ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Magnetic Resonance 164 (2003) 104–114

JMR Journal of Magnetic Resonance

www.elsevier.com/locate/jmr

Susceptibility corrections in solid-state NMR experiments with oriented membrane samples. Part I: applications

Ralf W. Glaser[a] and Anne S. Ulrich[b,*]

[a] *Institute of Molecular Biology, Friedrich-Schiller-Universität Jena, Winzerlaer Str. 10, D-07745 Jena, Germany*
[b] *Forschungszentrum Karlsruhe, IFIA, P.O. Box 3640, D-76021 Karlsruhe, Germany*

Received 21 February 2003; revised 27 May 2003

Abstract

Chemical shift referencing of solid-state NMR experiments on oriented membranes has to compensate for bulk magnetic susceptibility effects that are associated with the non-spherical sample shape, as described in the accompanying paper [J. Magn. Reson. 164 (2003) 115–127]. The resulting frequency deviations can be on the order of 10 ppm, which is serious for nuclei with a narrow chemical shift anisotropy such as ^{1}H or ^{13}C, and in some cases even ^{19}F. Two referencing schemes are proposed here to compensate for these effects: A flat (0.4 mm) glass container with an isotropic reference molecule dissolved in a thin film of liquid is stacked on top of the oriented membrane sample. Alternatively, the intrinsic proton signal of the hydrated lipid can be used for chemical shift referencing. Further aspects related to magnetic susceptibility are discussed, such as air gaps in susceptibility-matched probeheads, the benefits of shimming, and limitations in the accuracy of orientational constraints. A biological application is illustrated by a series of experiments on the antimicrobial peptide PGLa, aimed at understanding its concentration-dependent membranolytic effect. To address a wide range of molar peptide/lipid ratios between 1:3000 and 1:8, multilayers of hydrated DMPC containing a ^{19}F-labeled peptide were oriented between stacked glass plates. Maintaining an approximately constant amount of peptide gives rise to thick samples (18 plates) at low, and thin samples (3 plates) at high peptide/lipid ratio. Accurate referencing was critical to reveal a small but significant change over 5 ppm in the anisotropic chemical shift of the ^{19}F label on the peptide, indicative of a change in the orientation and/or dynamics of PGLa in the membrane.

Keywords: Chemical shift referencing; Bulk magnetic susceptibility; Lipid membranes; Amphiphilic antimicrobial peptides; ^{19}F solid-state NMR; Line broadening; Shimming

1. Introduction

The magnetic properties of a sample locally modify the magnetic field in which it is being measured. Consequently, the NMR frequency of an isotropic substance contained in a non-spherical sample depends on the sample shape and its orientation in the external magnetic field [1,2]. The magnitude of this effect is demonstrated by placing a capillary filled with water into the free-standing coil of an NMR probe. In the horizontal orientation (capillary axis normal to B_0) the resonance frequency is shifted by 4.5 ppm downfield compared to the vertical orientation (parallel to B_0). This difference corresponds to half the susceptibility χ of water,[1] as expected from theory [1,3]. When performing the same experiment with a flat "oriented" sample, i.e., with a thin layer of water trapped between two thin glass slides, the difference is about 8 ppm, nearly equal to the susceptibility of water. To correct for such chemical shift errors associated with sample shape and orientation, experiments have to be referenced. This is not a trivial task for static oriented lipid membranes, as they are

* Corresponding author. Visitor's address: Institute of Organic Chemistry, University of Karlsruhe, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany. Fax: +49-721-608-4823.
E-mail address: anne.ulrich@ifia.fzk.de (A.S. Ulrich).

[1] In SI units the susceptibility ($\chi = B/H - 1$) of water is $\chi = -9.05 \times 10^{-6}$, that of glass is $\chi \approx -12 \times 10^{-6}$, and of hexane $\chi = -7.1 \times 10^{-6}$. Note that in CGS-emu units, where $\chi = (B/H - 1)/4\pi$, the susceptibilities have different numerical values (i.e., for water $\chi = -0.72 \times 10^{-6}$), but we use SI units throughout.

1090-7807/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S1090-7807(03)00207-6

intrinsically anisotropic [2]. Here we describe possible ways of chemical shift referencing in the solid state, and illustrate its relevance with a biological example.

Many solid-state NMR applications are concerned with the structure analysis of peptides embedded in membranes. Typically, peptides with a selective isotope label (^{15}N or ^{13}C in the backbone, or $^{2}H_3$-Ala or ^{19}F-Phg as side chain) are reconstituted in macroscopically oriented phospholipid bilayers [4,5]. When supported on a glass plate, several thousand membranes spontaneously organize themselves parallel to the surface upon hydration above the lipid phase transition temperature [6]. The sample is assembled as a stack of several such glass plates. Together with the NMR coil it can be manually aligned at different tilt angles θ in the magnetic field. The anisotropic chemical shift recorded at $\theta = 0°$ (membrane normal parallel to B_0) and $\theta = 90°$ (perpendicular) reveals the orientation and dynamics of the labeled peptide segment in the membrane [4,5,7]. Accurate referencing becomes complicated when different sample geometries, such as the $\theta = 0°$ and 90° tilt angles, or thick and thin stacks, have to be compared.

Here we investigate the antimicrobial peptide "PGLa"[2] from the African claw frog [8], whose sequence NH_2-GMASKAGAIAGKIAKVALKALCONH_2 is closely related to the magainin family [9]. These peptides bind to lipid bilayers as an amphiphilic α-helix and act by selectively permeabilizing the bacterial plasma membrane, but the exact mechanism is still a matter of dispute [10]. Previous solid-state NMR investigations of ^{15}N-labeled PGLa in oriented lipid membranes suggested that the peptide is aligned with its helix axis parallel to the membrane surface [11]. However, in a number of peptides a transition between two different orientations was found depending on the lipid environment and peptide concentration [12,13]. These observations suggest a two-state model, where the amphiphilic helices are initially bound parallel to the membrane surface but can re-orient to form a pore or toroidal wormhole [14–17]. Leakage experiments and electrophysiological studies provided evidence for cooperativity between the peptides, suggesting a concerted re-alignment as oligomers [10]. Peptide concentration appears to be a critical factor for activity, and the level required for activity has been suggested for a peptide:lipid ratio between 1:30 and 1:120 [14,16], such threshold value depending on the type of peptide and experimental conditions.

Previous solid-state ^{15}N, ^{2}H, and ^{13}C NMR studies were not able to address peptide behavior over a wide range of concentrations because of the low sensitivity of these labels. Therefore, we used here the highly sensitive ^{19}F-nucleus as a reporter, which was incorporated into PGLa via the stiff side chain 4-fluoro-phenylglycine (4F-Phg) [18]. The peptide was reconstituted in oriented dimyristoylphosphatidyl-choline (DMPC) bilayers with molar peptide/lipid ratios from 1:3000 to 1:8. Over this wide range we were able to monitor by ^{19}F NMR whether PGLa undergoes a change in orientation and/or dynamics in the membrane. For sensitivity reasons a similar amount of peptide was used in each sample (0.02–0.2 μmol). The amount of phospholipid thus varied between 0.8 and 33 mg, and between three and 18 glass plates were required as supports. In this series, not only the intrinsic peptide concentration but also the sample geometry had effects on the ^{19}F resonance frequency on the order of a few ppm. Chemical shift referencing was thus essential to show that the orientation and/or dynamics of the peptide genuinely depend on the peptide/lipid ratio, thus ruling out artifacts from any systematic variations in sample geometry.

Analogous to high resolution NMR, different schemes of referencing can be employed: internal, external, and referencing by substitution. The latter, "traditional" method in solid-state NMR requires a separate reference specimen that is used to replace the sample. The reference must have the same geometry and susceptibility as the sample to be measured [1], but in many cases this is difficult to achieve. Internal referencing relies on a molecule in the same environment as the substance to be measured [2]. The reference molecule has to give a sharp resonance line that does not overlap with the signals of interest. For our ^{19}F NMR studies with oriented membranes we were unable to find a suitable isotropic molecule for internal referencing. $CFCl_3$, which defines zero on the chemical shift scale of ^{19}F, dissolves most glues and diffuses through parafilm. This compound is difficult to incorporate into membrane samples and hard to keep there during the experiment. Besides, any $CFCl_3$ leaking from the sample may increase the ^{19}F background signal of the probehead. Other molecules are also highly volatile and require impracticable sealing, or they orient themselves in the lipid bilayer, which renders them useless as a reference. Peptide samples often contain trifluoroacetic acid (TFA) remaining from resin cleavage or HPLC purification, which exhibits a triplet of narrow ^{19}F NMR peaks. However, these TFA signals are unsuitable for referencing as their chemical shift and dipolar coupling depend on the peptide and its concentration in the membrane [18].

We suggest to use an external reference in the form of a thin liquid "film" (~200 μm) sealed in a flat glass container. NaF dissolved in buffer is suitable for ^{19}F-referencing, and similar solutions may be employed for other nuclei. The reference is conveniently placed on top of the oriented membrane sample, from where it can be removed during data acquisition to avoid interference with the signals of interest or with the dynamic range of

[2] *Abbreviations used:* 4F-Phg, 4-fluoro-phenylglycine; PGLa, peptidyl-glycylleucine-carboxyamide antimicrobial peptide; DMPC, dimyristoylphosphatidyl-choline.

the experiment. Using this arrangement, however, three general problems have to be solved: (A) In the external position, on top of the stack, the reference may not truly reflect the local magnetic field in the bulk of the test sample. This error has to be evaluated before setting the reference frequency. (B) If the reference is removed for the actual data acquisition, the resulting change in stack height will modify the magnetic field in the membrane sample. This change also needs to be taken into account in the referencing procedure. (C) When the oriented sample is measured at different tilt angles, different chemical shift corrections for the above effects have to be applied.

All of these effects are calculated and experimentally verified here, to offer a robust procedure for referencing ^{19}F chemical shifts. The same scheme is applicable to other nuclei and will be important when their chemical shift anisotropy is narrow. Relevant cases would be ^{1}H NMR on peptides in oriented biomembranes [19], and to some extent also ^{13}C NMR [20]. Having established the autonomous external referencing procedure on ^{19}F with an error range of 0.1 ppm, we tested whether the ^{1}H frequencies of the hydrated lipid bilayer could also be used as an internal source of referencing information. By calibrating the ^{1}H chemical shifts against the absolute ^{19}F-frequencies, we found that the broad proton signals of a liquid crystalline membrane show no significant residual anisotropy beyond about 0.3 ppm. Hence the ^{1}H peaks of the hydrated lipids are quite useful for straightforward referencing of oriented membrane samples in general.

Bulk susceptibility effects do not only cause chemical shift changes, but they are also a major source of line broadening. Therefore, we examine the effects of shimming, susceptibility matching, and other practical modifications on the sample to improve the linewidth [2,3]. Finally, we discuss the consequences of the inhomogeneous and anisotropic bilayer environment at a molecular level. This paper is exclusively based on qualitative arguments and simple numerical simulations. A detailed theoretical treatment of susceptibility effects in samples of any general shape and composition is presented in the accompanying part II of this paper [1].

2. Experimental

2.1. NMR measurements

Measurements were performed on a 500 MHz wide-bore Unity Inova spectrometer (Varian, Palo Alto, CA). For ^{19}F pulses an external 470 MHz high power amplifier (Creative Electronics, Los Angeles, CA) was used. A ^{19}F/^{1}H double-tuned flatcoil probehead with a $9 \times 20 \times 2.4\,\text{mm}^3$ rectangular sample space in a susceptibility-matched ceramic housing (Doty Scientific, Columbia, SC, USA) [3] was used for ^{19}F and ^{1}H observation in the solid state. The central 10 mm of the sample space are surrounded by an Ω-shaped copper foil, forming a single turn RF coil. Sample and coil can jointly be rotated about the axis of the coil. The angle of rotation is denoted by θ, where $\theta = 0°$ indicates a horizontal orientation of the layered sample with the membrane normals aligned parallel to the B_0 field. ^{31}P NMR experiments were performed in a variable coil H/X solid state probehead (NMR Service M. Braun, Erfurt, Germany) and ^{19}F NMR experiments in solution with a standard 5 mm tunable ^{1}H probehead (Varian, Palo Alto, CA).

2.2. Assembly of external reference

Each reference specimen was assembled from two thin glass plates of the same kind that was used to orient the lipids, in our case $7.5 \times 18\,\text{mm}^2$ with a thickness of about 80 μm (Marienfeld, Germany). A glass rod was drawn to a diameter of less than 0.2 mm and cut into pieces of 7 and 17.5 mm length. These thin rods were coated with two-component epoxy glue (UHU GmbH, Germany) and positioned near the edges of one glass plate in the shape of a "U." The second glass plate was placed on top to produce a evenly flat sandwich with one short edge open. A heating plate with elevated temperature makes the glue more fluid and is helpful to produce thin and regular rims. When the glue was polymerized, the sample pocket was filled with about 20 μl of reference buffer (1 M NaCl, 10 mM phosphate buffer, pH 7) and closed with glue using one or two more glass rods. The sample should not contain any air bubbles. The total thickness is less than 0.4 mm.

2.3. Peptides and lipids, sample preparation

The peptide PGLa with a 4-fluorophenylglycine label in the Ala10 position was produced by solid phase Fmoc synthesis, and the all-L isomer was purified to >95% with HPLC [18]. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification.

For oriented membrane samples a peptide/lipid mixture was prepared and deposited on thin glass slides ($7.5 \times 18 \times 0.08\,\text{mm}^3$). The substrates had been cleaned by rinsing in ethanol and dried under vacuum. The peptide was dissolved in water and the lipid in methanol. Both components were mixed to give a 70% methanol solution, and 20 μl drops of the solution were spread onto one side of each glass plate. This yielded mass densities of lipid between 0.5 and 3 mg/cm^2. After initial evaporation the membrane films were dried under vacuum overnight. Any required number of coated glass plates, from 3 to 18, were stacked to form a block-shape sample. The samples were subsequently hydrated for 2

days at 48 °C in an atmosphere of 98% relative humidity over a saturated K_2SO_4 solution, such that the membranes spontaneously orient themselves [6]. The block was immediately wrapped in several layers of parafilm and polyethylene sheet (combined thickness 100–200 μm) to maintain the hydration of the sample during the NMR measurement. The height of the sample was between 0.6 and 2.4 mm (being the maximum height that fits into our NMR coil). The multilamellar lipid films prepared this way have a thickness in the range of 7–40 μm. With 80 μm glass plates, the volume filling factor of lipid material is about 8–33%. The degree of orientation of the lipid membranes was determined with ^{31}P NMR at $\theta = 0°$ tilt as described before [24]. Typically 80–90% of the lipid was well oriented parallel to the glass plates.

2.4. Simulation of magnetic field distribution

Numerical calculations of the magnetic flux density were performed with Mathematica (http://www.wolfram.com) as described in the accompanying paper [1]. The lineshapes shown in Fig. 4 were calculated as a histogram of field strength in a three-dimensional grid of $180 \times 150 \times 40$ equally spaced points in the simulated sample. The input for all Mathematica simulations mentioned in this paper is available under http://www.molebio.uni-jena.de/~rwg/refshift.html.

3. Results and discussion

3.1. Calculated field distribution in oriented membrane samples

Without any geometry-specific referencing, the intrinsic error in measuring the chemical shifts of oriented membranes on glass supports at different tilt angles can be up to about 9 ppm, corresponding to the susceptibility of the environment of the label. Therefore, we developed an external referencing procedure, using a thin container filled with a fluid reference substance that is stacked on top of the membrane sample within the NMR coil. To assess the validity of this procedure we had to find out how accurately the local magnetic field in the thin reference film at the surface reflects the field in the bulk interior of the membrane sample. As a first approximation we considered the sample as a homogeneous block with uniform susceptibility χ, neglecting the effects of its internal layered structure that are covered below and by the accompanying paper [1]. We numerically calculated the field distribution throughout the sample volume, to compare the chemical shift in the center with that at the surface. The distribution of the magnetic flux density B is specified with respect to the constant flux density $(1 - 2\chi/3)B_0$ in an ideal spherical sample. The difference $\Delta B = B - B_0$ is proportional to the external field B_0 and to the average susceptibility χ of the sample. We therefore describe the field distortion in units of χB_0. With the diamagnetic susceptibility of glass ($\chi \approx -12 \times 10^{-6}$, which makes the major contribution to the filling factor of our oriented membrane samples), $\chi B_0 = +1$ corresponds to an upfield shift of 12 ppm. The field distribution was evaluated specifically for the dimensions of the glass plates that fit into our coil, as a function of stack height. Note that only the relative proportions of the three-dimensional sample block are relevant here, but not its absolute sample size. The same calculations can be repeated for any other set-up to take the particular NMR coil geometry and the dimensions of other glass plates into account.

The relative field distortion ΔB at selected positions of an oriented sample aligned at $\theta = 0°$ or 90° is depicted in Fig. 1 for two situations: for an infinitely thin rectangular sample, and for a stack of $7.5 \times 18 \times 2.4\,\text{mm}^3$ (the maximum size that fits into our coil). Infinitely thin samples show a field difference of χB_0

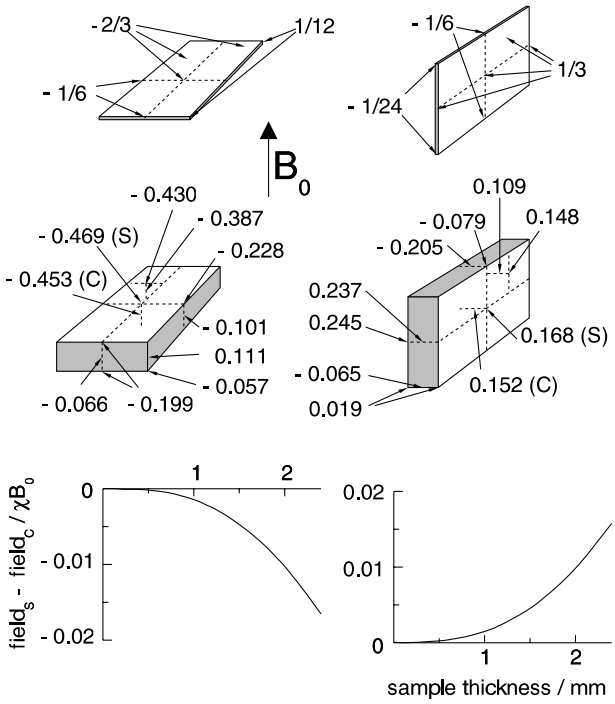

Fig. 1. The magnetic flux density in block-shaped samples is represented at several points by the numerical difference ΔB, which is expressed in units of χB_0 and specified relative to the homogeneous field that would be experienced by a spherical sample with the same susceptibility. Data are shown for an ideally thin sample (upper) and for a realistic sample of $7.5 \times 18 \times 2.4$ mm^3 (middle), when aligned at a tilt angle of $\theta = 0°$ and 90° between the sample normal and the external magnetic field B_0. The lower two panels show how the field at the surface (S) deviates from the bulk center (C) as a function of sample thickness for the two alignments.

between the vertical and horizontal sample alignment, and they experience a homogeneous field with distortions only at the very edges. In a thin "real" sample the field is still highly homogeneous over most of its volume, but significant field deviations appear near the edges in a thin region with dimensions on the order of the sample thickness. The thicker the sample, the more pronounced is the influence of these inhomogeneities at the edges on the bulk of the sample, and the higher is the proportion of regions where the field deviates significantly from that in the sample center. This leads to susceptibility-broadened lineshapes, as illustrated in Fig. 4 below, and explained in the accompanying paper [1]. In this theory paper we show that it is the central volume of the sample with a fairly homogeneous field that produces a distinct peak in the lineshape (see Figs. 2 and 4 in [1]). We will thus refer to this peak in defining the characteristic frequency of the NMR signal, for evaluating how it depends on the geometry and orientation of the sample. The calculations show that the flux density at the center of the sample (point "C" in Fig. 1) changes as a linear function of its total thickness, with deviations from linearity less than 0.0025 χB_0 (not shown). For our $7.5 \times 18\,\mathrm{mm}^2$ samples aligned at a tilt angle of $\theta = 0°$ the slope is 0.091 $\chi B_0/\mathrm{mm}$, and $-0.078\ \chi B_0/\mathrm{mm}$ for $\theta = 90°$. As expected, with increasing thickness of the sample the resonance frequency shifts from the extreme limit of $-2/3\ \chi B_0$ or $+1/3\ \chi B_0$ (Fig. 1) towards the value they would have in a spherical sample.

When we place a reference on top of the sample stack to measure and compensate this shift, we have to consider the difference between the magnetic field at point "S" on the sample surface that defines the reference signal and point "C" in the bulk center that defines the signal of the sample. For ideally thin samples this difference is zero, and it increases roughly with the third power of thickness, as illustrated in Fig. 1, bottom. The difference reaches $+0.016\ \chi B_0$ at a sample height of 2.4 mm at a tilt angle of $\theta = 0°$, which corresponds to -0.2×10^{-7} or a shift of 0.2 ppm upfield. The fact that the profile for $\theta = 90°$ appears nearly like an inversion of the profile for $\theta = 0°$ is a coincidence resulting from the particular proportions of our glass plates (i.e., length/width = 2.4/1). For square shaped supports the positive deviation $\Delta B/\chi B_0$ on the top or bottom surface of the sample at $\theta = 0°$ would be twice as large as the negative deviation for $\theta = 90°$ when the reference is placed at either side of such sample.

We conclude that a thin reference film on the surface of our kind of membrane samples will reflect the bulk chemical shift with a maximum referencing error of 0.2 ppm (upfield for $\theta = 0°$, downfield for $\theta = 90°$) for 2.4 mm thickness, while the error is only about 0.02 ppm for a sample of 1 mm thickness. These errors can be significantly reduced by susceptibility matching and suitable shimming as discussed below, hence they are not considered to be critical.

3.2. External referencing procedure

There are two options to determine the reference frequency: Either the reference sample is left in the coil throughout the data acquisition, or the position of the reference line is measured and then the reference is removed to acquire the spectrum of the membrane sample alone. To measure our labeled peptides we preferred the second possibility, as the signals of 4F-Phg and NaF show up in the same range around −120 ppm. When the reference is removed, however, the magnetic field in the membrane sample is modified, hence the resulting change in chemical shift has to be corrected for. We have derived above that the field in the center of the sample depends linearly on sample thickness. When placing the reference on top of the membranes, the total thickness of the stack increases by approximately 0.4 mm, namely by two 80 μm glass plates and an aqueous film of about 200 μm. Hence we evaluate for an oriented sample at $\theta = 0°$ that the field will change by $(0.16\,\mathrm{mm} \times -12 \times 10^{-6} + 0.2\,\mathrm{mm} \times -9 \times 10^{-6}) \times 0.091\ B_0/\mathrm{mm} = -0.34 \times 10^{-6}\ B_0$. This corresponds to a shift of −0.34 ppm (0.34 ppm upfield) for every NMR signal of the entire stack. When the reference is removed, all signals of the membrane sample will consequently shift downfield by the same amount. This implies the following referencing procedure: First, the reference is placed onto the membrane sample for measuring the resonance frequency of the reference solution. Then the reference is removed to acquire the spectrum of the membrane sample alone. The reference signal now has to be set to its chemical shift value of the first measurement plus 0.34 ppm for $\theta = 0°$ (or −0.29 ppm for $\theta = 90°$), because it would have moved 0.34 ppm downfield if it had been detectable during the actual data acquisition.

This referencing approach was experimentally verified. We placed a NaF reference onto an oriented membrane sample of 5% flufenamic acid in DMPC, which provides an intense and fairly sharp triplet [21]. The ^{19}F NMR spectrum was measured with and without the reference at $\theta = 0°$ and 90° tilt angles, to monitor the effect on the resonance frequencies of flufenamic acid. The intrinsic reproducibility of each one of these experiment was about ±0.07 ppm upon removing and reinserting the same sample in the coil. This experiment was repeated several times: with different individually constructed references, stacked on either on top or below the oriented membrane sample, or with several empty glass plates stacked between sample and reference. Basically, removal of the reference from the sample stack consistently gave rise to downfield shifts between 0.3 and 0.45 ppm at $\theta = 0°$, and upfield shifts

between 0.25 and 0.4 ppm at $\theta = 90°$. Within this range the shift in the resonance frequency correlated with the thickness of the reference sample. The experimentally determined deviations induced by our reference were thus in good agreement with the expected values.

For practical purposes there is a more straightforward way to set the reference frequency after removing the reference from the stack: we simply compensate for the difference in stack height by placing an appropriate number of glass plates (in our case three to four) without lipid onto the test sample. Then the reference frequency can be directly accepted as it appears in the spectrum. It is nonetheless essential to reference both spectra at $\theta = 0°$ and $\theta = 90°$ individually to obtain reliable orientational constraints. Although the variations in the magnetic field are small, knowledge about the influence of sample geometry on the reference frequency is important to avoid systematic misinterpretation of the data. In general, accurate chemical shifts are necessary to compare data from different probeheads and from samples in which materials other than glass were used to orient the membranes.

3.3. Chemical shift of the NaF reference relative to $CFCl_3$

The commonly accepted ^{19}F reference that defines $\delta = 0$, $CFCl_3$, is highly volatile and difficult to handle. Therefore, we filled our reference containers with NaF in aqueous phosphate buffer instead. The chemical shift of a NaF solution depends on temperature, concentration, and pH. The temperature- and concentration-dependence in neutral buffers relative to external $CFCl_3$ is shown in Fig. 2. Around neutral pH the resonance frequency is constant, and an upfield shift is only observed at low pH when HF is formed. The temperature-dependence of the chemical shift of $CFCl_3$ relative to the unlocked carrier frequency of the spectrometer was only 0.0029 ppm/K, which can be neglected for practical purposes in solid-state NMR spectroscopy. Therefore, we set the chemical shift scale for an oriented sample at one temperature only and kept it for all other temperatures without further correction.

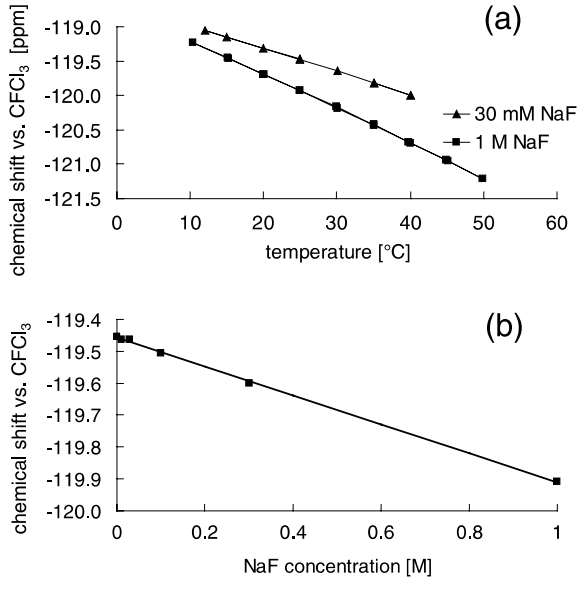

Fig. 2. Chemical shift of NaF reference solutions at neutral pH. Data are compiled for 30 mM and 1 M concentration at different temperatures (a), and at 25 °C for different NaF concentrations (b). Measurements were performed in standard 5 mm solution NMR tubes containing a central capillary filled with pure $CFCl_3$ as an external reference.

3.4. Proton spectra as reference

As an alternative to external referencing with a ^{19}F-containing sample we evaluated the potential of using the proton signal of the hydrated lipid. Solid-state ^{1}H NMR echo spectra of oriented membranes are shown in Fig. 3, where two broad peaks are usually resolved with variable intensities. The upfield peak contains contributions from the lipid fatty acyl chains and from the parafilm used for wrapping the sample. The downfield peak, which is usually less intense and has a slightly smaller linewidth, arises mainly from bound water but also contains the signals from the lipid headgroups. The shape and position of these peaks is thus expected to show slight variations with the composition of the sample, hydration, and wrapping procedure. Such variations were indeed observed, and they limit the accuracy of this referencing method.

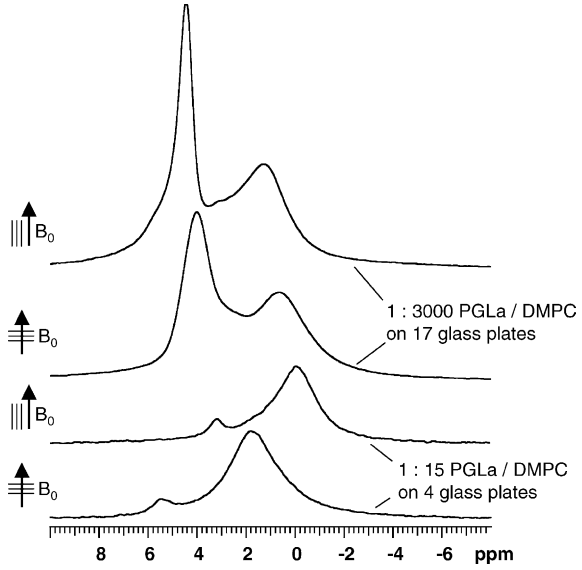

Fig. 3. Static solid-state ^{1}H NMR spectra of macroscopically oriented phospholipid samples at 98% hydration containing PGLa, acquired at 35 °C with 16 scans of an echo experiment. Samples with a molar peptide/lipid ratio of 1:3000 and 1:15 were measured at a tilt angle of $\theta = 0°$ and 90°. For demonstration purposes the chemical shift scale is *not* referenced for the individual samples, hence the peak positions reflect the significant modifications of the magnetic field by the sample. (Peak intensities vary with the sample composition as expected.)

To calibrate the solid-state ^{1}H NMR signals, we determined the ratio between the absolute frequencies of the corresponding reference substances TMS and $CFCl_3$. In a liquid sample containing 1% TMS and 1% $CFCl_3$ in $CHCl_3$ this ratio was 0.940 940 54. The difference of 0.85 ppm compared with the value of 0.940 939 74 given by Berger et al. [22] probably results from solvent effects. Using our value of 0.940 940 54 we determined the position of the two ^{1}H peaks in nine different membrane samples at 35 °C, based on the external NaF reference as described above. We found a proton chemical shift of 4.83 ± 0.21 ppm for the downfield peak, and 1.21 ± 0.18 ppm for the upfield peak. Considering the influence of the anisotropic molecular environment discussed below, this matches the expected values surprisingly well. The residual chemical shift anisotropy of water and the lipid acyl chains is sufficiently small in the liquid crystalline phase.

After this calibration it was possible to use the ^{1}H spectrum of the membrane to reference the ^{19}F chemical shift scale of the labeled peptide. In practice, we did not actually calculate the ^{19}F reference frequency from the ^{1}H spectrum for each sample. We used a pair of ^{1}H and ^{19}F experiments with the correct ratio of reference frequencies as a starting point, and shifted the ppm scale in the ^{19}F experiment by the same amount in ppm (not Hz) that was necessary to bring the two dominant ^{1}H peaks to about 4.83 and 1.21 ppm. This always requires some compromise, as the variation in the distance between downfield and upfield peak was ±0.24 ppm. As a consequence, the accuracy of chemical shift referencing via the ^{1}H NMR spectra is slightly lower than with an external reference, which gives a much sharper peak consisting of only one component. We had to use the ^{1}H NMR referencing method, however, when the membrane sample was so thick that an additional reference did not fit into the coil.

3.5. Practical aspects for improving the lineshape

The magnetic susceptibility difference between the sample and its environment not only shifts the resonance frequency, but it also leads to a considerable broadening of the lines. Using the approach outlined in the accompanying paper [1] we simulated in Fig. 4 the spectral lineshapes for a sample ($7.5 \times 18 \times 2\,mm^3$) tilted at $\theta = 0°$ (Figs. 4a and c) and $\theta = 90°$ (Figs. 4b and d). The advantages of shimming (Fig. 4c and d) and susceptibility matching (Figs. 4e, f, and g) are illustrated. The field distortions experienced by a hypothetical reference located on top of the sample surface are also depicted by the lineshapes drawn as thin lines, which represent only the uppermost 0.3 mm layer of the total stack height of 2 mm.

The linewidth at half height of a free-standing sample in air is on the order of 0.5 ppm (Figs. 4a and b), and the

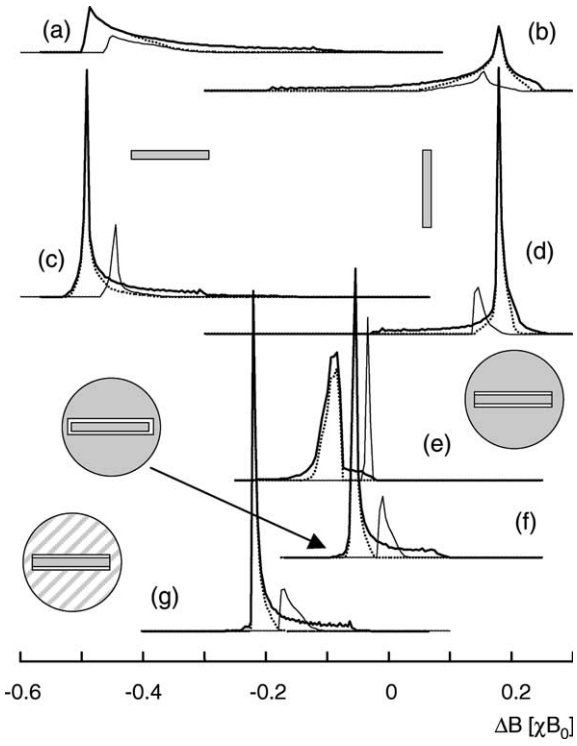

Fig. 4. Lineshapes of oriented samples were simulated to illustrate the magnetic field distortions due to susceptibility effects in various arrangements. Thick lines represent the signal of a rectangular sample of $7.5 \times 18 \times 2\,mm^3$; dotted lines arise from the central $5.5 \times 16 \times 2\,mm^3$ core of the sample; and thin lines represent the signal which would be experienced by a reference sample ($5.5 \times 16 \times 0.3$ mm^3 region) added on top of the stack. Data are shown for a free-standing sample in air, at a tilt angle of $\theta = 0°$ (a) and $\theta = 90°$ (b); the same sample with optimized x^2, y^2, and z^2 shims at $\theta = 0°$ (c) and 90° (d); the same oriented sample at $\theta = 0°$ in a susceptibility-matched sphere with air gaps in the rectangular sample chamber above and below the sample (e); the same with air gaps all around the sample (f); and the same as E but when the susceptibility of the spherical sample chamber is 2/3 of the sample susceptibility (g).

6 ppm wide "foot" arises from the edges of the sample. This "foot" would be considerably reduced if a 1 mm edge around the rim of the sample would not contribute to the signal, as indicated by the dotted lineshapes. A significant improvement in lineshape could in principle be accomplished by confining the lipid to the central part of the sample. In practice this is achieved by wrapping the sample with a soft susceptibility-matched material such as parafilm to cover the edges. This procedure is especially beneficial if the overall sample shape is made to resemble an ellipsoid.

As recently demonstrated by Soubias et al. [2], it is possible to compensate much of the magnetic field disturbance by the shim system of the magnet. In the central part of a thick sample the field can be roughly approximated by quadratic functions. This is simulated in Figs. 4c and d, where x^2, y^2, and z^2 shim fields are introduced and adjusted to set the sample center and the 6 points at half distance to the surface planes at the same field.

Shimming considerably narrows the susceptibility-induced linewidth to about 0.01 χB_0 or 0.1 ppm. Theoretically it could be improved further by optimizing higher-order shim gradients. Practically, however, besides the intrinsic T_2-limitations several facts prevent narrower lines: (i) the field distribution along a line through the sample cannot be described by a polynomial function; (ii) the field at each three-dimensional point in the sample is not a linear combination of x, y, and z profiles; (iii) extremely high shim fields are required—even the second-order shim field of about 1 T/m^2 in the simulation of Figs. 4c and d may be beyond the capacity of the shim system.

For practical applications, we suggest to shim on the sharp signal of the fluid reference, and then to replace it by three or four glass plates to maintain the same overall geometry for which the shims were optimized. The linewidth at half height of the reference was found between 80 and 170 Hz in the presence of membrane samples of various thickness. As the intrinsic linewidth of the ^{19}F-labeled peptide was at least 500 Hz due to T_2 relaxation, we usually made no further attempts to optimize the lineshape of the samples individually. Likewise, the ^{1}H NMR signals are much too broad for shimming (Fig. 3). As an alternative to overcome the problem that there are no intrinsic intense and narrow lines in macroscopically oriented samples, Soubias et al. [2] have suggested to incorporate ^{13}C-γ-methyl-labeled phosphatidylcholine lipids for shimming.

Another valuable approach to reduce linewidths and tilt-dependent susceptibility effects is the use of a susceptibility-matched probehead. The space around the sample is filled with a material of similar susceptibility as the sample itself, for example by a ceramic casing whose outer shape resembles an ellipsoid [3]. If the sample filled the cavity completely and if the susceptibility match was perfect, the field in the sample would become ideally homogeneous. Unfortunately, neither a perfect susceptibility match nor perfect filling can be achieved in practice. If the susceptibility match is not perfect but if the sample volume is completely filled, the signal looks very similar to Fig. 4a but scaled by a smaller susceptibility mismatch $\Delta\chi$ instead of the full susceptibility χ of the sample. If any air gaps remain in the sample cavity, however, the resulting lineshape is very sensitive to the size and position of these gaps. In Fig. 4e we simulated the case for two 0.5 mm air gaps above and below the sample, which disturb the field in the center of the sample and produce a lineshape without any sharp maximum. Allowing for an additional 0.5 mm air gap around the edges of the sample will partially compensate for the field disturbance by the gaps above and below, leading to an improved linewidth in Fig. 4f. We conclude that tiny changes in the distribution of material in a susceptibility-matched probehead have significant effects on the lineshape. It is therefore rather critical to optimize the distribution of the material and the wrapping in the immediate environment of the sample.

The arrangement depicted in Fig. 4g resembles a situation commonly encountered in our experiments. The NMR sample with the glass plates has a higher diamagnetic susceptibility than the material of the sample chamber, and the sample is thinner than the available space. In the numerical simulation the susceptibility of the sample chamber is 2/3 of that of the sample, which fills 2/3 of the chamber height. In this case the field disturbances are mostly balanced, and the lines are reasonably narrow besides the distinct "foot" resulting from mismatch at the edges. The susceptibility match reduces the differences in the reference frequency associated with sample orientation, but it has no effect on the shift resulting from different sample thickness or the deviations encountered upon removing the reference.

Another positive effect of both the susceptibility match and suitable shimming is, besides the narrower linewidth, that they reduce the field differences between the sample center and surface. The thin lines in Fig. 4 represent the local field in a reference sample that is additionally stacked on top of the sample. Comparison of these two lineshapes illustrates the procedure when the reference is first measured together with the sample, and then removed for the actual data acquisition. In the shimmed samples C and D and in the susceptibility-matched samples F and G the shift upon addition of the reference sample is in good agreement with the values calculated above. In the samples A and B the shift is somewhat smaller, because of the local field differences between sample center and surface shown in Fig. 1.

3.6. Oriented samples of ^{19}F-labeled PGLa at different peptide/lipid ratios

To study the concentration-dependent response of the antimicrobial peptide PGLa by ^{19}F NMR, we placed a 4F-Phg side chain in the position of Ala10. Samples were prepared with a wide range of peptide/lipid ratios to detect any signs of re-orientation or oligomerization from the anisotropic chemical shift of the ^{19}F label. Some representative spectra are shown below (Fig. 5, Table 1).

The minimum peptide/lipid ratio we could observe is limited by the sensitivity of the ^{19}F NMR measurement and by the maximum amount of lipid that can be accommodated in the oriented samples. With about 20 nmol of ^{19}F label we obtain sufficient signal/noise for a signal of 1–2 kHz linewidth in an overnight experiment (see Fig. 5), which corresponds to 0.04 mg of peptide. The optimum amount of lipid to produce well-oriented hydrated membranes is about 0.5–1.5 mg per glass plate. Beyond 2–3 mg lipid per plate the fraction of the well-oriented membranes rapidly drops below 70%, as observed by ^{31}P NMR spectra of DMPC bilayers. Using 60–80 μm thick glass plates in our coil with a maximum

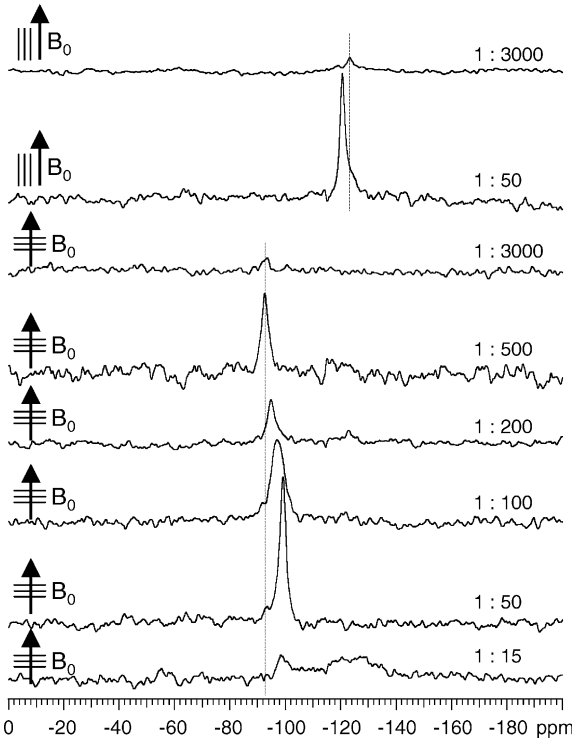

Fig. 5. Solid-state ^{19}F NMR spectra of macroscopically oriented DMPC membranes containing 4F-Phg-labeled PGLa (substituted at position Ala10). All spectra were recorded at 35 °C, with 15 kHz ^{1}H-decoupling and 3 s recycle delay. Details are given in Table 1.

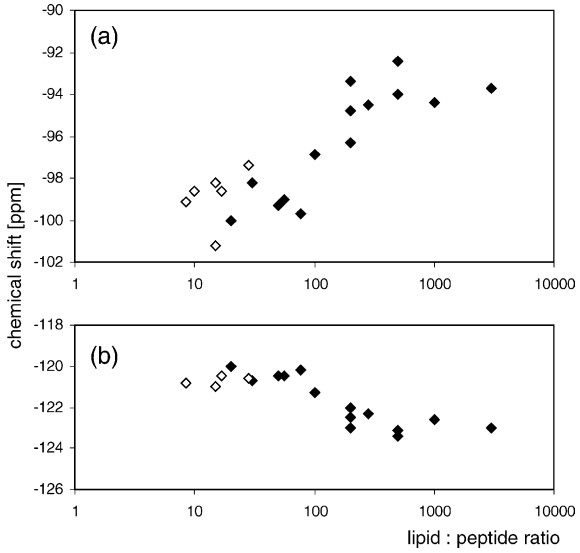

Fig. 6. Anisotropic ^{19}F NMR chemical shift of 4F-Phg-labeled PGLa in oriented DMPC samples with different lipid/peptide ratios, for a tilt angle of $\theta = 0°$ (a) and $\theta = 90°$ (b). Closed symbols represent samples with a single line of less than 2 kHz linewidth. For samples that showed an additional broad (15–20 kHz) component, the chemical shift of the sharp line is indicated with an open symbol.

stack height of 2.4 mm, the greatest amount of lipid that can be employed for oriented samples is 30–50 mg, distributed between 16 to 18 glass plates. Hence the minimum accessible peptide/lipid ratio is about 1:3000. Toward high peptide/lipid ratios the practical limit is set by peptide aggregation, as the peptide signal started to form a second, broad component, as illustrated by Fig. 5. In most samples, however, we could still distinguish a discrete peak up to a peptide/lipid ratio of 1:8. With 0.2–0.5 mg peptide per sample we obtain a good signal/noise within less than an hour. At the highest peptide/lipid ratios this corresponds to about 1 mg of lipid, which is spread over two or three glass plates resulting in a sample height of about 0.4 mm.

Figs. 5 and 6 demonstrate the small but significant change over ~5 ppm in the chemical shift of the ^{19}F-labeled PGLa. Given the variation in sample height, the spectra had to be carefully referenced. Otherwise the bulk susceptibility effects would have lead to chemical shift differences up to 2 ppm, thus introducing a 40% error. The genuine change in chemical shift is attributed to a concentration-dependent alteration of the peptide structure and/or dynamics. The smooth change in chemical shift suggests that both states of the peptide are in fast exchange with one another on the timescale of the NMR experiment (~2.5 kHz). The mid-point of our curve appears at a molar peptide/lipid ratio of about 1:200 in hydrated DMPC at 35 °C. At low peptide concentration the occurrence of narrow signals at both $\theta = 0°$ and $\theta = 90°$ means that the peptide helices are motionally averaged about the membrane normal, presumably as monomers or dimers [14]. We observe such narrow signals up to peptide/lipid ratios of 1:20, as indicated by the filled symbols of Fig. 6. With increasing peptide concentration, however, an additional broad component starts to appear at a ratio of 1:40 in some samples (see open symbols) and dominates all spectra at high concentration. The broad component represents a discrete population of immobilized PGLa peptides which have lost their high quality of alignment and no longer rotate within the membrane. This could be indicative either of ill-defined peptide aggregates or of discrete self-

Table 1
Sample composition and measurement times of the spectra shown in Fig. 5

Molar ratio peptide/lipid	1:3000	1:500	1:200	1:100	1:50	1:15
Total amount of peptide (μmol)	0.02	0.17	0.15	0.17	0.15	0.15
Measurement time for $\theta = 90°$	8 h				1 h	
Chemical shift at $\theta = 90°$ (ppm)	−123.0				−120.5	
Measurement time for $\theta = 0°$	8 h	2 h	2 h	5 h	4 h	4 h
Chemical shift at $\theta = 0°$ (ppm)	−94	−92.5	−94.8	−96.8	−99.3	−98.2

assembled oligomers, broadened by T_2-relaxation, but we cannot tell from this single 4F-Phg label alone. A more detailed structural interpretation of PGLa will be part of another paper, as here we have focused on the practical aspects of referencing the ^{19}F NMR spectra.

3.7. Susceptibility differences within the sample

Up to this point we have treated the sample as a homogeneous block, but we have to be aware that the internal layered structure of the membrane sample may influence the NMR measurements further. First, the glass plates, the lipid films, and the aqueous solution containing the reference molecule represent thin parallel layers with different susceptibilities. Second, at a molecular level the phospholipid membranes themselves may be considered as alternating layers of a hydrocarbon core, polar headgroups, and bound water. To assess the first situation, consider a test molecule and a reference molecule as spheres with susceptibilities χ_t and χ_r, embedded in thin slabs with the respective environment χ_T and χ_R. The difference between the magnetic flux density in the test molecule (B_t) and in the reference molecule (B_r) is $B_t - B_r = 2/3(\chi_t - \chi_r)B_0 - 2/3(\chi_T - \chi_R)B_0$ at $\theta = 0°$, and $B_t - B_r = 2/3(\chi_t - \chi_r)B_0 + 1/3(\chi_T - \chi_R)B_0$ at $\theta = 90°$. The susceptibility of the glass does not show up in these differences, as it affects only the absolute positions of both signals but not the difference between them. The contribution of the susceptibility difference of the molecules, $2/3(\chi_t - \chi_r)B_0$, is an integral part of the chemical shift and does not depend on the environment. The terms containing the susceptibility difference of the environment, $(\chi_T - \chi_R)B_0$, on the other hand, reflect the non-specific solvent effect known from external referencing in high resolution liquid state NMR. This solvent effect depends on the orientation of the solvent layer. Fortunately the susceptibilities of water (surrounding the reference molecule) and hydrated multilamellar phospholipids (surrounding the test molecule) are very similar, and it is not necessary to apply any corrections here. These considerations show, however, that there is no universally applicable referencing scheme. Anisotropic chemical shifts of molecules in oriented membrane samples may thus differ by more than 1 ppm depending on the method of referencing, even if the very same reference substance is used. The method by which a sample was referenced therefore has to be stated in detail to allow a comparison of the data.

Next consider a test molecule which can penetrate into different regions of the membrane with different magnetic susceptibilities (e.g., hydrocarbons versus headgroups). Its chemical shift may thus vary with its position in the lipid bilayer, as the resonance frequency depends on the susceptibility of the surrounding material in an orientation-dependent way. Furthermore, the peptide carrying the ^{19}F label is oriented amongst the lipids and disturbs their anisotropic alignment. Unlike the situation in an isotropic fluid, the magnetic dipoles on the lipid molecules do not average to zero. As a result, we expect to observe slightly different principal values for the CSA tensor of the same ^{19}F label when measured in a different environment and under different conditions, e.g., in a dry powder or in a frozen sample, or in different membranes where peptide orientation and dynamics are studied. When determining the orientation and/or dynamics of a label from its anisotropic chemical shift in the membrane, we need the three principal axis values of the CSA tensor of the label, which we can determine from the powder spectrum [23]. In a strict sense, however, we would need the powder spectrum of a molecule in different orientations *relative to* the membrane. Experimentally, however, we can only determine the powder spectrum of the molecule rotated *together with* the lipid bilayer. The difference between these two sets of values is on the same order as the susceptibility differences due to the local molecular environment, i.e., several ppm. In the case of a narrow CSA tensor, this uncertainty puts an additional limit to the accuracy of the molecular orientation and dynamics to be determined, and this intrinsic uncertainty cannot be reduced further by a more accurate measurement or referencing procedure.

In summary, the high sensitivity of ^{19}F NMR made it possible to measure anisotropic chemical shifts of about 1 μmol of peptide in an oriented membrane sample, with an intrinsic reproducibility down to 0.01 ppm. Taking the sample out of the probehead and measuring it upside down still gave a reproducibility of about 0.05 ppm. The external referencing procedure with a NaF solution had an accuracy of about 0.1 ppm, considering the maximum differences in the magnetic field between the bulk center and the surface regions of our samples. With this referencing procedure we could correct differences in the magnetic field due to variations in sample geometry that would have otherwise produced systematic errors up to 2 ppm. The remaining scatter amongst the individual data points in Fig. 6 of up to 2 ppm is due to the difficulty of preparing reproducible samples and measuring them under reproducible conditions. These considerations concern the distribution of material, the degree of hydration, and the wrapping procedure, hence it is worth focusing the attention on possibilities of how to reduce such variations in the future.

4. Conclusions

We recommend the following referencing procedure, if chemical shifts have to be determined in macroscopically oriented membrane samples within an accuracy of 1 ppm:

1. Prepare a thin reference sample with the appropriate glass plates that fit your sample and coil geometry.

2. Work with a susceptibility-matched coil, if possible. When wrapping the sample in parafilm to avoid drying in the first place, try to make it fit snugly into the sample cavity to reduce air gaps. When working with a free-standing solenoid coil, apply the wrapping to achieve an overall spheriodal sample shape.
3. Place the reference on top of the stack, and shim on the narrow reference signal.
4. Determine the reference frequency for each tilt angle separately.
5. If you replace the reference by an appropriate number of glass plates during the actual data acquisition, there is no need to correct for the chemical shifts associated with a change in stack height. Alternatively, you may wish to apply the appropriate correction terms that have been determined experimentally or numerically for each tilt angle, as outlined in the accompanying theory paper [1].
6. You may calculate the chemical shift error associated with the surface location of the reference for the particular proportions of your sample stack or its material properties χ [1], but usually these errors are sufficiently reduced by shimming and susceptibility matching.
7. If the sample is too thick to allow an additional reference to be fitted into the coil, use the ^{1}H signals of the lipid bilayer for chemical shift referencing. This method is more generally applicable although slightly less accurate.
8. Check the concentration- and temperature-dependence of your reference solution against the accepted standard, before setting the zero frequency.

Acknowledgments

We acknowledge helpful discussions with Jan Gimsa, and thank Waltraud Scheiding and Carsten Sachse for technical support and NMR measurements. The Deutsche Forschungsgemeinschaft is acknowledged for Grant TPB6/SFB604.

References

[1] R. Ulrich, R.W. Glaser, A.S. Ulrich, Susceptibility corrections in solid-state NMR experiments with oriented membranen samples. Part II: theory, J. Magn. Reson. 164 (2003) 115–127.
[2] O. Soubias, O. Saurel, V. Réat, A. Milon, High resolution ^{13}C spectra on oriented lipid bilayers: from quantifying the various sources of line broadening to performing 2D experiments with 0.2–0.3 ppm resolution in the carbon dimension, J. Biomol. NMR 24 (2002) 15–30.
[3] F.D. Doty, G. Entzminger, Y.A. Yang, Magnetism in high-resolution NMR probe design. I: general methods, Concepts Magn. Reson. 10 (1998) 133–156.
[4] J. Salgado, S.L. Grage, L.H. Kondejewski, R.S. Hodges, R.N. McElhaney, A.S. Ulrich, J. Biomol. NMR 21 (2001) 191–208.
[5] S.L. Grage, J. Wang, T.A. Cross, A.S. Ulrich, Solid state ^{19}F-NMR analysis of ^{19}F-labeled tryptophan in gramicidin A in oriented membranes, Biophys. J. 83 (2002) 3336–3350.
[6] T.M. Rothgeb, E. Oldfield, Nitrogen-14 nuclear magnetic resonance spectroscopy as a probe of lipid bilayer headgroup structure, J. Biol. Chem. 256 (1981) 6004–6009.
[7] T.A. Cross, S.J. Opella, Solid-state structural studies of peptides and proteins in membranes, Curr. Opin. Struct. Biol. 4 (1994) 574–581.
[8] W. Hoffmann, K. Richter, G. Kreil, A novel peptide designated PYLa and its precursor as predicted from cloned mRNA of *Xenopus laevis* skin, EMBO J. 2 (1983) 711–714.
[9] M. Zasloff, Magainines, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor, Proc. Natl. Acad. Sci. USA 84 (1987) 5449–5453.
[10] K. Matsuzaki, Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes, Biochim. Biophys. Acta 1462 (1999) 1–10.
[11] B. Bechinger, M. Zasloff, S.J. Opella, Structure and dynamics of the antibiotic peptide PGLa in membranes by solution and solid-state nuclear resonance spectroscopy, Biophys. J. 74 (1998) 981–987.
[12] Y. Hori, M. Demura, M. Iwadate, A.S. Ulrich, T. Niidome, H. Aoyagi, T. Asakura, Interaction of mastoparan with membranes studied by ^{1}H-NMR spectroscopy in detergent micelles and by solid-state ^{2}H-NMR and ^{15}N-NMR spectroscopy in oriented lipid bilayers, Eur. J. Biochem. 268 (2001) 302–309.
[13] J.A. Whiles, R. Brasseur, K.J. Glover, G. Melacini, E.A. Komives, R.R. Vold, Orientation and effects of mastoparan X on phospholipid bicelles, Biophys. J. 80 (2001) 280–293.
[14] O. Toke, R.D. O'Connor, W.L. Maloy, R.W. Glaser, A.S. Ulrich, J. Schaefer, Alignment of polypeptide chains in $(KIAGKIA)_3$ dimers in a phospholipid bilayer by solid-state NMR, Biophys. J., 2003, submitted.
[15] H.W. Huang, Action of antimicrobial peptides: two-state model, Biochemistry 39 (2000) 8347–8352.
[16] F.Y. Chen, M.T. Lee, H.W. Huang, Sigmoidal concentration dependence of antimicrobial peptide activities: a case study on alamethicin, Biophys. J. 82 (2002) 908–914.
[17] L. Yang, T.A. Harroun, T.M. Weiss, L. Ding, H.W. Huang, Barrel-stave model or toroidal pore? A case study on Melittin pores, Biophys. J. 81 (2001) 1475–1485.
[18] S. Afonin, R.W. Glaser, M. Berditchevskaia, P. Wadhwani, K.-H. Gührs, U. Möllmann, A. Perner, A.S. Ulrich, 4-Fluoro-phenylglycine as a label for ^{19}F-NMR studies of membrane-associated peptides, ChemBioChem, 2003, in press.
[19] A. Ramamoorthy, L.M. Gierasch, S.J. Opella, Three-dimensional solid-state NMR correlation experiment with 1H homonuclear spin exchange, J. Magn. Reson. B 111 (1996) 81–84.
[20] S. Yamaguchi, T. Hong, A. Waring, R.I. Lehrer, M. Hong, Solid-state NMR investigations of peptide–lipid interaction and orientation of a beta-sheet antimicrobial peptide, protegrin, Biochemistry 41 (2002) 9852–9862.
[21] S.L. Grage, A.S. Ulrich, Orientation-dependent ^{19}F dipolar couplings within a trifluoromethyl group are revealed by static multipulse NMR in the solid state, J. Magn. Reson. 146 (2000) 81–88.
[22] S. Berger, S. Braun, H.O. Kalinowski, NMR-Spektroskopie von Nichtmetallen, Band 4, ^{19}F-NMR-Spektroskopie, Georg Thieme Verlag, Stuttgart/New York, 1994.
[23] D.E. Warschawski, M. Traikia, P.F. Devaux, G. Bodenhausen, Solid-state NMR for the study of membrane systems: the use of anisotropic interactions, Biochimie 80 (1998) 437–450.
[24] A.S. Ulrich, M.P. Heyn, A. Watts, Structure determination of the cyclohexane ring of retinal in bacteriorhodopsin by solid state deuterium NMR, Biochemistry 31 (1992) 10390–10399.