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Switched-angle spinning applied to bicelles containing phospholipid-associated peptides

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Abstract

In a model study, the proton NMR spectrum of the opioid pentapeptide leucine-enkephalin associated with bicelles is investigated. The spectral resolution for a static sample is limited due to the large number of anisotropic interactions, in particular strong proton–proton couplings, but resolution is greatly improved by magic-angle sample spinning. Here we present two-dimensional switched-angle spinning NMR experiments, which correlate the high-resolution spectrum of the membrane-bound peptide under magic-angle spinning with its anisotropic spectrum, leading to well-resolved spectra. The two-dimensional spectrum allows the exploitation of the high resolution of the isotropic spectrum, while retaining the structural information imparted by the anisotropic interactions in the static spectrum. Furthermore, switched-angle spinning techniques are demonstrated that allow one to record the proton spectrum of ordered bicellar phases as a function of the angle between the rotor axis and the magnetic field direction, thereby scaling the dipolar interactions by a predefined factor.

Introduction

Bicellar phases can be employed as model membranes for NMR studies of membrane-associated biomolecules (Sanders and Landis, 1995; Glover et al., 2001a; Howard and Opella, 1996; Losonczi and Prestegard, 1998; Struppe et al., 1998). With bicelle concentrations of typically 15–33 w/v,** well aligned liquid-crystalline phases are formed. For membrane associated peptides this leads to a strong molecular alignment, with the anisotropy of the second rank alignment tensor of the order of 10^{-1}. As a consequence of this strong alignment, residual dipolar couplings are much larger than the J-couplings, causing strong-coupling effects and low spectral resolution.

The spectral resolution for bicelle-associated peptides and proteins is significantly lower than for biomolecules dissolved in the water part of liquid-crystalline bicellar phases. These bicelles are commonly used at concentrations of 3–7.5 w/v** to measure residual anisotropic interactions as structural constraints in liquid-state NMR spectroscopy (De Alba and Tjandra, 2002; Tjandra and Bax, 1997; Glover et al., 2001a; Ottiger and Bax, 1998; Freedberg, 2002). The dissolved biomolecules diffuse rather freely in the solvent, resulting in NMR spectra with similar spectral resolution to spectra from an isotropic phase (Tjandra and Bax, 1997; Bax and Tjandra, 1997). The anisotropy of the molecular alignment tensor obtained for the dissolved proteins typically has a magnitude of 10^{-3} (Tjandra and Bax, 1997; Ottiger and Bax, 1998), approximately two orders of magnitude smaller than the ones for membrane-associated proteins. The residual dipolar couplings are of the same order of magnitude as the J-couplings and the spectra can often be described in the weak coupling approximation.

One route to improve the spectral resolution for peptides bound to bicelles is the use of fast tumbling small bicelles (Vold et al., 1997; Luchette et al. 2001) with [DMPC]/[DHPC] = 0.1–1.0, although at the cost of having smaller diameter bicelles, which may be disadvantageous in particular for larger membrane-

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**The concentrations are expressed in grams of the lipids for 100 ml of the aqueous solvent.

associated proteins. In such systems high-resolution isotropic spectra have been observed (Glover et al., 2001b; Vold et al., 1997; Chou et al., 2002; Auger et al., 2002), however the information related to the anisotropic spin interactions is lost.

In the following, we discuss the application of macroscopically aligned bicelles as model membranes and we attempt to achieve high resolution by magic-angle sample spinning (MAS) of the bicellar phase (Tian et al., 1999; Zandomeneghi et al., 2003). With two-dimensional correlation spectroscopy, the anisotropic information (residual dipolar coupling and chemical shift anisotropy) is retained, maintaining the high resolution of the MAS spectrum. The experiments are based on the observation that the orientation of the liquid-crystalline director can be reoriented by sample-spinning techniques (Tian et al., 1999; Zandomeneghi et al., 2001). A detailed study of the bicelle orientation, in particular the mosaic spread, under different spinning conditions is presented in an accompanying paper (Zandomeneghi et al., 2003). If the experiments are designed properly, in particular if the spinning axis is oriented at, or close to, the magic angle only for a limited time interval, the liquid-crystalline ordering can be well preserved during the experiment (Zandomeneghi et al., 2001).

In this work we apply variable-angle spinning and switched-angle spinning ^{1}H NMR techniques to a bicelle sample containing leucine-enkephalin (Lenk, Tyr-Gly-Gly-Phe-Leu), an endogenous opioid peptide bound to the surface of the bicelles (Paterson et al., 1983; Sargent and Schwyzer, 1986; Milon et al., 1990). The main emphasis of the work is a demonstration of a novel technique. For a detailed structural study of Lenk, additional experiments are required.

Materials and methods

Sample preparation

Fifty milligrams of lipids (1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) and 1,2-Ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC), [DMPC]/[DHPC] = 3) were mixed with 140 μl of phosphate buffer in deuterated water (70 mM sodium phosphate, 30 mM KCl, pD = 7.0, pH meter not corrected for isotope effects). The sample was subjected to 3 cycles of vortexing (2 min), heating to 40 °C (20 min), vortexing (2 min), cooling to 0 °C (20 min). A 100 mM stock solution of leucine-enkephalin in buffer was prepared and 60 μl were added to the bicelle solution. The sample was again subjected to the procedure of vortexing, heating to 40 °C, vortexing and cooling to 0 °C. Lipids were purchased by Avanti Polar Lipids (Alabaster, AL) and D_2O from Cambridge Isotopes Lab (Cambridge, MA). Lenk was purchased from Sigma (Buchs, Switzerland) in the acetate salt form. The compound was dissolved in 0.1% trifluoroacetic acid and lyophilized. Freshly prepared bicelle solutions were used for all experiments.

NMR experiments

NMR experiments were performed on a Bruker DMX 400 spectrometer at a magnetic field of 9.4 Tesla. ^{1}H and ^{2}H spectra were obtained with a doubly-tuned home-built switched-angle spinning probehead (van Beek, 2002), using 4 mm zirconium MAS rotors. The orientation of the rotation axis was controlled by a servo motor (Schneider Automation, North Andover, MA), connected via Kevlar strings to the stator containing the Helmholtz coil. The angle-switching was done in 28 ms (for $\Delta\Theta = 54.7°$) and the setting of the spinning angle was precise to 1°. ^{1}H spectra were acquired using 90° pulses of 8 μs and a recycle delay of 4 s. Deuterium NMR experiments were performed with 90° pulses of 11.2 μs and a recycle delay of 2 s. Spectra have been processed with Felix 97.2 (Accelrys, CA).

Results and discussion

We have observed that the orientational behavior of the bicellar phase, as characterized in an accompanying paper (Zandomeneghi et al., 2003), is not substantially altered by the presence of Lenk at a molar ratio of [Lenk]/[DMPC]=1/10. This finding is consistent with the results from earlier studies (Sanders and Landis, 1994, 1995). Between 303.5 K and 323 K, the bicelles self-orient in the magnetic field, as witnessed by the appearance of two sharp lines in the ^{31}P NMR spectrum at positions of −9.13 ppm (DMPC) and −3.07 ppm (DHPC) at 308 K.

At 305.5 K the bicellar order parameter S_{Bic} (Sanders et al., 1994; Zandomeneghi et al., 2003) for the system bicelle/Lenk was measured to be $S_{Bic} = 0.538$. From a lineshape-analysis of the DHPC and DMPC signals, we have determined the mosaic spread of a static sample to be (Zandomeneghi et al., 2003) 3.3° ± 0.2°. The ^{2}H NMR residual quadrupole splitting of the solvent ^2H_2O is also a measure of the

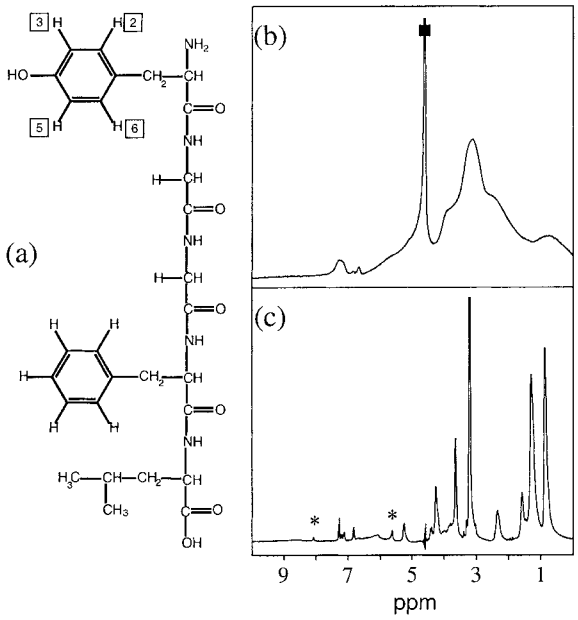

Figure 1. (a) Primary structure of leucine-enkephalin (Lenk) with nomenclature for the protons of the Tyr-1 ring. (b, c) ^{1}H NMR spectra of the DMPC/DHPC mixture containing Lenk taken at T = 305.5 K. (b) ^{1}H NMR of a static sample. The square-symbol indicates the signal of HDO. (c) ^{1}H NMR spectrum obtained under magic-angle spinning at 980 Hz. A selective irradiation of the water peak abolishes the solvent signal by saturation. The asterisks indicates the choline spinning sidebands.

orientation of the sample: at 305.5 K a splitting of 28 Hz is observed.

In Figure 1b the ^{1}H NMR spectrum of a static Lenk-containing sample at 305.5 K is reported. The spectral appearance is dominated by the high-intensity lipid signals. The resonances are relatively broad, mostly as a consequence of the homonuclear dipolar coupling (Forbes et al., 1988). Nevertheless, significant dynamics are present in the system and the anisotropic spin interactions within the lipids are much smaller than in a corresponding rigid sample (vide infra). The peaks in the region between 6.5 and 7.5 ppm arise from protons in the aromatic sidechains of Lenk (vide infra). It is known that Lenk is in a rapid dynamic equilibrium between a bilayer-associated and a dissolved state (Rinaldi et al., 1997; Deber and Behnam, 1984). The broad linewidth of the Lenk resonances suggests that the fraction of peptide associated to the bicelles is high, as reported earlier by Sanders and Landis (Sanders and Landis, 1994, 1995). From the ^{13}C chemical-shift position in isotropic bicelle solutions ([DMPC]/[DHPC]=0.5), measured as a function of the concentration ratio [Lenk]/([DMPC]+[DHPC])

Table 1. Assignment of ^{1}H NMR MAS spectrum of a sample containing bicelles and Lenk at 305.5 K

Signals	Chemical shift (ppm)
Lipids: Terminal C$\underline{H}_3$	0.94
Lipids: (C$\underline{H}_2$)$_n$	1.35
Lipids: C$\underline{H}_2$-C-CO	1.64
Lipids: C$\underline{H}_2$-CO	2.40
Lipids: C$\underline{H}_2$-O-CO	~4.5
Lipids: C$\underline{H}$-O-CO	5.33
Lipids: C$\underline{H}_2$-O-P	~4
Lipids: P-O-C$\underline{H}_2$	4.33
Lipids: C$\underline{H}_2$-N	3.71
Lipids: N(C$\underline{H}_3$)$_3$	3.27
Lenk: C$_\beta\underline{H}_2$/C$_\gamma\underline{H}$(Leu)	1.53
Lenk: C$_\alpha\underline{H}_2$(Gly2/Gly3)	~3.9
Lenk: Ar$\underline{H}_{3/5}$(Tyr)	6.89
Lenk: Ar$\underline{H}_{2/6}$(Tyr)	7.19
Lenk: Ar(Phe)	7.26
Lenk: Ar(Phe)	7.34

(Deber and Behnam, 1984), the fraction of membrane-associated Lenk at [Lenk]/([DMPC]+[DHPC])=1/13 was determined to be f = 0.91 ± 0.04.

The MAS spectrum of the same sample is reported in Figure 1c and shows much narrower lines. By comparison with the ^{1}H NMR spectra of Lenk in water (Milon et al., 1990) and in organic solvent solutions (Picone et al., 1990) a tentative assignment of the Lenk peaks was made (Table 1). The assignment of the lipids ^{1}H peaks, also reported in Table 1, is based on a TOCSY spectrum of a micellar solution of DHPC (spectrum not shown).

To supplement the high-resolution information contained in the MAS spectra with the correlated anisotropic information, two-dimensional switched-angle spinning (2D-SAS) spectra were recorded (Zandomeneghi et al., 2001). The experimental scheme is shown in Figure 2a: During t_1 the sample is rotated around the direction of the magnetic field ($\Theta = 0°$) and during t_2 around the magic angle. It is shown in a companion paper (Zandomeneghi et al., 2003) that the increase in mosaic spread is small under sample spinning, as long as the spinning angle is not close to the magic angle for a prolonged period of time. Therefore, the SAS experiment of Figure 2a was designed such that the sample is spun around the direction of the magnetic field for all time periods except for t_2,

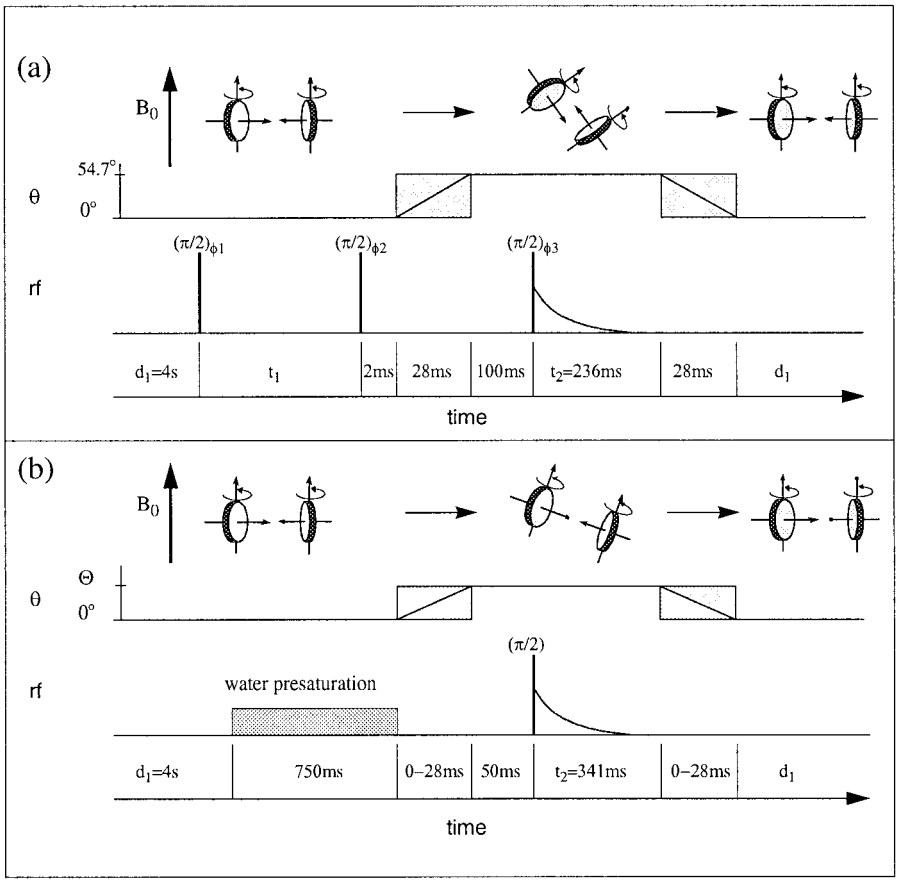

Figure 2. Scheme of (a) two-dimensional and (b) one-dimensional SAS experiments. Experiment (a) is acquired in phase-sensitive mode. Phases cycles are: ϕ_1: x, −x; ϕ_2: $(x)_8$, $(-x)_8$; ϕ_3: $(x)_2$, $(-x)_2$, $(y)_2$, $(-y)_2$; ϕ_{aq}: x, $(-x)_2$, x, y, $(-y)_2$, y, −x, $(x)_2$, −x, −y, $(y)_2$, −y). The phase cycle for the first pulse is incremented according to TPPI. In (a) the length of the evolution period was incremented in 256 steps of 56.8 μs each, giving a spectral width of 8.8 kHz in the ω_1 dimension. Spectral width in ω_2 dimension was 8.8 kHz with 2048 real and imaginary points. For each t_1 increment 16 transients were recorded. A delay of 50 ms between the angle-switching and the acquisition of the FID guaranteed a NMR signal free from distortions due to the mechanical flip. In (b) the duration of the flipping pulse depends on the angle Θ, varying between 0 and 28 msec (corresponding to $\Theta = 0°$ and $\Theta = 54.7°$).

when the FID is recorded. The resulting spectrum is shown in Figure 3.

For spinning at $\Theta = 0°$the bicellar director is oriented orthogonal to the magnetic field. Accordingly, the projection of the 2D-SAS spectrum onto the w_1 axis leads to the exact same spectrum as observed for a static sample (compare Figure 1b and Figure 3). During t_2, the sample rotates at the magic angle and the projection onto ω_2 leads to the high-resolution spectrum of Figure 1c with isotropic interactions only. Measurement of the ^{2}H NMR spectrum of the solvent ^2H_2O before and after the SAS experiment showed that the residual quadrupolar coupling and the lineshape have not changed, confirming that the order parameter and the mosaic spread of the system are not affected significantly by the SAS experiment.

For Lenk, as well as for the lipids, the two-dimensional correlation spectrum clearly contains more information than the 1D spectra of Figure 1. An expansion of the aromatic region of the SAS spectrum is shown in Figure 4. In the isotropic dimension the two doublets at 6.89 ppm and 7.19 ppm are assigned to the Tyr-1 aromatic protons of Lenk in positions 3, 5 and 2, 6, respectively. The splitting in the isotropic dimension, due to J-coupling between $^1H_2$ and $^1H_3$ and between $^1H_5$ and $^1H_6$, is 9 ± 2 Hz ($J_{2,3} = J_{5,6}$). In the anisotropic dimension a doublet with a splitting of 68 ± 10 Hz is present at the chemical shift of $^1H_{3/5}$. The peak due to $^1H_{2/6}$, with an isotropic chemical shift of 7.19 ppm, is also well resolved in the two-dimensional spectrum and appears in the anisotropic dimension as one line without a resolved splitting. The

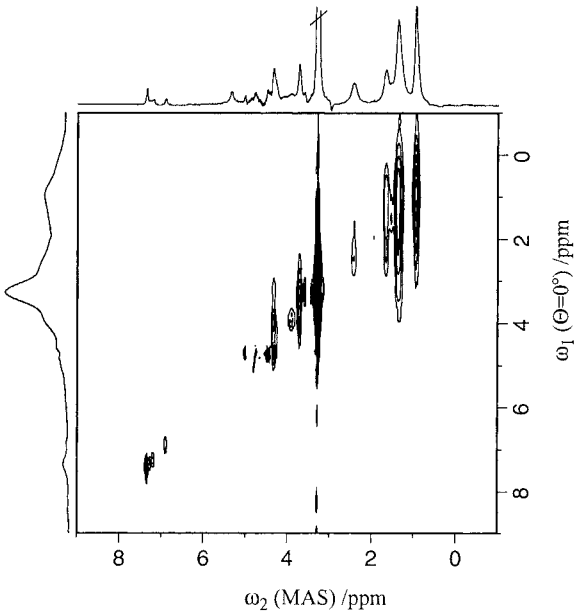

Figure 3. ^{1}H 2D-SAS spectrum. The spinning frequency was 130 Hz, the temperature 305.5 K. Other experimental conditions are described in Figure 2a. The water signal was suppressed in the processing of the data by time domain deconvolution.

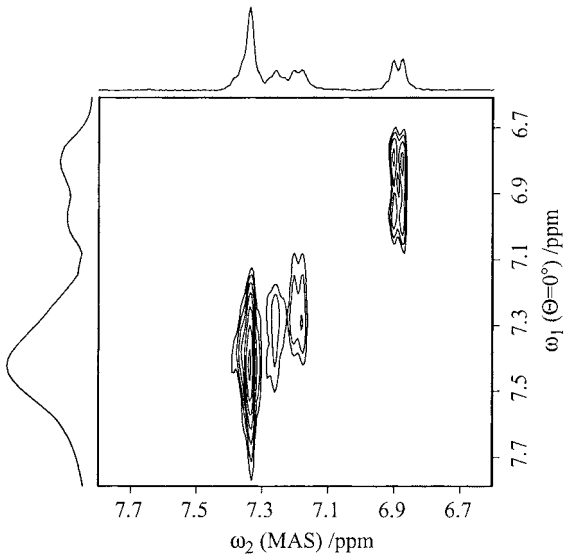

Figure 4. Expansion of the aromatic region in the spectrum reported in Figure 3.

resonances at 7.26 ppm and 7.34 ppm are assigned to the aromatic protons of Phe-4 and show no resolved splittings in the w_1 dimension. The signal of $^1H_2$ and $^1H_6$ cannot be interpreted as a simple doublet: The proximity with Tyr-1 $^1H_\beta$, $^1H_{\beta'}$ and with $^1H_3$, $^1H_5$ produces a complex spectrum, which appears as a single, unresolved line. In the w_1 aliphatic region, the $^1H_\beta$ and $^1H_\gamma$ of Leu-5 exhibit a narrow peak at 1.53 ppm. The multiplet with an isotropic shift of approximately 3.9 ppm is due to the alpha protons of the Gly-2, -3. In the w_1 dimension, the signal appears as a doublet with a splitting of about 100 Hz.

The lipids signals from to the phospholipid headgroup (POC$\underline{H}_2$ and C$\underline{H}_2$N with isotropic chemical shifts of 4.33 ppm and 3.71 ppm) exhibit broad doublets in the anisotropic dimension with splittings of 280 ± 20 Hz and 230 ± 20 Hz respectively, arising from the homonuclear dipolar interaction between the geminal protons (Seelig et al., 1977; Hong et al., 1995). The choline N(C$\underline{H}_3$)$_3$ signal at 3.27 ppm displays a single, symmetric, and relatively narrow signal in the anisotropic dimension because the free rotations around the C_β-N and N-C_γ bonds further reduce the anisotropic spin interactions (Seelig et al., 1977). Signals arising from the glycerol protons are broad due to the ^{1}H-^{1}H dipolar couplings, which are typically larger than those found in the more dynamic headgroup (Seelig et al., 1977).

The dependence of the Tyr-1 $^1H_{3/5}$ signals from Lenk on the spinning angle Θ in the range $0° \leq \Theta \leq 54.7°$ has been studied more extensively in a series of 1D-SAS experiments schematically described in Figure 2b. The sample is spun parallel to the magnetic field, then the spinning axis is flipped to the angle Θ and the NMR signal is recorded. The spinning angle Θ has varied between 0° and the magic angle. The bicelles director is maintained perpendicular to the spinning axis for each angle Θ. The aromatic region (6.0 to 8.5 ppm) of the 1D-SAS spectra is shown in Figure 5. The splitting of the Tyr-1 signals $^1H_{3/5}$ is strongly Θ-dependent. This dependence comes from the dipolar contribution to the splitting, which is the sum of scalar (J) and dipolar (D) couplings and has the angle dependence $\Delta\nu = J + D \cdot P_2(\cos\Theta)$, with the second-order Legendre polynomial $P_2(\cos\Theta) = (3\cos^2\Theta - 1)/2$.

The observed experimental splitting $\Delta\nu$ between protons 2,3 and 5,6 is plotted against $P_2(\cos\Theta)$ (for $\Theta < 44°$) in Figure 6. The experimental data can best be described with the function: $\Delta\nu = 5.5(1.3) - 68.6(1.7) \cdot P_2(\cos\Theta)$. The fitted J-coupling of 5.5 ± 1.3 Hz corresponds well to the observed splitting at the magic angle, 6.5 ± 1.5 Hz. The observed splittings give only the magnitude of $\Delta\nu$. Since the J-coupling is known to be positive (Hesse et al., 1991) we can immediately find the sign of the dipolar splitting to be negative.

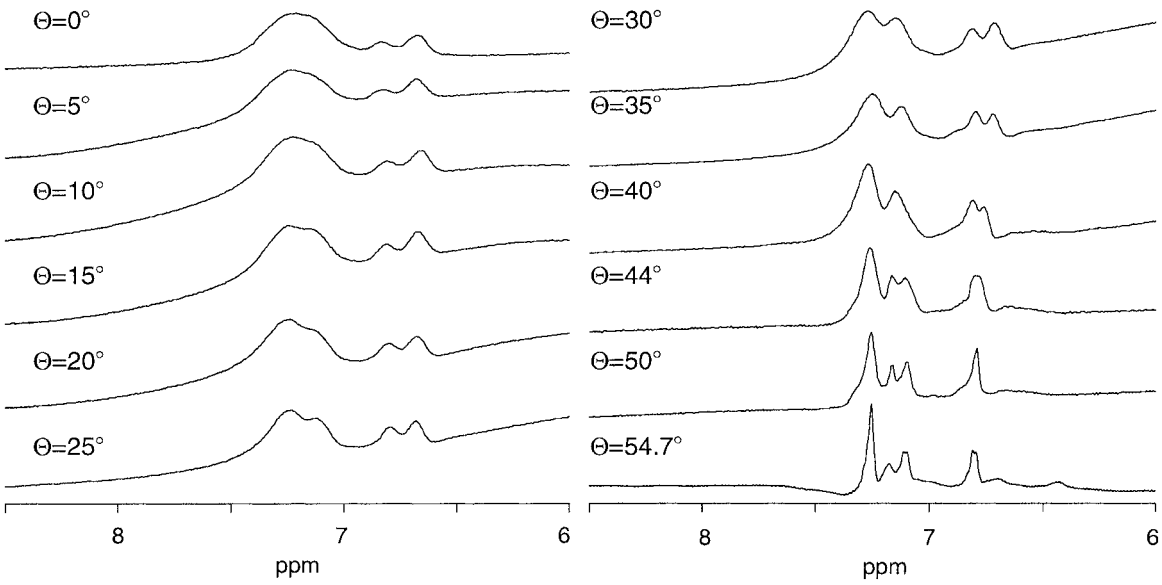

Figure 5. ^{1}H 1D-SAS spectra showing the aromatic region of Lenk as a function of the angle Θ. The spinning frequency was 700 Hz the temperature 305.5 K. Other experimental conditions are described in Figure 2b.

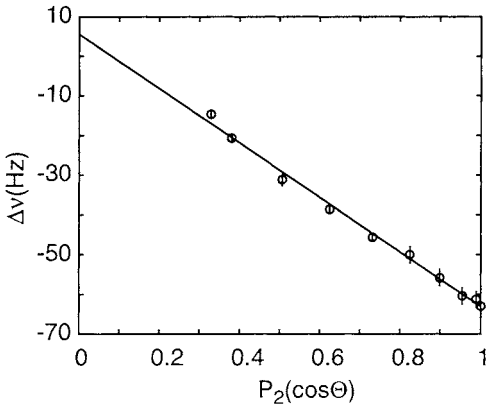

Figure 6. For each 1D-SAS experiment in Figure 5, for $\Theta < 44°$, the splittings of the tyrosine protons $^{1}H_3$ and $^{1}H_5$ are reported at the corresponding value of $P_2(\cos\Theta)$. The solid line represents $\Delta\nu = 5.5 - 68.6 \cdot P_2(\cos\Theta)$.

It should be noted that the data presented in Figure 5 can not be obtained by 1D experiments where the angle of the rotation axis is held at Θ. These VAS spectra showed a significant reduction in resolution and the data did not observe a linear dependence on $P_2(\cos\Theta)$, due to an increase in mosaic spread for small values of $P_2\cos(\Theta)$ (Zandomeneghi et al., 2003).

The value and the sign of $D_{2,3}$, $D_{5,6}$ can provide information about the orientation of the intramolecular vectors $^{1}H_3$-$^{1}H_2$ and $^{1}H_5$-$^{1}H_6$ and, therefore, of the tyrosine aromatic ring. The angle α_{ij} between the vector i-j and the bicellar director, is given by

$$D_{ij} = \frac{\mu_0}{8\pi^2}\frac{\gamma_H^2\bar{h}}{r_{ij}^3} f \cdot S_{Bic}\frac{\langle 3\cos\alpha_{ij}^2 - 1\rangle}{2}, \qquad (1)$$

where γ_H is the proton gyromagnetic ratio, $\hbar$ is the reduced Planck constant, r_{ij} is the internuclear distance between i and j, f is the fraction of peptide bound to the oriented bilayer, and the brackets $\langle\rangle$ denote time-averages. With $f = 0.91$, $r = 2.49$ Å and $S_{Bic} = 0.538$, we calculate $(\langle 3\cos\alpha_{ij}^2 - 1\rangle/2) = -0.018$.

The relatively small size of $D_{2,3}$ and $D_{5,6}$ indicates that one or several of the following conditions is fulfilled: (i) the angle between the internuclear vector and the bicelle normal is close to the magic angle, (ii) the tyrosil sidechain is mobile with respect to the Lenk backbone (Sanders and Landis, 1995) and (iii) there is considerable orientational mobility of Lenk with respect to the bicelle (Prosser et al., 1999).

The negative sign of $D_{3,2} = D_{5,6}$ and, therefore, the negative value of $\langle 3\cos\alpha_{ij}^2 - 1\rangle/2$ limits the set of possible orientations of the tyrosil ring with respect to the bicelle director but detailed molecular information can only be obtained by the simultaneous measurement of a significant number of coupling constants. The synthesis of ^{15}N and ^{13}C labelled compounds to perform these measurements is currently under way.

Conclusions

The macroscopically aligned liquid-crystalline bicellar phase composed of Lenk, DMPC and DHPC can be maintained under different director orientations with respect to the magnetic field direction. This orientational variation has been exploited to scale all second rank, anisotropic spin interactions by a factor of $P_2(\cos\Theta)$ compared to those observed in the static sample.

We have applied these properties in a two-dimensional switched-angle spinning experiment where the anisotropic and the isotropic spectra are correlated. In the 2D spectra it is possible to resolve the broad resonances due to anisotropic interactions of the lipids and to assign several resonances arising from Lenk in a static sample. ^{1}H-^{1}H dipolar couplings characterizing the headgroup of phospholipid and some residues of Lenk have been determined.

The experiments presented are mainly intended as a demonstration of a novel technique. As usual in the structure evaluation by residual dipolar couplings, the simultaneous observations of many couplings is necessary to obtain a structure.

These switched-angle spinning methods can easily be extended to heteronuclear and three-dimensional experiments, e.g. a ^{15}N-^{1}H HSQC with an additional ^{1}H dimension recorded at a different angle. Applications to ^{13}C and ^{15}N spectroscopy are also possible and these experiments could be particularly useful in systems with strong ^{1}H-^{1}H interactions.

Similar methods may also be applicable to a wide variety of macroscopically aligned systems: for instance, small bicelles with ([DMPC]/[DHPC] = 0.1–1.0.), which do not orient spontaneously but can be oriented using polyacrylamide gels (Tycko et al., 2000; Chou et al., 2001, 2002), lipid membrane mechanically aligned on glass plates (Glaubitz and Watts, 1998) or filamentous bacteriophage particles (Hansen et al., 1998). In such samples, 1D SAS experiments can select the optimum ratio of dipolar and J-interactions and 2D-SAS experiments can be used to reduce spectral overlap.

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