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Orientation-Dependent ^{19}F Dipolar Couplings within a Trifluoromethyl Group Are Revealed by Static Multipulse NMR in the Solid State

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The homonuclear dipolar coupling between the three equivalent ^{19}F-spins of a trifluoromethyl group, rotating about its threefold symmetry axis, was studied by multipulse solid-state NMR. A modified CPMG sequence was used first to resolve the dipolar splitting of a powder sample, and then to follow its orientation-dependence in uniaxially aligned samples. Our aim is to employ the CF_3-group as a highly sensitive reporter to describe the mobility and spacial alignment of ^{19}F-labeled molecules in biomembranes. As an example, the fluorinated anti-inflammatory drug, flufenamic acid, was embedded as a guest compound in lipid bilayers. Undistorted ^{19}F dipolar spectra of its CF_3-group were obtained without ^{1}H-decoupling, revealing a sharp triplet lineshape. When an oriented membrane sample was tilted in the magnetic field, the change in dipolar splittings confirmed that the guest molecule is motionally averaged about the membrane normal, as expected. A different behavior of flufenamic acid, however, was observed under conditions of low bilayer hydration. From this set of orientation-dependent lineshapes we conclude that the axis of motional averaging becomes aligned perpendicular to the sample normal. It thus appears that flufenamic acid induces a hexagonal phase in the membrane at low hydration. Finally, the dipolar ^{19}F NMR experiments were extended to frozen samples, where no molecular diffusion occurs besides the fast rotation about the CF_3-axis. Also under these conditions, the CPMG experiment with composite pulses could successfully resolve the dipolar coupling between the three ^{19}F-nuclei.

Key Words: **three-spin system; oriented membranes; flufenamic acid; homonuclear dipolar interaction; fluorine CPMG.**

INTRODUCTION

Because of its high sensitivity, ^{19}F NMR is increasingly being used to study macromolecules in the solid state (*1*). Especially in biological systems, where fluorine is not naturally present, background-free selective labeling is possible (*2*). The high gyromagnetic ratio of ^{19}F gives rise to strong dipolar interactions, which can be exploited to determine specific local structural parameters. For example, REDOR distance measurements between an ^{19}F label and a neighboring ^{13}C or ^{31}P in frozen enzymes have provided sufficient structural details to allow conclusions about their functional mechanisms (*3–6*). It would be highly desirable to make use of the strong ^{19}F dipolar couplings also in uniaxially aligned samples, such as biomembranes or fibers, because the angle-dependence yields additional information about the orientations of the interacting spins (*7–11*). To this aim, static NMR experiments need to be applied, rather than any of the popular dipolar recoupling techniques under magic-angle spinning (MAS) (*12, 13*). It is indeed possible to acquire pure dipolar spectra of spin pairs with the aid of a static Carr–Purcell–Meiboom–Gill (CPMG) sequence and related multipulse experiments (*14–17*). Based on this approach, we have recently recorded and analyzed the orientation-dependence of the dipolar interaction between two ^{19}F labels in uniaxially aligned lipid bilayers (*18*). As a next step, we investigate here the homonuclear dipolar couplings within a three-spin system. We illustrate how the pure dipolar spectrum of a trifluoromethyl group can be readily obtained by static multipulse NMR, both in a powder and in oriented samples.

It has been previously demonstrated that the homonuclear dipolar coupling of a CF_3-group does not emerge in a number of indirect NMR experiments. Indeed, when ^{19}F is used as a dephasing nucleus in a REDOR experiment, the dipolar interactions within a rotating CF_3-group do not have to be taken into account (*6, 19*). This is because the fast rotation of the CF_3-group about its threefold symmetry axis renders the three spins chemically and magnetically equivalent, and their interactions become inhomogenous. This situation changes, however, when ^{19}F itself is the observed nucleus. Then, unlike the scalar J-coupling between equivalent spins, the homonuclear dipolar interaction is effective in a rotating CF_3-group, and it will produce a triplet in the directly observed static NMR spectrum.

The dipolar interaction between three spins of $I = \frac{1}{2}$ has been addressed in previous publications in various ways, for example, by observing the refocusing behavior in echo experiments (*20–22*), as a superimposed oscillation ("slow beat") of echo amplitudes (*23*), or by multiple quantum experiments (*24–26*). However, the lineshape of a pure dipolar spectrum has not yet been observed experimentally for a three-spin system, to our knowledge.

Here, a CPMG-type experiment is used to obtain pure homo-

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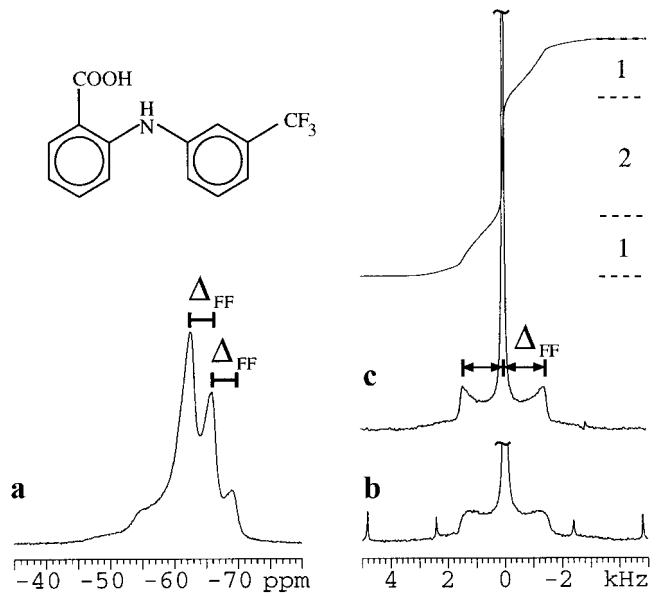

FIG. 1. (a) The single-pulse ^{19}F NMR spectrum (512 scans, 35 kHz ^{1}H-decoupling) of flufenamic acid (FFA, top left) in DMPC membranes at 35°C is dominated by homonuclear dipolar and chemical shift interactions. The dipolar splitting of $\Delta_{FF} = 1.6$ kHz is resolved under ^{1}H-decoupling. (b) The CPMG sequence (pulse repetition time 60 μs, 1024 scans, without decoupling) removes the chemical shift interaction and yields the pure dipolar spectrum. (c) Replacing the 180° inversion pulses by 90°–$180^{\circ}_{90^{\circ}}$–90° composite pulses improves the lineshape, and the powder spectrum shows the expected 1:2:1 intensity ratio of a three-spin triplet.

nuclear dipolar spectra, by suppressing the chemical shift anisotropy, the heteronuclear dipolar couplings, and field inhomogenities. The CF_3-groups in a nonoriented sample will give rise to a powder lineshape with three peaks, like that of a quadrupolar spin with $I = \frac{3}{2}$. Any molecular motions besides the rapid CF_3-rotation will reduce the width and splitting of the powder spectrum according to the geometry of averaging. The dynamic behavior of a membrane-embedded molecule can thus be described in terms of an order parameter. Furthermore, the CF_3-group serves as a sensitive probe of segmental orientation in uniaxially aligned samples, such as membranes or fibers. Since the dipolar splitting depends on the orientation of the C–CF_3 vector with respect to the external magnetic field, we are using the CF_3-label to determine the alignment of a guest molecule in a lipid matrix. Finally, we will show that the dipolar splitting can be measured even in frozen membranes or crystalline samples, without the need for ^{1}H-decoupling. Even though fast ^{19}F-relaxation and spin diffusion to surrounding protons tend to be unfavorable without decoupling, this problem is less severe in CF_3-groups as long as they retain their intrinsic mobility.

The nonsteroidal anti-inflammatory drug flufenamic acid (FFA) is used here to illustrate some applications of CF_3-labels in biomembranes. In addition to the trifluoromethyl group, FFA carries a carboxylic acid group (see Fig. 1). Depending on its protonation state, the molecule is soluble in either aqueous or hydrophobic environments. The behavior of this compound in lipid bilayers is of pharmacological interest, because the activity of FFA relies on its diffusion across cellular membranes (e.g., when orally administered) or across the stratum corneum (when topically applied). FFA is a potent inhibitor of several classes of membrane proteins, e.g., nonselective cation channels, certain chloride channels, and the erythrocyte anion exchanger band-3 (*27–30*). FFA has also been shown to prevent the formation of amyloid fibrils in some neurodegenerative diseases (*31*). Furthermore, the anti-inflammatory effect of FFA has been attributed to an inhibition of prostaglandin synthesis by blocking cyclooxygenase (*32*).

THEORETICAL BACKGROUND

Dipolar Interaction between Three Equivalent Spins

From solution-state NMR, it is well known that the scalar J-coupling within a group of equivalent spins is not present in the directly acquired spectrum (*33*, p. 481). Like the J-coupling, the dipolar coupling Hamiltonian H_D among three equivalent spins of $I = \frac{1}{2}$ commutes with the remaining part of the Hamiltonian H_R (including the chemical shift, heteronuclear couplings, and J-couplings). However, whereas the J-coupling commutes also with the observable I_x or I_y (or with the initial spin density $\rho_0 \sim I_x$ or I_y), the dipolar interaction does not commute, even if the spins are equivalent. Hence, for the dipolar interaction among three equivalent spins of $I = \frac{1}{2}$, the dependence on the dipolar interaction H_D does not vanish in the observed signal $\langle I_{x,y} \rangle = \mathrm{Tr}(e^{-iH_R t} e^{-iH_D t} \rho_0 e^{iH_D t} e^{iH_R t} I_{x,y})$.

The pure dipolar spectrum of a rotating CF_3-group consists of a triplet with relative intensities of 1:2:1. This behavior is reminiscent of an axially symmetric, first-order quadrupolar spectrum of a nucleus with spin $I = \frac{3}{2}$ (*21, 22, 34*). The positions of the outer two lines depend on the angle θ between the C–CF_3 axis and the static magnetic field direction according to $(3 \cos^2 \theta - 1)/2$, in the same way as the doublet arising from two coupled spins of $I = \frac{1}{2}$ or from a quadrupolar spin of $I = 1$ (*18, 35*). Since the central line is invariant to θ, the powder spectrum of a CF_3-group consists of a Pake doublet with a pronounced center peak. The intrinsic splitting Δ^0_{FF} between the central line and either of the 90°-edges of the powder spectrum is given by

$$\Delta^0_{FF} = \frac{1}{2} \frac{3\gamma^2\hbar}{r^3_{FF}}. \qquad [1]$$

Here, γ is the gyromagnetic ratio of ^{19}F, r_{FF} is the internuclear ^{19}F–^{19}F distance, and the factor $\frac{1}{2}$ accounts for the CF_3-group rotation. In the crystal structures of two polymorphs of FFA the average internuclear fluorine distances are $r_{FF} = 2.094$ Å and $r_{FF} = 2.091$ Å, respectively (*36, 37*). With these distances, the theoretical dipolar coupling of a CF_3-group, which is rotating about its threefold symmetry axis, is $\Delta^0_{FF} = 8.7$ kHz.

Any additional motions of a rotating CF_3-group (e.g., long axial rotation in a liquid crystal, segmental wobble, or molecular diffusion) reduce Δ^0_{FF} to a splitting Δ_{FF} in a powder spectrum, which is scaled by an order parameter S_{CF3}:

$$\Delta_{FF} = S_{CF3}\Delta^0_{FF}. \quad [2]$$

In a liquid crystalline membrane, the lipid molecules and any small guest compounds tend to be motionally averaged around the bilayer normal. In other words, the time-averaged director of such a molecule is usually aligned parallel to the bilayer normal (but see Results and Discussion, below). When the bilayers are uniformly aligned in an oriented sample, the pure dipolar spectrum will consist of three narrow lines. The splitting then depends on the angle α between the macroscopic sample normal and the static magnetic field direction (*38*):

$$\Delta^{\text{oriented}}_{FF}(\alpha) = \Delta_{FF} \cdot (3\cos^2\alpha - 1). \quad [3]$$

MATERIALS

Lipid Dispersion Sample

A total of 5 mg of flufenamic acid (Sigma) was cosolubilized in chloroform or isopropanol with 95 mg of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) from Avanti Polar Lipids (Alabama). After the solvent was evaporated with N_2, the sample was dried under vacuum overnight. The mixture was then hydrated with 100 μl Millipore water at a temperature above the lipid phase transition and was homogenized by five freeze–thaw cycles.

Oriented Sample

A total of 1.2 mg of flufenamic acid was cosolubilized in 300 μl isopropanol with 22.8 mg DMPC. Oriented membrane films were obtained by spreading the solution onto glass cover slips (Marienfeld) with dimensions of 8 mm $\times$ 20 mm $\times$ 0.07 mm. Aliquots of 25 μl were distributed over 12 plates, corresponding to 2.0 mg of dry material per glass plate. After evaporation of the solvent, the glass plates were dried under vacuum overnight. Each plate was then hydrated to saturation by exposing the lipid film to warm steam over boiling water (*18*). The plates were then immediately placed face-down onto a base plate to form a stack. The stack was sealed at the edges with epoxide glue to prevent the plates from slipping, and it was then wrapped in cling film. After it was placed into another plastic bag, the sample was incubated for 24 h at 35°C to anneal, and then it was measured immediately.

Samples at Low Hydration

A remarkable change in the NMR-lineshapes and an altered phase behavior were observed in the oriented sample after storage (several months) due to dehydration (see Results and Discussion). To confirm this interpretation and to reproduce the observed effects, several dispersion samples of FFA/DMPC with well-defined low water contents were prepared in a way similar to that described above. The required amount of water was added to the dry lipid mixture with a 10-μl Hamilton syringe (*39*). The resulting samples had total water contents of 2, 3, 4, 5, or 6 $\pm$ 0.5 H_2O molecules per DMPC molecule, on the assumption that 1 H_2O/DMPC had remained bound to the lipid even after overnight vacuum. The successive changes in the phase transition temperatures of these samples were observed by ^{31}P and ^{19}F NMR. From this series of data, it was concluded that the "low" hydration level of the oriented sample corresponds to 3.0 $\pm$ 0.5 H_2O/DMPC. The phase behavior and NMR data were entirely reproducible under these conditions.

METHODS

A Unity Inova widebore 500-MHz NMR spectrometer (Varian, Palo Alto, CA) was used at a ^{19}F-frequency of 470 MHz, supplemented with a ^{19}F high-power amplifier (Creative Electronics, Northridge, CA). The experiments were performed with a flat-coil (2.5 mm $\times$ 9 mm) double-tuned $^1H/^{19}F$ probe (Doty Scientific Inc., Columbia, SC), equipped with a goniometer to orient the sample at a variable angle. At 35°C, single-pulse ^{19}F spectra were obtained with ^1H-decoupling at 35 kHz, using band-selective filters (FSY Microwave, Columbia, SC) as well as band-stop filters (Doty Scientific) for both ^1H and ^{19}F. At -60°C we used the quadrupole-echo sequence with 25-μs delay and 60-kHz ^1H-decoupling. The 90° pulse length was 2.0–2.5 μs, and the acquisition time was 20 ms. About 800 transients were acquired with a recycle delay of 3 s. A linebroadening of 50 Hz was applied, and spectra were referenced against $CFCl_3$.

Pure dipolar spectra were acquired with a modified Carr-Purcell-Meiboom-Gill (CPMG) experiment (*15, 18, 40, 41*). Instead of 180° pulses we used composite pulses with 90°–$180^\circ_{90^\circ}$–90° (*42, 43*). An xy8 phase cycling scheme was employed for samples in the liquid crystalline phase at 35°C (*41, 44*). To avoid sideband artifacts in the much broader spectrum at -60°C, xy8 phase cycling was omitted there. The mid-to-mid pulse distance in the multipulse train was 30 μs if not stated otherwise. The echo trains had a duration of 50–100 ms, with a typical duty cycle of about 30%. Between 800 and 1600 transients were averaged, and no linebroadening was applied to the CPMG spectra.

RESULTS AND DISCUSSION

Dipolar Spectrum of FFA in DMPC

Our aim is to use the ^{19}F homonuclear dipolar coupling within the trifluoromethyl group of flufenamic acid (FFA; see inset of Fig. 1) to characterize the molecule's alignment and motional behavior in a lipid bilayer. When the membrane-

permeant drug is incorporated at 5 wt% in dimyristoyl-phosphatidylcholine (DMPC), the fully hydrated sample consists of smooth multilamellar liposomes, as confirmed by freeze fracture electron microscopy (data not shown). Static solid-state ^{19}F NMR spectra of nonoriented FFA/DMPC samples were recorded at 35°C, where the membrane is in the liquid crystalline state. The conventional single-pulse spectrum in Fig. 1a was acquired with ^{1}H-decoupling; hence it is dominated by the chemical shift anisotropy (CSA) and homonuclear dipolar interactions. Since the CF_3-group is rotating rapidly around its threefold symmetry axis, this makes the three spins equivalent. The spectrum can be understood in terms of axially averaged CSA and dipolar tensors that are aligned along the C–CF_3 axis. The lineshape reflects a CSA powder pattern of about 12 ppm width, centered around an isotropic chemical shift at −62 ppm. Additionally, each position of the CSA (corresponding to a certain angle θ) is subject to an orientation-dependent dipolar splitting that is proportional to $(3\cos^2\theta - 1)/2$. The prominent triplet with a peak-to-peak splitting of $\Delta_{FF} \approx 1.6$ kHz thus represents the ^{19}F–^{19}F dipolar coupling at the 90°-edges of the powder spectrum. This splitting is less than the static value of $\Delta_{FF}^{0} = 8.7$ kHz for a CF_3-group rotating only about its threefold axis (Eq. [1]). Hence the reduced coupling is attributed to some further motional averaging of the FFA molecule in DMPC, and the same averaging also applies to the ^{19}F CSA. Using the concept of an order parameter, this means that the dipolar splitting and the CSA are both reduced by the factor $S_{CF3} \approx 0.18$.

The pure dipolar ^{19}F NMR spectrum of FFA in DMPC (at 35°C) without any contribution from the CSA is shown in Fig. 1b. It was acquired using the CPMG sequence, modified with a compensating xy8-phase cycling scheme. The effect of the multipulse train is to refocus the chemical shift and heteronuclear dipolar interactions, as well as the magnetic field inhomogeneities, and there is no need for ^{1}H-decoupling. Even though the xy8-phase cycling is known to improve the basic CPMG experiment (*18, 44*), the spectrum in Fig. 1b still suffers from distortions due to pulse imperfections. The dipolar lineshape could be further improved by applying composite 90°_{x}–180°_{y}–90°_{x} inversion pulses, instead of the simple 180°_{y} pulses. The increased excitation profile of the composite pulses helps to reduce offset-effects, which otherwise tend to accumulate in multipulse spectra of ^{19}F due to the comparatively wide chemical shift range.

The dipolar spectrum in Fig. 1c exhibits a sharp axially symmetric powder pattern, as expected for three equivalent spins of $I = \frac{1}{2}$. The integrated intensities of the three components possess the correct ratio of 1:2:1, as shown by the integral in Fig. 1c. The dipolar splitting at 35°C is $\Delta_{FF} = 1.5$ kHz, in good agreement with the value obtained from the corresponding single-pulse spectrum in Fig. 1a. Note that the intense central component is a genuine feature of the lineshape, rather than an artifact as sometimes observed for static CPMG-type spectra at high field. Unlike the outer two components of the triplet, which display the characteristic orientation-dependence of a Pake pattern, the central component is invariant to the angle θ.

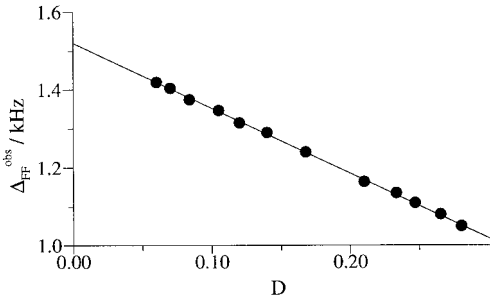

FIG. 2. The directly observed, unscaled dipolar splitting $\Delta_{FF}^{obs}(D)$ depends linearly on the duty cycle D of the multipulse train, with a slope of $1.10 \cdot \Delta_{FF}^{obs}(0)$. Extrapolation to $D = 0$ yields the true dipolar coupling of $\Delta_{FF} = 1.53$ kHz.

Scaling Behavior

The widths of the dipolar spectra in Fig. 1 have been scaled to compensate for the finite pulse widths of the multipulse sequence. These data and all following dipolar spectra are shown to reflect the true dipolar frequencies. The scaling factor for the CPMG experiment with composite pulses was determined experimentally by acquiring a series of spectra with varying duty cycle D (pulse repetition times between 30 and 140 μs) (*40*). The dipolar coupling of FFA stays constant over a broad temperature range in the gel phase of DMPC below 23°C. Therefore, spectra were recorded at 15°C to ensure that any potential differences in sample heating at different duty cycles did not affect the dipolar splitting. Figure 2 shows the measured dipolar splittings $\Delta_{FF}^{obs}(D)$ as a function of the duty cycle D. The scaling factor $\lambda = \Delta_{FF}^{obs}(D)/\Delta_{FF}^{obs}(0)$ follows a linear dependence on D, even up to fairly large values of $D \approx 30\%$, with a slope of 1.10. This slope is used for scaling also the other CPMG spectra under different conditions. Extrapolation of $\Delta_{FF}^{obs}(D)$ to $D = 0$ yields the corrected value for the dipolar spitting at 15°C of $\Delta_{FF} = 1.53$ kHz.

FFA in Oriented Membranes

Information about the alignment of a labeled segment in a membrane can be derived from its dipolar splitting in an oriented sample.

Figure 3 shows conventional ^{19}F NMR spectra (on the left) and multipulse spectra (on the right) of FFA in oriented DMPC membranes (5% w/w), acquired at various tilt angles α between the sample normal and the magnetic field. In the left series of spectra the chemical shift and the dipolar splitting of the triplet change with the factor $(3\cos^2\alpha - 1)$ (Eq. [3]). This behavior is in accordance with the expected alignment of the molecule's director axis parallel to the sample normal. The dipolar spectra on the right-hand side show the same angular

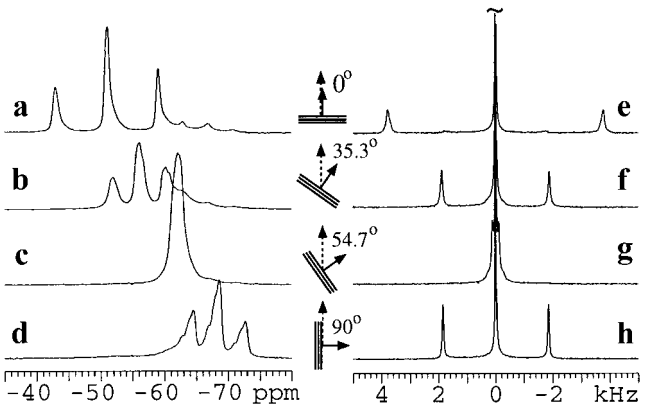

FIG. 3. Uniaxially oriented membranes (5% FFA in DMPC, fully hydrated) were measured at different sample orientations α, using single-pulse ^{19}F NMR (a–d) and CPMG experiments (e–h). In both series of spectra, the dipolar splitting Δ_{FF} varies according to $(3\cos^2\alpha - 1)/2$, as expected for an alignment of the FFA director parallel to the sample normal. The resolution enhancement by CPMG is about one order of magnitude.

dependence, albeit at a much better resolution and without being affected by the CSA. While the conventional ^1H-decoupled ^{19}F-wideline spectra suffer from magnetic field inhomogeneity, giving linewidths between 400 and 1000 Hz, they are narrowed by the CPMG sequence to around 50 to 150 Hz even without ^1H-decoupling. This considerable resolution enhancement is readily visualized by referring to Fig. 3g, where a misalignment of the sample by about 1° off the magic angle gives rise to a small splitting of 100 Hz that is easily resolved by CPMG.

Having confirmed the expected alignment of the molecule's long axis in the bilayer, additional information is accessible from the size of the dipolar coupling. We note that the splitting in the oriented system is about 20% larger than in the corresponding nonoriented sample, probably due to a lack of bilayer undulations in the former, as has been previously observed in oriented samples between glass plates (*18*). Nonetheless, the splittings of the single-pulse spectra agree well with the CPMG data. At an alignment of $\alpha = 90°$ the splitting is $\Delta_{FF} = 1.9$ kHz, and at $\alpha = 0°$ it is twice this value (3.8 kHz).

Hexagonal Lipid Phase Induced by FFA at Low Hydration

Besides illustrating nicely the orientation-dependence of the dipolar interaction, the use of oriented samples provided us with intrinsically new information about samples of FFA at low hydration. Specifically, it was observed that the director axis alignment deviates from the "trivial" orientation along the sample normal. The dipolar spectra of an oriented sample with a water/lipid molar ratio of 3/1 are shown in Figs. 4a–4d. At a sample alignment of $\alpha = 0°$, the lineshape consists of a triplet (Fig. 4a), which is consistent with a unique angle between the time-averaged director axis of FFA and the macroscopic sample normal. However, upon tilting the sample away from $\alpha = 0°$ the lineshapes become broad and more complex (Figs. 4b–4d). These data indicate that the director orientations of FFA are distributed over a distinct range of angles with respect to the magnetic field, meaning that they are no longer aligned parallel to the macroscopic sample normal.

To determine the angle between the time-averaged FFA director and the macroscopic sample normal, the experimental spectra (Figs. 4a–4d) can be analyzed by simulation. The best-fit lineshapes are shown in Figs. 4e–4h. They correspond to the situation where the time-averaged director axes are uniformly distributed within the plane of the sample, i.e., radially and at a right angle around the macroscopic sample normal. The lineshapes $P(\nu)$ for a planar distribution of directors in a sample, tilted at an angle α, are calculated according to (*7–11, 38*)

$$P(\nu) = \Theta[(\nu/\Delta_{FF} + 1)(-\nu/\Delta_{FF} + (3\sin^2\alpha - 1))]P_+(\nu) + \Theta[(-\nu/\Delta_{FF} + 1)(\nu/\Delta_{FF} + (3\sin^2\alpha - 1))] \times P_-(\nu) + \frac{1}{2}\delta(\nu) \quad [4]$$

with

$$P_\pm(\nu) = \frac{1}{2\pi\nu_0} \frac{1}{\sqrt{(\pm\nu/\Delta_{FF} + 1)(\mp\nu/\Delta_{FF} + (3\sin^2\alpha - 1))}} \quad [5]$$

and $\Theta[x] = 1$ or 0 for positive or negative x, respectively. A residual dipolar coupling of $\Delta_{FF} = 320$ Hz and a Lorenzian linebroadening of 100 Hz were used to simulate the dipolar spectra in Figs. 4e–4h.

The unusual dipolar lineshapes in Fig. 4 can be explained by

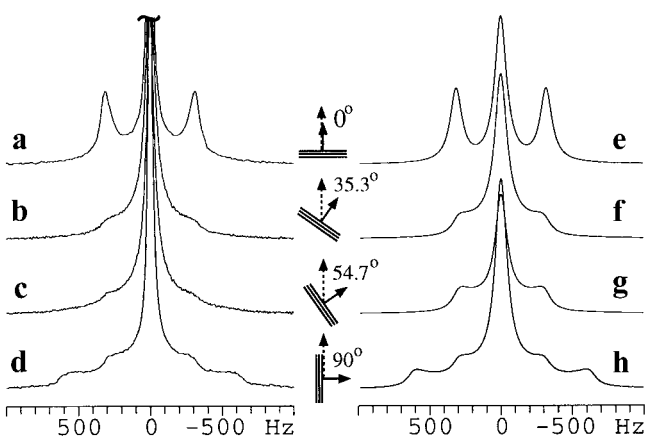

FIG. 4. The angle-dependent dipolar spectra of the oriented sample at low hydration are different from those at full hydration (cf. Figs. 3e–3h). The observed lineshapes (a–d) are well reproduced by simulation (e–h, splitting of 320 Hz, linebroadening by 100 Hz), assuming a radial distribution of director axes. Hence, FFA is motional averaged by rotation around an axis that lies at a right angle to the macroscopic sample normal.

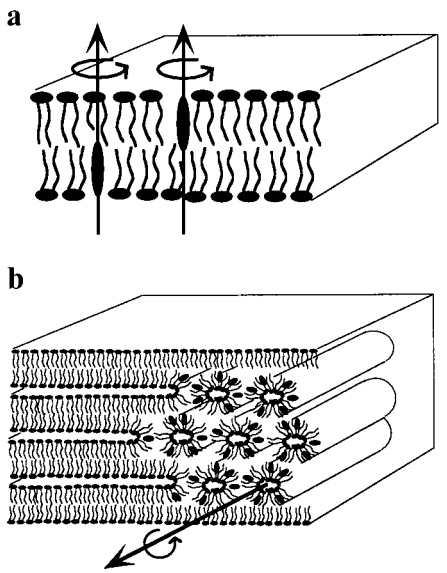

FIG. 5. A different behavior of FFA in DMPC is observed at different levels of hydration. (a) At full hydration the guest molecule rotates about an axis parallel to the membrane normal, as expected for a liquid crystalline bilayer. (b) At low water content, on the other hand, FFA induces a hexagonal morphology in DMPC, where it is averaged around an axis parallel to the planar solid support.

a hexagonal morphology of the lipid system, induced by FFA at low levels of hydration, as illustrated in Fig. 5. Part of the lipids seem to assemble with the FFA molecules in tubes or cylindrical structures, which are packed in a hexagonal lattice. Motional averaging of the molecules by rapid diffusion around the cylinders causes the FFA director to point along the cylinder axis. In the oriented sample, the cylinders are oriented within the plane of the glass plates with a local hexagonal packing but lacking any long-range translational order. This picture is fully supported by a series of ^{31}P NMR spectra of the phospholipids and by electron microscopy images of nonoriented samples containing FFA (*45*).

An inverted hexagonal phase, H_{II}, is common for a range of phospholipids, depending on the water content and temperature of the sample. Nevertheless, since an H_{II} phase is usually associated with a small lipid headgroup, it would not have been expected to occur in DMPC, even at low hydration. Hence, the presence of 5% FFA induces a significant change in the lipid chain packing, which favors the formation of hexagonal structures at low hydration. Whether this effect has any pharmacological relevance for the activity of FFA in a cell membrane remains open to speculation.

At low water content, the CF_3-group on FFA shows a significantly smaller splitting than in the fully hydrated samples, even when the additional spectral narrowing by a factor of $\frac{1}{2}$ due to the cylindrical averaging is taken into account. This smaller value is therefore attributed to a higher degree of wobbling or, more likely, to a change in the orientation and localization of FFA with respect to the lipid chains and headgroups.

We note that the standard single-pulse spectra of FFA in DMPC at low hydration did not reveal any orientation-dependence, as they consisted of a broad (3 ppm) featureless line at all sample orientations (data not shown). Only after the dramatic simplification and resolution enhancement by the CPMG multipulse sequence did the residual dipolar interaction become apparent. Without the combined use of oriented samples and CPMG, it would not have been possible to observe and interpret the morphological transition induced by FFA in DMPC.

FFA in Frozen Membranes

The previous spectra were acquired at 35°C, where the DMPC bilayer is in the liquid crystalline phase and allows a high mobility of the FFA guest molecules. It is usually more difficult to acquire CPMG spectra of fluorinated compounds in less mobile sites because the relaxation times and range of frequencies are less favorable when no motional averaging occurs. However, the intrinsic CF_3-rotation facilitates studies even under conditions where the molecule itself is immobilized, such as in frozen or crystalline samples. Figure 6 shows ^{19}F NMR spectra of FFA in DMPC membranes at −60°C. Essentially the same splitting is also observed in crystalline FFA (data not shown). These wideline spectra have a width of over 100 ppm, since the dipolar coupling and chemical shift dispersion are no longer subject to motional averaging besides the CF_3-rotation. For the conventional spectra (Figs. 6a and 6b) high-power ^{1}H-decoupling was essential to resolve the dipolar splittings of $\Delta_{FF} \approx 8$ kHz. The CPMG experiment with composite pulses (Fig. 6c), on the other hand, is able to suppress the chemical shift interaction and the heteronuclear ^{19}F–^{1}H

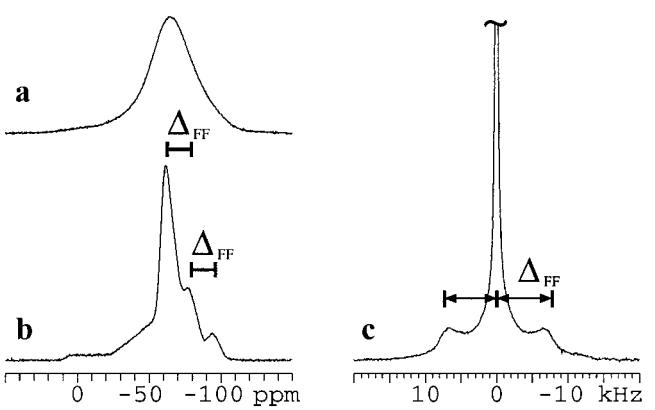

FIG. 6. The molecular diffusion of FFA in DMPC is frozen out at −60°C, but the CF_3-group is still engaged in fast rotation. The conventional ^{19}F NMR spectrum (a) is extensively broadened by the CSA and strong dipolar couplings; hence (b) ^{1}H-decoupling (60 kHz) is needed to resolve the dipolar splitting of $\Delta_{FF} = 8.0$ kHz. The pure dipolar spectrum (c) is resolved by CPMG multipulse narrowing without ^{1}H-decoupling.

coupling per se. The dipolar powder spectrum is still slightly broadened, but the splittings and the expected lineshape are clearly visible. The dipolar coupling of 8 kHz is close to the limiting value expected from the crystal structure of FFA (Δ^0_{FF} = 8.7 kHz).

CONCLUSIONS

The CF_3-group is a highly sensitive NMR label for studying the structure and dynamics of guest molecules in biomembranes. Fast rotation around the CF_3-axis renders the three spins chemically and magnetically equivalent. The homonuclear dipolar coupling and the CSA both contribute to conventional ^{1}H-decoupled ^{19}F NMR spectra in the solid state. Powder samples produce relatively complex lineshapes, but in uniaxially aligned membranes a simple 1:2:1 triplet is observed, whose position varies according to the CSA and whose dipole splitting, too, changes with the sample orientation.

Using a CPMG-type experiment, the CSA can be fully suppressed and the pure dipolar spectrum extracted. We have improved the basic multipulse sequence with an xy8 phase cycle and composite 90°_x–180°_y–90°_x inversion pulses, so that there was no need for ^{1}H-decoupling. We have measured accurately the ^{19}F–^{19}F dipolar splitting within the CF_3-group on flufenamic acid (FFA) under various conditions. When this pharmacologically active compound is embedded at 5% in DMPC, the dipolar splitting of the powder spectrum reveals the motional averaging of FFA in the lipid membrane.

In combination with oriented samples, a remarkable resolution enhancement by an order of magnitude was achieved by the multipulse narrowing. Furthermore, the angle-dependent lineshapes of FFA revealed two distinct morphologies in the lipid under different levels of hydration. In the fully hydrated sample the molecule is motionally averaged about the macroscopic sample normal, as expected for uniaxially aligned liquid crystalline bilayers. At low hydration, on the other hand, the axis of averaging is flipped into the plane of the sample, being indicative of a hexagonal phase. Finally, we have illustrated that even in immobilized molecules the favorable motional properties of the CF_3-group facilitate multipulse narrowing.

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REFERENCES

1. A. S. Ulrich, High resolution solid state NMR, 1H, 19F, *in* "Encyclopedia of Spectroscopy and Spectrometry" (J. Lindon, G. Tranter, and J. Holmes, Eds.), pp. 813–825, Academic Press, London (2000).
2. J. T. Gerig, Fluorine NMR, *in* "Biophysics Textbook On-Line" (V. Bloomfield, Ed.), NMR section, Chap. 24, biosci.umn.edu/biophys/OTLB/NMR.html#24 (1998).
3. L. M. McDowell, S. M. Holl, S. Qian, E. Li, and J. Schaefer, Intertryptophan distances in rat cellular retinol binding protein II by solid-state NMR, *Biochem.* **32,** 4560–4563 (1993).
4. L. M. McDowell, M. Lee, R. A. McKay, K. S. Anderson, and J. Schaefer, Intersubunit communication in tryptophan synthase by carbon-13 and fluorine-19 REDOR NMR, *Biochem.* **35,** 3328–3334 (1996).
5. D. R. Studelska, C. A. Klug, D. D. Beusen, L. M. McDowell, and J. Schaefer, Long-range distance measurements of protein binding sites by rotational-echo double-resonance NMR, *J. Am. Chem. Soc.* **118,** 5476–5477 (1996).
6. J. M. Goetz, B. Poliks, D. R. Studelska, M. Fischer, K. Kegelbrey, A. Bacher, M. Cushman, and J. Schaefer, Investigation of the binding of fluorolumazines to the 1-MDa capsid of lumazine synthase by ^{15}N {^{19}F} REDOR NMR, *J. Am. Chem. Soc.* **121,** 7500–7508 (1999).
7. A. S. Ulrich, M. P. Heyn, and A. Watts, Structure determination of the cyclohexene ring of retinal in bacteriorhodopsin by solid-state deuterium NMR, *Biochem.* **31,** 10390–10399 (1992).
8. A. S. Ulrich and A. Watts, 2H NMR lineshapes of immobilized uniaxially oriented membrane proteins, *Solid State NMR* **2,** 21–36 (1993).
9. A. S. Ulrich, A. Watts, I. Wallat, and M. P. Heyn, Distorted structure of the retinal chromophore in bacteriorhodopsin resolved by 2H NMR, *Biochem.* **33,** 5370–5375 (1994).
10. A. S. Ulrich, I. Wallat, M. P. Heyn, and A. Watts, Re-alignment of the retinal chromophore in the M-photointermediate of bacteriorhodopsin, *Nature Struct. Biol.* **2,** 190–192 (1995).
11. T. Asakura, M. Minami, R. Shimada, M. Demura, M. Osanai, T. Fujito, M. Imanari, and A. S. Ulrich, 2H-labeling of Bombyx mori silk fibers and their structural characterization by solid state 2H NMR, *Macromolecules* **30,** 2429–2435 (1997).
12. D. P. Raleigh, M. H. Levitt, and R. G. Griffin, Rotational resonance in solid state NMR, *Chem. Phys. Lett.* **146,** 71–76 (1988).
13. T. Gullion and J. Schaefer, Detection of weak heteronuclear dipolar coupling by rotational-echo double-resonance nuclear magnetic resonance, *Adv. Magn. Reson.* **13,** 57–83 (1989).
14. J. W. Emsley and D. L. Turner, Simplification of deuterium spectra of liquid crystals by the refocussing of proton–deuterium dipolar couplings, *J. Chem. Soc. Faraday Trans. 2* **77,** 1493–1508 (1981).
15. J. F. M. Post, B. W. Cook, S. R. Dowd, I. J. Lowe, and C. Ho, Fluorine-19 nuclear magnetic resonance investigation of fluorine-19-labeled phospholipids. 1. A multiple-pulse study, *Biochem.* **23,** 6138–6141 (1984).
16. T. Terao, Structural measurements by solid-state NMR, *J. Mol. Struct.* **441,** 283–294 (1998).
17. A. Nordon, E. Hughes, R. K. Harris, L. Yeo, and K. D. M. Harris, Direct measurement of the distance between adjacent guest molecules in a disordered solid inclusion compound using solid-state F19–H1 NMR spectroscopy, *Chem. Phys. Lett.* **289,** 25–29 (1998).
18. S. L. Grage and A. S. Ulrich, Structural parameters from 19F homonuclear dipolar couplings by multipulse solid-state NMR on static and oriented systems, *J. Magn. Reson.* **138,** 98–106 (1999).
19. J. M. Goetz and J. Schaefer, REDOR dephasing by multiple spins in the presence of molecular motion, *J. Magn. Reson.* **127,** 147–154 (1997).
20. P. S. Allen, W. Harding, and P. Mansfield, Quasiquadrupolar echoes from solids containing only $I = \frac{1}{2}$ spins, *J. Phys. C* **5,** L89–L93 (1972).

21. Y. N. Moskvich, N. A. Sergeev, and G. I. Dotsenko, Quadrupolar echos in systems with dipole–dipole interactions, *Fiz. Tverd. Tela* **15,** 2854–2857 (1973).

22. Y. N. Moskvich, N. A. Sergeev, and G. I. Dotsenko, Two-pulse echo in solids containing three-spin systems, *Phys. Status Solidi A* **30,** 409–418 (1975).

23. K. Sakaie, C. P. Slichter, and J. H. Sinfelt, Methods using the nuclear quadrupolar interaction to determine the structure of randomly oriented molecules, *J. Magn. Reson. A* **119,** 235–239 (1996).

24. A. K. Roy and K. K. Gleason, Analytical solutions for multiple-quantum-coherence dynamics among two or three dipolar-coupled, spin-$\frac{1}{2}$ nuclei, *J. Magn. Reson. A* **120,** 139–147 (1996).

25. U. Friedrich, I. Schnell, S. P. Brown, A. Lupulescu, D. E. Demco, and H. W. Spiess, Spinning-sideband patterns in multiple-quantum magic-angle spinning NMR spectroscopy, *Mol. Physics* **95,** 1209–1227 (1998).

26. U. Friedrich, I. Schnell, D. E. Demco, and H. W. Spiess, Triple-quantum NMR spectroscopy in dipolar solids, *Chem. Phys. Lett.* **285,** 49–58 (1998).

27. H. Kankaanranta and E. Moilanen, Flufenamic and tolfenamic acids inhibit calcium influx in human polymorphonuclear leukocytes, *Mol. Parmacol.* **47,** 1006–1013 (1995).

28. C. Eder, R. Klee, and U. Heinemann, Involvement of stretch-activated Cl^- channels in ramification of murine microglia, *J. Neurosci.* **18,** 7127–7137 (1998).

29. H. Gögelein, D. Dahlem, H. C. Englert, and H. J. Lang, Flufenamic acid, mefenamic acid and nifenamic acid inhibit single nonselective cation channels in the rat, *FEBS Lett.* **268,** 79–82 (1990).

30. J.-L. Cousin and R. Motais, Inhibition of anion transport in the red blood cell by anionic amphiphilic compounds. 1. Determination of the flufenamate-binding site by proteolytic dissection of the band 3 protein, *BBA* **687,** 147–155 (1982).

31. S. A. Peterson, T. Klabunde, H. A. Lashuel, H. Purkey, and J. C. Sacchettini, Inhibiting transthyretin conformational changes that lead to amyloid fibril formation, *Proc. Nat. Acad. Sci. USA* **95,** 12956–12960 (1998).

32. M. Ouellet and M. D. Percival, Effect of inhibitor time-dependency on selectivity towards cyclooxygenase, *Biochem. J.* **306,** 247–251 (1995).

33. A. Abragam, "Principles of Nuclear Magnetism," Clarendon, Oxford (1961).

34. E. R. Andrew and R. Bersohn, Nuclear magnetic resonance line shape for a triangular configuration of nuclei, *J. Chem. Phys.* **18,** 159–161 (1950).

35. A. S. Ulrich and S. L. Grage, 2H NMR, *in* "Solid State NMR" (I. Ando and T. Asakura, Eds.), Elsevier Science, Amsterdam (1998).

36. J. F. McConnell, 3′-trifluoromethyldiphenylamine-2-carboxylic acid, $C_{14}H_{10}F_3NO_2$, flufenamic acid, *Cryst. Struct. Commun.* **2,** 459 (1973).

37. H. M. Krishna-Murthy, T. N. Bhat, and M. Vijayan, Structure of a new crystal form of 2-{[3-(trifluoromethyl)phenyl]amino} benzoic acid (flufenamic acid), *Acta Crystallogr. Sect. B* **38,** 315 (1982).

38. J. Seelig, Deuterium magnetic resonance: Theory and application to lipid membranes, *Q. Rev. Biophys.* **10,** 353–418 (1977).

39. A. S. Ulrich and A. Watts, Molecular response of the lipid headgroup to bilayer hydration monitored by 2H NMR, *Biophys. J.* **66,** 1441–1449 (1994).

40. M. Engelsberg and C. S. Yannoni, The determination of bond lengths in solids using the Carr–Purcell sequence, *J. Magn. Reson.* **88,** 393–400 (1990).

41. M. J. Lizak, T. Gullion, and M. S. Conradi, Measurements of like-spin dipole couplings, *J. Magn. Reson.* **91,** 254–260 (1991).

42. M. H. Levitt and R. Freeman, NMR population inversion using a composite pulse, *J. Magn. Reson.* **33,** 473–476 (1979).

43. R. Freeman, S. P. Kempsell, and M. H. Levitt, Radiofrequency pulse sequences which compensate their own imperfections, *J. Magn. Reson.* **38,** 453–479 (1980).

44. T. Gullion, D. B. Baker, and M. S. Conradi, New, compensated Carr–Purcell sequences, *J. Magn. Reson.* **89,** 479–484 (1990).

45. S. L. Grage, D. Gauger, C. Selle, W. Pohle, W. Richter, and A. S. Ulrich, The amphiphilic drug flufenamic acid can induce hexagonal lipid phases in DMPC—A^{31}P- and ^{19}F-NMR study, *Phys. Chem Chem. Phys.* (2000), in press.