$^{13}$C Selective Polarization and Spin Diffusion in a Lipid Bilayer-Bound Polypeptide by Solid-State NMR

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While $^{15}$N solid-state NMR has proven to be very advantageous for the development of structural biological methods, $^{13}$C spectroscopy has increased sensitivity and spectral dispersion. However, large natural abundance signals and homonuclear dipolar interactions pose significant problems. Here we have used a pair of $^{13}$C-labeled sites in a lipid-solubilized polypeptide to show the selective polarization can be used in combination with spin diffusion to achieve simplified spectra. Both unoriented and oriented samples have been used, with the latter providing a well-resolved homonuclear dipolar splitting.

Key Words: solid-state NMR; selective polarization; spin diffusion; polypeptide; orientational constraints.

Resolution in solid-state $^{13}$C NMR spectra of specific site labels in a background of natural abundance is very challenging for all but the smallest biological molecules. Particularly, the observation of a labeled polypeptide in unoriented and oriented lipid bilayer preparations poses a very severe problem. Yet in comparison with $^{15}$N, $^{13}$C is a relatively sensitive nucleus and it possesses favorable relaxation properties for homonuclear spin diffusion. While spin diffusion is the scourge of many NMR experiments here it can be used to observe homonuclear dipolar interactions and to potentially make resonance assignments. In the present work, a combination of selective polarization and spin exchange techniques has been applied to observe $^{13}$C$_a$–$^{13}$C$_i$ homonuclear dipolar splitting from a membrane-bound polypeptide while suppressing natural abundance signals from the lipid carbonyl carbon resonances.

A standard pulse sequence for two-dimensional (2D) spin diffusion experiments with cross-polarization preparation is shown in Fig. 1. However, a contact time of a few tens of microseconds instead of a few milliseconds has been used to selectively polarize the protonated versus nonprotonated carbons. Fortunately, such a relatively short contact time may lead to better sensitivity for the protonated carbons because of the strong couplings between the carbons and the attached protons, especially for systems having a short proton $T_1$ relaxation.

This is based on transient dipolar oscillation phenomena as described before (1, 2). For the selectively $^{13}$C$_a$–$^{13}$C$_i$-labeled polypeptide, $^{13}$C$_a$ but not $^{13}$C$_i$ is polarized. Following cross-polarization the $^{13}$C$_a$ magnetization evolves during the evolution period $t_1$. A 90° pulse is applied to restore the $^{13}$C$_a$ magnetization along the $z$ axis before the mixing time $\tau_m$, during which the magnetization is transferred from $^{13}$C$_a$ to $^{13}$C$_i$ through proton-driven carbon–carbon spin diffusion in the absence of proton decoupling. The spin–lattice relaxation effect during the mixing time is alleviated by storing the magnetization alternatively along the $+Z$ and $-Z$ directions (3). Another 90° pulse brings the longitudinal $^{13}$C$_a$ magnetization back into the transverse plane for signal observation during the detection period $t_2$. For one-dimensional experiments, the evolution period $t_1$ is set to zero.

The selectivity of polarizing protonated carbon versus nonprotonated carbon through transient oscillation depends on the magnitude difference of the dipolar interaction between the protonated carbon and the bonded protons and that between the nonprotonated carbon and nearby protons. For the protonated carbon (e.g., $^{13}$C$_a$) where strong heteronuclear proton–carbon dipolar interactions are present, the cross-polarized $^{13}$C$_a$ magnetization experiences a dipolar oscillation during the cross-polarization in these hydrated bilayer preparations (2). The buildup of the $^{13}$C$_a$ magnetization can be characterized by $T_{1\text{ep}}$, proton–proton spin diffusion rate $R$ and the static magnitude of the dipolar interaction $b$ (I):

$$M(t) = M_0 e^{-\gamma b t/2} \left( 1 - 0.5 e^{-R t} - 0.5 e^{-1.5 R t} \cos \left( \frac{bt}{2} \right) \right). \quad [1]$$

On the other hand, because the protons involved in the cross-polarization are further away from the carbonyl carbon, the buildup of the cross-polarized $^{13}$C$_i$ magnetization is rather slow and no dipolar oscillations are expected during cross-polarization. The polarized $^{13}$C$_i$ magnetization can be described using the following expression (4) with the assumption of a very long $^{13}$C$_i$ longitudinal relaxation time in the doubly rotating frame:
Here, \( \lambda = 1 - T_{C_{13}H}/T_{1p}^{H} \) and \( T_{C_{13}H} \) is the cross-relaxation time between the \( ^{13}\text{C}_{1} \) and bulk protons involved in the cross-polarization process. In the system studied here, the \( ^{13}\text{C}_{a} \text{–H} \) heteronuclear dipolar coupling for glycine residues is 22.5 kHz. We can reasonably assume that \( T_{1r}^{H} = 5.4 \text{ ms} \) and \( R = 280 \mu s \) as obtained before (2), and \( T_{C_{13}H} \) is considered to be \( \sim 450 \mu s \) (5). Thus, a factor of 10 in selectivity for the protonated site can be achieved by using a 15-\( \mu \text{ s} \) contact time. A longer contact time will deteriorate the selectivity. For instance, a 25-\( \mu \text{ s} \) contact time generates a factor of 7.5 in selectivity. Although the selectivity is not great, it is sufficient to suppress the lipid carbonyl carbon resonances. This may be partially due to the high peptide:lipid molar ratio (1:8) used in the sample preparations. For systems where more dilute peptide:lipid ratios are needed, the signals from the lipid carbonyl carbons can be further suppressed by incorporating other selective excitation techniques. For example, a shaped pulse can be easily applied because the carbonyl carbon resonances are more than 100 ppm away from the \( ^{13}\text{C}_{a} \) resonance (6).

Chemical shift spectra of a labeled \( ^{13}\text{C}_{13} \text{–Gly} \), gramicidin A in hydrated unoriented dimyristoylphosphatidylcholine (DMPC) bilayers recorded at 36°C are shown in Fig. 2. A spectrum using a standard cross-polarization experiment is presented in Fig. 2A. The \( ^{13}\text{C}_{a} \) signal is between 40 and 60 ppm, buried in the natural abundance \( ^{13}\text{C} \) signals from the lipids. The signals around 170 ppm result from the superposition of the chemical shift powder pattern from the lipid backbone and isotopically labeled carbonyl carbons in the peptide. Both chemical shift tensors are extensively averaged by fast global motions of the polypeptide and lipid about the bilayer normal (7) which is parallel to the applied magnetic field. For the Gly\(_{2} \) site in gramicidin the static chemical shift tensor has the \( \sigma_{11} = 242 \text{ ppm element in the peptide plane with } \sigma_{22} = 173 \text{ ppm also in the plane and making an angle of 20° with respect to the carbonyl bond, while } \sigma_{33} = 91 \text{ ppm is perpendicular to the peptide plane (8). With a peptide:lipid molar ratio of 1:8, approximately 70% of the carbonyl signal is from the single-site label while the natural abundant signal is contributed in equal parts from the polypeptide and lipid. Furthermore, this carbonyl region is complicated by the \( ^{13}\text{C}_{a} \text{–}^{13}\text{C}_{1} \) homonuclear dipolar interaction. However, from the known structure of the polypeptide the \( ^{13}\text{C}_{a} \text{–}^{13}\text{C}_{1} \) bond of the glycine residue makes an angle of 56° with respect to the channel axis, corresponding to a predicted dipolar splitting of 236 Hz (2.4 ppm at 9.4 T) for the parallel component based on a \( ^{13}\text{C}_{a} \text{–}^{13}\text{C}_{1} \) dipolar coupling.

\[ M(t) = M_0 e^{-\lambda t} \left( 1 - e^{-\lambda T_{C_{13}H}} \right). \]
constant of 2.12 kHz using a C–C bond length of 1.53 Å. Such a weak coupling results in an unresolved dipolar powder pattern superimposed on a broadening of the chemical shift powder pattern. No evidence exists in this spectrum for a splitting of 1.9 kHz previously reported for this site (9). This latter study faced considerable challenges using oriented samples and suffered from linewidths of 2 kHz. The data may have been overinterpreted.

Figure 2B is a stacked plot of spectral series with different mixing times for spin exchange recorded using the pulse sequence shown in Fig. 1 (t1 = 0). A 25-μs contact time was used for selective polarization of the protonated carbons. The carbonyl signal was not observed when the mixing time was very short (e.g., 100 μs), indicating that the carbonyl carbons were barely cross-polarized. However, a carbonyl signal does build up when the mixing time is increased as shown in Fig. 2B. The exchange process was nearly completed at a mixing time of ~100 ms. Since the intrinsic longitudinal relaxation rates for 13Ca and 13Cb in this sample were not measured, the experimental data were not simulated (10, 11). Spin diffusion from the methylene carbons toward the carbonyl carbons along the lipid chain is not visible with the mixing times used in these experiments (spectra not shown) because the natural abundance occurrence of any specific 13C–13C pairs is 10^-4. Furthermore, the polypeptide backbone is effectively separated from the lipid chain by its side chains so that polarization transfer from the labeled 13C–Gly2 gramicidin A to the carbonyl carbons in the lipid bilayers cannot occur during the given mixing times. Consequently, the carbonyl intensity in Fig. 2B is undoubtedly that from the labeled site, despite the relatively weak 13C–13C dipolar interaction for this site. The spin diffusion rate between two spins depends on their distance, the orientation of the internuclear vector with respect to the magnetic field direction, and the overlap of the two proton-coupled resonances (12). The very efficient carbon–carbon spin diffusion could potentially be applied to assign the resonances in uniformly labeled samples. In fact, 13C–13C spin diffusion has been incorporated to assign a fully 13C/15N labeled polypeptide in a recent MAS study recently (13).

In the 2D spin exchange experiments, the intensities of the diagonal and cross-peaks can be written as (14)

\[ I_{AA}(\tau_m) = \frac{1}{2} \left[ \left( 1 - \frac{\delta}{D} \right) + \left( 1 + \frac{\delta}{D} \right) \exp(-2D\tau_m) \right] \times \exp[-(\sigma - D)\tau_m]M_0(A), \]

\[ I_{BB}(\tau_m) = \frac{1}{2} \left[ \left( 1 - \frac{\delta}{D} \right) + \left( 1 + \frac{\delta}{D} \right) \exp(-2D\tau_m) \right] \times \exp[-(\sigma - D)\tau_m]M_0(B), \]

\[ I_{AB}(\tau_m) = \frac{1}{2} \left[ \left( 1 - \frac{\delta}{D} \right) + \left( 1 + \frac{\delta}{D} \right) \exp(-2D\tau_m) \right] \times \exp[-(\sigma - D)\tau_m]M_0(B) \times \exp[-(\sigma - D)\tau_m]M_0(A), \]

where \( \sigma = \frac{1}{2} (2k + R_1^+, R_1^-) \), \( \delta = \frac{1}{2} (R_1^+ - R_1^-) \), \( D = [\delta^2 + k^2]^{1/2} \), \( M_0 \) is the initial transverse magnetization after cross-polarization, \( \tau_m \) is the mixing time, and \( R_1 \) is the spin–lattice relaxation rate while \( k \) is the spin diffusion rate between spins A and B. Here A and B stand for 13C and 13C1, respectively. A 2D spectrum with selective cross-polarization is shown in Fig. 3, further indicating that the observed carbonyl signal in Fig. 2B comes from spin exchange. Since the carbonyl carbons were not polarized, i.e., \( M_0(B) = 0 \), no diagonal peak for the carbonyl carbon resonance (i.e., \( I_{BB} = 0 \)) was observed, resulting in the lack of the lower right cross-peak in the 2D contour (\( I_{BA} = 0 \)). In fact, the absence of the strong, uninform-
The measured chemical shifts provide only limits to the orientational range. The efficient spin diffusion between adjacent carbon sites is in contrast to the relatively inefficient spin diffusion between $^{15}$N sites in the polypeptide backbone (20) and so this more efficient spin diffusion may potentially be useful for resonance assignments in uniformly labeled samples.

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