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NMR spectroscopy approach to study the structure, orientation, and mechanism of the multidrug exporter EmrE

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Abstract

Multidrug exporters are a class of membrane proteins that remove antibiotics from the cytoplasm of bacteria and in the process confer multidrug resistance to the organism. This chapter outlines the sample preparation and optimization of oriented solid-state NMR experiments applied to the study of structure and dynamics for the model transporter EmrE from the small multidrug resistance (SMR) family.

1. INTRODUCTION

Drug efflux by membrane transport proteins is one of the primary mechanisms bacteria use to confer resistance to antiseptics and antibiotics (1–3). These multidrug transporters bind and efflux lethal compounds from the cytoplasm and in the process reduce the toxicity to the organism. Several transporter structures have been determined using X-ray crystallography which have provided detailed insight into the transport process (reviewed in (4)). A complementary method for resolving the mechanism of ion-coupled transport is through the use of nuclear magnetic resonance (NMR) spectroscopy. One major advantage of this approach is to study efflux pumps under conditions that better mimic the native membrane environment. This chapter will describe how oriented solid-state NMR (O-SSNMR) spectroscopy is used to directly interrogate the structure of the drug transporter EmrE from the small multidrug resistance (SMR) family. The anisotropic observables offered from these experiments (e.g., chemical shifts and dipolar couplings) are used as restraints in structure determination and are excellent reporters of the tilt and rotation angles for transmembrane proteins within the lipid bilayer (Figure 1) (5–11).

The prerequisite for using O-SSNMR to characterize membrane protein structures is to align the samples within the magnetic field. This is most commonly accomplished using mechanical alignment of lipid bilayers absorbed onto glass plates (12,13) or magnetic alignment with lipid bicelles (14). The former approach has been successfully applied for multiple membrane proteins including M2 from the influenza A virus (15) and the muscle regulatory protein phospholamban (16). For EmrE, we utilize lipid bicelle technology due to the excellent control of sample hydration and improved alignment with respect to the magnetic field (17). Specifically, our experiments use a bicelle composition of DMPC (14:0)

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and DHPC (6:0) at a 3.2/1 molar ratio, which readily forms aligned liquid crystals at 37 °C (18–21). Replacing the DMPC component with a shorter (12:0) or longer (16:0) chain phosphatidylcholine lipid can be used to lower or raise the alignment temperature, respectively (22–24). In addition, the usage of POPC in combination with DMPC can also reduce the alignment temperature to 25 °C (17). One of the most important requirements of bicelle selection is to ensure protein stability in the chosen lipid composition (20). Finally, since the quality of protein alignment can be protein dependent, the lipid to protein ratio should be optimized in an empirical fashion to ensure bicelle alignment in the magnetic field. For EmrE, we find that a lipid to protein ratio of 150:1 (mol:mol) is effective to achieve good signal to noise without compromising the overall alignment with respect to the magnetic field.

Below we discuss the procedure of bicelle sample preparation containing EmrE and the set-up of the polarization spin exchange at the magic angle (PISEMA) experiment (25,26), which is a sensitive way to probe secondary structural information with respect to the membrane. Using this experiment and others, we have resolved the asymmetric monomer subunits within the EmrE homodimer and quantified conformational exchange dynamics of the transporter that are required for drug efflux (27,28).

2. MATERIALS

2.1 Sample Preparation

1 Isotopically ^{15}N enriched protein: modify the expression conditions for your favorite protein with the addition of $^{15}NH_4Cl$ to M9 minimal media to obtain uniformly ^{15}N enriched proteins or the addition of one or several ^{15}N labeled amino acids for selectively enriched proteins.

2 n-dodecyl-β-D-maltoside (DDM)

3 n-octyl-β-D-glucoside (OG)

4 Bio-Beads™ SM-2 resin, Bio-Rad Laboratories

5 Ultracentrifuge: instrument should reach centrifugal forces of at least 150,000 x g.

6 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), Avanti Polar Lipids, Inc.

7 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), Avanti Polar Lipids, Inc.

8 Ytterbium(III) chloride ($YbCl_3$)

2.2 NMR Instrumentation and Data Analysis

9 Bicelle sample holder assembly (design by Peter Gor'kov at NHMFL, Tallahassee, FL):

- **a.** 5 mm open-ended glass tube, New Era Enterprises, part No. NE-RG5-P-17-OE-BOE.

b. B5 plugs with and without fill hole, Revolution NMR, part No. MP4763-001 and MP4764-001.

10 Solid-state NMR spectrometer: our experiments were carried out using an Agilent DD2 spectrometer operating at a ^{1}H Larmor frequency of 600 MHz.

11 Solid-state NMR probes capable of ^{31}P and ^{15}N detection. Our experiments use the following:

a. BioStatic H-X probe with 5 mm bicelle coil tuned to ^{31}P on the X-channel (Agilent).

b. *Low-E* ^{1}H-^{15}N probe with 5 mm bicelle coil for ^{15}N detection (probe design by Peter Gor'kov) (29). This probe technology is commercially available from Revolution NMR, LLC (www.revolutionnmr.com).

12 NMR data processing software: NMRPipe (30) and Sparky v3.113 (T.D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

3. METHODS

3.1 Sample Preparation

EmrE is expressed from a pMAL™ vector (New England Biolabs) where maltose binding protein (MBP) is positioned on the N-terminal side of the EmrE gene. The expression is performed in *E. coli* BL21(DE3) cells in minimal media using selectively labeled ^{15}N amino acids or $^{15}NH_4Cl$ to uniformly enrich the protein. EmrE is purified with an amylose affinity column, cleaved with TEV protease to remove MBP, and passed over a size exclusion column as previously described (27,28). Purified EmrE in DDM detergent micelles is then reconstituted into DMPC lipid bilayers. Below is the reconstitution procedure:

1. 28 mg DMPC is hydrated with 1 mL of 20 mM sodium phosphate and 20 mM NaCl and subjected to multiple cycles of flash freezing.

2. Bath sonicate the sample for 15 min.

3. Add 5.6 mg OG to the mixture and equilibrate for 10 min.

4. Add 7 mL EmrE at a concentration of 0.5 mg/mL in DDM detergent to the sonicated lipids. Additional DDM may be added to ensure the lipids are solubilized, which will be evident when the mixture becomes clear. Typically, a total of ~30 mg DDM is added including the detergent with EmrE.

5. Incubate the sample for 1 hour at room temperature.

6. Add 2.25 grams of Bio-Beads to give a final ratio of 75 mg per mg of detergent.

7. Gently stir for 12 hours at 4 °C. Note that the removal of detergent by the Bio-Beads will instigate formation of proteoliposomes and lead to a slightly cloudy solution.

8. Remove Bio-Beads from the suspension.

9. Centrifuge proteoliposomes in a Beckman Optima MAX_XP TLA 110 rotor at 100,000 x g for 1.5 hrs.

10. Resuspend the liposome pellets in 100 mM HEPES and 20 mM NaCl (pH 7) buffer and freeze thaw 5–10 times to exchange the buffer.

11. Centrifuge the sample in the TLA 110 rotor for 2.5 hrs at 150,000 x g.

12. Add 25 μL of 425 mM DHPC in water to the proteoliposome pellet. This corresponds to a DMPC:DHPC molar ratio ("q value") of ~3.2–3.5 (see Note 1).

13. Mix thoroughly by a combination of vortexing, flash freezing with liquid nitrogen, and heating to 37 °C. After the mixing is complete, the sample should be fluid on ice and a viscous gel at 37 °C (see Note 2).

14. Centrifuge the sample in a 1.5 mL microcentrifuge tube for 2 min at 1,500 x g using a benchtop centrifuge and place the supernatant in a new microcentrifuge tube.

15. For unflipped bicelle samples (bicelle normal perpendicular to magnetic field), proceed to *step 15*. For flipped samples (bicelle normal parallel to magnetic field), add 6 μL of 100 mM $YbCl_3$ to give a final concentration of 4.5 mM (see Note 3).

16. Place the sample in the bicelle sample holder for O-SSNMR experiments.

3.2 Check Bicelle Alignment with ^{31}P NMR

The alignment of the bicelle in the magnetic field is checked by recording a one-pulse ^{31}P experiment optimized for a 90° tilt angle followed by acquisition of the free induction decay under ^{1}H decoupling with SPINAL-64 (31) This experiment is a sensitive way to check the sample since ^{31}P is 100% natural abundant, has a relatively high gyromagnetic ratio, and is present in both DMPC and DHPC headgroups. Representative ^{31}P spectra of flipped, isotropic and unflipped bicelles are shown in Figure 2A, B, C, respectively (see Note 4).

3.3 Optimization of the polarization inversion spin exchange at the magic angle (PISEMA) experiment

PISEMA is a separated local field experiment to correlate dipolar couplings with anisotropic chemical shifts (25,26). The spectral patterns from PISEMA are directly sensitive to the tilt and rotation angles of the protein secondary structures with respect to the magnetic field

[1]The ability of a sample to align is very dependent on the molar ratio of DMPC:DHPC ("q-value"). If the q-value is significantly below 3.2 the bicelle will be isotropic.

[2]The final volume of the sample should be 125 μL to ensure that the total lipid concentration is at least 25% (w/v). If the lipid concentration is too low the bicelles will not align.

[3]The sample will readily align at 37 °C with the bilayer normal perpendicular with respect to the direction of the magnetic field (i.e., unflipped bicelles). Some proteins that undergo rapid uniaxial rotational diffusion can be characterized under these conditions (50). However, our experiments show that EmrE's rotational diffusion is not sufficiently rapid to average out the residual anisotropy (51). For this reason, the bicelle normal is "flipped" by 90° using $YbCl_3$, which orients the bilayer normal parallel to the magnetic field. These paramagnetic ions bind to the phosphate within the lipid headgroup and change the magnetic susceptibility of the bicelle (19,21).

[4]It is important to note that these spectra do not guarantee that the protein will be properly aligned in the magnetic field. However, when the bicelle containing protein is unaligned or partially aligned, our experience is that the protein's ^{15}N spectrum will give a powder pattern or be of low quality.

(7,9). PISEMA begins with a cross-polarization (CP) sequence to transfer magnetization from ^{1}H to ^{15}N spins in order to increase the sensitivity of the experiment. Homonuclear dipolar decoupling in the indirect dimension is achieved by frequency-switched or phase-modulated Lee-Goldburg (FSLG or PMLG) (32–35). Both of these sequences enable evolution of a scaled heteronuclear dipolar coupling in the absence of ^{15}N chemical shift evolution. After evolving the dipolar coupling in the indirect dimension, the PISEMA experiment detects the free precession of the X nucleus under high-power ^{1}H decoupling. The most common application of PISEMA is to enrich the protein with ^{15}N labeling and correlate the ^{1}H-^{15}N dipolar coupling with the ^{15}N anisotropic chemical shift (see Note 5). Advancements in the original PISEMA experiment include developments to reduce the ^{1}H offset dependence of the indirect dimension (36), a sensitivity-enhanced version to improve signal-to-noise (37,38) and a constant-time experiment to improve the resolution in the dipolar coupling dimension (39).

Prior to data acquisition on the protein sample, it is recommended to first optimize the pulse sequence parameters with two model compounds: (1) an aqueous buffer composed of 100 mM HEPES and 20 mM NaCl (pH 7.0) and (2) a single crystal of ^{15}N-labeled *N*-acetyl-leucine (NAL).

3.3.1 Insert buffer sample to calibrate ^{1}H 90° pulse parameters—If the probe requires pulse length calibration for the first time, it is necessary to optimize ^{1}H 90° pulses for a given pulse duration that is within the voltage handling of the probe circuit. This is typically specified by a minimum 90° pulse width that can be applied without damage to the probe. We typically optimize for 5 and 6 μsec pulse lengths on the water signal of a sample of 100 mM HEPES and 20 mM NaCl (pH 7.0) by carrying out a full nutation curve where the pulse length is varied at a constant power (Figure 3A). Next, the pulse power is more finely calibrated to the desired 360° pulse (e.g., 20 or 24 μsec, respectively) by adjusting the power level to identify the corresponding null in the spectrum (see Note 6). An example of the calibration is shown in Figure 3B.

3.3.2 Insert the *N*-acetyl leucine single crystal

1. *Optimize cross-polarization (CP) from ^{1}H to ^{15}N.* There are a few variants of this sequence that provide efficient polarization transfers, including linear and adiabatic ramps as well as CP-MOIST (40,41) (see Note 7).
2. *Optimize the 90° pulse for ^{15}N.* The experiment employs a 90° ^{15}N pulse after the CP from ^{1}H to ^{15}N. The signal on ^{15}N is detected under ^{1}H decoupling. The flip back pulse is set to the desired length with the power adjusted in an iterative

[5]The solid-state NMR probe for PISEMA spectroscopy needs to include a sensitive detection coil optimized for ^{15}N detection. The probe designed by Gor'kov *et al.* (29) is the most sensitive available and uses a loop-gap resonator for the ^{1}H channel that minimizes sample heating and uses a ^{15}N solenoid for detection. The inner solenoid coil is positioned closest to the sample in order to maximize the filling factor and sensitivity for ^{15}N detection.

[6]It is important to acquire a full nutation curve when first optimizing power levels to ensure accurate measurement of the 360° pulse.

[7]The optimal match condition for the single crystal is typically not the best for the protein sample; however, it should be optimized to ensure sufficient signal-to-noise for calibration of the ^{15}N pulse and PISEMA experiment. The contact time can also be adjusted iteratively, but typically ~2 msec will give the highest signal-to-noise.

manner. The null in the spectrum gives the optimal power corresponding to the pulse length.

3. *Calculate the parameters for the indirect dimension of the PISEMA experiment.* For the SEMA portion of PISEMA (i.e., t_1 period), the effective frequency on ^{15}N must match the effective frequency of ^{1}H (i.e., $\omega_{eff,15N} = \omega_{eff,1H}$). The ^{15}N spin-lock during t_1 evolution is on resonance so the effective frequency is equal to applied frequency ($\omega_{1,15N} = \omega_{eff,15N}$) (see Note 8). Unlike the ^{15}N effective frequency, the ^{1}H effective frequency during FSLG or PMLG is not equal to the applied frequency. For this reason, the frequency offset ($\Delta\omega$) and applied frequency (ω_1) need to be set so that the effective frequency of ^{1}H will match that of ^{15}N ($\omega_{eff,15N} = \omega_{eff,1H}$). A second requirement of PMLG or FSLG is that the effective frequency on ^{1}H is applied at the magic angle in order to average out homonuclear dipolar couplings. Using the trigonometric relationships given in Eqns. 1 and 2 and the desired effective frequencies for ^{15}N and ^{1}H, $\Delta\omega$ and ω_1 can be calculated for ^{1}H (see Note 9).

i. $$\tan\theta = \frac{\omega_1}{\Delta\omega} \ (\theta = 54.7) \quad (1)$$

ii. $$\omega_{eff}^2 = \omega_1^2 + \Delta\omega^2 \quad (2)$$

4. *Collect the 2D PISEMA experiment.* The PISEMA experiment is collected using the optimized pulse sequence parameters and Fourier transformed to give the resulting two-dimensional spectrum (see Note 10).

5. *Adjust ^{1}H offset to minimize the zero-frequency.* Since the dipolar coupling frequency measured in the indirect dimension is sensitive to the ^{1}H offset, one can adjust the transmitter frequency for ^{1}H to minimize the zero-frequency signals for desired peaks in the single crystal sample. The optimal ^{1}H offset may be different for the protein sample.

3.3.3 Acquire PISEMA spectrum on membrane protein sample

1. *Optimize the ^{1}H 90° pulse*. Similar to the pulse calibration on the buffer sample, we optimize for 5 and 6 μsec 90° pulse lengths on the water signal in the protein/

[8]Note that the applied nutation frequency can be calculated by $\omega_1/2\pi = 250/t_{90}$, where t_{90} is equal to the length of the 90° pulse in μsec and $\omega_1/2\pi$ is given in kHz. For example, a 6 μsec pulse corresponds to a frequency of 41.7 kHz.

[9]We commonly employ an effective ^{1}H frequency of $\omega_{eff}/2\pi = 41.7$ kHz, an offset $\Delta\omega/2\pi = 24.1$ kHz, and an applied frequency of $\omega_1/2\pi = 34.0$ kHz. Unlike most multidimensional NMR experiments, the dwell time during the t_1 evolution period cannot be set independently from the FSLG or PMLG parameters. Specifically, the minimum t_1 dwell time is the application of two 360° pulses. This works out to give a minimum dwell time equal to $8\times250/(\omega_{eff}/2\pi)$. For example, with an effective frequency of $\omega_{eff}/2\pi = 41.7$ kHz, the dwell time is 48 μsec.

[10]The indirect ^{1}H-^{15}N dipolar coupling is scaled due to the Lee-Goldburg sequence and needs to be adjusted by the scaling factor (0.82) to give the correct couplings (26). For example, a dwell time of 48 μsec during FSLG or PMLG gives a corrected spectral width of 25.4 kHz.

bicelle sample. As described above and shown in Figure 3B, the pulse length is set to 4 times the 90° pulse with the power levels adjusted to find the null.

2. *Optimize the CP condition for protein*. In order to find optimal matching conditions for CP, it is recommended to have a uniformly ^{15}N labeled sample that gives at least 10:1 signal-to-noise in ~256–512 scans. Similar to that described for the single crystal, the CP values can be optimized directly on the sample by adjusting the ^{1}H power level (Figure 4) (see Note 11).

3. *Optimize the length of the CP contact time*. In addition to the power levels, the contact time of the CP should be iteratively adjusted to maximize signal-to-noise. EmrE in aligned bicelles shows an optimal value of ~0.75 msec.

4. *^{1}H offset and recycle delay.* The ^{1}H offset must be set correctly in order to reduce the zero-frequency peaks. For transmembrane helical proteins in flipped bicelles, a ^{1}H offset in the range of 4.7 to 7 ppm will minimize the zero-frequency signals (37).

5. *Find optimal recycle delay.* The recycle delay should be set to 3–5 times the ^{1}H T_1, which can be measured using an inversion recovery experiment on the protein. For EmrE, we normally use a delay of 3 or 4 sec.

6. *Acquisition of the PISEMA experiment*. Using the calibrated ^{15}N pulse powers from the single crystal and the ^{1}H pulse power from the protein sample, one can calculate the applied frequency for ^{1}H during FSLG or PMLG to match the desired effective frequency. Since the maximum ^{1}H-^{15}N dipolar couplings are ~10 kHz, we typically employ a t_1 dwell time of 48 μsec for protein samples in flipped bicelles, which corresponds to an effective frequency $\omega_{eff}/2\pi$ = 41.7 kHz. This spectral width is sufficient to cover the entire breadth of dipolar couplings in the indirect dimension. An example PISEMA spectrum for selectively labeled EmrE with ^{15}N methionine is shown in Figure 5.

7. *Assignment of PISEMA spectra.* The acquired spectrum needs to be assigned in order to obtain site-resolved structural information. There are several viable approaches including the usage of site-directed mutagenesis (27), selectively labeled samples in combination with periodic wheel approximations (7,9,42,43), and spectroscopic methods that make use of magnetization transfers (44,45). Once the assignments are obtained, these can be used as restraints in structural refinement calculations.

We have successfully employed PISEMA to show that both monomers in the functional EmrE dimer give a separate set of peaks (see Figure 5). These results validated the asymmetric dimer structural models available from X-ray crystallography and cryoelectron microscopy (46,47,27). Furthermore, we recently applied the pure exchange (PUREX) method (48) to investigate the conformational dynamics of EmrE in aligned bicelles that can be applied to other membrane protein systems (28). Notably, we discovered that the change

[11]For selectively labeled samples, the signal-to-noise often prevents detailed optimization of pulse parameters. However, since the buffer composition is the same as that of the uniformly ^{15}N labeled sample, the optimal values typically do not vary significantly.

in protonation state of a conserved glutamate residue alters the conformational exchange rate of EmrE that plays a direct role in drug efflux (49).

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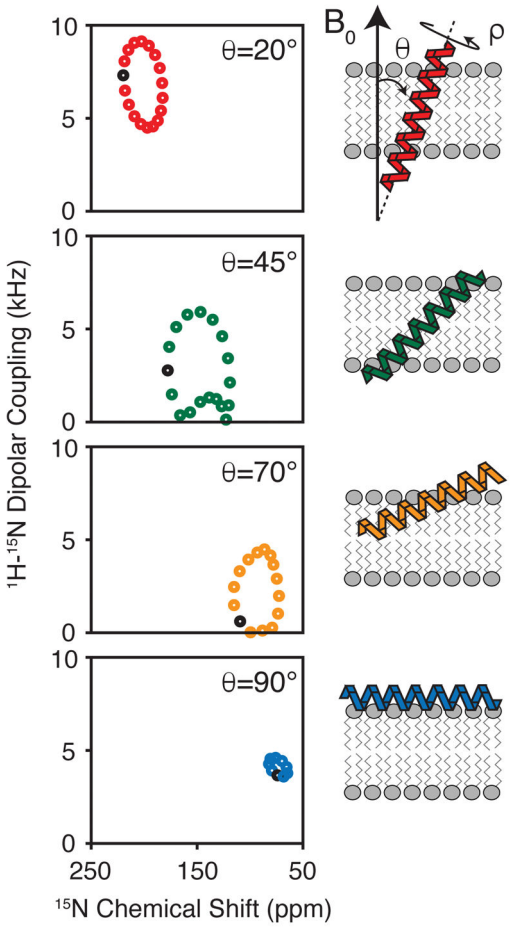

Figure 1.

Calculated separated local field spectra of ^{1}H-^{15}N dipolar coupling vs. ^{15}N chemical shift for an ideal helix. The periodic spectral patterns are sensitive to the tilt and rotation angles of the secondary structures with respect to the magnetic field. These patterns have been termed PISA wheels (9,7).

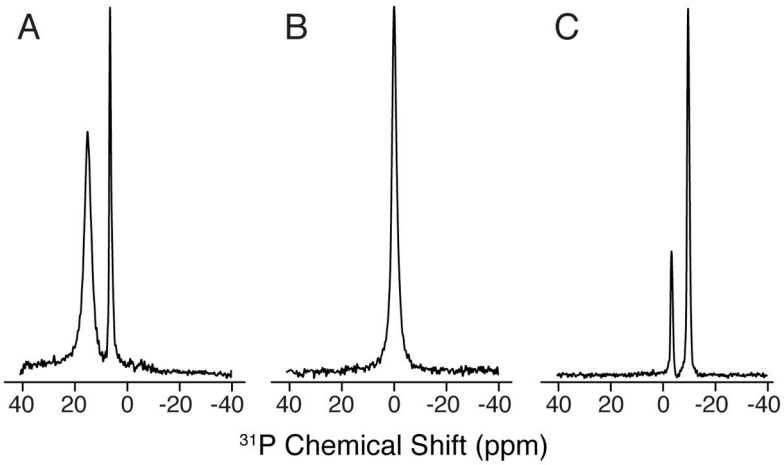

Figure 2.

^{31}P spectra of lipid bicelle samples. The panels show representative spectra for (A) flipped, (B) isotropic, and (C) unflipped bicelles. The flipped spectra were obtained by the addition of $YbCl_3$.

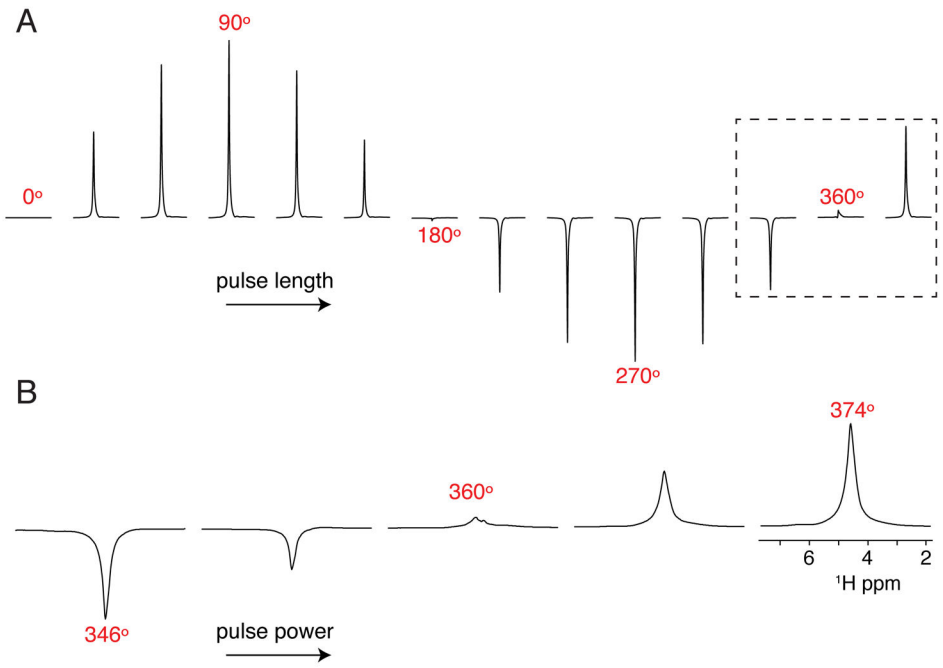

Figure 3.

Optimization of the ^{1}H 90° pulse on the water peak. (A) Nutation curve by varying the pulse length at a fixed B_1 amplitude to determine an accurate 360° pulse. (B) Fine tune calibration of the 360° pulse by setting the pulse length to four times the desired 90° pulse and increasing the power level from left to right. The null value corresponds to the 360° pulse.

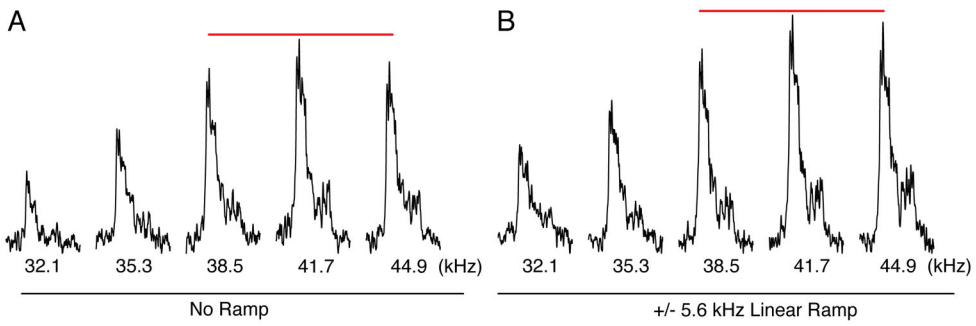

Figure 4.

Optimization of ^{1}H to ^{15}N cross-polarization as demonstrated for EmrE in lipid bicelles. (A) The ^{1}H power is arrayed around a constant ^{15}N amplitude used for cross-polarization. As seen from the spectra, a linear ramp of 41.7 ± 5.6 kHz gave the highest signal/noise for all experiments. These data show that a linear ramp improves the signal/noise and emphasizes the importance of optimizing cross-polarization.

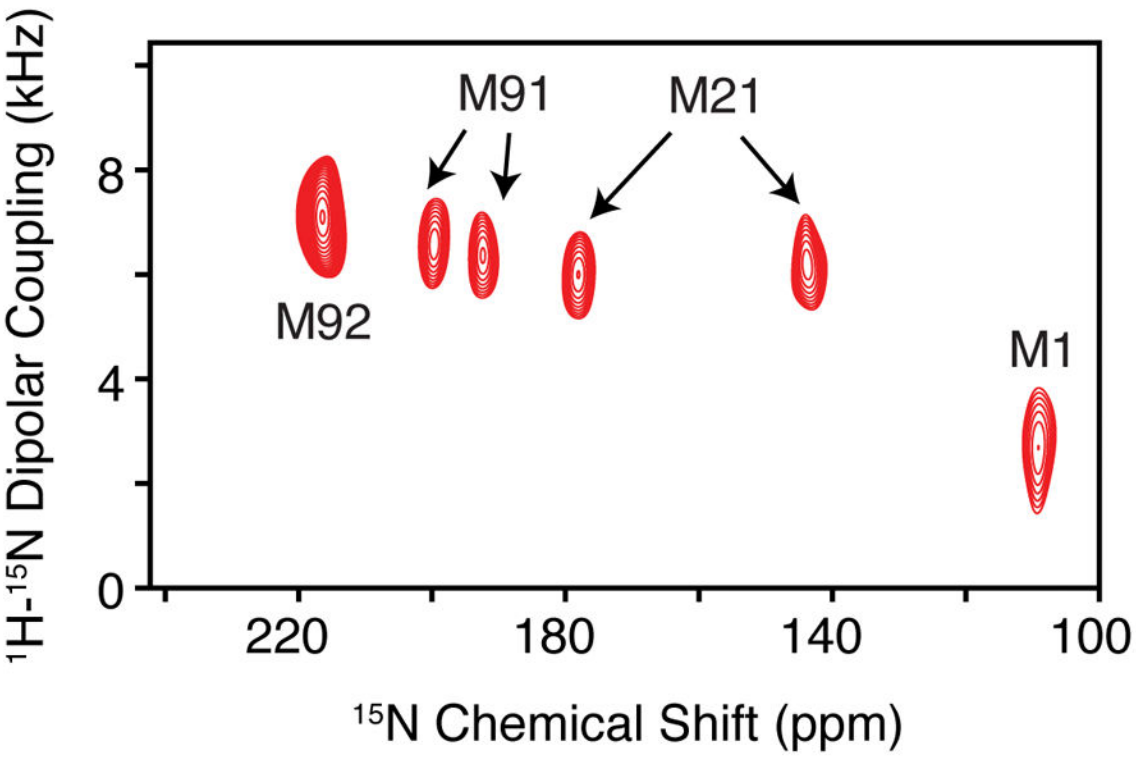

Figure 5.
PISEMA of ^{15}N methionine labeled EmrE in DMPC/DHPC aligned bicelles. Note that M21 and M91 show clear doubling in the spectrum indicative of an asymmetric dimer. (Reproduced with permission from Ref. (27). © WILEY-VCH Verlag GmbH & Co. KgaA, Weinheim, 2013).