Communication

# A novel oriented system made of fatty acid hexagonal phases with tuneable orientation

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## ARTICLE INFO

## ABSTRACT

There is a strong demand in the field of solid state NMR for oriented lipid supramolecular assemblies. This is mainly devoted to biophysical structural studies or materials chemistry because the NMR signal depends on the orientation. Here we report a novel system made of a fatty acid hexagonal phase which self orient in the magnetic field. The orientation occurs within the magnetic field upon cooling the system from its isotropic phase. The cylinders of the hexagonal phase are then oriented parallel to the field. We take advantage that the hexagonal phase is a gel, i.e., the orientation is maintained fixed within the sample tube to investigate the orientational dependence of the deuterium solid state NMR signal using deuterated fatty acids and $D_2O$ by manually rotating the sample tube within the coil probe. As expected, the oriented signal follows the low $|3\cos^2\theta - 1|$ where $\theta$ is the angle between the long cylindrical axis and the field. We expect this system to be of interest in materials chemistry and structural biology.

## 1. Introduction

There is a growing interest to build surfactant assemblies that can self orient. The orientation can be achieved upon shearing or confinement between for instance glass plates or spontaneously in a strong magnetic field. In materials chemistry, this should allow to produce oriented materials (by templating) which could exhibit optical or other particular properties. In biology for the NMR community, this task is mainly devoted to the determination of membrane protein structure or at least, for gaining information on how a protein can orient within the lipid assembly [1–23]. Indeed, the solid state NMR signal is angle dependent [24] and the position of the peak allows the determination of for instance, the orientation of a protein helix within or at the surface of the bilayer [2]. For this, most of the systems investigated to date have been oriented lipid bilayers between glass plates [3,25] because they can ideally probe biological cell membranes. Recently, there has been a growing interest for bicelles (flat bilayer discs) which self orient in the magnetic field and can also mimic cell membranes [1,26–43]. However, one limitation here is that the orientation can either be parallel or perpendicular to the field and other orientations are difficult to obtain. Oriented materials are also useful for determining residual dipolar interactions of soluble molecules [44,45]. This may help for the determination of protein structures.

Recently, we have shown that hexagonal phases made of fatty acids can also self orient in a magnetic field [46]. Direct hexagonal phases are made of cylinders (elongated rigid micelles) stacked in an hexagonal array [47]. In those fatty acid mixtures, self orientation occured by heating the sample in the isotropic phase and then, cooling it back within the magnetic field. The long axis of the hexagonal cylinders were shown to be oriented parallel to the field. Here, we take advantage that the hexagonal phase is a gel. As a consequence, the orientation of the cylinders is maintained fixed within the sample tube. By manually rotating the sample tube in the coil probe, we can then investigate all the orientations as can be done with samples oriented between glass plates.

## 2. Results and discussion

Our previous system was composed of concentrated palmitic acid–tetrabutyl ammonium hydroxide (TBAOH) at different molar ratio in water [46]. In the hexagonal phase region of the phase diagram, we had shown that self orientation occurred in the magnetic field. Here, we investigate another system forming hexagonal phase by adding a long chain alcohol, tetradecanol. Indeed, alcohols are often used as cosurfactants in liquid crystalline phases made of synthetic surfactants [48,49]. The complete phase diagram is still under investigation but we report here several samples for a given composition (see material and methods). The dispersion was a birefringent transparent gel and the formation of the hexagonal phase was proven by both cross polarized microscopy and X-ray scattering (Fig. 1). The texture of the micrograph is typical of such a phase and moreover, the 3 diffraction peaks occur at $2\theta = 1.7°$, $3.0°$ and $3.4°$, respectively, i.e., exactly in a ratio $1:\sqrt{3}:2$ what demonstrate the existence of a hexagonal phase by SAXS.
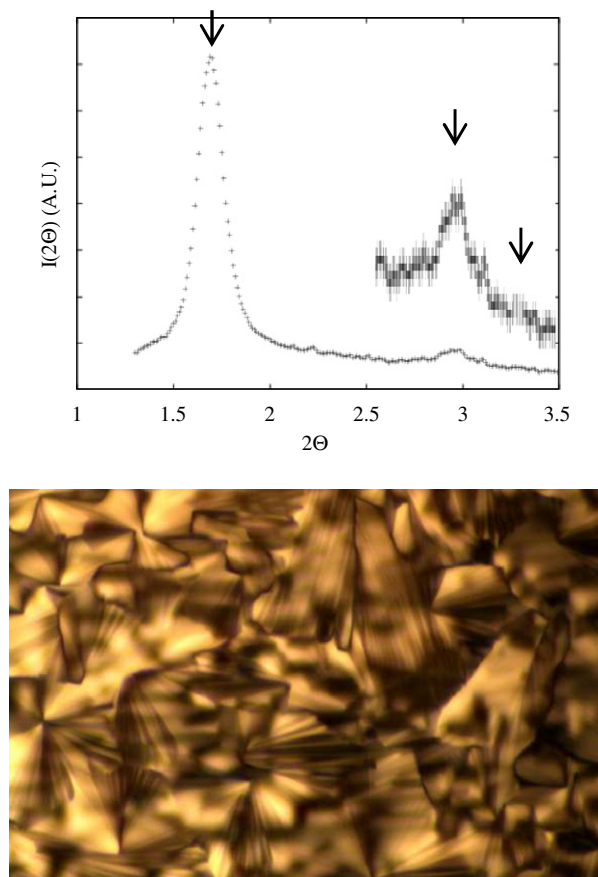
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Fig. 1. Cross polarized micrograph (bottom) and X-ray scattering experiments (top) of the sample at room temperature. The texture and the position of the three diffraction peaks in a ratio $1:\sqrt{3}:2$ (arrows) demonstrate the existence of the hexagonal phase. The X-ray signal is amplified to better visualize the second and third order.

The hexagonal phase was further characterized by deuterium solid state NMR using per-deuterated palmitic acid. This represents a strong advantage of using fatty acids as liquid crystals. Indeed, other surfactants or phospholipids are rarely available in their per-deuterated form. This could be a good opportunity for obtaining structural information of host molecules within such phases by proton NMR and even small angle neutron scattering. Both these techniques would only probe the protonated host molecule. However, we will have to search in the future for other counter-ions than TBAOH that are also commercially available under their per-deuterated form. Beside, we have previously shown in dilute aqueous systems that fatty acids mixed with organic counter-ions exhibit a large polymorphism [50–53]. Then, we can also expect in concentrated systems a large variety of liquid crystal phases upon changing the nature of the counter-ion.

The NMR signal of the present sample was very similar to that obtained with the previous system [46], i.e., a typical powder pattern of lipids embedded in a hexagonal phase [54,55] at room temperature and an isotropic line in the isotropic phase at 310 K (not shown). Upon cooling back to room temperature, the signal was characteristic of the hexagonal phase oriented with its long cylindrical axis parallel to the field (Fig. 2). It must be reminded here that the self orientation occurs spontaneously. Moreover, the cooling procedure took only 15 min and yielded a very well oriented signal.

At 0°, the signal is composed of well resolved peaks and over the 15 labeled positions of palmitic acid, 14 peaks can be easily assigned. The assignment can be made following an order profile in which the smaller the quadrupolar splitting, the higher the carbon position along the chain [56]. The smallest splitting stands for the methyl position [24]. Note that the largest quadrupolar splitting can be assigned without ambiguity to that of C2 (C1 being the carboxylic group) by comparison with the spectrum at 0° using the selectively labeled palmitic acid on position 2 (not shown). The well resolved peaks are separated by a quadrupolar splitting $\Delta\nu_0$ (where 0 denotes the angle between the axis of the cylinders and the magnetic field, see Fig. 2). The splittings are relatively small compared to those obtained for fatty acids embedded in bilayers [54,57], showing that fast rotation along the cylinder axis still occurs [55]. As mentioned above, the hexagonal phase is a gel, that is to say, the position of the cylinders is fixed within the sample tube. By manually turning the sample tube, we should now be capable of
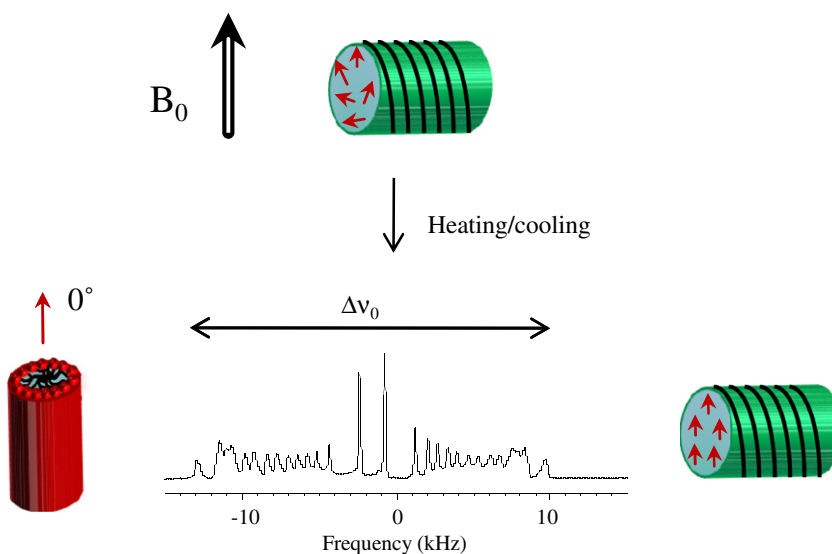


Fig. 2. Schematic representation of the sample tube (green) in the coil probe. The red arrows stand for the long axis of the cylinders of the hexagonal phase. They are oriented randomly and after heating and cooling the sample within the magnetic field ($B_0$), they become oriented parallel to it, i.e., at 0°. The oriented spectrum at 0° is shown and the quadrupolar splitting, $\Delta\nu_0$, for position C2 is reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

investigating all the possible orientations what is further demonstrated. Using a home made goniometer allowing to measure angles at ±2°, we then turned the sample tube from $\theta = 0°$ to 90° in the coil probe and recorded a spectrum for each orientation as depicted Fig. 3.

Upon increasing the angle $\theta$, we observed two main features. First, there was a marked broadening of the peaks for orientations different than 0 and 90°. When one even slightly deviates from the orientation at 0°, the linewidth markedly increases. However, the linewidth did not increase for the methyl group (smallest splitting). Moreover, for positions closer to the methyl end group (small splittings), the peaks were more resolved for angles higher than the magic angle. These findings show that the broadening depends on the carbon position and the orientation. This broadening then arises from the variation of the relaxation times with the carbon position and the orientation. It is known that the alkyl chains exhibit higher mobility at the end of the alkyl chain [24] and then, different relaxation times than for positions close to the carboxylic group. Such a feature has already been observed for phospholipids embedded in a hexagonal phase [55].

Second as expected, the quadrupolar splitting varied with the orientation. It decreased and vanished at the magic angle (isotropic line). A further increase of $\theta$ yielded a re-increase of the splitting until the value of $\Delta v_{90}$ which is half $\Delta v_0$. Since the methyl group is weakly affected by the relaxation time, we plotted the corresponding quadrupolar splitting as a function of $\theta$ (Fig. 4) which shows the expected angular dependence [24] following the well known low $\Delta v \sim |3\cos^2\theta - 1|$.

We also performed similar experiments using deuterated water since this molecule exhibits different motions than alkyl chains and then, its signal should be differently affected by the relaxation time. The spectra obtained for that sample are shown on Fig. 5 as a function of the orientation with respect to the magnetic field. First, the spectra are not composed of an isotropic line (except at the magic angle) but exhibit a quadrupolar splitting showing that water is very confined between the cylinders. Next, this confinement is such that water molecules are also oriented in average so that the signal also follows the angular dependence upon rotation of the sample within the coil probe (Figs. 4 and 5). This shows that such systems could be used for inducing weak
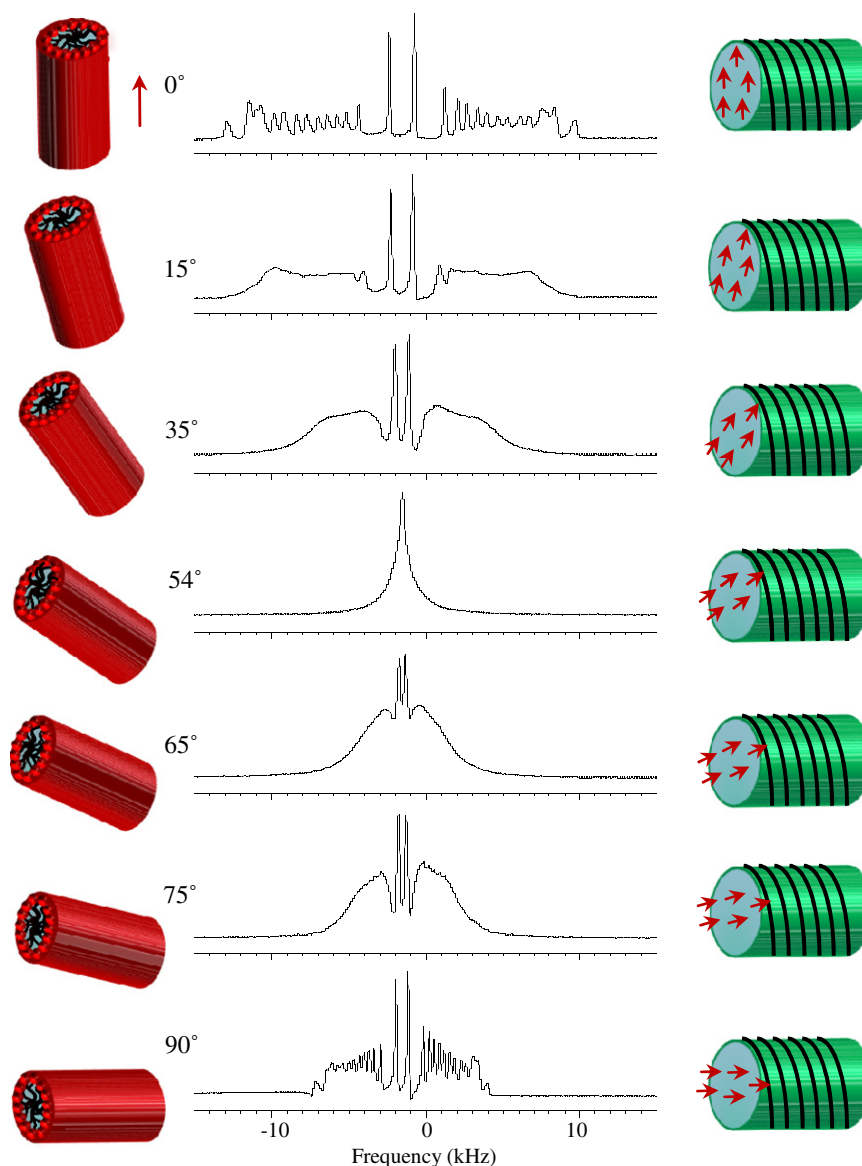


**Fig. 3.** Selected deuterium solid state NMR spectra for the oriented hexagonal phase at 296 K as a function of the angle $\theta$. A sketch of a cylinder of the hexagonal phase is also depicted, showing the orientation (red arrows, $\theta$) within the sample tube (green, right) with respect to the magnetic field which is vertical. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
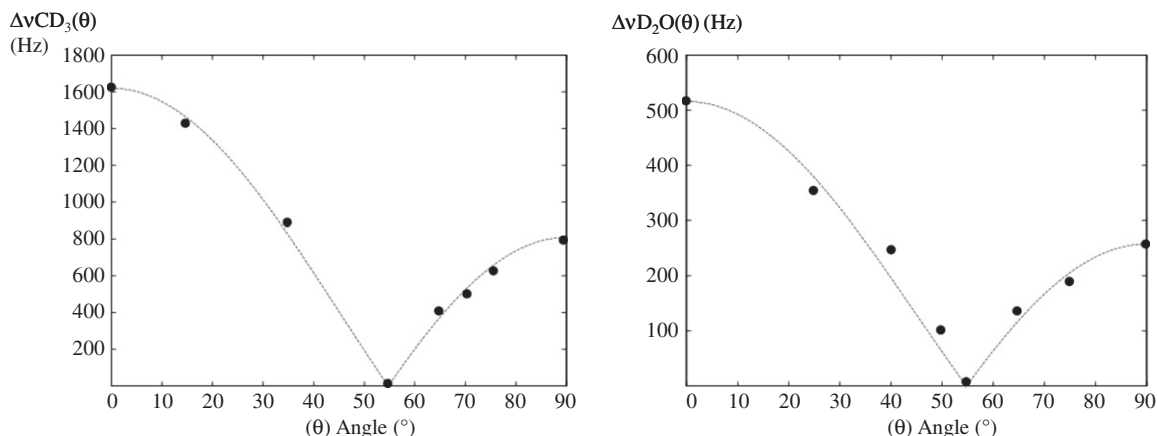
**Fig. 4.** (Left) Angular dependence of the quadrupolar splittings for the methyl position which were measured from data obtained, a part of which are represented in Fig. 3. (B). (Right) Angular dependence of the quadrupolar splitting for the deuterated water signal which were measured from data obtained, a part of which are represented in Fig. 5. The solid line shows the theoretical dependence following the low $\nu\Delta \sim |3\cos^2\theta - 1|$.

alignment of soluble host molecules for measuring residual dipolar interactions [44].

The fact that the angular dependence of the quadrupolar splitting is in agreement with the theoretical expectation is not so tremendous. However, this demonstrates that the cylinders are indeed 'immobilized' within the gel what represents a novel system for structural studies of host molecules. Interestingly, we have performed experiments with various samples (both using $D_2O$ or per-deuterated palmitic acid) in order to study the possible disorientation of the hexagonal phase as a function of time. The sample was initially oriented at 0° by the heating/cooling procedure. Then, the sample was rotated by hand at 90° and a first spectrum was recorded. The samples remained in the magnetic field with that 90° orientation for 24 h. A second spectrum was recorded and shown to be perfectly superimposable to the first one (not shown) demonstrating that there is no re-orientation of the cylinders of the hexagonal phase during that period of time. Such a lap of time is sufficient to perform structural studies of host molecules. Moreover, since the sample is a 'solid' gel, we can expect that no re-orientation occurs for longer period of time. Since those mixtures are made of fatty acids and that the hexagonal phase occurs for low amounts of water, those systems may not be adapted for biophysical studies. These may be more attractive in materials chemistry for the synthesis of oriented materials or for studying the orientational dependence of host organic or inorganic molecules.

In summary, it is presently shown that saturated long chain fatty acids such as palmitic acid may form hexagonal phases that self orient in a magnetic field. The self orientation procedure is fast, easy and only requires that the sample is heated in its isotropic phase and further cooled within the magnetic field. Since the hexagonal phase is a gel, the orientation is maintained fixed within the sample tube for at list 1 day allowing the investigation of the orientational dependence by solid state NMR. This represents a novel system for studying the orientation of host molecules within the cylinders of the hexagonal phase. Moreover, this system could also be useful for inducing weak alignment of host soluble molecules for solution structure determination.

# 3. Materials and methods

## 3.1. Sample preparation

Protonated (Sigma–Aldrich, both 99% purity), perdeuterated (Eurisotop) palmitic acid (PA) and selectively labelled PA on position 2 (Sigma) were used. For the NMR experiments, the preparation of samples was according to the following. For the per-deutrated system, 200 mg of PA was dispersed in TBAOH 1 M (molar ratio of 1) and then, 32 mg of tetradecanol was added. For the selectively labelled system, 200 mg of PA were neutralized with TBAOH and then, 35 mg of tetradecanol were incorporated. For the third sample, 210 mg of protonated PA was neutralized with TBAOH and then, 36 mg of tetradecanol were incorporated. Finally, 10 μL of deuterated water (Eurisotop) was added. The samples were further heated (80 °C) and vigorously vortexed to fully disperse the lipid material. By cooling the sample at room temperature, we checked that a hexagonal phase was formed by visual inspection and microscopy between crossed polarisers.

As previously mentioned, the phase diagram of this system is still in progress and will be reported elsewhere, however, the hexagonal phase was obtained for a large variety of mixtures. For instance, the hexagonal phase also occurs for mixtures of palmitic acid (+TBAOH) and dodecanol or decanol, except that the phase transition to the isotropic phase occurred at lower temperature. By contrast, that phase transition was increased by using stearic acid and alcohols from C12 to C16 chain length. In the same way, tetradecanoic acid (+TBAOH) and tetradecanol or dodecanol also form hexagonal phases in a large domain of molar ratio. The characterization of all those systems by NMR is still under study but all the hexagonal phases obtained were shown to self orient in the magnetic field upon cooling form their isotropic phase.

## 3.2. Solid state NMR

Deuterium solid state NMR experiments were performed on a 400 MHz Bruker spectrometer operating at 61 MHz for deuterium using a static double channel probe. The sample coil of the probe was adapted to load a 7 mm rotor such as those used for magic angle spinning probes equipped with a stretched stator. Typically, lipid dispersions were previously heated to 60 °C and a volume of *ca.* 500 μL transferred into the rotor which was sealed and then end-capped. A Hahn quadrupolar echo sequence [19] was used with an inter pulse delay of 40 μs. Height $k$ points in 1k accumulations (every 2 s) were done with a 90 pulse and spectral width of 5 μs and 125 kHz, respectively. Free induction decay signal were zero-filled to 16k points prior to Fourier transform after a broad line exponential multiplication of 10 Hz.

For deuterium spectroscopy, the general theory for lipid systems can be found in the literature [24]. Briefly, the deuterium NMR signal is composed of doublets with a splitting, $\Delta\nu$ which depends on the orientation of the C—D bond with respect to the
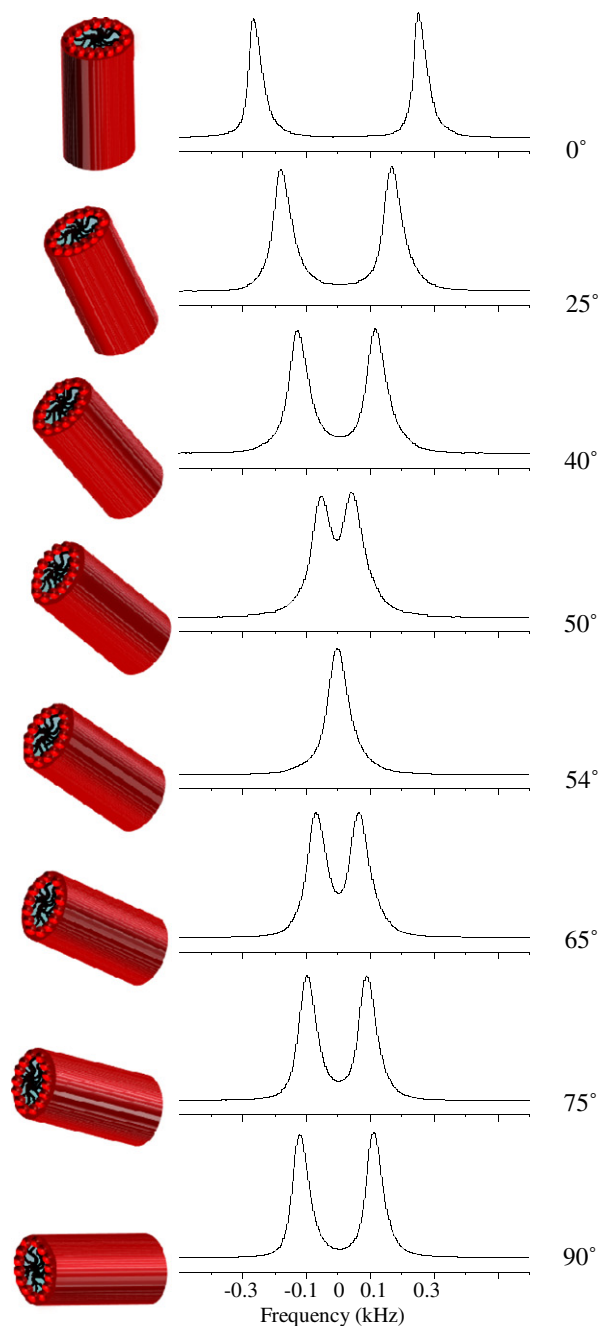
**Fig. 5.** Deuterium solid state NMR spectra of the oriented hexagonal phase (using $D_2O$) at 296 K as a function of the angle $\theta$ which is reported on the right.

magnetic field. In an anisotropic but *disoriented* medium, all the orientations are allowed and these doublets are superimposed to form a powder spectrum having two main peaks with an increased intensity corresponding to the 90 orientation, separated by $\Delta v_{90}$. The edge of the spectrum corresponds to the 0 orientation, with a splitting $\Delta v_0$ equal to twice $\Delta v_{90}$. In the case of perdeuterated systems, the spectrum is composed by the superimposition of signals from each labeled position.

### 3.3. Cross polarized microscopy

Observations were made at room temperature at $20\times$ magnification using an optical microscope in the cross polarized mode (Nikon Eclipse E-400, Tokyo, Japan) equipped with a 3-CCD JVC camera allowing digital images ($768 \times 512$ pixels) to be collected.

A drop of the lipid dispersion (about 20 μL) was deposited on the glass slide surface ($76 \times 26 \times 1.1$ mm, RS France) and covered with a cover slide ($22 \times 22$ mm, Menzel-Glaser, Germany). The glass slides were previously cleaned with ethanol and acetone.

### 3.4. X-ray

Diffraction diagrams were monitored by recording X-ray diffraction diagrams every 30 min on a Bruker D8 Discover diffractometer. Cu KR1 radiation (Cu KR1) 1.5405 Å), produced in a sealed tube at 40 kV and 40 mA was selected and parallelized using a Göbel mirror parallel optics system and collimated to produce a 500 mm beam diameter. Samples were transferred into a 1.5 diameter capillary which was further sealed.

### Acknowledgments

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