

Using solution-phase NMR spectroscopy to study the folding and supramolecular assembly of conformationally constrained β -sheet peptides

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Abstract

Conformationally constrained β -sheet peptides are a valuable model system for studying the folding and supramolecular assembly of aggregation-prone peptides. Understanding how these peptides assemble into soluble oligomers is central to understanding the molecular assembly of amyloid diseases. This chapter describes how to study the folding and supramolecular assembly of β -sheet peptides by solution-phase NMR spectroscopy. First, the chapter describes how to prepare an NMR sample and acquire NMR data. Then, the chapter describes how to assign residues and identify spectroscopic hallmarks of β -sheet folding. Next, the chapter describes how to use isotopic labeling to verify key intermolecular contact. The chapter concludes with the spectroscopic hallmarks of supramolecular assembly. This chapter focuses on the β -sheet peptides but can be generalized to the studying the folding and supramolecular assembly of a wide variety of peptides.

Abbreviations

COSY	Correlation spectroscopy
DSA	Sodium 4,4-dimethyl-4-silapentane-1-sulfonate
DSS	4,4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate
DOSY	Diffusion Ordered Spectroscopy
EXSY	Exchange Spectroscopy
FID	Free Induction Decay
HSQC	Heteronuclear single-quantum correlation spectroscopy
MW	Molecular weight
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
ROESY	Rotating-frame nuclear Overhauser effect spectroscopy
SPPS	Solid-phase peptide synthesis

S/N	Signal-to-noise
TFA	Trifluoroacetic acid
TOCSY	Total correlation spectroscopy
TSP-d₄	Sodium 3-trimethylsilyltetraduteriopropionate
VT	Variable temperature
WATERGATE	Water suppression by gradient tailored excitation
2D	Two-dimensional
3D	Three-dimensional
δ^{Orn}	δ -linked ornithine turn unit



Equipment & reagent list

- ACS grade HCl (CAS # 7647-01-0)
- Analytical balance
- Barnstead GenPure Pro water purification system (ThermoFisher, cat. # 50131922)
- Bruker NMR spectrometer (500 MHz or greater)
- Büchner funnel (200 mL)
- Deionized water from water purification system ($\geq 18 \text{ M}\Omega\cdot\text{cm}$)
- D₂O ampules (0.7 mL, 99.9 %) (Cambridge Isotopes, cat. # DLM-4-10 $\times$ 0.7)
- Eppendorf tubes (1.7 mL) (Corning, cat. # MCT-175-L-C)
- Eppendorf pipettes (P200, P1000)
- Filter paper
- Fmoc-Osu (CAS # 82911-69-1)
- HPLC grade acetonitrile (CAS # 75-05-08)
- HPLC grade TFA (CAS # 76-05-1)
- Isotopically labeled amino acids (Cambridge Isotopes, cat. # DLM-250-1, DLM-372-1, NLM-142-0.5, NLM-202-1, NLM-454-0.5, NLM-752-0.5)
- Lyophilizer (Labconco, cat. # 710401000)
- NMR tube caps (Wilmad, cat # 521-BLK-100)
- Oven for drying NMR tubes
- Pipette tips (VWR, cat. # 76322-522, 76323-946)
- Precision 5 mm NMR tubes (Wilmad, cat. # 528-PP-7-5, 535-PP-7-5, 541-PP-7-5, 542-PP-7-5)
- Static gun (Fisher, cat. Z108812)
- Sodium 3-trimethylsilyltetraduteriopropionate
- Sodium 4,4-dimethyl-4-silapentane-1-sulfonate
- Triethylamine ($\geq 99.5\%$) (CAS # 121-44-8)

- TopSpin
- Vortexer (Fisher, cat. # 02215414)
- 3-button mouse
- 4,4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate

1. Introduction

Our laboratory has utilized solution-phase NMR spectroscopy to study the folding and supramolecular assembly of conformationally constrained β -sheet peptides (Cheng et al., 2012; Khakshoor et al., 2007; Li et al., 2022; Pham et al., 2014; Truex et al., 2016; Truex & Nowick, 2016; Woods et al., 2007; Zhu et al., 2025). We are especially interested in using these peptides as model systems to mimic A β oligomers involved in neurodegeneration in Alzheimer's disease. A β oligomers are difficult to characterize because they are metastable and often not conformationally defined. By incorporating conformational constraints into these peptides — such as β -strand templates, linking the *N*- and *C*-termini together with a δ -linked ornithine turn unit (δ Orn), and adding disulfide staples — we reduce oligomer heterogeneity and enable high-resolution studies on A β oligomer mimetics (Fig. 1) (Nowick et al., 2002; Nowick & Brower, 2003; Samdin et al., 2023).

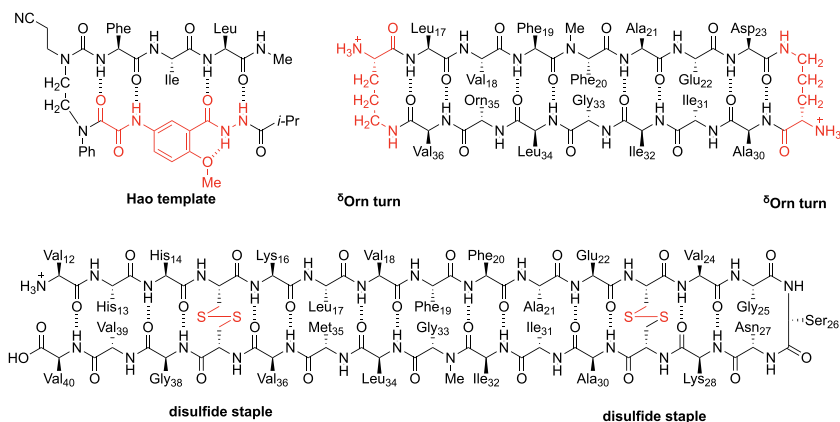
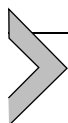


Fig. 1 Different types of conformational constraints our laboratory has utilized to promote β -sheet secondary structure (Nowick et al., 2002; Truex et al., 2016; Samdin et al., 2023).

Constrained peptides are typically prepared by chemical synthesis, rather than expression, so it is generally not feasible to incorporate full ^{15}N and ^{13}C isotopic labeling. For this reason, many of the heteronuclear 2D and 3D experiments routinely used in protein NMR are not applicable to the study of constrained peptides. Homonuclear 2D NMR experiments therefore become the primary working tools of peptide NMR spectroscopy.

Here, we describe the study of conformationally constrained β -sheet peptides using solution-phase NMR spectroscopy. We detail how to prepare a sample, run an experiment, and process the NMR data. Based on the acquired data, we describe how to systematically generate peak assignments and utilize those peak assignments to identify spectroscopic hallmarks of β -sheet folding. We then detail the identification and validation of intermolecular contacts using isotopic labeling. Lastly, we conclude by illustrating the spectroscopic hallmarks of β -sheet folding and supramolecular assembly. These methods can be adapted to the study of the folding and supramolecular assembly of other peptides by solution-phase NMR spectroscopy.



2. Preparing a solution-phase NMR sample

Solution-phase NMR samples are prepared from purified ($\geq 95\%$) lyophilized dry peptide synthesized through Fmoc-based solid-phase peptide synthesis (SPPS) protocols previously published by our laboratory (Guaglianone et al., 2021). These peptides are purified in binary acetonitrile–water mixtures with 0.1 % trifluoroacetic acid (TFA), which provides trifluoroacetate counterions and improves separation of impurities. The molecular weight of the peptides is calculated as TFA salts, with side chains such as arginine, lysine, and aspartic acid assumed to be protonated. Use either D_2O or 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$ for NMR studies. Peptide samples run in D_2O provide information about the $\text{H}\alpha$ protons while peptide samples run in 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$ provide complementary information about the amide NH protons.

2.1 Field strength, sample tubes, and solvent selection

Use NMR spectrometers with a field strength of ≥ 500 MHz for greater dispersion of peaks. Wilmad 5 mm Precision NMR tubes (528, 535, 541, 542) are recommended. NMR tubes should be stored in a 60°C oven to remove adventitious water when not in use. D_2O should be sourced from

freshly opened ampules ($\geq 99.9\%$) to minimize the size of the residual HOD peak. For samples run in 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$, use deionized water ($\geq 18\text{ M}\Omega\cdot\text{cm}$) from a Barnstead GenPure Pro water purification system, or alternatively a Milli-Q water purification system.

Note

- The oven temperature must remain below 70°C to prevent warping of the NMR tubes.

2.2 Sample preparation

1. Calculate the target mass of lyophilized peptide required for a $550\text{ }\mu\text{L}$ sample at your desired peptide concentration. A concentration of 1 or 2 mM is good starting point.
2. Weigh out the calculated mass of the lyophilized peptide in a low-binding Eppendorf tube on an analytical balance. Discharge static with a static gun prior to weighing.
3. Dissolve the lyophilized peptide in either D_2O or 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$. Adjust the volume of solvent as needed to achieve the desired peptide concentration. (For dilute solutions, dissolve the peptide in $550\text{ }\mu\text{L}$ and assume the effect of the peptide on the total volume of solution is negligible.)
4. Vortex the Eppendorf tube until all lyophilized peptide has dissolved.
5. Transfer the solution to an oven-dried 5 mm NMR tube and cap immediately.

Notes:

- When weighing, err on the side of adding slightly more than the target mass required for a $550\text{ }\mu\text{L}$ sample. Insufficient sample volume can cause undesired distortions to NMR spectra.
- To let the amide NHs exchange out, let the sample stand at room temperature for 24 hr.

2.3 Internal standard (optional)

Several NMR standards are commonly used for peptide NMR such as sodium 3-trimethylsilyltetraduteriopropionate (TSP- d_4), sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), and 4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA) (Nowick et al., 2003). These standards can be used to reference chemical shifts. DSS is typically used for anionic peptides, as the negatively charged sulfonate group can interact undesirably with cationic peptides (Shimizu et al., 1994). DSA replaces the negatively charged sulfonate

with a positive charged ammonium group, which does not interact with cationic peptides. To add an internal standard, make a concentrated stock of the NMR standard in D_2O and titrate in as needed.

1. Weigh out approximately 10 mg of the NMR standard. Record the exact mass on the analytical balance.
2. Dissolve the standard in D_2O to make a 10 mM stock solution.
3. Add $\sim 1\text{--}5\ \mu\text{L}$ of the 10 mM stock solution to the NMR sample. The slight ($<1\%$) dilution of the peptide that occurs on adding the stock solution can be ignored.



3. Selecting, loading, and running NMR experiments

Solution-phase NMR spectroscopy contains numerous experiments that can be used to study the folding and supramolecular assembly of conformationally constrained β -sheet peptides. Below are the experiments that have proven to be the most useful in our studies: ^1H NMR, TOCSY, NOESY, ROESY, EXSY, DOSY, $^1\text{H},^{15}\text{N}$ HSQC, and ^{15}N -edited NOESY. These NMR experiments collectively provide insight into the folding and supramolecular assembly of peptides. In this section, we describe what information each experiment provides and how our laboratory sets up and processes NMR experiments.

3.1 Selecting an NMR experiment

^1H NMR

^1H NMR provides an overview of the behavior of the conformationally constrained peptide in aqueous solution. Amide NH, $\text{H}\alpha$, and aliphatic methyl side chains all have characteristic chemical shifts. Significant deviation from these characteristic chemical shifts can reveal information about the folding and supramolecular assembly.

Total correlation spectroscopy (TOCSY)

Total correlation spectroscopy (Total Correlation Spectroscopy) is useful for identifying intraresidue correlations associated with amino acid side chains. For peptide NMR, TOCSY is superior to the correlation spectroscopy (COSY) because the TOCSY experiment illuminates longer range correlations between protons within the same spin system that are not observed in the COSY experiment. A key parameter in the TOCSY experiment is the mixing time, with longer mixing times (e.g. 150–200

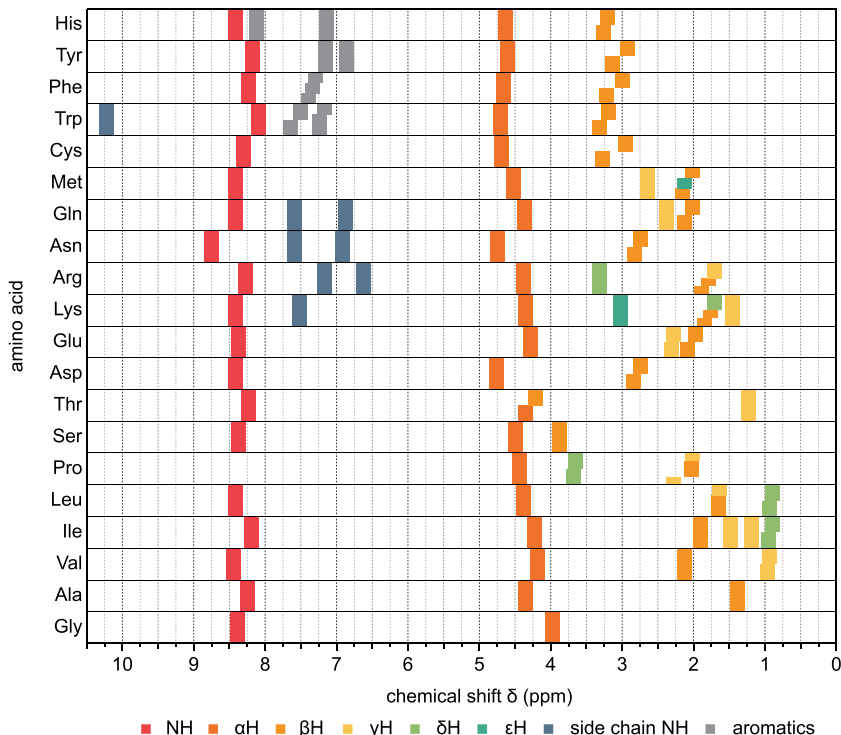


Fig. 2 Representative chemical shifts for the 20 canonical amino acids in aqueous solution. Chemical shifts based on values reported by Wüthrich (Wüthrich, 1991).

msec) being needed to achieve full correlation within longer spin systems, such as Lys. The additional longer-range correlations simplify the identification of longer amino acid side chains, such as Leu, Ile, and Lys, which are often difficult to identify by COSY. In the TOCSY spectrum, the 20 canonical amino acids have characteristic chemical shifts and patterns which are illustrated in Fig. 2.

Nuclear Overhauser effect (NOE) experiments: NOESY and ROESY

Nuclear Overhauser Effect spectroscopy (NOESY) and rotating frame Overhauser effect spectroscopy (ROESY) provide structural information on which protons are close in space based on the enhancement observed through the nuclear Overhauser effect (NOE). The strength of the NOE decays with an r^{-6} relationship, where r is the distance between the two protons. The upper limit for a detectable H–H NOE is approximately 5 Å. Table 1 lists typical distances for β -sheet contacts and their relative NOE strength.

Table 1 Typical distances and relative NOE strength for β -sheet peptides.

H – H distance (Å)	Description	Relative NOE strength
1.8	Geminal CH ₂	7.2
2.2	H α to NH (interresidue)	2.2
2.5	H α to H α (interstrand)	1
3.0	NH to H α (intraresidue)	0.3
3.2	NH to H α (interstrand)	0.2
3.3	NH to NH (interstrand)	0.2
5	Upper limit of detection	0.02

While the NOESY and ROESY experiments both provide distance constraints, the two experiments are not fully interchangeable. NOESY measures the buildup of NOEs in a fixed frame of reference, while ROESY measures the buildup of NOEs in a rotating frame. A practical consequence of this distinction is that the sign and intensity of NOE crosspeaks in the NOESY experiment depend strongly on the rate of molecular tumbling, which correlates with molecular weight (MW). NOE crosspeaks appear positively phased in the NOESY spectrum for small molecules (MW < ca. 1000 Da) and negatively phased for large molecules (MW > ca. 2000 Da). For intermediately sized molecules (ca. 2000 > MW > ca. 1000), NOESY spectra will give weak to no NOE crosspeaks. Therefore, ROESY is recommended for intermediately sized molecules. In the ROESY experiment, NOE crosspeaks always appear positively phased opposite of the diagonal, irrespective of the molecular weight. However, the ROESY experiment is less sensitive than the NOESY experiment for large molecules (MW > ca. 2000).

For supramolecular assemblies of β -sheet peptides, we recommend running both the NOESY and ROESY experiments. The lower sensitivity of the ROESY experiments makes it easier to identify larger NOEs that typically correlate with intramolecular contacts useful for peak assignments. Once peak assignments are complete, the NOESY spectra can then be used to identify intermolecular contacts. For very large molecules or supramolecular assemblies (MW > ca. 5000 Da), which are in the negative NOE regime, the mixing time of NOESY experiments should be decreased

to < 200 msec to limit spin diffusion which can convolute the analysis of the NOESY spectra.

Small molecular weight ($MW < \text{ca. } 1000 \text{ Da}$)

- NOESY is recommended, because NOESY crosspeaks are strong than ROESY crosspeaks in this size regime.

Intermediate molecular weight ($\text{ca. } 1000 \text{ Da} < MW < \text{ca. } 2000 \text{ Da}$)

- ROESY is recommended, since NOESY crosspeaks are weak or absent in the intermediate molecular weight regime.

Large molecular weight ($MW > \text{ca. } 2000 \text{ Da}$)

- Both NOESY and ROESY are recommended.
- Use the ROESY to assign peaks and identify intramolecular contacts.
- Use the NOESY to identify intermolecular contacts.

Exchange spectroscopy (EXSY)

Some peptides exhibit multiple sets of peaks because two (or more) conformers are in slow equilibrium or because monomeric and oligomeric species are in a slow equilibrium. These peptides can exhibit EXSY crosspeaks in the NOESY or ROESY spectra

If a peptide adopts multiple conformations that interconvert slowly, EXSY crosspeaks may appear in the NOESY or ROESY spectrum. EXSY crosspeaks can also appear if a peptide monomer is in slow equilibrium with an oligomer. These crosspeaks show correlation between identical protons in the different species. An EXSY experiment identifies which protons that are correlated through either chemical or conformational exchange. EXSY experiments are NOESY or ROESY experiments used for identifying EXSY crosspeaks. To run an EXSY experiment, run a NOESY or ROESY experiment and look for peaks associated with exchange.

For EXSY crosspeaks to appear, the rate of exchange must occur on the NMR timescale, typically on the order of a hundred milliseconds. If exchange is substantially faster than tens of milliseconds, individual peaks are not seen for the different species. If exchange is substantially slower, than a few seconds, exchange crosspeaks are not observed. Variable-temperature (VT) NMR can be used to modulate the rate of exchange to occur on the order of hundreds of milliseconds.

Depending on the NMR experiment and molecular weight, the NOE crosspeak can either be the same or oppositely phased as that of EXSY crosspeaks (Table 2). In NOESY experiments on small molecules ($MW < \text{ca. } 1000$), EXSY crosspeaks appear opposite in phase to NOE

Table 2 Relative phases of NOE and EXSY crosspeaks in the NOESY and ROESY spectrum.

	Molecular weight (Da)	Crosspeak	Phase (+/-)
NOESY	MW < ca. 1000 (small)	NOE	+
		EXSY	-
	ca. 2000 > MW > ca. 1000 (intermediate)	NOE	~ 0
		EXSY	~ 0
	MW > ca. 2000 (large)	NOE	-
		EXSY	-
ROESY	MW < ca. 1000 (small)	NOE	+
		EXSY	-
	ca. 2000 > MW > ca. 1000 (intermediate)	NOE	+
		EXSY	-
	MW > ca. 2000 (large)	NOE	+
		EXSY	-

crosspeaks and in the same phase as the diagonal. For large molecules (MW > ca. 2000), the EXSY and NOE crosspeaks and the diagonal all appear in the same phase. In ROESY experiments, the EXSY crosspeaks always appear opposite in phase to the NOE crosspeaks and the diagonal. The phasing of the EXSY crosspeaks relative to the NOE crosspeaks. Because the exchange crosspeaks are always oppositely phased relative to NOE crosspeaks, ROESY is generally preferred for EXSY experiments.

Notes:

- The diagonal of both NOE experiments should be negatively phased.
- TOCSY artifacts appear in the ROESY spectra in the same phase as that of EXSY crosspeaks.

Diffusion ordered spectroscopy (DOSY)

The diffusion ordered spectroscopy experiment (DOSY) provides information about the translational diffusion coefficient of molecules in aqueous solution. DOSY measures the decay of signal from molecular

Table 3 Typical diffusion coefficient quotients for small oligomers.

Oligomer	Quotient (relative to monomer)
Monomer	1
Dimer	0.79
Trimer	0.69
Tetramer	0.63
Hexamer	0.55
Dodecamer	0.46
Hexadecamer	0.44

diffusion according to the Stejskal–Tanner equation, where I is the signal intensity, γ is the gyromagnetic ratio, G is the gradient strength (gpz6), D is the diffusion coefficient, δ is the gradient duration (p30), and Δ is the diffusion time (d20).

Stejskal–Tanner Equation:

$$I_G = I_{G=0}^{-\left(\gamma\delta G\right)^2D\left(\Delta-\frac{\delta}{3}\right)}$$

DOSY can be used to assess the oligomerization state and size of supramolecular assemblies formed by constrained β -sheet peptides. Assuming that peptides can be approximated as compact (spherical) structures, the diffusion coefficient scales proportionally with inverse cube of the molecular weight, $D \propto MW^{-1/3}$. (The compact-structure assumption applies well to the cyclic peptides that our laboratory typically studies.) Therefore, the quotient of the diffusion coefficient for an oligomer relative to the diffusion coefficient of monomer provides an estimation of the oligomerization state (Table 3). While distinguishing a dimer from a monomer is simple, distinguishing the oligomerization state for higher-order oligomers ($n > 6$) can be difficult to discern because of the smaller changes in the diffusion coefficient quotient.

3.2 Loading an NMR sample

1. Place the NMR tube in the sample spinner and adjust the height of the tube according to the depth gauge. Ensure that the solvent meniscus is above the recommended fill line; if it is slightly below, adjust the NMR tube to split the difference.

2. Load the NMR sample into the magnet using the *ij* command.
Caution: Remove keys, wallet, and other magnetic items from your pockets before approaching the magnet.
3. Set up a ^1H NMR experiment on the computer using the *edcp* command.
4. Check that the sample is set to the correct temperature, typically 298 K, using the *edte* command and that the sample spinning is set to the appropriate position (*ON/OFF*). Spin the NMR sample for 1D experiments and do not spin for 2D experiments. If the sample spinning setting is changed, the sample must be reshimmied.
5. Match and tune the magnet by first typing the *wobb* command. Manually adjust the ^1H match and tune pins located underneath the magnet while observing the wobble curve on the computer screen. Adjust the tune pin before adjusting the match pin. Alternate between both pins until the magnet is matched and tuned. Alternatively, use the *atma* command if the spectrometer can match and tune automatically.
6. Lock to the solvent of the NMR sample.
7. Shim the magnet using the *lockdisp* command. Adjust the $[Z^1]$ and $[Z^2]$ shims to maximize the lock signal.
8. Adjust the $[X, Z^0]$ and $[Y, Z^0]$ shims to further maximize the lock signal.
9. Repeat steps 6 and 7 until no further increases to the lock signal can be obtained. Then press $[STD BY]$. Alternatively, use the *optshim* command to automatically optimize the shims.

3.3 Running NMR experiments

1. Set the receiver gain using the *rga* command and run an 8 scan ^1H NMR experiment using the *zg* command. Wait until the experiment completes.
2. Use the *ft* and *apks* commands to process the ^1H NMR spectra to verify the shims. Inspect the HOD peaks to check the shims.
3. Set up another experiment using the *iexpno 2* command. For each subsequent experiment, increment the experiment number by 1.
4. Set the receiver gain using the *rga* command and optimize the parameters corresponding to the NMR experiment(s) shown in [Table 4](#).
5. Run the experiment using the *zg* command.

Always double check the acquisition parameters save before running the experiment!

Table 4 Summary of key acquisition parameters.

Experiment	Key parameters
^1H NMR	ns = 64 scans sw = 12 ppm o1p = 4.79 ppm experiment time = 8 min
TOCSY (gradient)	ns = 4 scans sw = 12 ppm o1p = 4.79 ppm td = 512 increments d9 = 25, 50, or 200 msec experiment time = 1 hour 20 min Note: Use the same receiver gain used for the proton NMR to avoid ADC overflow.
NOESY (gradient)	ns = 4 scans sw = 12 ppm o1p = 4.79 ppm d8 = 1 sec td = 512 increments experiment time = 2 hours
ROESY	ns = 4 scans sw = 12 ppm o1p = 4.79 td = 512 p15 = 200 msec experiment time = 1 hour 30 min
DOSY	sw = 12 ppm o1p = 4.79 ppm gpz6 = 2 or 95 % d1 = 5 sec p1 = variable, typically about 10 μsec d20 = variable, typically 50–100 msec p30 = variable, typically 1200–2500 μsec experiment time = 1 hour
$^1\text{H}, ^{15}\text{N}$ HSQC	ns = 4 scans sw = 12 ppm o1p = 4.79 ppm d1 = 1 sec td (f_2, f_1) = 2048, 512 experiment time = 45 min

^1H , ^{15}N -edited	ns = 4 scans
NOESY	sw = 12 ppm
	d8 = 120 msec
	o1p = 4.79 ppm
	td (f_3, f_2, f_1) = 2048, 1, 1024
	experiment time = 1 hour 30 min

The acquisition parameters listed in [Table 4](#) are general recommendations and can be adjusted to maximize spectral resolution and signal-to-noise (S/N) ratio. To maximize spectral resolution and S/N, we recommend the following:

1. Narrow the spectral window (sw, o1p) to maximize resolution of the f_2 dimension.
2. For 2D experiments, increase the number of increments (td) in the f_1 dimension to reduce the distortion of crosspeaks in the f_1 dimension.
3. Select the appropriate mixing time maximizes the observed signal. The mixing time (d8, d9, or p15 depending on the experiment) refers to the time between applying a pulse and observing the resulting FID.

Multiple experiments

To minimize the burden on the NMR facilities, we recommend queueing up experiments overnight.

1. Follow steps 1–5 in the Running NMR experiments section.
2. Use the *iexpno* command to create a new NMR experiment and set up the parameters corresponding to the experiment.
3. Use the *rga* command to set the receiver gain for the experiment.
4. Repeat Steps 2 and 3 as needed for each new experiment.
5. Run the experiments using the *multizg* command. The computer will prompt which experiment number to start on.

Solvent suppression

Water suppression by gradient tailored excitation (WATERGATE) is required for all samples run in 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$.

1. Follow steps 1–3 in the Running NMR experiments section.
2. Use the *cop 2* command to create a copy of the ^1H NMR experiment.
3. Use the *setsfo1* and *keepsfo1* commands to set and load the observation frequency to the water peak into the solvent suppressed ^1H NMR.

Make sure to select *h1sup.** experiment prior to closing the experiment window.

4. Set the acquisition, *aq*, to 1.5 and the relaxation delay, *d1*, to 5.
5. Use the *aqcu* command to go to the acquisition window and type *gs*.
6. Adjust the *o1* frequency to minimize the size of the FID. Save the frequency and stop the pulsing.
7. Verify that the spectral width, *sw*, covers the entire spectral window.
8. Use the *rgazg* command to set the receiver gain and start the experiment.
9. For subsequent 2D experiments that require solvent suppression, select the corresponding experiment with the solvent suppression suffix.

Note:

- The WATERGATE solvent suppression also suppresses peaks within ~ 1 ppm of the HOD peak.

3.4 Variable temperature (VT)

1. Follow steps 1–3 in the Running NMR experiments section.
2. Use the *edte* command to open the temperature controller.
3. Change the *Target temp.* to the desired setpoint. Once the *Sample temp.* has reached the desired setpoint, wait five minutes to allow the sample to thermally equilibrate.
4. Use the *iexpno #* command to create a new experiment. Set the desired acquisition parameters and run with *rgazg*.
5. For each additional temperature, the *Target temp.* needs to be reset and equilibrated.
6. Once all variable temperature experiments are complete, change the *Target temp* back to 298 K and wait until the sample equilibrates.
7. Use the *ej* command to eject the sample.

Notes

- Do not heat or cool the NMR sample past the boiling and freezing point. Note that D₂O freezes at 3.8 °C and boils at 101.4 °C. Always leave a 10 °C margin of error when setting *Target temp.*
- Because the chemical shift of the HOD peak varies with temperature, VT NMR can be used to walk the water peak to enable analysis of the H α protons buried underneath the residual HOD peak.
- VT NMR is also useful for studying the exchange rates of EXSY crosspeaks.

DOSY

1. Run an 8-scan ^1H NMR to check shims. Verify sample spinning is OFF.
2. Use *iexpno 2* command and change the pulse program from *zg30* to *zg0*. Vary *p0* value to calibrate the pulse time (default: 32 μsec).
3. Type *edcp* to create a new experiment and select the *dosy1D* experiment to calibrate the diffusion time and diffusion gradient length.
4. Set *p1* to equal *p0/4*. Set Δ (*d20*) to 75 ms, δ (*p30*) to 1200 μs , and gradient strength, *gpz6* to 2 %. Use the *etde* command to verify that the parameters saved. Run the experiment with *rgazg*.
5. Run the 1D DOSY experiment and process using the FID using the *efp* command. Phase-correct the ^1H NMR spectra as needed.
6. Use *iexpno 4* command to create an identical instance of the *dosy1D* experiment. Set the gradient strength, *gpz6* to 95 %. Run the experiment with *zg* (do not use *rgazg* because the receiver gain must remain constant between both 1D DOSY experiments). Phase correct the ^1H NMR spectra as needed.
7. Create a stacked plot of both *dosy1D* spectra. The attenuation of signal between both *dosy1D* experiments should be 20x. If the attenuation is not 20, rerun both *dosy1D* experiments while varying *d20* and/or *p30* until the 20x signal attenuation is achieved.
8. Use the *makedosy2d* to set up a pseudo two dimensional DOSY experiment. Use the *edte* command to check *p1*, *d20*, and *p30* saved.
9. Run the pseudo 2D DOSY experiment using *dosy 2 95 161 y y*.

Notes:

- Accurate DOSY measurements require calibration of *p1*, Δ , and δ . Increasing either Δ or δ leads to greater signal attenuation.
- To minimize the number of scans required for adequate S/N at high gradient strengths (*gpz6* = 95 %), the ^1H NMR spectra can be processed with increased line broadening, *lb* to increase the S/N. Increase *lb* from 0.3 Hz to 5 Hz for both 1D DOSY experiments.
- **The gradient duty cycle, $p30/(d1+aq) \leq 0.05$, must never exceed 5 %. Increasing the gradient duty cycle above 5 % can damage NMR hardware.**

^1H , ^{15}N HSQC and ^{15}N -edited NOESY

1. On TopSpin click on the *Acquire* Tab, go to *More*, and open *BioTop*.

2. In Sample Information Tab, enter in the molecular size (kDa), isotopic labeling, solvent, and tube. Select the check box for the *Temperature*, *Lock automatically*, *Tune/Match Automatically*, and *Shim Automatically*.
3. In the *Optimization* tab, select the check box for *Auto opt.* corresponding to the isotopes present in your sample. Press *Start optimization* at the bottom of the Menu. The automated optimization takes around five minutes, and you may encounter an ADC overflow warning as the program is optimizing the solvent suppression parameters.
4. In the *Experiments* tab, select the ^1H NMR experiment with WATERGATE solvent suppression (ZGGPW5), add the experiment to the queue, and press *Run Experiments*. Once the ^1H NMR experiment is complete, process the spectrum to verify the shims.
5. To set up a ^1H , ^{15}N HSQC experiment, select ^{15}N HSQC and adjust the acquisition parameters (see Table 4 for recommended parameters).
6. To set up a ^{15}N -edited NOESY experiment, select the 2D WaterGATE NOESY experiment and adjust the acquisition parameters (see Table 4 for recommended parameters).
7. Press Run Experiments to run the isotope edited experiments.



4. Processing NMR experiments

4.1 1D Experiments

1. Type *ft* to Fourier Transform the FID and *apks* to phase correct.
2. In the *Processing* menu, select *Adjust Phase* to manually phase correct the ^1H NMR spectrum. Iterate between applying zero-order (*p0*) and 1st-order (*p1*) phase corrections until all peaks appear symmetrical. Press the *Return & Saved Spectrum* button.
3. In the *Processing* menu, select the *baseline correction* option. Use the polynomial baseline correction up to the third term (Cx^2). Press the *Return, save regions* button.
4. In the *Processing* Tab, select the *Calib. Axis*. For peptide samples run in D_2O , reference to the solvent residual at 4.79 ppm for samples run at 298 K. For peptide samples run in 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$ reference to a diagnostic peak such as a methyl singlet for the same peptide run at the same concentration and temperature in D_2O .
5. In the *Analysis* tab, integrate all observed protons and pick peaks.

Notes

- As a rule of thumb, the integrations for peptide NMR should be within 5–10 % for a properly phased and baseline corrected ^1H NMR spectrum.
- Normalize integrals to a proton of known integration such as an aromatic resonance, $\text{H}\alpha$ proton, or distinct methyl group.
- The integration of the residual HOD peak should be smaller than 100 mM for D_2O samples. Estimate this by comparing the integration of the HOD peak at 4.79 ppm for samples run at 298 K to the integration of peptide proton or known NMR standard.

4.2 2D Homonuclear experiments

1. Type *xfb* to Fourier Transform and *abs2* to phase correct.
2. In the Processing menu, select Adjust Phase and pick three points along the diagonal to phase correct. Phase correct the Rows by iterating between applying zero-order (*p0*) and 1st-order (*p1*) phase corrections until all peaks appear symmetric. Press the *Return & Save phased spectrum*. Then, phase correct on the columns. Press the *Return* button.
3. In the *Processing* menu, calibrate the 2D homonuclear experiment to either a solvent residual or to the same peak used to calibrate the reference ^1H NMR spectrum.
4. Click on f_2 projection above the 2D homonuclear spectrum and right click to open a dropdown menu. Select *External Projection* and specify the *External Projection* to point to the processed ^1H NMR spectrum. Repeat the same process to set an *External Projection* in the f_1 axis.

4.3 2D Heteronuclear experiments

$^1\text{H}, ^{15}\text{N}$ HSQC

1. Type *xfb* to Fourier Transform and *abs2* to phase correct.
2. In the Processing menu, select *Adjust Phase* and pick three points along the diagonal to phase correct. Phase-correct the *rows* by iterating between applying 0 order (*p0*) and 1st order (*p1*) phase corrections until all peaks appear symmetric. Press the *Return & Save phased spectrum*. Repeat the Phase correction on the *columns*. Then press the *Return* button.
3. Use the *xiref* command to reference the peaks.
4. Click on the f_2 and f_1 projection around the 2D heteronuclear spectra reference the $^1\text{H}, ^{15}\text{N}$ HSQC to the corresponding NMR spectra for the unlabeled peptide at the same concentration and temperature.

¹⁵N-edited NOESY

1. Type *xfb* to Fourier Transform and specify a new experiment number to save the 2D data. Then type *abs2* to phase correct the 2D spectrum.
2. In the *Processing* menu, select *Adjust Phase* and pick three amide NHs along the diagonal to phase correct. Phase-correct the rows by iterating between applying zero-order (*p0*) and 1st-order (*p1*) phase corrections until all peaks appear symmetric. Press *Return & Save phased spectrum*. Repeat the phase-correction on the columns. Then press the *Save for nD Spectrum* button.
3. Use the *xiref* command to reference the peaks.
4. Click on *f₂* projection above the 2D homonuclear spectrum and right click to open a dropdown menu. Select *External Projection* and specify the *External Projection* to point to the processed ¹H NMR spectrum. Repeat the same process to set an external projection in the *f₁* axis. We recommend referencing the ¹⁵N-edited NOESY to the unlabeled peptide in 9:1 H₂O/D₂O for the *f₂* axis and the unlabeled peptide in either D₂O or 9:1 H₂O/D₂O for the *f₁* axis.

4.4 DOSY

1. Type *rser* to phase correct *FID# 1* and name it *EXPNO 999*. Press *Okay*.
2. Process the ¹H NMR spectrum using the *efp* command.
3. In the *Process Tab*, select *adjust phase* and manually phase correct the ¹H NMR spectrum. Press the *Save for nD spectrum* button, then press *Return*.
4. Return to the 2D DOSY experiment. Type *xf2* and *abs2* to Fourier Transform and baseline correct.
5. Type *setdiffparm* to prepare the DOSY parameters for processing.
6. Type *eddosy* to view the processing parameters with DOSY. We recommend windowing the *DISPmin* and *DISPmax* parameters between -12 and -8, respectively.
7. Type *dosy2d* to process the 2D DOSY experiment.
8. Calibrate the diffusion coefficient of the residual HOD peak (4.79, -8.7212) (Longworth, 1960).
9. To calculate the translational diffusion coefficient, identify a horizontal track in the DOSY track and record the *f₁* coordinates. For example, a track at -9.008 in the *f₁* axis would correspond to a diffusion coefficient of $10^{-9.008} = 9.81 \times 10^{-10} \text{ m}^2/\text{s}$.

5. Generating peak assignments

Peak assignments are essential for studying the folding and assembly of β -sheet peptides.

Here, we describe a systematic approach for generating peak assignments (Fig. 3). We recommend first identifying residue-specific tracks in the TOCSY spectra. Once these residue-specific tracks are identified, analyze the ROESY spectra to identify adjacent residues. Finally, generate sequence specific assignments by analyzing the TOCSY in conjunction with the ROESY data.

5.1 TOCSY

1. Analyze the TOCSY spectra of your peptide in 9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$ with WATERGATE solvent suppression between 10 – 7 ppm in the f_2 dimension and 10 – 7 ppm in the f_1 dimension.
2. Draw a vertical line corresponding to each amide NH observed in the NMR spectra and verify that the number of tracks corresponds with the number of residues in the peptide sequence.
3. Inspect each vertical TOCSY track and compare the observed cross-peaks to the typical chemical shifts for the 20 canonical amino acid (Fig. 2).
4. For each vertical TOCSY track, make tentative assignments based on the peptide sequence and observed crosspeaks. It is often helpful to

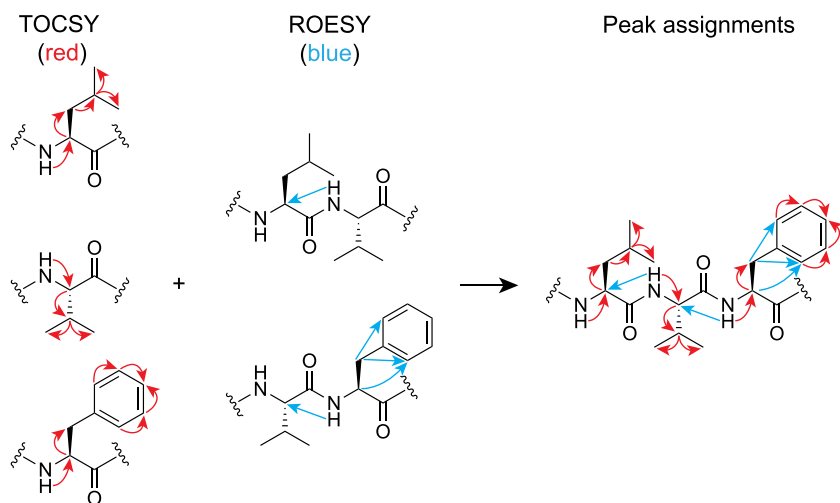


Fig. 3 Overview of generating peak assignments using TOCSY and ROESY NMR.

group and assign residues by asking a series of diagnostic questions. Ambiguous residues can be left unassigned at this stage and revisited later.

- a. Does this residue lack side chain protons (Gly) or an amide NH (Pro)?
 - b. Does this residue contain an aliphatic methyl group (Ala, Val, Leu, Ile)?
 - i. How many methyl groups does this aliphatic residue contain?
 - ii. What is the splitting for the aliphatic residues?
 - c. Does this residue contain aromatic side chains (Phe, Tyr, Trp, His)?
 - i. What is the integration and splitting for the aromatic side chains?
 - d. Does this residue contain a side chain NH (Trp, Gln, Asn, Arg, Lys)?
 - i. How many methylenes does the side chain contain and how many are observed?
 - e. Does this residue contain an aliphatic alcohol (Ser, Thr)?
 - f. Based on process of elimination, can this residue be one of the remaining amino acids (Met, Glu, Asp, Cys)?
 - i. How many methylene protons are observed?
5. Once all the TOCSY tracks have been analyzed, proceed onwards with analyzing the ROESY spectra.

Notes:

- The chemical shifts for the 20 canonical amino acids shown in [Fig. 2](#) should be taken as a guideline because the shifts can be affected by secondary structure and supramolecular assembly. The resulting changes in chemical shift can affect the chemical shifts of resonances by up to 1 ppm, or more in some cases.
- It is particularly challenging to assign residues when multiple resonances overlap (≤ 0.02 ppm). Make sure to consider different possibilities for the amino acids that are involved, as it is easy to make mistakes.
- If you are unsure about an assignment, record all possible residues assignments and revisit later.

5.2 ROESY

1. Analyze the ROESY spectra of your peptide in 9:1 H₂O/D₂O with WATERGATE solvent suppression between 10 – 7 ppm in the f_2 dimension and 6 – 4 ppm in the f_1 dimension.
2. Draw a vertical line corresponding to each amide NH observed in the ROESY spectra.
3. For each ROESY track, analyze the NH to H α crosspeaks based on your tentative peak assignments. Within a β -strand, the strongest NOE

should correspond to an interresidue NH to H α contact between adjacent residues.

4. For residues containing aromatic side chains, analyze the ROESY spectra for intraresidue NOE crosspeaks between the aromatic protons ($\sim 7 - 8$ ppm) to the adjacent methylene (~ 3 ppm) or H α proton ($\sim 4 - 6$ ppm).
5. Once all the ROESY tracks have been analyzed, revisit the TOCSY tracks corresponding to unassigned residues. Iterate between analyzing the peptide sequence, TOCSY, and ROESY spectra until all the residues have been assigned.

Note

- Additional weaker intraresidue NOE crosspeaks associated with the NH-H α protons may be observed as well as NOEs associated with intermolecular contacts (discussed in a subsequent section).

5.3 Peak assignments

1. Inspect the peptide sequence to identify entry points to generate sequence specific assignments. An entry point can be any residue that can be unambiguously assigned. Useful entry points often include *N*-methylated amino acids, glycine, and any residue that appears once in the peptide sequence.
2. For each entry point, use the ROESY spectra to identify adjacent residues and build peptide fragments. The TOCSY provides intraresidue NH to H α correlations while the ROESY provides interresidue NH to H α correlations for building out peptide fragments.
3. TOCSY and ROESY spectra can be chained to perform an NH to H α walk. We recommend performing an NH to H α walk starting from the *N*-terminal residue to the *C*-terminal residue to check the sequence specific peak assignments. For cyclic peptides, systematically walk around the peptide using both the TOCSY and ROESY spectra.

Note

- Accurate peak assignments are the foundation of all peptide NMR studies. We strongly recommend using a variety of different methods to validate peak assignments including isotope-edited experiments (partial deuteration, ^{15}N -labeling), considering pairwise swaps of residues with similar chemical shifts (e.g., Cys and Met), and carefully analyzing the peak assignments in areas of heavy spectral overlap.

6. Identifying spectroscopic hallmarks of β -sheet folding

The chemical shifts of the $H\alpha$ protons, interstrand NOEs, and magnetic anisotropy of the δ Orn turn units are three spectroscopic signs of folding in aqueous solution (Fig. 4). Pioneering work by Wishart identified that the chemical shifts of $H\alpha$ protons correlate with the secondary structure in peptides and proteins (Wishart & Sykes, 1994). Upfield shifts of the $H\alpha$ resonances suggest α -helical secondary structure while downfield shifts of $H\alpha$ resonances suggest β -sheet secondary structure. Specifically, downfield shifts of ≥ 0.2 ppm relative to random coil values is characteristic of β -sheet secondary structure. Even greater downfield shift often occurs if there is further supramolecular assembly through β -sheet interactions. The NOESY spectra reveal networks of interstrand contacts when cyclic peptides fold in aqueous solution. These interstrand contacts typically occur between NH-NH, NH- $H\alpha$, and $H\alpha$ - $H\alpha$ protons. Previous spectroscopic studies established that the magnetic anisotropy of the diastereotopic δ -linked ornithine (δ Orn) δ -protons is a spectroscopic probe for folding (Nowick & Brower, 2003). For β -sheet peptides, a magnetic anisotropy of 0.6–0.7 ppm indicates complete folding, while a magnetic anisotropy of ≤ 0.1 indicates little or no folding. Intermediate values reflect partial folding.

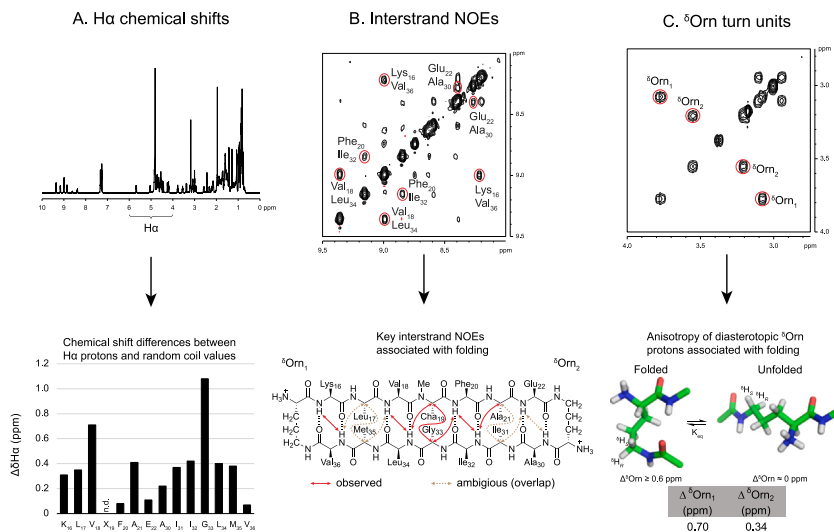


Fig. 4 Spectroscopic hallmarks of β -sheet folding. (A) Downfield shifting of $H\alpha$ resonances relative to random coil values. (B) Interstrand NOEs associated with folding. (C) Magnetic anisotropy of the diastereotopic δ Orn protons.

6.1 Chemical shifts of H α protons

1. Tabulate the chemical shifts of all the assigned H α proton.
2. Calculate the difference between the observed H α chemicals and random coil values. We recommend the chemical shifts reported by Wishardt, Sykes, and Richards ([Wishart et al., 1991, 1992](#)).
3. Plot the chemical shifts differences between the observed and random coil values to visualize the secondary structure.

6.2 Interstrand NOEs

1. Analyze in the NOESY spectra of your peptide in D₂O between 6–4 ppm in the f_2 dimension and 6–4 ppm in the f_1 dimension. Using the sequence-specific peak assignments, identify H α –H α NOE crosspeaks consistent with β -sheet folding.
2. Analyze the NOESY spectra of your peptide in 9:1 H₂O/D₂O with WATERGATE solvent suppression between 10–7 ppm in the f_2 dimension and 10–7 ppm in the f_1 dimension. Based on the sequence specific peak assignments, identify NH–NH NOE crosspeaks consistent with β -sheet folding.
3. Analyze the NOESY spectra of your peptide in 9:1 H₂O/D₂O with WATERGATE solvent suppression between 10–7 ppm in the f_2 dimension and 6–4 ppm in the f_1 dimension. Identify NH–H α NOE crosspeaks consistent with folding.

6.3 δ -Linked ornithine turn unit

1. Inspect the ¹H NMR spectra in D₂O between 4–2.75 ppm to identify the *pro*-S δ Orn proton(s).
2. Analyze in the NOESY spectra of your peptide in D₂O between 4–2.75 ppm in the f_2 dimension and 4–2.75 ppm in the f_1 dimension. Identify the strong crosspeak between the diastereotopic *pro*-S and *pro*-R δ Orn protons.
3. Calculate the magnetic anisotropy based on the difference in chemical shift between the f_2 and f_1 dimensions to assess the extent of β -sheet folding.



7. Identifying intermolecular contacts

The appearance of intermolecular NOEs is a hallmark of the supramolecular assembly of constrained β -sheet peptides. The following workflow outlines a systematic approach for identifying intermolecular

NOEs in the homonuclear 2D NOESY spectra and utilizing those NOE crosspeaks to elucidate the close contacts between molecules.

1. Generate sequence-specific peak assignments using the TOCSY and ROESY spectra.
2. Analyze the homonuclear 2D NOESY spectra for NH-NH, NH-H α , and H α -H α contacts. Annotate all observed crosspeaks.
3. Identify all intramolecular contacts associated with residue specific peak assignments and folding. Additionally, note any crosspeaks arising from chemical exchange. Mark off all NOE crosspeaks that correspond to intramolecular contacts.
4. Analyze the remaining NOE crosspeaks to identify the intermolecular contacts. Outer-facing amide NH and H α protons form intermolecular contacts. Inner-facing amide NH and H α protons form interstrand intramolecular contacts.
5. Categorize the intermolecular contacts based on crosspeak strength and look for patterns. For each intermolecular contact, examine adjacent NH or H α protons for additional intermolecular NOEs. Typically, a minimum of three intermolecular contacts is used to confirm the formation of a stable intermolecular interface.

Notes

- Contacts between identical protons at the same chemical shift cannot be identified by NOESY or ROESY experiments.
- NOEs that can either be intermolecular or intramolecular cannot be used to unambiguously identify intermolecular contacts.



8. Confirming intermolecular contacts with isotopic labeling

While homonuclear 2D NMR experiments are the primary tools for studying constrained β -sheet peptides by solution phase NMR spectroscopy, heteronuclear 2D NMR experiments can also be useful to study the supramolecular assembly of peptides. The incorporation of an isotope aids in the confirmation of intermolecular contacts. We have found ^{15}N labeling of selected amino acids to be a powerful tool for identifying key contacts in constrained β -sheet peptides. Deuterium labeling can also be useful. Isotope edited experiments should be performed to address specific ambiguous crosspeaks after 2D homonuclear NMR experiments have been

fully analyzed. We have found isotopic labeling to be useful in dealing with mixtures of monomer and complex oligomers in which the NMR spectra is heavily overlapped. Here, we describe how to design an isotope labeling scheme, preparing the suitably protected amino acid, and analyzing the resulting heteronuclear 2D NMR experiment.

8.1 Isotope labeling design

1. Analyze intermolecular interfaces identified in determining the intermolecular NOE crosspeaks and list two protons involved in each intermolecular contact.
2. For intermolecular contacts involving amide NHs, consider using a ^{15}N -labeled amino acid. For intermolecular contacts involving H α protons, consider using specifically or fully deuterated amino acids.
3. Identify key intermolecular contacts along each interface and design suitable isotopic labeling scheme(s) by selecting one of the protons involved in an intermolecular contact to isotopically label. At minimum, label the strongest contact along each interface.

Note

- The pricing and availability of isotopically labeled amino acids may influence the design of the isotope labeling scheme.

8.2 Fmoc-protection of isotopically labeled amino acids

1. Purchase the corresponding isotopically labeled amino acid from a commercial vendor, such as Cambridge Isotope Laboratories. ^{15}N -Labeled amino acids without functional groups in the side chains (Gly, Ala, Phe, Leu, and to a lesser extent Met and Ile) are relatively economical and are good choices for isotopic labeling. Some deuterated amino acids are also available albeit at higher cost.
2. Weigh out the isotopically labeled amino acid in a round bottom flask. Add a stir bar. Then add 0.95 equivalents of Fmoc-Osu to a round bottom flask.
3. Add 1:1 acetonitrile/ H_2O to the round bottom flask. We recommend adding 5 mL of solvent per 100 mg of amino acid.
4. Dissolve the solids in 1:1 acetonitrile/ H_2O . Add 5 mL of solvent per 100 mg of amino acid. The solids won't dissolve right away.
5. Then add 1 equivalent of neat Et_3N and let the reaction stir for 15 minutes.

6. Add additional Et_3N slowly, dropwise via syringe, as needed until the solution becomes homogenous. Do not add a gross excess of Et_3N because Fmoc is base-labile. Check and record the pH of the solution.
7. Let the reaction stir for 1 hr at room temperature.
8. Precipitate the Fmoc-protected amino acid by the solution to 1 M HCl while stirring vigorously. We recommend 25 mL of 1 M HCl per 100 mg of amino acid.
9. Filter the Fmoc-protected amino acid using filter paper and a Büchner funnel.
10. Assess the purity of Fmoc-protected amino acid by NMR and mass spectrometry. Pay special attention to $^1J_{\text{NH}}$ splitting for ^{15}N amino acids and the isotopic pattern in the mass spec for deuterated amino acids.
11. Typical yields of the Fmoc-protected amino acid are $\geq 85\%$.

Note:

- We recommend purchasing the unprotected amino acid and doing the Fmoc protection in-house, because the reaction is high yielding and does not require purification by column chromatography prior to use. Fmoc-protected isotopically labeled amino acids are significantly more expensive than their unprotected counterparts.

8.3 Deuteration

Deuterated peptides can be analyzed by running the standard homonuclear 2D NMR experiment. If a proton involved in an intermolecular contact is deuterated, the corresponding NOE disappears from the NOESY spectra. Additionally, the ^1H NMR spectra of a deuterated peptide can also be used to cross-check peak assignments.

8.4 $^1\text{H}, ^{15}\text{N}$ HSQC

Heteronuclear HSQC experiments can be analyzed comparing the chemical shifts and number of crosspeaks to the peak assignments for the unlabeled peptide. The $^1\text{H}, ^{15}\text{N}$ HSQC experiment can be useful for quickly discerning the number of distinct oligomer species present when constrained peptides self-assemble because each peak corresponds to a single species.

8.5 ^{15}N -edited NOESY

The ^{15}N -edited NOESY can be an essential experiment for verifying key intermolecular contacts and resolving heavy overlap in the amide NH region. The extra dispersion afforded by access to an orthogonal dimension

(^{15}N) greatly simplifies the NOESY spectra by greatly reducing the observed number of crosspeaks. A limitation of the ^{15}N -edited NOESY experiment is that it cannot resolve overlapping resonances in the f_1 dimension. Distinguishing ambiguous crosspeaks from spectral overlap in the f_1 dimension requires chemical intuition and knowledge of sequence-specific peak assignments.

9. Elucidating spectroscopic hallmarks of supramolecular assembly

The supramolecular assembly of constrained β -sheet peptides is characterized by a number of spectroscopic hallmarks of assembly in solution-phase NMR spectroscopy (Fig. 5). These characteristics include peak broadening, further downfield shifting of oligomer $\text{H}\alpha$ resonances, upfield shifting of some aromatic and aliphatic methyl protons, new peaks associated with oligomer, and a specific reduction in the diffusion coefficient. Here, we describe how to identify the spectroscopic hallmarks of supramolecular assembly.

9.1 Discrete new peaks associated with oligomer

1. Inspect the ^1H NMR stacked plot and look for the appearance of new peaks associated with oligomer. These new peaks should be clearly distinct in chemical shift from the monomer resonances
2. Analyze the homonuclear NOESY and ^{15}N -edited NOESY to identify intermolecular contacts.

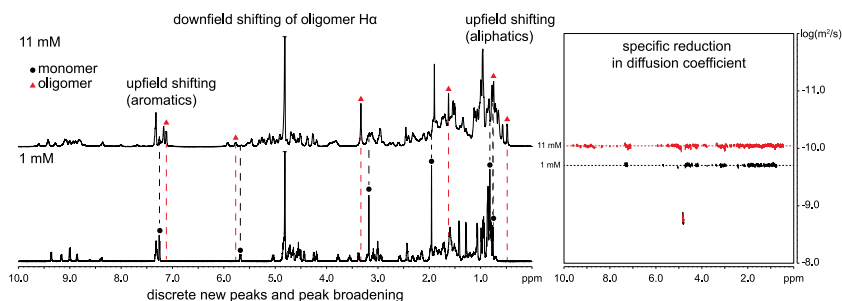


Fig. 5 Spectroscopic hallmarks of supramolecular assembly observed in the ^1H NMR and DOSY spectra. These hallmarks are discrete new peaks associated with oligomer, downfield shifting of oligomer $\text{H}\alpha$ protons, upfield shifting of some aromatic and aliphatic resonances, peak broadening, and a specific reduction in diffusion coefficient.

Notes

- If a peptide oligomerizes through transient, nonspecific interactions, the ^1H NMR spectrum at high concentration may look virtually identical to the ^1H NMR spectrum at low concentrations but significantly broadened.
- At intermediate concentrations, multiple sets of peaks may appear, corresponding to both monomer and oligomer species.

9.2 Downfield shifting of $\text{H}\alpha$ resonances associated with oligomer

1. Look at the stacked plot of your constrained β -sheet peptide, paying special attention to the $\text{H}\alpha$ region around 4 – 6 ppm.
2. Look for downfield shifting of the $\text{H}\alpha$ oligomer resonances with increasing peptide concentration. As a rule of thumb, $\text{H}\alpha$ chemical shifts downfield of 5 ppm are significant.
3. Compare the $\text{H}\alpha$ chemical shifts of the monomer relative to the oligomer. The supramolecular assembly of constrained peptides into well-defined oligomers often reinforces β -sheet secondary structure, which is reflected as further downfield shifting of $\text{H}\alpha$ resonances for the oligomer relative to the monomer. This can further be corroborated with the magnetic anisotropy of the diastereotopic δ^{Orn} protons.

9.3 Upfield shifting of some aromatic and aliphatic resonances

1. Examine the stacked ^1H NMR plot, focusing on the aromatic region around 7 – 8 ppm and the aliphatic region around 0 – 2 ppm.
2. Look in the stacked plot and look for upfield shifting of aromatic resonances and appearance of methyl groups upfield of 0.5 ppm.
3. Corroborate the upfield shifting of the upfield shifted resonances with NOE crosspeaks in the NOESY spectra. The presence of these NOE crosspeaks often indicates hydrophobic packing associated with the formation of a well-defined oligomer.

9.4 Peak broadening

1. Generate a stacked plot of your constrained β -sheet peptide at a variety of different peptide concentrations. We recommend 1–10 mM in D_2O as a starting point.
2. Identify distinct peaks in the ^1H NMR spectrum at your lowest concentration. Some examples would include isolated methyl singlets, $\text{H}\alpha$ protons, and the *pro-S* δ^{Orn} proton.

3. Inspect the appearance of the peaks in the NMR spectra with increasing peptide concentration. Look for the disappearance of splitting associated with H α protons and aliphatic methyl groups, indicative of peak broadening.

Note

- The slower rate of tumbling for larger species, such as oligomer, leads to more rapid T_2 relaxation and peak broadening in the ^1H NMR spectra. Part of the inherent challenge with studying supramolecular assembly by solution-phase NMR spectroscopy is in designing constrained β -sheet peptides to oligomerize without uncontrollably aggregating into large NMR-invisible aggregates.

9.5 Specific reduction in the diffusion coefficient

1. Run DOSY NMR of your constrained peptide β -sheet peptide at several different peptide concentrations in D_2O . Make sure to calibrate the DOSY spectra to the residual HOD peak before calculating the diffusion coefficient.
2. Look for a specific reduction in diffusion coefficient corresponding to the formation of a well-defined oligomer. Calculate the quotient of the oligomer diffusion coefficient relative to monomer to estimate the oligomer size.

Notes:

- You may observe the appearance of multiple tracks at intermediate concentrations in between that of the monomer and oligomer. This reflects exchange between monomer and oligomer occurring on the NMR timescale of the DOSY experiment.
- If the diffusion coefficient continuously decreases with increasing peptide concentration, it may suggest that the peptide is oligomerizing through transient non-specific interactions rather than forming a well-defined oligomer.



10. Summary

In this chapter, we have detailed how to use solution-phase NMR spectroscopy to study the folding and supramolecular assembly of constrained β -sheet peptides. These constrained β -sheet peptides are designed to assemble into well-defined soluble oligomers through the incorporation

of ^8Orn turn linkers that double as a spectroscopic probe for folding and a single *N*-methylated amino acid to prevent fibril formation. We describe how to prepare an NMR sample, run a variety of NMR experiments (^1H NMR, TOCSY, NOESY, ROESY, DOSY), and process NMR spectra. We then detail how to systematically process the NMR data to generate sequence specific peak assignment that further serve as the foundation for identifying intermolecular contacts. These intermolecular contacts can be further verified through isotope-edited experiments discussed in this protocol. Lastly, we conclude with spectroscopic hallmarks of supramolecular assembly that can be observed in aqueous solution. We anticipate that the protocols described herein can be adapted to studying the folding and supramolecular assembly of other peptides by solution-phase NMR spectroscopy.

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