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Probing the atomic structure of transient protein contacts by paramagnetic relaxation enhancement solution NMR:

Transient protein structure by PRE NMR

Vincenzo Venditti^{1,2} and Nicolas L. Fawzi³

¹Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States

²Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa 50011, United States

³Department of Molecular Pharmacology, Physiology, and Biotechnology, Brown University, Providence, Rhode Island 02912, United States

Abstract

Important biological processes, including enzyme catalysis, signaling and protein folding, proceed through lowly populated (< 5%) states that elude structural characterization by conventional techniques. Here we describe the steps required for visualization of these sparsely populated conformations and transient protein-protein interactions using paramagnetic relaxation enhancement solution NMR. We describe experimental design, sample preparation, data acquisition and processing, and the basics of data analysis of structural ensembles.

Keywords

transient interactions; protein-protein interactions; NMR spectroscopy; site-directed spin-labeling; encounter complex; lowly populated states; dark states

1. Introduction

The history of Paramagnetic Relaxation Enhancement (PRE) in solution NMR applications dates back to the early work of Solomon and Bloembergen who established that, in paramagnetic solutions, the dipolar coupling between a nucleus and an unpaired electron results in an increase in the nuclear spin relaxation rates [1,2]. The PRE is proportional to the population-averaged $\langle r^{-6} \rangle$ distance between the paramagnetic center and the nucleus. Owing to the large magnitude of the electron magnetic moment, the PRE can extend for distances up to ~35 Å from the paramagnetic center, making it an important source of long-range distance restraints in structure calculation protocols. Due to this very strong dipolar interaction and very steep distance dependence, PREs are also uniquely able to detect and characterize lowly populated (< 5%) conformational states [3]. Although they are sampled only transiently, these lowly populated states underlie a range of crucial biological

processes, acting as encounter complexes between protein interaction partners and as intermediates in enzyme catalysis, protein folding, and aggregation [4,5]. In order to be detected by PRE experiments, the minor (invisible to conventional NMR techniques) state must undergo rapid exchange with the major (NMR visible) state, and the distance between the paramagnetic center and the nuclei of interest must be shorter in the minor state than in the major state [3]. When these conditions are satisfied, because a nucleus in close proximity to a paramagnetic center exhibits an extremely large PRE ($>1000 \text{ s}^{-1}$), the contribution from the close-proximity state will dominate the overall PRE measured for that nucleus, even if it constitutes a minor percentage of the total population. Therefore, PRE NMR for transient states takes advantage of the strong dipolar interaction and r^{-6} (i.e. steep) distance dependence. Currently, PRE is the technique of choice to provide direct structural information on lowly populated conformational states and transient encounter complexes.

Despite its ability to provide unique structural information, for about 50 years the use of paramagnetic NMR had been restricted primarily to metalloproteins, where a paramagnetic center can be easily introduced by replacing the natural metal cation with a paramagnetic one [6,7]. PRE has become a routine tool in biomolecular NMR spectroscopy only in the last two decades after the introduction of straightforward biochemical methods for site-specific incorporation of paramagnetic labels in proteins and nucleic acids [8-10] and the development of a robust theoretical framework to account for the intrinsic flexibility of artificially introduced paramagnetic tags in back-calculation of the PRE from atomic-resolution structures [11,12]. Here, we describe in detail a protocol that takes advantage of the most recent technical advances in paramagnetic NMR spectroscopy to characterize transient intramolecular protein contacts (e.g. for proteins with a flexible hinge [13]). The notes describe modifications of the protocol necessary to detect transient intermolecular contacts.

2. Materials

Prepare all solutions using deionized water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Preparation of protein and labeling material

1. Protein stock solution: ~0.1 mM (for one NMR sample) of purified protein in 2 mL of any buffer in H_2O . The protein should be uniformly ^{15}N labeled (e.g. grown in M9 minimal media with $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source) (see Note ¹ regarding experiments to detect transient intermolecular contacts). Protein hydrogen atoms may be deuterated at carbon-attached positions for large proteins

¹This protocol describes the use of PRE NMR to observe transient intramolecular contacts. A parallel approach can be used to detect transient intermolecular contacts. The changes to the protocol are briefly described here for clarity. For intermolecular contacts, NMR samples constitute both an NMR silent (e.g. ^{14}N , natural abundance) / paramagnetically labeled species with an NMR visible (^{15}N isotopically labeled) species. Preparation of the paramagnetically labeled species is unchanged from this protocol. By positioning the label at several locations, intermolecular PREs can be used to define the surfaces mediating intermolecular contacts. However, quantitative interpretation of intermolecular PREs requires accurate positioning of the label on the protein surface, which is best accomplished by the intramolecular PRE approach described in this protocol (e.g. measurement of intramolecular PREs on a sample simultaneously and uniformly MTSL-labeled and ^{15}N -labeled using the protocol described in the **Methods** section is advised as a precondition for intermolecular PREs). Additional considerations concerning intermolecular PREs can be found in previous review articles [3,14].

to improve the quality of spectra. Store at 4 °C. The protein should contain a single surface cysteine for spin-labeling. We recommend purifying and storing the protein stock in a buffer containing a reducing agent (e.g. 2 mM DTT). Data from several single cysteine variants may be necessary to characterize structure. Care must be taken when selecting a labeling site that is close to the interface being interrogated to ensure that labeling with MTSL does not disrupt binding or create label-dependent binding. Comparing spectra of diamagnetically labeled protein (i.e. the MTSL-labeled protein with the unpaired electron reduced by ascorbate addition, or the protein labeled with a diamagnetic analogue of the paramagnetic tag) to the wild type protein (see below) can be used to ensure the site is appropriate.

2. MTSL labeling agent: (1-Oxyl-2,2,5,5-tetramethyl- 3-pyrroline-3-methyl) Methanethiosulfonate (Toronto Research Chemicals, O875000) (See Note ²). We typically use 10 mg vial size to have a fresh label stock. Stock solution: 100 mM solution in ethanol. Store at –20 °C (see Note ³).
3. Dithiothreitol (DTT) stock solution: 0.5 M in protein buffer (See Note ⁴). Store at –20 °C.
4. Desalting buffer: 20 mM Tris·HCl (pH 7.4 – pH 8.0) (see Note ⁵)
5. Ascorbate stock solution: 100 mM in NMR buffer, re-adjust pH after dissolution.

2.2 NMR sample preparation

1. NMR tube: wash a conventional NMR tube (do not use Shigemi – see Note ⁶) with concentrated nitric acid (see Note ⁷). Then rinse the tube with 200 mL of water. Completely dry the tube before usage.

²Several choices for spin label exist. The most flexible and best characterized is the stabilized nitroxide radical MTSL which attaches via a single disulfide bond. A variety of MTSL derivatives with substituent groups [20] and two disulfide bond sites [21] exist if a more rigid label is desired. An acetylated diamagnetic analogue of MTSL, (1-Acetoxy-2,2,5,5-tetramethyl-6-3-pyrroline-3-methyl) Methanesulfonate (Toronto Research Chemicals, A167880) is available to aid characterization of the diamagnetic state. If the hydrophobic character of the spin-label results in label-dependent contact formation or structural changes, a functionalized form of EDTA chelated with a paramagnetic metal ion such as Mn²⁺ provides a potential alternative [12]. In this case, a diamagnetic metal chelate (e.g. Ca²⁺ or Mg²⁺) can be used as a diamagnetic control. For proteins where a single surface free cysteine cannot be engineered, spin labels can also be attached at non-natural amino acids using bioorthogonal labeling [22] though special protocols must be used for labeling as well as protein expression in minimal media for isotopic labeling [23]. To determine if the combination of spin label and position are appropriate for the system, the NMR spectra, titration, or NMR dynamical observables of the diamagnetic form (with ascorbate or with the acetylated MTSL analogue) can be compared to the wild type protein.

³The stock of MTSL can be aliquoted and kept at –20 °C for a week or flash frozen and stored at –80 °C. Because MTSL is used in excess, slight precipitation of the compound in the stock is not of high concern.

⁴Any reducing agent can be chosen including TCEP. However, we prefer DTT to β-mercaptoethanol (βME) because DTT is a stronger reducing agent and βME can form adducts at cysteine positions. Although some studies report that TCEP will not interfere with disulfide bond formation by methanethiosulfonate labeling reagents, this has not been our experience.

⁵Spin-labeling can be performed in any sulfhydryl-free buffer including phosphate and Good's buffers. However, the spin-labeling reaction via disulfide linkage to a cysteine residue requires deprotonation of the cysteine side-chain and is faster at basic pH. Therefore, we recommend running the reaction at pH > 7.0. We have had good experience with pH 8.0.

⁶Plugs in Shigemi tubes are optimized to match the magnetic susceptibility of pure water. Paramagnetic centers substantially alter the magnetic susceptibility of the NMR sample. The use of Shigemi tubes with paramagnetic samples may render the shimming step challenging.

⁷NMR tubes – even the brand new ones – may contain trace paramagnetic heavy metal contamination. Rinsing the tubes with nitric acid followed by standard cleaning with water and ethanol ensures removal of these contaminants that may introduce unwanted paramagnetic ions in the sample.

2. NMR buffer: prepare 100 mL (or more) of the working NMR buffer (i.e. the buffer that results in the best quality ^1H - ^{15}N HSQC spectra of the protein of interest). If possible, we recommend to use a slightly acidic buffer (i.e. pH 6.5 instead of 7.4) (see Note ⁸). Do not add reducing agents to the NMR buffer (see Note ⁹). Purify the buffer from any residual ion using Chelex-100 (Sigma). We suggest filtering the NMR buffer with a vacuum filtration unit on whose membrane a thin layer of Chelex-100 has been applied. Rinse the Chelex-100 with 300 mL of water (twice) before filtering the NMR buffer.

2.3 NMR data acquisition

1. Pulse sequence: any pulse sequence for measuring ^1H transverse relaxation rate constants can be used to collect PRE data. We suggest the sequences used by Clore and coworkers [14].
2. Data processing: any processing software can be used for post-acquisition processing of the NMR spectra. Processing parameters are strongly dependent on the NMR pulse program.

2.4 Fitting structural models to PRE data

1. $^1\text{H}/^{15}\text{N}$ spectral assignments of protein of interest in the experimental conditions used to acquire the PRE data.
2. XPLOR-NIH software and processing scripts. XPLOR-NIH software is available at this site for academic download and use: <http://nmr.cit.nih.gov/xplor-nih> [15,16] Scripts for back calculation of PREs from 3D structural models, and for refinement of protein 3D structures against PRE data are available at <https://spin.niddk.nih.gov/clore/on> the Software page at the Spin Label Build link.

3. Methods

3.1 Site specific labeling reaction

1. Prepare the $^1\text{H}/^{15}\text{N}$ labeled protein for labeling by adding DTT to a final concentration of 10 mM to ensure any intermolecular disulfide bonds are reduced. To enable completion of the following two desalting steps without need to concentrate the sample, keep the protein sample volume to 15 mL or less. Incubate for at least 1 hour at room temperature or at 4°C overnight.

⁸Detection of PREs using $^1\text{H}_\text{N}$ positions is convenient due to the large gyromagnetic ratio of hydrogen and the excellent spectral dispersion of backbone amide positions, which results in single residue resolution. However, base-catalyzed exchange of H_N positions with water hydrogens is rapid at high temperatures and high pH. These water exchange events are therefore an additional relaxation mechanism for proton transverse magnetization, resulting in an increase the background $^1\text{H}_\text{N}$ - R_2 . In addition, slight changes in pH can have a large effect on rates at these conditions. Exchange with water may also cause a reduction in the intensity or, in the most extreme cases, the complete disappearance of NMR cross-peaks, especially at H_N positions that are exposed to the solvent and not involved in intramolecular hydrogen bonds. Therefore, where possible, we recommend to run PRE experiments at acidic pH (e.g. pH 6.5 or lower – the minimum exchange is at pH 3 to pH 4) and low temperature. We also recommend avoiding Tris buffer which has a high temperature dependence on pH. Predicted exchange rates for disordered regions lacking stable hydrogen bonds can be made using the program Sphere from the Roder group (<http://landing.foxchase.org/research/labs/roder/sphere/sphere.html>)

⁹There are commercially available spin-labels that bind the protein using non-reducible covalent linkers. Reducing agents should not be used in PRE experiments, even if using these class of spin-label compounds. Indeed, reducing agents can reduce the paramagnetic center and hence quench the PRE.

2. Equilibrate the HiPrep 26/10 desalting column with the labeling buffer (without reducing agent).
3. To remove the DTT, pass 7 mL (or less) of the protein/DTT mixture through the equilibrated HiPrep 26/10 Desalting column. Use a flow rate of 7 mL/minute. Collect the fractions containing the protein and discard all the fractions containing DTT. If a UV detector is connected to the chromatographic system in use, the protein fractions can be identified by inspecting the chromatogram. Otherwise, gel electrophoresis can be used to locate the protein fractions. It is important to completely separate the protein from DTT. DTT has a small absorbance at 280 nm and can be detected in the UV chromatogram. In any case, due to the small molecular size, DTT elutes from a 26/10 desalting column at volume > 25–30 mL. In order to facilitate the following steps of the labeling protocol, the recollected protein sample should be <15 mL.
4. Immediately add the dissolved MTSL label stock to the tube containing the desalted protein. The label should be added in 5× (or higher) excess to the protein and should have a final concentration 1 mM.
5. Incubate the reaction at room temperature in the dark for 2 hours.
6. During the reaction, equilibrate the desalting column with the experimental buffer (without reducing agent).
7. To end the reactions, pass the protein/MTSL mixture through the HiPrep 26/10 desalting column at 7 mL/minute. Collect all fractions containing the protein and discard later fractions containing the unreacted MTSL (see 3. above). Like in the case of DTT, MTSL has a weak UV absorbance at 280 nm. Therefore the MTSL peak can be detected if an UV detector is connected to the chromatographic apparatus. Note that small contamination from unreacted MTSL may be tolerated because it will be further eliminated during the buffer exchange step (see 8. below).
8. Concentrate and buffer exchange the protein into the NMR buffer of interest. We suggest using Amicon Ultra-15 centrifugal filter units (EMD Millipore) of the appropriate molecular weight cutoff.
9. To confirm labeling efficiency, total mass determined by LC/MS for a sample after labeling can be compared to a sample before labeling. MTSL addition at a cysteine position (forming the “R1” sidechain using the terminology of Hubbell and coworkers [9]) adds 184 Da.

2.4 NMR sample preparation

1. In a 1.6 mL tube, prepare a 500 µl sample of up to 200 µM (See Note ¹⁰) of the MTSL labeled protein in NMR buffer including up to 10% D₂O for NMR lock

¹⁰If the concentration of the spin labeled species is too high, so called “solvent PRE” [24] effects can dominate the observed paramagnetic relaxation enhancements. These PREs arise from random intermolecular collisions rather than physiologically relevant transient interactions. Typical advice is therefore to limit samples to 300 µM or less, though this value will be system dependent.

(See Note ¹¹ regarding estimating protein concentration). Transfer to a standard NMR tube.

2. Prepare a 500 μL sample with half of the concentration which will be used to check for the presence of intermolecular interactions.

2.5 NMR data collection

1. Record a two-dimensional, $^1\text{H}/^{15}\text{N}$ correlation spectrum (HSQC or TROSY) and compare the signal intensities (or signal to noise ratios) to unlabeled reference spectra. Confirm that no significant chemical shift differences are present.
2. Using the high concentration sample, set up a $^1\text{H}_\text{N}$ - R_2 NMR experiment. For quantitative interpretation of PRE data, we strongly recommend measurement of $^1\text{H}_\text{N}$ - R_2 using a two-time-point approach as opposed to attempting to measure PREs by quantifying intensity attenuations in two dimensional correlation data – see discussion in reference [14]. Adjust the length of the relaxation delay for the second time-point (T_b) such that T_b is $1.15/({}^1\text{H}_\text{N}$ - $R_{2,\text{dia}} + R_2)$, where R_2 is the PRE value that should be measured with greatest accuracy (see details in Iwahara et al. [14]). A value of 15 ms is common for anticipated R_2 values up to 50 s^{-1} . In practice, choice of T_b with 50% signal intensity compared to intensity at T_a , the reference delay (= 0 ms) time point, is a good starting place. It is recommended to check the lower concentration sample at this point to ensure that a significant concentration dependence of $^1\text{H}_\text{N}$ - $R_{2,\text{para}}$ is not already observed in the first increments, which would suggest contribution of intermolecular interactions.
3. Record the full $^1\text{H}_\text{N}$ - R_2 two-time-point experiment with sufficient signal to noise (typically using 32 scans or more) as described previously [14].
4. Record the full $^1\text{H}_\text{N}$ - R_2 two-time-point experiment for the sample at half concentration, if desired.
5. To make the diamagnetic reference sample, add concentrated ascorbate stock solution to a final concentration of 2 mM, incubate at room temperature for 1 hour to reduce the nitroxide radical.
6. Record the full $^1\text{H}_\text{N}$ - R_2 two-time-point experiment on the reduced sample with sufficient signal to noise. The delay T_b must be kept the same due to the contribution of $^3J_{\text{HN/HA}}$ scalar coupling to the evolution of the NMR signal. We recommend using the exact same experimental parameters, including number of transients, as for the paramagnetic sample.

Proteins which self-assemble or favorably interact with the paramagnetic tag will show intermolecular PREs at lower concentrations [25].

¹¹The labels are attached by a disulfide bond which adds a small contribution ($\sim 125\text{ M}^{-1}\text{cm}^{-1}$) to the absorbance at 280 nm, A_{280} . Delocalization of the radical in MTSL adds approximately $700\text{ M}^{-1}\text{cm}^{-1}$ at 280 nm (note the compound is yellow) based on our empirical tests. This additional absorbance can be significant contribution to A_{280} in proteins without tryptophan residues. In our experience, acetylated diamagnetic control labels are colorless and do not absorb at 280 nm, aside from the disulfide linker.

2.5 NMR data processing

1. Process the interleaved NMR relaxation spectra with the appropriate spectral parameters using nmrPipe [17] or another equivalent approach. The [series.com](https://spin.niddk.nih.gov/bax/software/NMRPipe/index.html) script of the nmrPipe distribution is a convenient approach (<https://spin.niddk.nih.gov/bax/software/NMRPipe/index.html>) for processing data from NMR relaxation experiments. Alternatively, any other software package that performs similar tasks may be used.
2. Transfer known assignments to measure peak intensities in the paramagnetic and diamagnetic sample data. The [ipap.com](https://www.ipap.com) script of the nmrPipe distribution is convenient for this process.
3. Measure the peak intensities in the two time point spectra for the paramagnetic and diamagnetic conditions.
4. Measure the standard deviation of the noise (σ), for example using the statistics function in nmrDraw.
5. Compute the values of Γ_2 , the paramagnetic relaxation enhancement values, at each H/N position from the intensities using Equation 5 in Iwahara et al. [14]

$$\Gamma_2 = R_{2,\text{para}} - R_{2,\text{dia}} = \frac{1}{T_b - T_a} \ln \frac{I_{\text{dia}}(T_b) I_{\text{para}}(T_a)}{I_{\text{dia}}(T_a) I_{\text{para}}(T_b)}$$

where $I_{\text{dia}}(T_a)$ and $I_{\text{para}}(T_a)$ are the peak intensities in the diamagnetic and paramagnetic spectra, respectively, at time point T_a , and $I_{\text{dia}}(T_b)$ and $I_{\text{para}}(T_b)$ are the peak intensities in the diamagnetic and paramagnetic spectra, respectively, at time point T_b .

6. Compute the values of $\sigma(\Gamma_2)$, the uncertainty or standard deviation of the paramagnetic relaxation enhancements, at each H/N position from the intensities using Equation 6 in Iwahara et al. [14]

$$\sigma(\Gamma_2) = \frac{1}{T_b - T_a} \sqrt{\left\{ \frac{\sigma_{\text{dia}}}{I_{\text{dia}}(T_a)} \right\}^2 \left\{ \frac{\sigma_{\text{dia}}}{I_{\text{dia}}(T_b)} \right\}^2 \left\{ \frac{\sigma_{\text{para}}}{I_{\text{para}}(T_a)} \right\}^2 \left\{ \frac{\sigma_{\text{para}}}{I_{\text{para}}(T_b)} \right\}^2}$$

where σ_{dia} and σ_{para} are the standard deviation of the noise measured for the diamagnetic and paramagnetic spectra, respectively (see point 3).

7. Verify that PREs are large for backbone H/N groups within 5–10 Å of the paramagnetic center. Also, remove unreliable PREs from the list before further analysis. A convenient approach to judge reliability of experimental PREs is to plot the experimental PREs versus $\log(I_{\text{dia}}(T_a)/I_{\text{para}}(T_a))$. A linear correlation is expected. Outliers must be removed at this point from the list of experimental PREs [3].

2.6 Structural fit of tag positions to intra-domain data

1. Create a list of residues within the same domain as the tag position (i.e. that are not expected to undergo backbone motion relative to the label site).
2. For these residues, create a file `fittag.tbl` specifying the Γ_2 and $\sigma(\Gamma_2)$ at each position using the following format, where `res id` is the residue number and the last two values are Γ_2 and $\sigma(\Gamma_2)$:

```
assign (name NS1) (resid 32 and name HN) 15.4939 2.3295
```

More details and an example data file are described in the Spin Label Build README file.

3. Using XPLOR-NIH, follow the procedure described in the Spin Label Build README file to: 1) set the protein rotational correlation time in `fit.py` and `calc.py`, 2) rename the tagged residue name to “CYSP” in the `pdb` file, 3) automatically generate a `psf` file and add any necessary atoms to the `pdb` file (`xplor -py buildtag.py`), and 4) build a 5 label conformer model (`xplor make5conf.inp` – see Note ¹²).
4. Edit the `fit.py` file to replace the single instance of `data.tbl` with your file name `fittag.tbl` from above (see 2. above).
5. Calculate a structural ensemble of the five tag positions that best reproduce the observed intra-domain PRE data, as described in the Spin Label Build README file (`xplor -py fit.py > fit.out`).

2.7 Calculate and analyze predicted PRE data for the entire structure

1. Calculate the PREs for all the backbone H/N groups using the reference `pdb` file including the optimized five-conformers representation of the paramagnetic tag (`xplor -py calc.py > calc.out`).
2. Extract the predicted PREs from the output file using the script `GrabPREs.awk` provided by the Spin Label Build package (`GrabPREs.awk > GrabPREs.out`).

¹²The optimal number of conformations for the spin-label depends on numerous experimental factors, such as the chosen paramagnetic tag, the labeling site, the temperature, the pH, and others. When determining the ensemble of label positions that best reproduce the experimental data, as a general rule we perform several optimization runs on simulated systems with increasing number of spin-label conformers (typically ranging from 1 to 5). For each system the agreement between experimental and back-calculated PREs is evaluated by χ^2 statistic or by the Q -factor [11,14], which is analogous to the crystallographic R -factor:

$$Q = \sqrt{\frac{\sum_i (\Gamma_2^{obs}(i) - \Gamma_2^{calc}(i))^2}{\sum_i \Gamma_2^{obs}(i)^2}}$$

In general, increasing the number of spin-label conformers results in a lower Q -factor, indicative of better agreement between experimental and back-calculated PREs. However, when the number of conformers is sufficient to fully account for spin-label flexibility, further increasing the ensemble size will not cause a significant reduction of Q -factor.

3. Graph the predicted (i.e. calculated) and observed (i.e. experimental) PREs vs residue number. Because the experimental values were used to optimize the spin label positions for back calculation, the predicted and observed PREs should match within experimental error for the intra-domain PREs (i.e. all the experimental PREs listed in the `fittag.tbl` file). For all other PREs, deviations of the experimental values from those predicted from the reference pdb file suggest transient protein conformational change. For example, higher than predicted PREs suggest transient approach of the tag to these positions.
4. Repeat the protocol for each tag position of interest to validate the observed transient conformational change. For example, given elevated PREs observed near region A when the label is placed in region B, a sample with the label placed in region B should show elevated PREs in region A, confirming that the system samples a closer approach of the two regions than is represented by the static structure in the reference pdb file.
5. The inter-domain PREs from several label positions can also be simultaneously used to generate a dynamic ensemble of structures quantitatively consistent with all experimental data (see the approach of Tang et al. [18]). This approach requires advanced use of XPLOR-NIH with scripts available on the software website. The most faithful representation of the solution structural ensemble can be achieved by combining PREs with structural restraints from complementary solution-phase techniques, such as small and wide angle x-ray scattering (SAXS/WAXS) data and NMR residual dipolar couplings [19].

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