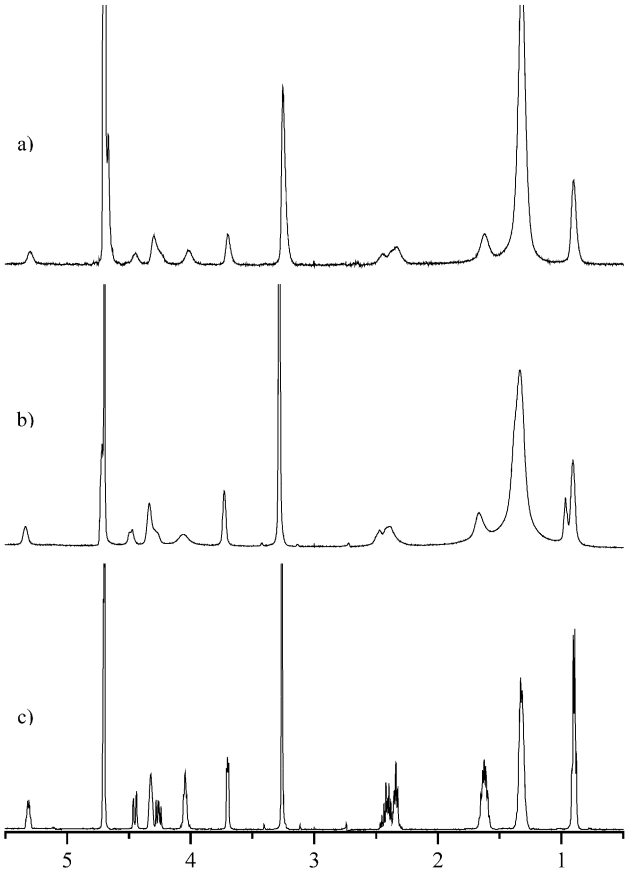

Fig. 2. ^{1}H-NMR spectra (recorded at 500 MHz) of (a) DMPC liposomes (80% hydration in D_2O), (b) DMPC/DCPC bicelles (75:25 molar ratio, 80% hydration in D_2O), (c) DCPC micelles (80% hydration in D_2O). The magic angle spinning speed is 10 kHz in all cases. Acquisition conditions are described in Materials and methods. Temperature inside the rotor is 35 °C after correction for heating due to air friction at 10 kHz.

lysolipids were detected, the data was not used. Research grade KCl was purchased from Sigma (St. Quentin-Fallavier, France). The 35 aa transmembrane segment, neu_{tm35}, of the tyrosine kinase receptor *neu* was synthesized according to published procedures [15].

2.2. Sample preparation

Liposomes (water-to-lipid dispersions, also called multilamellar vesicles) were prepared by mixing 25 mg of egg PC with 100 μl of water (80% hydration). Several cycles of vigorous shaking in a vortex mixer, freezing and thawing led to a milky fluid suspension characteristic of multilamellar vesicles of micrometer size. Bicelles were prepared by weighting the appropriate quantities of DMPC and DCPC to obtain $X = 85\%$, 75% and 66% DMPC mole fractions into a centrifuge vial. One-hundred microliters of a 100-mM KCl water solution were added to the total 25 mg of lipids, hydration (v/w) was therefore 80%. The sample was then vortexed at 2700 rpm, centrifuged at 7500 rpm for 5 min, frozen in liquid nitrogen, and warmed up in a 50 °C water bath for 10 min. This cycle was repeated between 5 and 10 times until a highly viscous, transparent gel was obtained at room temperature [16]. Micelles were obtained using the same preparation steps as for bicelles except that the DMPC mole fractions were 50% and 0% (pure DCPC). Incorporation of neu_{tm35} into $\{^2H_{72}\}$DMPC/DCPC bicelles was accomplished according to Ref. [17]. The molar ratio of neu_{tm35} vs. lipids is 1/200.

2.3. NMR spectroscopy

Magnetically orienting bicelles, which are characterized by two sharp ^{31}P NMR lines at ca. -4 and -12 ppm (with 85% H_3PO_4 as reference), were controlled with a Bruker Avance 400 NB spectrometer operating at 162 MHz [16]. A phase-cycled Hahn-echo pulse sequence with gated broadband proton decoupling was used [18]. Deuterium (D_2O) lock was used and 128 acquisitions were recorded. Experimental parameters were: spectral width 40 kHz, $\pi/2$ pulse width of 5.8 μs, interpulse delay of 50 μs and recycle delays of 5 s. ^2H-NMR experiments were carried out on a Bruker Avance 500 WB US spectrometer operating at 76.7 MHz. Experiments on perdeuterated *sn*-2 DMPC chains were performed using a phase-cycled quadrupolar echo sequence and 1 k transients were acquired [19]. Experimental parameters were: spectral width of 500 kHz, $\pi/2$ pulse width of 2.8 μs, interpulse delay of 50 μs and recycle delay of 1 s. ^1H-NMR MAS spectra were recorded on a Bruker MSL 200 WB, Avance 500 WB US and Avance 800 SB spectrometers. Probes were either CP or HR MAS type with an intrinsic resolution ≤ 1 Hz. Deuterium lock was achieved either on the lock channel of the HR MAS probe or using the X-channel tuned to deuterium frequency. Experimental

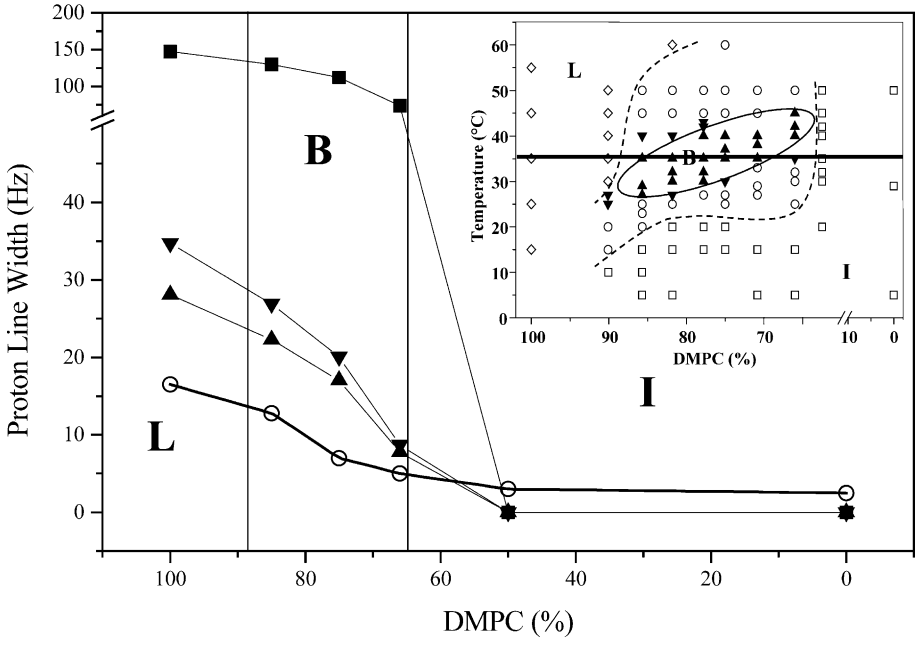

Fig. 3. ^1H-NMR line width at half maximum for the 3.5 ppm choline resonance (recorded at 500 MHz) MAS rate 10 kHz as a function of DMPC mole fraction in the DMPC/DCPC (80% hydration) system (○). The letters L, B and I refer to lamellar, magnetically orienting bicelles and isotropic (micelles) as reported in the temperature composition diagram of Raffard et al. [16] shown in the inset. (■) Line width calculated according to Eq. (4) of text by taking the main chain order parameter, $S^{CD} \approx S^{HH}$ is plotted as filled squares. S^{CD} values are -0.142, -0.125, -0.108, -0.071, 0, 0 for DMPC contents of 100%, $85S^H$, $75S^H$, $66S^H$, $50S^H$, 0%, respectively. (▼) Theoretical line width according to Eq. (5) with $\tau_c = 1 \times 10^{-6}$ s and the above values for S^{HH}. (▲) Line width using Eqs. (4) and (5). See text for details.

parameters: spectral width of 10 ppm, $\pi/2$ pulse width of 3–5 μs and recycle delay of 3 s. When used, presaturation of water signal was applied during the recycling delay.

3. Results

3.1. High-resolution magic angle sample spinning ^{1}H-NMR of lipidic biomembranes

Experiments were performed both on natural lipid membranes made of egg lecithin water dispersions and on synthetic lipid membranes (MLV, bicelles and micelles). Fig. 1 reports egg lecithin liposome spectra acquired in spectrometers operating at proton Larmor frequencies of 200, 500 and 800 MHz. The spinning speed was set to 10 kHz and the temperature regulated to 25 °C. Isotropic resonances are clearly resolved: the larger the field, the higher the resolution. Line widths at half height of representative resonances along the lipid structure were measured. For the chain methyl terminal (0.89 ppm), the choline protons (3.25 ppm), and the glycerol CH_2 (4.02 ppm), it was respectively found 20, 15 and 30 Hz, independently of the magnetic field strength. Increasing the spinning speed above 7.5 kHz and up to 15 kHz did not bring appreciable line narrowing. Resonance attribution, as shown in Fig. 1 (bottom), was performed with COSY experiments and by making use of literature data.

Similar experiments were performed at 500 MHz only by varying the membrane nature, according to the composition–temperature diagram of the DMPC/DCPC bicellar system [16], as shown in the inset of Fig. 3. The temperature was set to be 35 °C inside rotor for a MAS speed of 10 kHz. Calibration curves were previously recorded with an ethylene glycol sample to monitor the speed-driven increase in temperature inside the rotor and to correct for it. Several sample compositions were measured along the DMPC dilution line shown by the bold horizontal line in the inset of Fig. 3. Representative spectra are presented in Fig. 2. Fig. 2c displays the 100% DCPC micellar spectrum where a liquids-like resolution can be observed. In Fig. 2a, the 100% DMPC lamellar spectrum shows features that are akin to those of Fig. 1, i.e., with the same spectral resolution. The spectrum of a mixture of DMPC/DCPC (75:25 molar ratio), which is otherwise known to form bicelles that magnetically orient in the field at 35 °C, is shown in Fig. 2b. There is a marked increase in resolution as compared to Fig. 2a. However, the J coupling constants for indirect spin–spin interaction are still not detected. Nonetheless, it is noteworthy that the chain terminal methyl resonances for DCPC and DMPC are clearly resolved at 0.97 and 0.90 ppm, respectively. The line width at half height for the choline protons (~ 3.25 ppm) is plotted as a function of composition in Fig. 3 (empty circles). This figure is vertically separated into three regions, labeled L, B, I as in the phase diagram inset, and standing respectively for lamellar (liposomes), bicellar, and isotropic (micellar) phases. The line width gradually decreases on going from lamellar to isotropic phases. Of interest is the fact that in the bicelle domain, close to the isotropic phase, the line width is about 5 Hz.

3.2. Solid state deuterium NMR of biomembranes

In order to gain further understanding in the phenomenon of line width reduction while decreasing the DMPC content in the bicelle system, a series of deuterium NMR spectra have been obtained with different membrane compositions. Fig. 4 reports solid state, wide line, ^{2}H-NMR spectra for pure $\{^2H_{27}\}$DMPC (100%) and for 84%, 75%, 66% of $\{^2H_{27}\}$DMPC in the DMPC/DCPC system at 35 °C. Below 60% DMPC content, the spectrum is composed of an isotropic line (not shown) whatever the temperature [16]. Fig. 4d displays the characteristic axially symmetric powder pattern of a lipid undergoing fast anisotropic reorientation along its molecular long axis [20,21]. Spectra of Fig. 4a–c are relative to bicelles that self-orient in the magnetic field with the disc plane parallel to the field direction. It is clearly

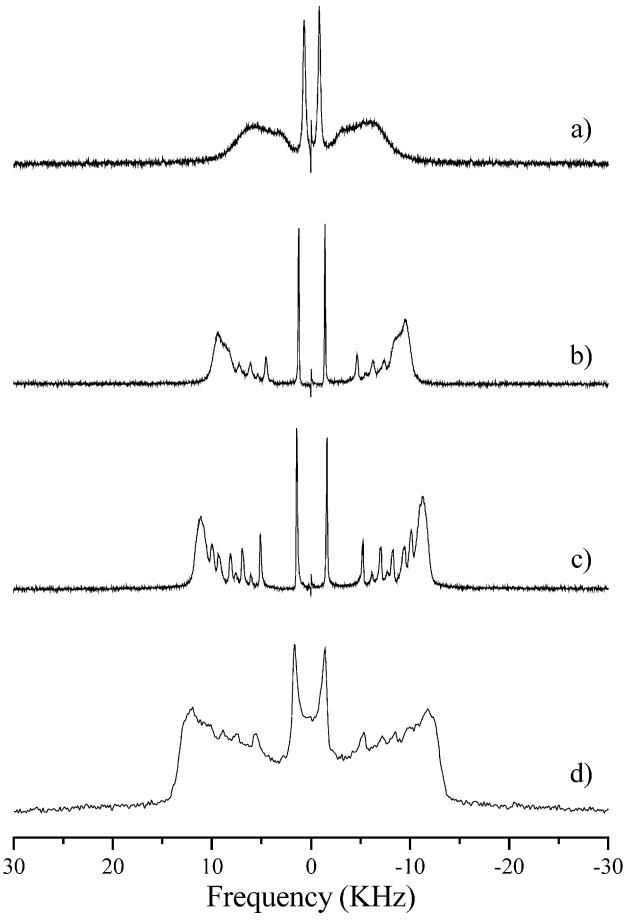

Fig. 4. ^{2}H-NMR spectra of DMPC/DCPC bicelles for several DMPC molar fractions. (a) 66% DMPC, (b) 75% DMPC, (c) 85% DMPC and 100% DMPC. The long chain lipid is deuterium-labeled on the entire *sn*-2 chain. Temperature is 35 °C.

seen that the widths of the patterns decrease on decreasing the DMPC content. An important line broadening is observed for spectrum of Fig. 4a suggesting a possible exchange situation between bicelles and micelles, the sample composition being very close to the boundary. From such deuterium patterns, the S^{CD} order parameter relative to each of the C–D bonds along the lipid chain can be measured. An average S^{CD} order parameter for the entire chain can also be extracted from such a set of individual S^{CD}'s. One finds -0.142, -0.125, -0.108 and -0.071, respectively for pure DMPC, 84%, 75% and 66% DMPC. It is seen that there is an overall decrease by a factor 2 on going from pure DMPC liposomes to bicelles with 66% DMPC content.

3.3. Magic angle spinning ^{2}H-NMR of bicelle membranes

The orientational properties of bicelles (78% DMPC content) under magic angle spinning conditions were also followed. Fig. 5 shows deuterium NMR spectra acquired at

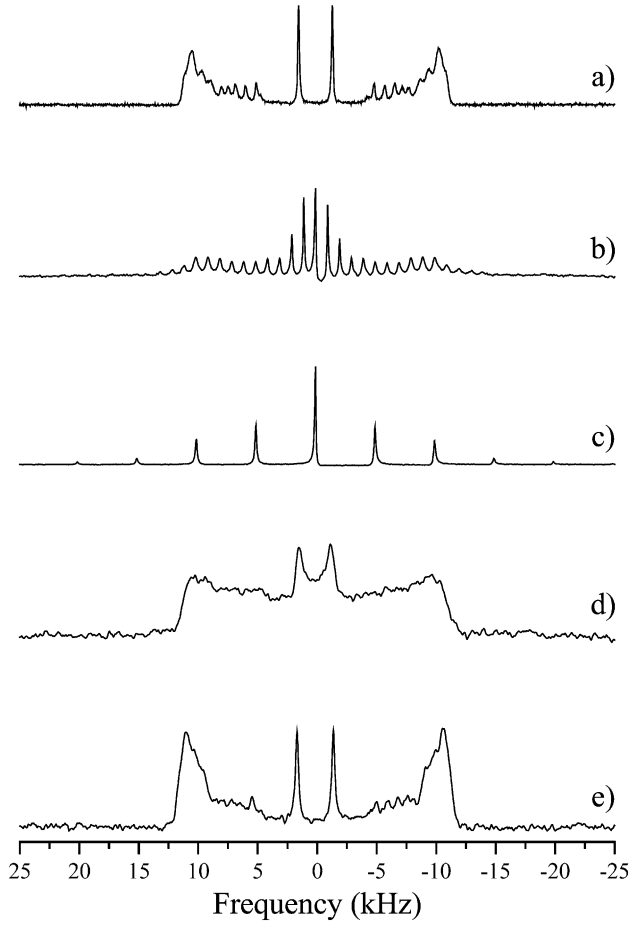

Fig. 5. ^{2}H-NMR spectra of DMPC/DCPC bicelles (78:22 molar ratio, 80% hydration) under static and MAS conditions. The long chain lipid is deuterium-labeled on the entire *sn*-2 chain. Temperature is 35 °C. (a) Static sample, (b) spinning speed 1 kHz, (c) spinning speed 5 kHz, (d) static sample, spectrum taken just after stopping MAS, (e) static sample, spectrum taken 3 h after stopping MAS.

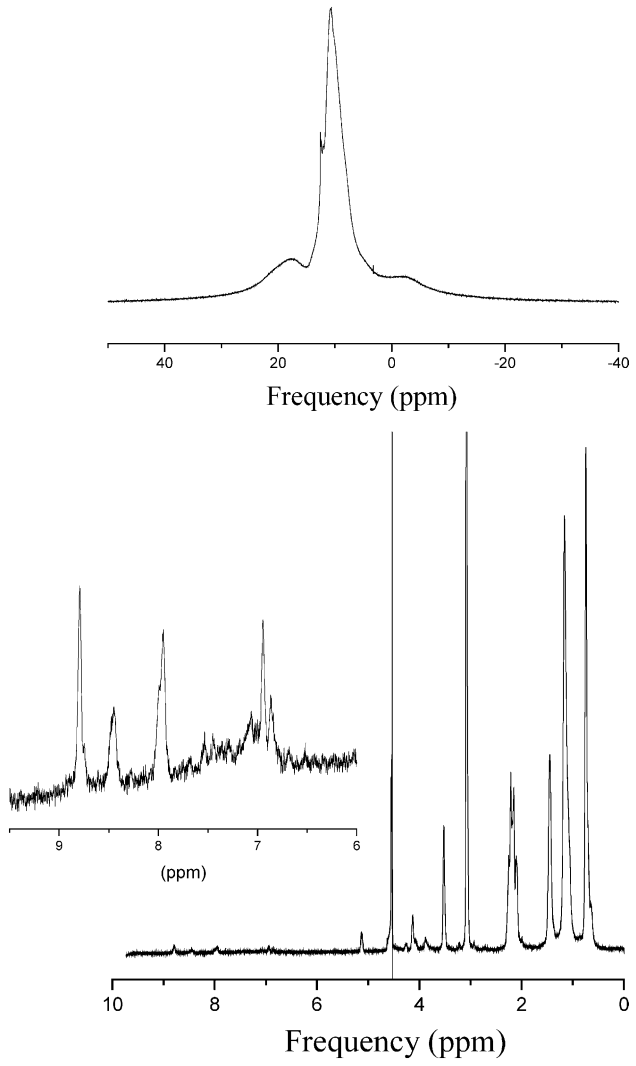

Fig. 6. ^{1}H-NMR MAS spectra (recorded at 500 MHz) of the protein neu_{tm35} embedded in $\{^2H_{72}\}$DMPC/DCPC bicelles (75:25 molar ratio, 80% hydration (H_2O/D_2O). Molar ratio of protein vs. lipids is 1:200. The long chain lipid is entirely deuterated. Temperature is 35 °C. Top spectrum: static sample. Bottom spectrum: spinning speed 10 kHz, the insert is a vertical expansion of the 6–9 ppm zone. Water presaturation was utilized here to reduce the huge water signal that would otherwise occur at 4.7 ppm. Acquisition conditions are as described in Materials and methods.

several spinning speeds at 35 °C. Fig. 5a displays the regular non-spinning ^{2}H-NMR spectrum of DMPC-$^2H_{27}$-containing bicelles. The spectrum is characteristic of sample macroscopic orientation with the disc plane parallel to the magnetic field direction [10,16]. There are no frequencies detected beyond ± 12 kHz. Spectra of Fig. 5b and c are acquired under MAS conditions at 1 and 5 kHz, respectively. Besides the classical spinning side bands pattern that delineate spectrum of Fig. 5a, there are spinning side bands up to ± 20 kHz. Just after stopping the magic angle spinning, one obtains the spectrum of Fig. 5d, which displays features of so-called powder spectra, i.e., of material that is randomly oriented with respect to the magnetic field direction. After waiting for 3 h without spinning, one

obtains the spectrum of Fig. 5e. Again macroscopic orientation similar to that of Fig. 5a is detected. Interestingly, when the sample of Fig. 5d is taken to 15 °C, where it is very fluid, and then brought back to 35 °C, one immediately obtains the spectrum of Fig. 5a.

3.4. High-resolution magic angle sample spinning ^{1}H-NMR of neu_{tm35} in bicelles

The 35 aa tyrosine kinase transmembrane segment has been incorporated into bicelles where the perdeuterated DMPC mole fraction was $X = 75\%$. The sample was hydrated in H_2O/(10% D_2O) to obtain water content vs. lipids (w/v) of 80%. Fig. 6 displays the proton spectrum both for static and MAS (10 kHz) conditions. The static spectrum has no resolution and it is clearly dominated by the strong dipolar interaction (ca. 20 ppm). Under MAS conditions, it shows a remarkable resolution both in the region where the resonances of protonated DCPC hide those of the protein (1–5 ppm) and in the (6.5–9 ppm) region where the protein resonances are exclusively detected. Line widths of the order of 20 Hz are detected for the NH and aromatics resonances. Similar experiments performed with liposomes would give larger line widths (data not shown).

4. Discussion

From the above results, it appears that there are two ways to increase the proton spectrum resolution in lipid membranes rotating at the magic angle, (i) increase the field strength and (ii) work with membranes with high disorder. These two aspects will be discussed in the following. We will also discuss the possible extension of our results to proteins embedded in membranes.

4.1. Proton HR MAS on liposome membranes at high field

The line width values we find when spinning egg PC liposomes at 10 kHz are in the range 30–15 Hz, i.e., in very good agreement with those reported by Ref. [5] for a membrane made of POPC. The values do not depend on the magnetic field strength, within the experimental error (Fig. 1) and do not change for spinning speeds greater than 7.5 kHz, also in agreement with the above cited authors. This suggests that there is little contribution from the chemical shielding anisotropy to the residual line width, and that probably the dipolar proton spin–spin relaxation is responsible for most of the residual broadening (vide infra). Also of interest is the fact that moderate speeds are sufficient to obtain line narrowing. This is in part accounted for by the quite small S^{HH} order parameter of proton pairs in the lipid, which is equal for CH_2 groups to the methylene C–D order parameter as measured with deuterium NMR. The dipolar interaction (Eq. (1)) available for broadening is thus reduced by internal molecular motions by about a factor 5 (S^{HH} value of the order of 0.2), which appears to be sufficient to afford line narrowing with spinning speeds at the magic angle of the order of 7 kHz. Fig. 1 clearly indicates the advantage of working at 800 MHz: the resolution increases from 0.150–0.075 ppm at 200 MHz to 0.037–0.019 ppm at 800 MHz for the various protons of the lipid moiety. It must be mentioned here that only the resonance at 3.25 ppm is a singlet, the others at 0.89 and 4.02 ppm should, in principle, be triplets with a $^3J_{HH}$ coupling constant of 7 Hz. The resolution should therefore be discussed on the resonance at 3.25 ppm. At 800 MHz, it is 0.019 ppm and akin to that of liquids.

4.2. Proton HR MAS on bicelle membranes

The line width of the 3.25 ppm resonance strongly decreases when changing the membrane nature across the bicellar phase diagram (Figs. 2 and 3). Not surprisingly, in the isotropic (micellar) state (region I in Fig. 3, 50% and 0% DMPC), the line width is comparable to that obtained in liquids. It must be mentioned here that not much is gained by spinning a micellar suspension at the magic angle, the same kind of line width could be attained without spinning (not shown). The only advantage is to work with small quantities of material, inside the rotor (20–50 μl), compared to the regular 400 μl in a regular 5-mm tube. In the bicellar phase (B in Fig. 3), spinning at the magic angle is essential; the line width would be of the order of several hundred hertz in the absence of MAS. The line width markedly decreases when decreasing the amount of long chain lipid in the bicelle domain. This is again to be related to the residual orientational ordering of the lipids inside the membrane because the average S^{CD} chain order parameter concomitantly decreases when decreasing the amount of long chain lipid. A rationale for such an effect can be obtained by estimating the contribution of the homogeneous broadening to the line width. Following Davis et al. [5], the line width of a proton resonance can be written as:

$$\Delta\nu_H \approx \frac{\delta_{\omega_0}}{\omega_{MAS}} \sqrt{(S^{HH})^2 \Delta M_2} \quad (4)$$

where $\delta_{\omega 0} = 500$ Hz (1 ppm at 500 MHz), $\omega_{MAS} = 10$ kHz and S^{HH} the order parameter of proton pairs. $\Delta M_2 \approx 1.7 \times 10^{10}$ s^{-2} is the rigid lattice second moment, M_2, of methylene chains in the all-trans conformations [13,22]. In determining such a value, it was assumed that the terminal methyl groups undergo rapid rotation around their C_3 axis. We do not have the S^{HH} order parameter of the choline head group, but as a rough estimate one can inject in Eq. (4) the mean S^{CD} order parameters we have measured on the deuterated chains. As already commented above and as was remarked by Higgs and MacKay [23], S^{CD}s and S^{HH}s are not expected to differ markedly in the lipid L_α phase. The result is plotted in Fig. 3 (empty squares). A decrease of the residual line width is indeed observed on increasing the

DCPC content in the system, but the obtained values are two orders of magnitude larger than the experimental values. This indicates that the homogeneous broadening does not account in total to the residual proton line width. As suggested by Davis et al. [5], the intermediate time scale motions have to be taken into account. Such motions whose correlation time is in the microsecond range are akin to the collective modes of motion of lipids in a membrane. Hydrodynamic undulations of the bilayer surface such as bending or twisting have been shown to be present in such systems in the L_α phase. In addition, oscillations of the bicelle itself with respect to the magnetic field direction may also be present. Following Haeberlin and Waugh [24], the line width of a proton line under MAS conditions, in the presence of intermediate time scale motions, can be written as:

$$\frac{1}{\pi T_2^{rot}} = \frac{\langle \Delta M_2 \rangle}{3\pi^2} \times \left\{ \frac{2\tau_c}{1+(\omega_{\mathrm{MAS}}\tau_c)^2} + \frac{\tau_c}{1+4(\omega_{\mathrm{MAS}}\tau_c)^2} \right\} \qquad (5)$$

where τ_c is the correlation time of a given motional mode and $\langle \Delta M_2 \rangle$ the dipolar second moment that is averaged by the intermediate time scale motions. By making the approximation that $\langle \Delta M_2 \rangle = \Delta M_2 \{S^{\mathrm{HH}}\}^2$ and using the mean S^{CD} as the choline S^{HH} one can calculate Eq. (5) for given values of τ_c. As we have already shown for lipid dispersions, collective modes of motion may range from 10^{-8} to 10^{-3} s [21]. We performed a calculation by taking $\tau_c = 1 \times 10^{-6}$ s in Eq. (5). The result is plotted in Fig. 3 (down triangles). It is remarkable that the experimental value is overestimated by only a factor 2. Of course, an almost perfect fit could be obtained by taking $\tau_c = 0.5 \times 10^{-6}$ s but we have no experimental data to support such a value. The purpose of the calculation using Eq. (5) was just to show that indeed the intermediate time scale motions, in the microsecond time range, are of first importance in line narrowing processes. We also plotted the residual line width due both to the homogeneous broadening and to the intermediate time scale motions (up triangles). It is seen, as already remarked by Davis et al. [5], that the homogeneous dipolar contribution is of limited effect in line narrowing processes.

It is remarkable that despite the rough approximations we have made in using Eq. (4), one describes almost quantitatively the experimental behavior of the proton line width. This strongly suggests that the orientational disorder of the bicelle is of first importance to obtain further narrowing of the proton lines at reasonable spinning speeds. At this level of discussion, it is interesting to understand what is happening to the bicelles under magic angle spinning. The MAS spectra of Fig. 5 clearly indicate that there are spinning side bands at ± 20 kHz, i.e., outside the deuterium spectrum of oriented bicelles (resonances up to ± 12 kHz). This demonstrates that under MAS conditions, the nematic order of bicelles is destroyed. This is further demonstrated by the static spectrum recorded just after stopping spinning: it is a randomly oriented (powder) pattern. In other words, the bicelles do not orient along the magic angle axis, as this could have been invoked as an extra line narrowing process. It thus appears that the key factor for line narrowing is the specific additional orientational disorder that is present in the bicelles.

4.3. Towards proton HR MAS of hydrophobic proteins embedded in membranes

Membrane-inserted hydrophobic helical peptides or helix bundles as encountered in transmembrane proteins may be subjected to anisotropic axial rotation in the microsecond range and lead to high-resolution proton spectra under MAS of liposomal membranes. Large proteins that may be anchored to the membrane by strong electrostatic interactions may even have slower motional averaging processes that may not be sufficient to reduce the huge H–H dipolar interaction. However, embedding or associating these proteins with bicelles and spinning the whole system at the magic angle has proven to narrow protein resonance lines. As demonstrated in Fig. 6, residual lipid lines do not alter the crucial 5–9 ppm region. This region is of special interest because it is used for multidimensional experiments to assign and determine distance constraints in solving protein structure. Of course, the resolution is not as good as in solution NMR but the combination of high field, MAS and bicelles may lead to solve membrane protein structure. The bicelle membrane offers the unique advantage of a flat phospholipid bilayer, which is supposed to preserve the native structure, at converse to micelles where the high curvature radius may alter protein folding. Bicelles nonetheless have a dynamics close to that of micelles which helps in providing sufficient motional averaging to get sharp lines under MAS conditions.

As a conclusion, it seems possible to obtain liquids-like resolution in membrane samples by spinning bicelle membranes at the magic angle and operating at high NMR field.

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