Communication

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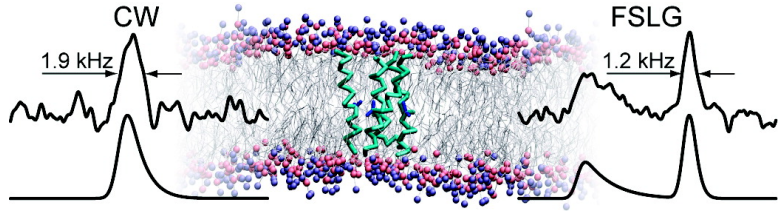

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Resolution Enhancement in Solid-State NMR of Oriented Membrane Proteins by Anisotropic Differential Linebroadening

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Over the past decades, solid-state NMR spectroscopy on macroscopically oriented lipid bilayer or magnetically oriented bicelle samples has attracted considerable attention for determination of the structure of membrane-bound proteins.[1,2] So far, most studies have addressed smaller membrane proteins and peptides for which it has been possible to obtain well-resolved peaks in 2D separated local field (SLF) spectra correlating the amide $^{1}H-^{15}N$ dipole–dipole couplings and ^{15}N chemical shifts. In less favorable cases, in particular, for larger proteins and high protein/lipid ratios, the resonances are substantially broader and display significant overlap.[2–4] Despite large efforts in sample preparation,[3,5] it is believed that a major cause of line broadening is imperfect sample alignment (e.g., mosaic spread), which in many cases appears to be an intrinsic and unavoidable property of the system. The inevitable consequences are low signal-to-noise ratio and increased risk for spectral overlap.

In this Communication, we demonstrate a new method that may significantly improve the resolution in the ^{15}N dimension of 1D ^{15}N and 2D $^{1}H-^{15}N$ SLF experiments for oriented membrane protein systems. The method, relying on orientation disorder induced differential line broadening, bears resemblance to the TROSY-type experiments[6] where differential line broadening, induced indirectly from anisotropic interactions by relaxation, offered new capabilities for studying large proteins by liquid-state NMR. The *orientation-induced* differential line broadening occurs when an inhomogeneous broadening from one anisotropic interaction (e.g., chemical shift) is partly canceled by inhomogeneous broadening from another anisotropic interaction (e.g., heteronuclear dipolar coupling) with opposite sign. In the present work, this effect is observed in 1D and 2D ^{15}N experiments using ^{1}H homonuclear dipolar decoupling instead of standard ^{1}H heteronuclear decoupling.

To experimentally demonstrate the spectral effects of orientational disorder in oriented lipid bilayer samples, we prepared a sample of ^{15}N-Aib$_8$ alamethicin in oriented DMPC bilayers. With the high peptide/lipid ratio (1:15 molar ratio) used here, the resonances in a 1D spectrum (detected with ^{1}H heteronuclear SPINAL-64[7] decoupling) display significant line broadening as evidenced by the spectrum in Figure 1a. Indeed, the spectrum shows accumulation of intensity at ~200 ppm which is characteristic for oriented peptides with a transmembrane α-helical conformation.[8] The measured line width of 1.9 kHz, however, is significantly larger than the values observed for perfectly oriented peptides. Figure 1d illustrates the appearance of the ^{15}N spectrum

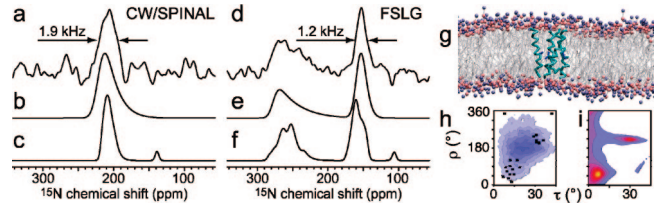

Figure 1. (a,d) Experimental and (b,c,e,f) simulated 16.45 T $^{1}H-^{15}N$ spectra for a sample of ^{15}N-Aib$_8$ alamethicin in oriented lipid bilayers. The spectra are obtained using (a–c) heteronuclear SPINAL-64 ^{1}H decoupling and (d–f) ^{1}H homonuclear FSLG decoupling. (b,e) Simulations assuming a single molecular conformation (corresponding to an α-helix conformation of τ,ρ = 7.85°, 52°) and $\Delta\beta = 18°$ for the mosaic spread. (c,f) Simulations obtained using the average chemical shift and dipole–dipole coupling frequencies for the 25 peptides in the MD simulation. (g) Snapshot of an alamethicin channel-like aggregate with four alamethicin molecules resulting from the MD simulation. (h) Distribution of helix conformations in the MD simulation. Blue contours represent conformation counts, while the black points represent average conformations back-calculated from averaging the nuclear spin interaction frequencies for each peptide conformation. (i) Restriction plot showing peptide conformations in agreement with the FSLG spectrum (d), assuming an ideal α-helical secondary structure.

recorded using ^{1}H homonuclear frequency-switched Lee–Goldburg (FSLG) decoupling.[9] The spectrum shows the expected dipolar doublet, the peak at ~150 ppm is relatively sharp (1.2 kHz line width) compared to the regular 1D spectrum, while the peak at ~270 ppm is much broader and reaches only about half the peak height with a pronounced upfield tail extending down to ~200 ppm.

The observation that the FSLG-decoupled spectrum displays a resonance with only about half the line width of the regular 1D spectrum renders homonuclear decoupling a very appealing alternative to standard ^{1}H heteronuclear decoupling in the design of new experiments for samples with orientational disorder, provided we can justify the origin of the differential line broadening effect. Because of the asymmetric line shape of the downfield peak in the present spectrum, we can rule out transverse relaxation as a main reason for the spectral appearance. Relaxation would only broaden the resonances but cannot account for the line shape of the downfield peak. On the other hand, considering that the two peaks appear at the frequency of the orientation-dependent chemical shift plus or minus the scaled dipolar coupling, the two peaks will be influenced by the sum or difference, respectively, of an inhomogeneous distribution of *anisotropic* parts of the chemical shift and dipolar coupling, leading to different net anisotropies for the two peaks.

To get insight into the behavior of alamethicin in a bilayer at high peptide concentrations, we performed a coarse-grained (CG) molecular dynamics (MD) simulation including an ensemble of

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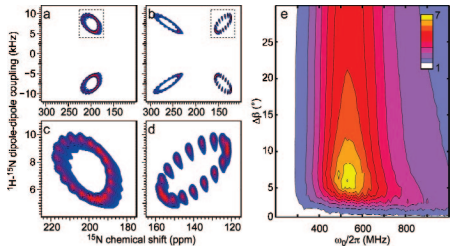

Figure 2. Simulated 16.45 T ^{15}N SLF spectra for an ideal uniformly ^{15}N-labeled α-helix with a tilt of $\tau = 15°$ relative to the bilayer normal and with $\Delta\beta = 8°$. (a,c) Regular SLF spectrum and (b,d) SLF spectrum using ^{1}H FSLG decoupling during acquisition. The representations in (c,d) are expansions illustrated by dashed boxes in (a,b). (e) Average resolution enhancement for the 18 residues of an ideal α-helix ($\tau = 15°$) obtained using FSLG decoupling as compared to standard heteronuclear decoupling as a function of the mosaic spread and magnetic field strength (^{1}H MHz).

25 alamethicin peptides in a bilayer consisting of 330 DMPC lipids (1:13.2 peptide/lipid ratio). Figure 1g shows a snapshot from this simulation, which illustrates the channel-like assembly of four of the peptides. For this snapshot, we make two interesting observations. First, the four monomers do not have identical conformations, and second, the bilayer is not perfectly aligned. On this basis, we will use two models for the orientational disorder assuming (*i*) that all peptide conformations average to a single conformation in the bilayer on the time scale of the NMR experiment, with a static disorder (mosaic spread) of the bilayer and (*ii*) the presence of multiple different molecular conformations in the bilayer.

The conformation-dependent nuclear spin interactions will be averaged around the local bilayer normal due to fast rotational diffusion at the present temperature well above the DMPC phase transition.[10] The local bilayer normals are assumed to have a Gaussian distribution of width $\Delta\beta$ with respect to the external magnetic field due to mosaic spread.[4,11] The NMR spectra, simulated using SIMPSON and SIMMOL,[12] and for the first model using the peptide conformation and mosaic spread ($\Delta\beta$) as free variables are shown in Figure 1b,e. The corresponding restriction plot, representing peptide conformations being in agreement with the experimental spectrum, is shown in Figure 1i. We observe a good agreement between experimental and simulated spectra, which supports the validity of this model.

For the second model, we use the molecular conformations from the MD simulation. To eliminate the effects of fast fluctuations, which would not be directly observed in the NMR experiments, we have calculated the time-averaged ^{15}N chemical shift and effective ^{1}H−^{15}N dipole–dipole coupling for each of the 25 alamethicin molecules and used these parameters with additional 10° mosaic spread as input for the simulations in Figure 1c,f. Again, we observe a good agreement with the experimental data, although the spectra are quite ragged due to the small number of alamethicin peptides in the MD simulation. The two models support each other by the presence of more than a single unique peptide conformation. This is further substantiated in Figure 1h, which shows (with black dots) the back-calculated peptide conformations from the 25 average nuclear spin interaction parameters. These conformations compare well with the restrictions found by the first model (Figure 1i).

On the basis of the good agreement between the experimental and simulated spectra, it is evident that direct effects from differential line broadening can be rationalized and used experimentally by recording spectra with homonuclear instead of heteronuclear decoupling. It is relevant to exploit this for multidimensional experiments, which are indispensable for multiple labeled samples. The immediate gain of homonuclear decoupling is demonstrated in Figure 2 by simulated 2D SLF spectra for a uniformly ^{15}N-labeled α-helical peptide with a mosaic spread of $\Delta\beta = 8°$ using standard heteronuclear (CW or SPINAL-64) or FSLG decoupling during acquisition. While both spectra show the expected wheel-like patterns for the resonances, the FSLG-decoupled spectrum splits into a doublet in the ^{15}N chemical shift dimension due to the ^{1}H−^{15}N dipolar coupling and, more importantly, displays significantly better resolution than the standard SLF spectrum. We note that only the horizontal ^{15}N chemical shift dimension is affected by the homonuclear decoupling. Nonetheless, while the standard SLF experiments display severe overlap of all resonances, the FSLG spectrum shows significantly better resolution. A more systematic investigation of the gain in resolution is presented in Figure 2e, which shows the relative line width of the narrow peak in the ^{15}N dimension of the FSLG spectrum versus the line width in the CW-decoupled spectrum as a function of the mosaic spread and magnetic field strength. This demonstrates that it is possible to achieve up to 7-fold gain in resolution by using homonuclear decoupling.

In conclusion, we have demonstrated that significant improvement in the resolution of spectra for inhomogeneously disordered oriented samples may be obtained by recording spectra with homonuclear proton decoupling instead of conventional heteronuclear proton decoupling. This resolution enhancement may be essential for assignment and structural exploitation of spectra for large oriented membrane proteins.

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Supporting Information Available: Details on the sample preparation, the experimental setup of the solid-state NMR experiments, the numerical simulations, and the MD simulation. This material is available free of charge via the Internet at http://pubs.acs.org.

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