

# Manipulation of the Director in Bicellar Mesophases by Sample Spinning: A New Tool for NMR Spectroscopy

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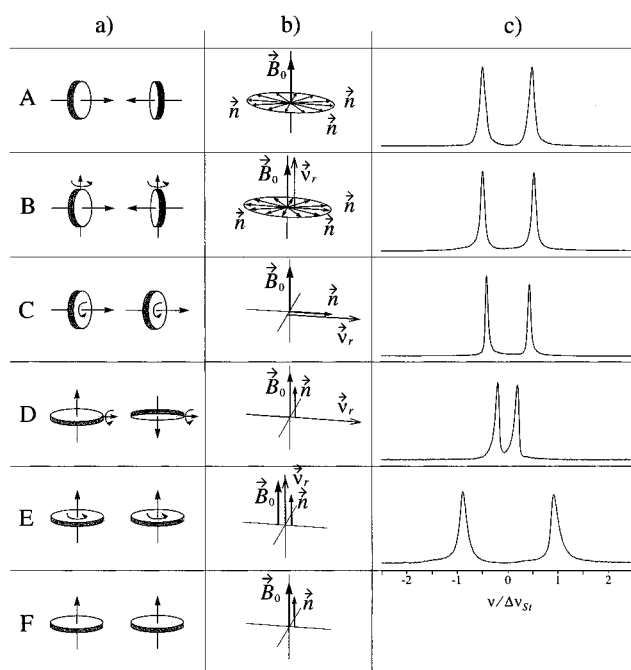
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**Abstract:** It is shown that bicellar nematic liquid-crystalline phases can be oriented with the director (the normal to the bicellar plane) at an arbitrary angle to the applied magnetic field by sample rotation around one axis (variable-angle sample spinning) or around two axes successively (switched-angle spinning). This promises to open novel possibilities for NMR studies of bicelles and proteins incorporated into bicelles or dissolved in a solution containing bicelles, including the correlation of several orientations in a two-dimensional NMR experiment.

## Introduction

Over the past few years bicelles were introduced as model membranes for the study of structural and dynamic properties of membrane-bound molecules, e.g., proteins.<sup>1</sup> Bicelles are disk-shaped structures formed by two phospholipids with different chain length: a bilayer of long-chain phospholipids forms the plane of the disk whose edge is covered by short-chain phospholipids. We used samples with 33% w/v lipids in aqueous solution with a molar ratio of the “long chain” 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) to the “short chain” 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) of 3.5. Around 35 °C a lyotropic liquid-crystalline phase of bicelles is formed.<sup>1</sup> Due to the negative anisotropy of the diamagnetic susceptibility,  $\Delta\chi$ , the energetically favorable orientation of the liquid-crystalline director  $\vec{n}$  is perpendicular to the magnetic field  $\vec{B}_0$ .<sup>1</sup> For NMR spectroscopy these magnetically oriented systems are a promising alternative to oriented bilayers on glass plates.<sup>2</sup> The latter can, however, easily be oriented with  $\vec{n}$  at an arbitrary angle with respect to  $\vec{B}_0$  while the “natural” bicelle orientation is  $\vec{n} \perp \vec{B}_0$  (Figure 1A). This is not always a desirable orientation and for the NMR study of membrane-bound molecules it is beneficial to investigate several orientations with the single most important being the one with  $\vec{n} \parallel \vec{B}_0$  (Figure 1F).<sup>3</sup> The addition of trivalent paramagnetic lanthanide ions (with a positive  $\Delta\chi$ ) induces the orientation  $\vec{n} \parallel \vec{B}_0$ .<sup>3,4</sup> Adding lanthanides, however, can cause paramagnetic shifts and broadening of resonance lines.

From studies of liquid-crystalline phases it is known that  $\vec{n}$  can be reoriented by sample rotation around an axis  $\vec{v}_r$  inclined by an angle  $\Theta$  with respect to  $\vec{B}_0$ .<sup>5</sup> For spinning frequencies  $\nu_r$  larger than a critical frequency,<sup>6</sup> which depends on the strength



**Figure 1.** Column a: View of the bicelle orientation in the respective phase discussed. Different disks represent the preferred bicelle orientation for coexisting domains. Column b: Orientation of the magnetic field  $\vec{B}_0$ , the director  $\vec{n}$ , and the sample spinning axis  $\vec{v}_r$ . Column c: Experimental  $D_2O$  spectra. In the rotating and the static experiments the samples were allowed to equilibrate until a constant order parameter was obtained. Its value depends slightly on the exact temperature and the sample history. Therefore, the frequency scale is given in units of the quadrupole splitting  $\Delta\nu_{Si}$  measured in a static experiment immediately after the spectrum shown. Rows A to F refer to a static sample (A), a rotating sample at  $\Theta = 0^\circ$  (B), a rotating sample at  $\Theta = 80^\circ$  with  $\nu_r$  smaller than the second critical frequency (C), the same as row C but rotating faster or the metastable phase after a flip from  $0^\circ$  to  $80^\circ$  (spectrum shown obtained after a flip) (D), and the metastable phase obtained after a change from  $\Theta = 80^\circ$  to  $0^\circ$  (E). This phase has the same orientation as a static sample doped with lanthanide ions (F). The spectra are plotted on a reduced frequency scale  $\Delta\nu/\Delta\nu_{Si}$ . Spectra were recorded at a  $^2H$  resonance frequency of 61.4 MHz. For the schematic representation in column a and b the angle of  $\Theta = 80^\circ$  was drawn as  $90^\circ$ .

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of the magnetic field, on  $\Delta\chi$ , and on the Leslie viscosity coefficient,<sup>7</sup>  $\vec{n}$  orients such that the magnetic energy per unit volume, averaged over a rotor cycle,  $E_{\text{mag}}(\Theta, \delta)$ , is minimized:<sup>5</sup>

$$E_{\text{mag}}(\Theta, \delta) = -\frac{\Delta\chi}{3\mu_0} B_0^2 \cdot \left( \frac{3 \cos^2 \delta - 1}{2} \right) \left( \frac{3 \cos^2 \Theta - 1}{2} \right) \quad (1)$$

$\delta$  is the angle between  $\vec{v}_r$  and  $\vec{n}$ , and  $\mu_0$  is the vacuum permeability. The critical spinning frequency is typically found to be smaller than 100 Hz.<sup>5,6</sup> For  $\Delta\chi < 0$ , two regimes must be distinguished: in the range  $0^\circ \leq \Theta < 54.7^\circ$  the energy is minimized for  $\delta = 90^\circ$  ( $\vec{n} \perp \vec{v}_r$ ). For the range  $54.7^\circ < \Theta \leq 90^\circ$  the minimum is at  $\delta = 0^\circ$  ( $\vec{n} \parallel \vec{v}_r$ ). At the magic angle, the magnetic energy is independent of the director orientation and no magnetic torque exists that would tend to orient the bicelles. There can be a distribution of angles  $\delta$ ,<sup>8</sup> which, however, will be neglected in the following. The effect of sample spinning on the magnetic order of bicellar systems was experimentally confirmed for angles  $\Theta < 54.7^\circ$ .<sup>8</sup>

Here we report a  $^2\text{H}$  NMR study on bicellar liquid-crystalline phases in a sample spun at different angles  $\Theta$  with respect to  $B_0$ , in particular also for the range  $\Theta \geq 54.7^\circ$ . We investigate the possibility of orienting the bicelles with  $\vec{n}$  at an arbitrary orientation with respect to  $B_0$ . Switched-angle spinning experiments (SAS)<sup>9</sup> are used which allow one to investigate phases with a metastable director orientation.

## Experimental Section

**Sample Preparation.** Fifty milligrams of lipids (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), [DMPC]/[DHPC] = 3.5) were mixed with 150  $\mu\text{L}$  of  $\text{D}_2\text{O}$ . The following procedure was repeated 3 times: vortexing (2 min), curing at 40  $^\circ\text{C}$  (20 min), vortexing (2 min), curing at 0  $^\circ\text{C}$  (20 min). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and  $\text{D}_2\text{O}$  from Cambridge Isotopes Lab (Cambridge, MA).

**NMR Experiments.**  $^2\text{H}$  NMR measurements were performed at magnetic fields of 9.4 and 7 T. The experiments at 9.4 T ( $^2\text{H}$  resonance frequency of 61.4 MHz) were done on a Bruker DMX 400 spectrometer, using a home-built switched-angle spinning probehead with 4 mm plexiglass MAS rotors. The  $90^\circ$  pulse length was set to 16  $\mu\text{s}$ . The pulse length is the same at all angles  $\Theta$  because a Helmholtz-type coil arrangement is used. Four scans were accumulated per spectrum with a 3 s repetition delay, except for the SAS experiments where a single scan was Fourier transformed. In the SAS experiment the angle switching was done in 50 ms (40 ms switching time and 10 ms settling time), using a servo motor (Schneider Automation, North Andover, MA) controlled by the pulse programmer. Experiments at 7 T ( $^2\text{H}$  resonance frequency of 45.7 MHz) were performed on a home-built spectrometer<sup>10</sup> with a home-built SAS probehead,<sup>11</sup> using 6 mm plexiglass rotors with a volume restricted to 20  $\mu\text{L}$ .  $90^\circ$  pulses of 26  $\mu\text{s}$  were used, produced by a Helmholtz-type coil. All measurements were done at 35  $^\circ\text{C}$ .

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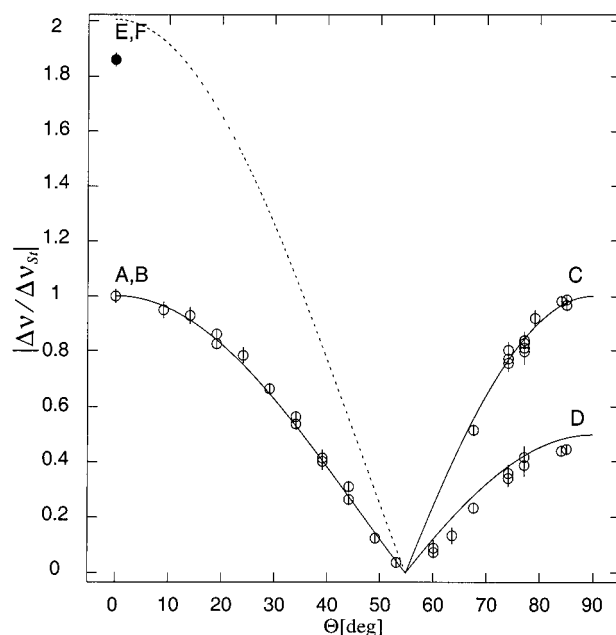
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**Figure 2.** Dependence of the experimental quadrupole splitting (in units of the static splitting  $\Delta\nu_{\text{st}}$ ) under identical conditions as a function of the angle  $\Theta$  between the spinning axis and the magnetic field. Experimental data points measured at a  $^2\text{H}$  resonance frequency of 45.7 MHz are given by open circles. The filled circle is from a SAS experiment, recorded at a  $^2\text{H}$  resonance frequency of 61.4 MHz (see text). The solid lines denote the theoretical curves mentioned in the text, the dashed line the theoretical curve for a lanthanide-doped sample or a sample prepared by SAS. Letters A to F refer to the phases of Figure 1.

## Results and Discussion

The deuterium quadrupole splitting of the solvent  $\text{D}_2\text{O}$  was used as a probe for the bicelle orientation. The solvent water becomes partly oriented through interaction with the oriented bicelles.<sup>12</sup> Therefore, the  $^2\text{H}$  NMR spectrum of  $\text{D}_2\text{O}$  in oriented static phases,  $\vec{n} \perp B_0$ , consists of a doublet split by

$$\Delta\nu_{\text{st}} = -\frac{3}{8\pi} Q_{33} S_{33} \quad (2)$$

where  $Q_{33}$  is the component of the motionally averaged nuclear–quadrupole interaction tensor along the director  $\vec{n}$ <sup>13</sup> and  $S_{33}$  is the order parameter.<sup>14</sup> For a rotating sample the splitting is<sup>12</sup>

$$\Delta\nu = \frac{3}{4\pi} Q_{33} S_{33} P_2(\cos \delta) P_2(\cos \Theta) \quad (3)$$

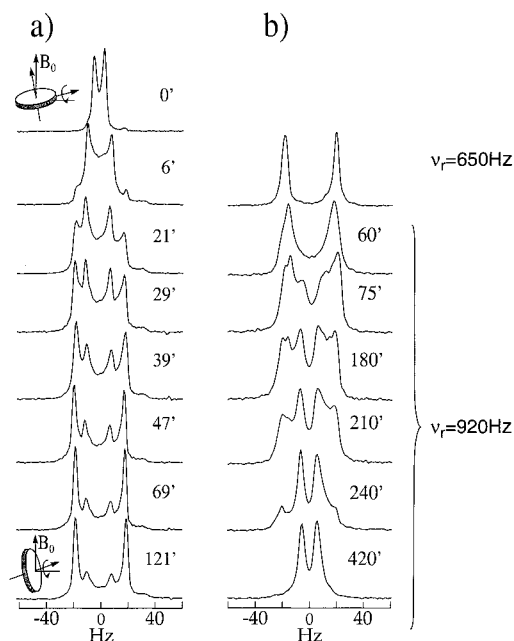
where  $P_2(\cos \gamma) = (3 \cos^2 \gamma - 1)/2$  is the second-order Legendre polynomial.

In Figure 2 we plot the experimental quadrupole splitting  $\Delta\nu$  as a function of  $\Theta$ , relative to the splitting for the static sample ( $\Delta\nu_{\text{st}}$ ) under identical conditions. Spectra were recorded after the phase was allowed to equilibrate until the splitting became time-independent. For spinning frequencies in the range of 150–900 Hz, the splitting was found to be independent of the spinning frequency, within experimental error. For a sample spinning at  $0^\circ$  (which does not influence the spin Hamiltonian)

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**Figure 3.** (a) Time dependence of the experimental spectrum at  $\Theta = 79^\circ$  with  $\nu_r = 620$  Hz. The freshly prepared sample was in the orientation of Figure 1D. With time (indicated in minutes), this phase slowly vanished while the phase corresponding to Figure 1C grows in. (b) Spinning frequency dependence. The sample is prepared by overnight spinning with  $\nu_r = 650$  Hz leading to the director orientation of Figure 1C. Increasing  $\nu_r$  to 920 Hz leads progressively to the orientation of Figure 1D but the phase behavior is less than ideal. The times (indicated in minutes) refer to the time after the increase of the spinning frequency. Spectra were recorded at a  $^2\text{H}$  resonance frequency of 45.7 MHz.

the ratio  $\Delta\nu/\Delta\nu_{\text{St}}$  was found to be one within experimental error (see also Figure 1, rows B and A, respectively) suggesting that the order parameter does not change upon sample rotation. Therefore, eq 3 simplifies to  $\Delta\nu = P_2(\cos \Theta) \cdot \Delta\nu_{\text{St}}$  (solid line, in Figure 2). The experimental points follow the theoretical curve within experimental error.

For  $\Theta > 54.7^\circ$  our experimental spectra at spinning frequencies below 900 Hz consisted of either one or two doublets, depending on the condition and sample history. When two doublets were detected, their splitting differed by a factor of 2 (Figure 2). The splittings follow quite closely either  $\Delta\nu = -2P_2(\cos \Theta) \cdot \Delta\nu_{\text{St}}$  or  $\Delta\nu = P_2(\cos \Theta) \cdot \Delta\nu_{\text{St}}$ , which are special cases of eq 3 for  $\delta = 0^\circ$  and  $90^\circ$ , respectively.

Spectra recorded with  $\Theta = 79^\circ$  are shown in Figure 3a) as a representative example for the behavior observed for angles  $\Theta$  larger than the magic angle. A freshly prepared sample, rotating with 620 Hz, consisted of a single phase (corresponding to  $\delta = 90^\circ$ ). With time, a second phase with twice the splitting (corresponding to  $\delta = 0^\circ$ ) begins to appear. Also seen in the spectra is an initial increase in doublet splitting within the first 20 min after the sample has been inserted into the magnet. This ordering is a consequence of an increase in order parameter with time. After this initial period, the splittings (and the order parameter) stay constant but the relative contribution of the two phases varies. The spectra of Figure 2 were taken in the regime with a constant order parameter. The growth of the  $\delta = 0^\circ$  phase is described by an exponential with a time constant of 22 min and a single phase is obtained after some hours. The spectra of the two phases are always consistent with a director orientation as shown in Figures 1C ( $\delta = 0^\circ$ ) and 1D ( $\delta = 90^\circ$ ) and a constant order parameter. For angles  $70^\circ < \Theta \leq 90^\circ$  and spinning frequencies up to 900 Hz, the parallel orientation was

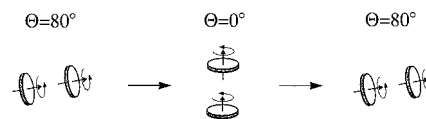
found to be the equilibrium one. It was observed that the evolution toward the orientation  $\delta = 0^\circ$  shown in Figure 3a is slower at smaller angles (but still exceeding the magic angle). A preliminary  $^{31}\text{P}$  chemical shift study fully corroborates the  $^2\text{H}$  results presented here. The  $^{31}\text{P}$  spectra should also allow one to assess the orientational distribution of the director neglected here.

Increasing the spinning frequency from 650 to 920 Hz leads to a gradual reconversion to the initial phase (Figure 3b). The phase behavior is less than ideal: spectra are more complicated than the sum of two doublets and the splitting after 420 min is significantly smaller than expected for  $\delta = 90^\circ$ .

Our observations are consistent with previous studies<sup>5,15</sup> on lyotropic liquid crystals with negative  $\Delta\chi$ . There it is also found that, for  $54.7^\circ < \Theta \leq 90^\circ$ ,  $\vec{n}$  is parallel to  $\vec{v}_r$ , when the spinning frequency is higher than the critical one but lower than a second "critical frequency". Upon exceeding the second critical frequency, the orientation of  $\vec{n}$  becomes perpendicular to  $\vec{v}_r$ . Sometimes, the two phases can coexist. This behavior could be explained by an additional torque that depends on the square of the spinning frequency. This torque is thought to stabilize the  $\delta = 90^\circ$  orientation. For angles  $0^\circ < \Theta \leq 54.7^\circ$  both torques would stabilize the same orientation. For angles  $54.7^\circ < \Theta < 90^\circ$  the second torque, which dominates at high spinning frequencies, would stabilize another orientation than the magnetic torque and lead to the second-phase transition. The effect is, however, not understood at present.<sup>15,16</sup> We have found, in our system, that this second torque prevails at angles  $\Theta$  where the magnetic torque, proportional to  $P_2(\cos \Theta)$ , is weak. In addition, we have observed that the second torque becomes smaller with the age of the sample (over days to weeks). This change is possibly connected with a decrease in the viscosity of the DMPC/DHPC system.

Our results show that the bicelles director can be manipulated by sample spinning. The orientation with respect to the spinning axis is  $\delta = 0^\circ$  or  $90^\circ$ . Only the  $\delta = 0^\circ$  orientation corresponds to a time-independent director orientation with respect to  $\vec{B}_0$ . An interesting case is  $\delta = 0^\circ$  and  $\Theta = 90^\circ$  (Figure 1C): as for a static sample,  $\vec{n}$  is orthogonal to  $\vec{B}_0$  but, in contrast to the static phase where  $\vec{n}$  is randomly oriented in the plane orthogonal to  $\vec{B}_0$ , it is now aligned with the rotation axis.

Despite the wealth of possible orientations that can be prepared by variable-angle sample spinning experiments, it is not possible to orient the bicelles with  $\vec{n} \parallel \vec{B}_0$  as in lanthanide-doped samples. However, the VAS results suggest how this can be achieved by switched-angle spinning experiments. After preparing the  $\delta = 0^\circ$  phase (Figure 1C), while spinning at  $\Theta_0 > 54.7^\circ$  (in the present experiments  $\Theta_0 = 80^\circ$ ), the spinning axis is switched to a direction parallel to  $\vec{B}_0$ : the bicelles end up in a metastable phase with  $\vec{n} \parallel \vec{B}_0$  (Figure 1E). During the switch, the magnetization can be stored along the magnetic field direction. We have found that the evolution of the orientation of the director from the metastable phase  $\vec{n} \parallel \vec{B}_0$  toward the stable phase  $\vec{n} \perp \vec{B}_0$  can be described by a double exponential, the shorter time constant being 0.6 s. The lifetime of the metastable phase is thus sufficiently long to record an NMR signal and switch back to  $\Theta_0 = 80^\circ$ , where the bicelles find themselves again in the stable orientation.



The spectrum obtained at  $\Theta = 0^\circ$  using the SAS scheme is

given in Figure 1E. The experimental quadrupole splitting ( $\Delta\nu = 100 \pm 2$  Hz) is slightly less (about 8%) than twice the splitting of the static sample ( $\Delta\nu_{\text{st}} = 54 \pm 1$  Hz) and corresponds to the metastable  $\delta = 0^\circ$  phase. The expected splitting for this phase is given in Figure 2 as a dashed line, the experimental data point is given by a filled circle. We attribute the slight difference between experiment and expectation to a decreased order parameter. We have accumulated 1024 spectra with the SAS technique (with a waiting time between scans of 20 s) and observed no further decrease in  $S_{33}$ . This finding indicates that the SAS technique on bicelles can also be employed if many transients must be accumulated. The line width of the  $^2\text{H}$  NMR signal in the metastable phase is about 13 Hz, probably determined by a mosaic spread in the orientation of the director around  $\delta = 0^\circ$ .<sup>8</sup> This spread already exists at  $\Theta_0 = 80^\circ$  where, however, it leads to a smaller broadening due to the smaller value of the second Legendre polynomial. At this line width, the finite lifetime (0.6 s) of the metastable phase does not influence the NMR line shape. For considerably narrower lines, the finite lifetime could lead to line broadening and the appearance of dispersive line shapes which can, in a simple approximation, be described with a two-site exchange model,<sup>17</sup> with only one site populated at time zero. For possible applications to proteins, this effect will probably only be relevant if the proteins are in the liquid phase (not membrane bound). Optimizing the bicelle system may allow one to achieve a metastable orientation with a longer lifetime.

Preliminary experiments showed that the addition of an integral membrane peptide (M4 transmembrane domain of the acetylcholine receptor with a weight ratio of peptide:DMPC = 4:30) to the bicellar solution does not change substantially the

properties of the system. In particular, it preserves the sign of the anisotropy of the susceptibility and all the experiments described above can be performed with this sample. Work toward the characterization of the protein resonances is currently underway.

## Conclusions

It is shown that it is possible to orient the bicelles with the director  $\vec{n}$  along the rotor axis and at an arbitrary and time-independent orientation with respect to  $\vec{B}_0$ . Furthermore, the director can be oriented perpendicular to the rotation axis at arbitrary angles between the rotation axis and the magnetic field. The (metastable) orientation with the director along the magnetic field (Figure 1E) is, up to an inconsequential rotation around  $\vec{B}_0$ , the same as the one observed after adding lanthanides but does not suffer from the drawbacks associated with having paramagnetic ions present. Furthermore, the SAS scheme allows one to correlate the resonance frequencies at different bicelle director orientations and should be useful in the context of two-dimensional experiments. Applications to membrane-bound proteins can be envisioned. The angle switching can take place in 50 ms (or less if the design is optimized) and SAS experiment can be applied to systems with  $T_1$  relaxation times of 50 ms or longer in experiments where the magnetization is stored along the  $z$  direction during the flip. For proteins dissolved in a solution oriented by bicelles, VAS experiments can potentially distinguish between scalar and dipolar couplings while SAS experiments can correlate anisotropic and isotropic spectra.

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