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Utilization of ^{13}C-labeled amino acids to probe the α-helical local secondary structure of a membrane peptide using electron spin echo envelope modulation (ESEEM) spectroscopy

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Electron spin echo envelope modulation (ESEEM) spectroscopy in combination with site-directed spin labeling (SDSL) has been established as a valuable biophysical technique to provide site-specific local secondary structure of membrane proteins. This pulsed electron paramagnetic resonance (EPR) method can successfully distinguish between α-helices, β-sheets, and 3_{10}-helices by strategically using ^{2}H-labeled amino acids and SDSL. In this study, we have explored the use of ^{13}C-labeled residues as the NMR active nuclei for this approach for the first time. ^{13}C-labeled d_5-valine (Val) or ^{13}C-labeled d_6-leucine (Leu) were substituted at a specific Val or Leu residue (*i*), and a nitroxide spin label was positioned 2 or 3 residues away (denoted *i*-2 and *i*-3) on the acetylcholine receptor M2δ (AChR M2δ) in a lipid bilayer. The ^{13}C ESEEM peaks in the FT frequency domain data were observed for the *i*-3 samples, and no ^{13}C peaks were observed in the *i*-2 samples. The resulting spectra were indicative of the α-helical local secondary structure of AChR M2δ in bicelles. This study provides more versatility and alternative options when using this ESEEM approach to study the more challenging recombinant membrane protein secondary structures.

1. Introduction

Membrane proteins are responsible for performing vital biological functions that ensure the survival of living organisms [1]. Despite their physiological importance, minimal structural information is currently available as a result of limited biophysical techniques for studying these challenging protein systems [2,3]. More than 70% of membrane proteins with solved 3-D structures contain α-helical secondary structural motifs, which play important roles in assembly, packing, and membrane protein interactions [4]. Several established spectroscopic methods for studying secondary structural motifs like these include circular dichroism (CD), solid-state nuclear magnetic resonance (ss-NMR) spectroscopy, FT-IR, and FT-Raman [5–10]. However, these techniques are not without limitations. CD is only able to provide global secondary structural information and thus, makes it a challenge to identify local secondary structural motifs within a protein or peptide. Additionally, ss-NMR requires large sample concentrations, which can often be unobtainable for membrane proteins [3]. Aside from CD, the results obtained from these biophysical methods are often ambiguous and require extensive data analysis [8–10].

The Lorigan lab has established a powerful electron spin echo envelope modulation (ESEEM) approach coupled with site-directed spin labeling (SDSL) to probe the local secondary structure of membrane proteins that is advantageous compared to other structural biology approaches because of the high sensitivity and short data acquisition.

times required [1,4]. ESEEM spectroscopy can detect weakly-coupled NMR active nuclei to nearby unpaired electron spins. Traditionally, ^{2}H-labeled amino acids act as the NMR active nuclei; however, ^{13}C-labeled amino acids have been used for the first time in this study. An unpaired electron is introduced via the most common spin label (SL), 1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl methanethiosulfonate (MTSL).

If the ^{2}H and SL are within the appropriate detection limit (8 Å for ^{2}H), the weak dipolar coupling will produce ^{2}H modulation in the time domain data after which a Fourier transformation will yield a peak at the corresponding ^{2}H Larmor frequency. Alternatively, for ^{13}C, the theoretical estimated distance will be a maximum of up to 7 Å based on the nuclear spin and gyromagnetic ratio of ^{13}C compared to the detectable ^{2}H ESEEM modulation. Thus, the use of ^{13}C-labeled amino acids should show similar patterns to ^{2}H labeled amino acids. Distinct patterns are observed from the ESEEM data because of the unique turn periodicities of various secondary structural components, which

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Abbreviations: EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; SDSL, site-directed spin labeling

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provide insights into the type of secondary structures present in membrane proteins. Previously, we utilized this pulsed EPR method to identify site-specific secondary structural motifs such as α-helices [1,4,11], β-sheets [12], and more recently, 3_{10}-helices [13]. Site-specific secondary structural information provides a better understanding of the functions, dynamics, and protein-lipid interactions of membrane proteins [4,14]. Not only has this ESEEM approach been successful for model peptides, it has also been utilized in over-expression systems to probe α-helical secondary structural motifs in both a water-soluble protein and a membrane protein, demonstrating the versatility of this technique [15,16].

Despite this, over-expression systems are extremely challenging to express and purify because site-specific isotopic labeling is tedious and can suffer from scrambling [17]. In addition, incorporation rates of deuterium into selected sites in a recombinant protein system can be as low as 56% [18]. Alternatively, higher incorporation rates of up to 95% can be achieved when using other common isotopes such as ^{13}C [18]. The use of ^{13}C has been better characterized, specifically for use in a variety of NMR spectroscopic techniques, which is beneficial in avoiding long periods of optimization that would be required when using ^{2}H [19–23].

This work explores the use ^{13}C as the NMR-active nucleus for this ESEEM spectroscopic technique for the first time. The α-helical M2δ subunit of the acetylcholine receptor (AChR M2δ) was the model membrane peptide used in this study. AChR M2δ was selectively labeled with $^{13}C_5$-labeled valine (Val) or $^{13}C_6$-labeled leucine (Leu) (i) and the SL was placed either 2 or 3 amino acid residues away (*i*-2 and *i*-3). Here, we have successfully demonstrated that other NMR active nuclei such as ^{13}C can be utilized for membrane protein local secondary structural determination. This ESEEM approach is comparable to rotational echo double resonance (REDOR) solid-state NMR spectroscopy, which can probe an α-helical secondary structure by measuring dipolar coupling between ^{13}C or ^{15}N nuclei, however, does not suffer from the limitations of large sample concentrations and long data acquisition times [11]. ESEEM spectroscopy can be conducted using small sample concentrations (μM) and uses short data acquisition times (~30 min). This method provides an alternative approach to this biophysical technique which extends the application of ESEEM spectroscopy to study more challenging recombinant membrane proteins in the future.

2. Materials and methods

2.1. Solid phase peptide synthesis

The M2δ subunit of the acetylcholine receptor (AChR) was the α-helical transmembrane model peptide utilized for this study. All peptides were chemically synthesized using Fmoc-solid phase peptide chemistry on a CEM Liberty Blue microwave solid-phase synthesizer [24]. This method is suitable for proteins of up to 70 amino acid residues or less [24]. Seven unique peptides were designed including the wild type (WT) peptide with the following amino acid sequence, EKMSTAISVLLAQAVFLLLTSQR. $^{13}C_5$- labeled valine at position 15 or $^{13}C_6$- labeled leucine at position 11 were introduced (*i*) and a single cysteine residue (X) was substituted 2 and 3 successive positions away (*i*-3 and *i*-2) for subsequent MTSL attachment. Additionally, a sample containing no ^{13}C-labeled valine or leucine for the *i*-3 positions were synthesized for each set of peptides as a control. One full set of peptides are as follows: *i*–3 (EKMSTAISVLL**X**QA***i***FLLLTSQR), *i*-2 (EKMSTAISVLLA**X**A***i***FLLLTSQR), and *i*-3 control (EKMSTAISVLL**X**QAVFLLLTSQR).

The peptides were cleaved from their solid support using trifluoroacetic acid (TFA), anisole, triisopropylsilane (TIPS), and H_2O (85/5/5/5). Following evaporation under N_2 gas, peptides were precipitated using methyl-tertbutyl ether. Reverse-phase HPLC was used for purification using a C4 preparation column with a gradient of 5% to 95% solvent B (90% acetonitrile) [4]. The purified peptides were labeled with 5-fold excess of MTSL (Toronto Research Chemicals) in dimethylsulfoxide (DMSO) for ~16 h. Excess MTSL was removed using RP-HPLC on a C4 semi-preparative column. MALDI-TOF was used to confirm purity and accurate molecular weight of the target peptides. All peptides were lyophilized and stored as a powder at −20 °C. The spin labeled AChR M2δ peptides were then incorporated into DMPC/DHPC (3.5/1) bicelles at a 1:1000 M ratio following the previously described method to observe the peptides in a native mimetic environment [11]. Lipid bicelles were chosen because they provide a bilayer environment that is less heterogeneous than that of liposomes. Liposome samples have much shorter phase memory times, $T_{m,}$ due to an uneven distribution of the spin labeled peptides within the membrane resulting in local inhomogeneous pockets of high spin concentration [13]. Thus, the use of bicelles avoids the effects of poorer signal-to-noise ratios for ESEEM signals that are observed when using liposomes.

2.2. Circular dichroism spectroscopy

The wild type AChR and labeled AChR constructs were solubilized in trifluoroethanol (TFE) at a concentration of 100 μM. CD spectroscopy was performed on an Aviv Circular Dichroism Spectrometer Model 435 in a rectangular 0.1 cm quartz cuvette. Data was collected from 185 nm to 260 nm with an average of 5 scans per sample and 1 nm bandwidth at 25 °C.

2.3. Three-pulse ESEEM spectroscopy

X-band CW-EPR (~9 GHz) spectroscopy was used to measure spin concentrations of the MTSL-labeled M2δ peptides (150 μM). All samples showed 80–90% labeling efficiency and the three-line EPR spectrum showed significant lineshape broadening which indicated successful incorporation of peptides into the lipid bilayer. Three-pulse ESEEM measurements were performed on a Bruker ELEXSYS E580 with an ER4188X MS3 resonator. Initially, tau (τ) optimization experiments were conducted to determine the optimal τ value to suppress ^{1}H modulation and enhance ^{13}C modulation. Three different τ values were run at 198 ns, 268 ns, and 408 ns. Following this, all subsequent measurements used a 408 ns τ value to suppress ^{1}H modulation while enhancing ^{13}C modulation. Three-Pulse ESEEM data were collected at ~9.279 GHz at 80 K. A starting T of 386 ns and 512 points in 12 ns increments were used to collect the ESEEM spectra. Approximately 40 uL of bicelle-peptide sample were used to collect the data.

The original ESEEM time domain data were normalized by division through a polynomial fit and subsequent subtraction of unity as described previously [15,25–27]. The LPSVP algorithm was used for back-prediction of the missing data points [28]. The data were then processed further using Hamming apodization and zero filling [29]. A cross-term averaged Fourier Transformation (FT) was then used on the resulting spectrum to generate the corresponding frequency domain with minimized dead time artifacts as has been previously established [14]. The ^{13}C peaks were observed at 3.5 MHz representing the ^{13}C Larmor frequency.

3. Results and discussion

3.1. Global secondary structure

Circular dichroism (CD) spectroscopy was utilized to examine the global secondary structure of the model peptide, AChR M2δ. Fig. 1 shows the CD spectra for the WT AChR M2δ as well as the CD spectra for the peptide constructs labeled with either $^{13}C_5$- labeled Val or $^{13}C_6$-labeled Leu and the attached MTSL label. Double minima were observed at 220 nm and 208 nm, which is indicative of α-helical global secondary structure for both the WT AChR and the labeled peptides [5,6]. Additionally, there was not a significant difference in the overall spectra for the peptide constructs that had been labeled. This ensures that the addition of ^{13}C in a single Val or Leu amino acid side chain and

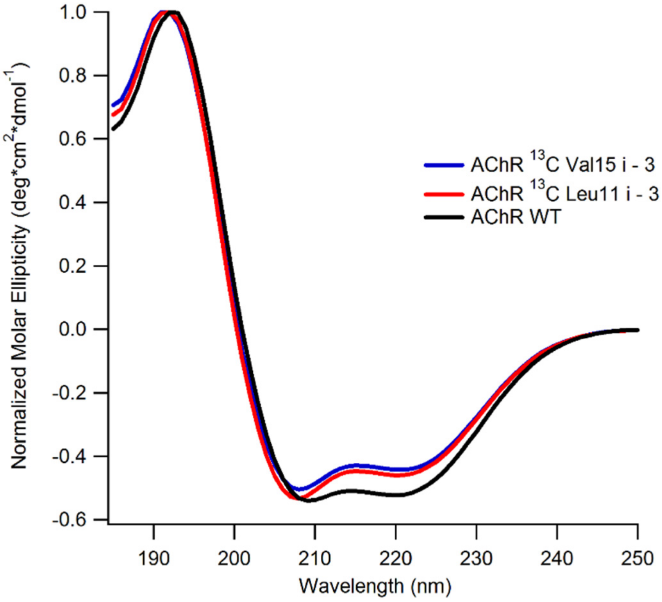

Fig. 1. Circular Dichroism of AChR $^{13}C_5$-labeled Vali15 i-3 and AChR $^{13}C_6$-labeled Leu11 i-3 compared to WT AChR in Trifluoroethanol (TFE).

the addition of the cysteine residue with the attached spin label did not significantly perturb the secondary structural integrity of these peptides. Due to light scattering, it was difficult to collect the CD spectra for the bicelles, and thus TFE, an α-helical inducing solvent, was used to examine the global secondary structure.

3.2. Tau optimization

Fig. 2 represents the various experimental τ values used for these ESEEM experiments. In three-pulse ESEEM experiments, the peak amplitudes and modulation depth are dependent upon the tau (τ) value [30]. The τ value is the constant delay time between the first and the second microwave pulses in the three-pulse ESEEM experiment (π/2- τ -π/2-T-π/2-τ + T-echo) [1]. Optimal τ values vary depending on the field and frequency at which the data were collected [1]. Typical three-pulse ESEEM experiments for ^{2}H nuclei are run around a τ value of 200 ns; however because this technique is using ^{13}C nuclei for the first time, the τ value of the experiment had to first be optimized.

In Fig. 2, three different τ values were examined. The τ value at 198 ns, which is typical of ^{2}H three-pulse ESEEM experiments, had both a peak at the ^{1}H Larmor frequency at 14 MHz as well as the peak for the ^{13}C nuclei at 3.5 MHz. In an effort to suppress both the ^{13}C peak and the ^{1}H peak, a τ of 268 ns was chosen. Here, the ^{13}C Larmor frequency peak was completely suppressed, and the ^{1}H peak was slightly enhanced. Ultimately, a τ value of 408 ns was used. This τ value was able to successfully enhance the ^{13}C modulation. For these three-pulse ESEEM experiments, choosing a τ value that will enhance the Larmor frequency of the nuclei being observed is the ideal experimental set-up. In addition, at these various τ values and another minor peak was also observed at 5.7 MHz. This peak is from the ^{31}P nuclei that are present in the DMPC/DHPC bicelles that is weakly coupled to the electron spin of the SL attached to the peptides [29]. The ^{13}C modulation could not be easily observed in the time domain data, however, the FT frequency domain was able to show the ^{13}C signal as seen in Fig. 2. The three pulse ESEEM time domain data for AChR ^{13}C labeled Valine15 at the i-3 position in DMPC/DHPC bicelles are shown in supporting information (Fig. S1).

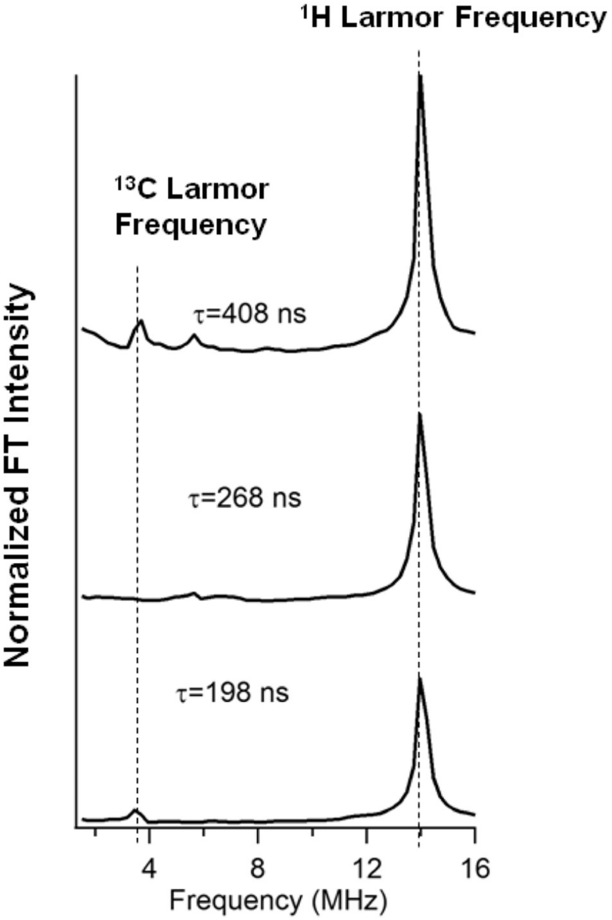

Fig. 2. Frequency domain data collected for AChR ^{13}C labeled Valine15 at the i-3 position in DMPC/DHPC bicelles at several τ values. 408 ns is the optimal τ value where the ^{13}C peak at 3.5 MHz (dotted line) is at a maximum. At 268 ns, the suppression of both ^{13}C and ^{1}H is observed. 198 ns did not maximize the ^{13}C peak. The minor peak observed at 5.7 MHz is from the ^{13}P nuclei in the lipid bicelles.

3.3. Three-pulse ESEEM for local α-helical secondary structure characterization

A three-pulse ESEEM experiment was performed using ^{13}C as the NMR-active nuclei in exploring the local secondary structure of the α-helical model membrane peptide, AChR M2δ in DMPC/DHPC bicelles. Fig. 3 shows the ESEEM frequency domain data for $^{13}C_5$ -labeled valine at position 15 *(i)* and the MTSL attached to a cysteine residue (X) either 2 or 3 amino acids away (denoted *i*-2 and *i*-3). Here, a peak was observed at the ^{13}C Larmor frequency at 3.5 MHz for the *i*-3 sample. For the *i*-2 sample, no peak was observed because it is outside the detection limit. In addition, in a control sample with no ^{13}C labeled Val, no peak was observed since there are no ^{13}C nuclei. This type of pattern is similar to what has been found for ^{2}H labeled amino acids and are strongly indicative of the α-helical local secondary structure of this model peptide [1,11,13,15].

In previous studies, ^{2}H-labeled Leu has been shown to provide better signal to noise when compared to ^{2}H labeled valine [11]. We explored the use of ^{13}C further by also collecting three-pulse ESEEM spectra for $^{13}C_6$-labeled Leu at position 11 on this model peptide as shown in Fig. 4. For the ^{13}C labeled Leu, a peak was observed at 3.5 MHz for the *i*-3 sample representing the ^{13}C Larmor frequency. No peak was observed for the *i*-2 sample or the control sample with no ^{13}C. This pattern again matched with the expectation of the known α-helical secondary structure for this peptide. The peak for the *i*-3 for ^{13}C labeled

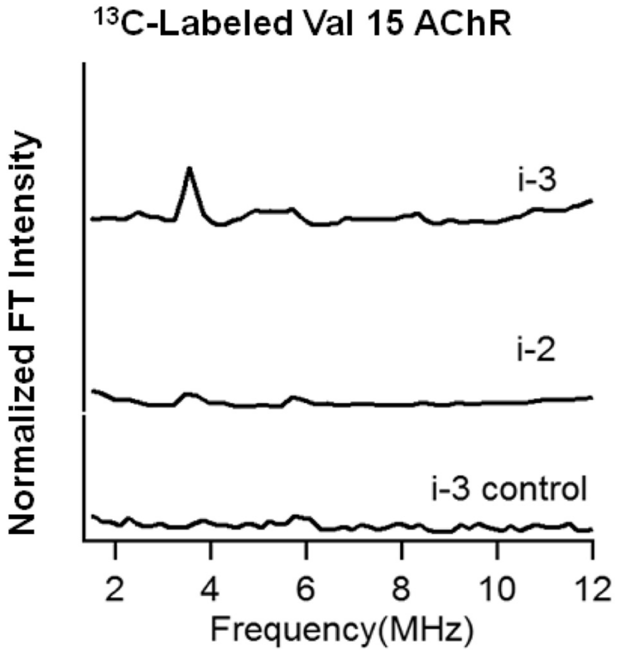

Fig. 3. Three-Pulse ESEEM Frequency domain data of AChR ^{13}C labeled Val15 in DMPC/DHPC bicelles. The i-3 sample shows a peak at the ^{13}C Larmor frequency at 3.5 MHz. No peaks are observed for the control sample without ^{13}C Valine or the i-2 sample.

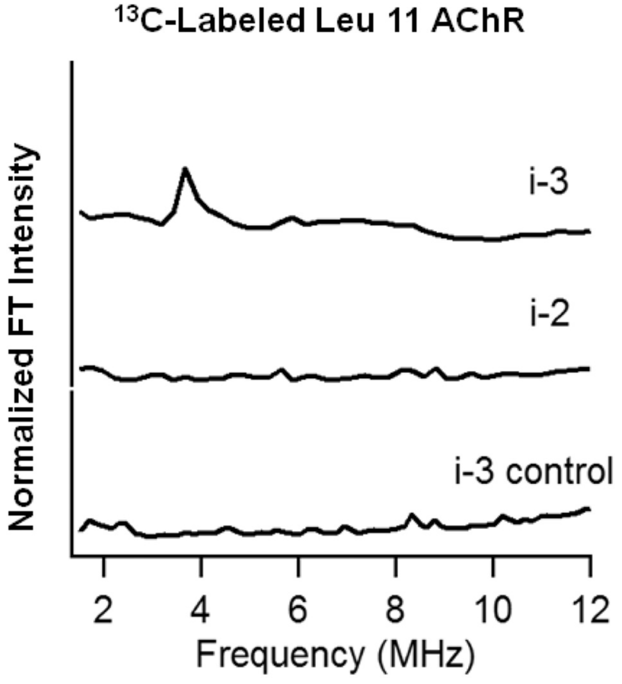

Fig. 4. Three-Pulse ESEEM Frequency domain data of AChR ^{13}C labeled Leu11 in DMPC/DHPC bicelles. The i-3 sample shows a peak at the ^{13}C Larmor frequency at 3.5 MHz. No peaks are observed for the control sample without ^{13}C Leu or the i-2 sample.

Leu sample was similar to what was observed for the ^{13}C labeled Val. It is likely that ^{13}C-labeled Leu does not show an improvement in signal intensity like ^{2}H labeled leucine amino acids because the ^{13}C nuclei are in the backbone of the amino acids when compared to ^{2}H, which are on the perimeter and are more mobile and likely to come closer in distance. Since the modulation depth for ESEEM is proportional to $1/r^6$, this is less variable for ^{13}C-labeled amino acids compared to ^{2}H labeled amino acids[11]. In addition, Leu only has one more carbon atom than Val whereas Leu has two more ^{2}H atoms than Val. The more nuclei present, the better the signal intensity will be. As a result, for ^{13}C-labeled amino acids, the signal observed is similar for both ^{13}C-labeled Val and ^{13}C-labeled Leu.

These data verify that ^{13}C can be used successfully as the NMR active nuclei for this ESEEM approach to determine local secondary structure. However, it is clear from these samples that the signal intensities and signal to noise ratios when using ^{13}C is much less than that of ^{2}H-labeled amino acids. This is because ^{13}C has nuclear spin (I) = 1/2, whereas ^{2}H has a nuclear spin (I) = 1. This means that ^{13}C is approximately three times less sensitive than ^{2}H which results in lower signal intensity and poorer signal to noise. Since the sensitivity of ESEEM experiments is mainly dictated by the modulation depth of these experiments (k), for I = 1/2, nuclei the FT peak amplitude is lowered significantly, resulting in a loss of sensitivity when using ^{13}C compared to ^{2}H [31–33]. Taking into account the nuclear spin and the gyromagnetic ratio of ^{13}C, the maximum distance detectable is a maximum of 7 Å, whereas for ^{2}H the detection limit is up to 8 Å. For ^{13}C, however, the experimental modulation depth depends upon the choice of amino acid site and the nature of the sample preparation. Thus, the detection limit may slightly vary from the theoretical distance estimated using the detectable ^{2}H ESEEM modulation. Additionally, there are fewer ^{13}C nuclei in amino acids than there are ^{2}H atoms when using these labeled amino acids, so there are fewer NMR active nuclei to interact with the electron spin when using ^{13}C compared to ^{2}H. Taken together, ^{13}C-labeled amino acids are not as sensitive when compared to ^{2}H labeled residues; however, this technique still provides pertinent local secondary structural information while overcoming the limitations of comparable solid-state NMR experiments. This also provides an alternative approach that could be useful for studying more challenging recombinant membrane proteins.

4. Conclusions

In summary, while the I = 1/2 spin system suffers from lower sensitivity for this three-pulse ESEEM technique when compared to the I = 1 for ^{2}H, ^{13}C has been able to be successfully used as the NMR-active nuclei in order to determine local secondary structure of a model membrane peptide for the first time. This technique using ^{13}C-labeled amino acids is comparable to REDOR solid-state NMR spectroscopy techniques; however, it requires less sample concentration (μM) and shorter data acquisition times (~30 min) making it ideal for studying membrane protein secondary structure [34,35]. This method provides an alternative approach to this biophysical technique in order to provide more versatility and options when used to study a variety of secondary structures.

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Transparency document

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