

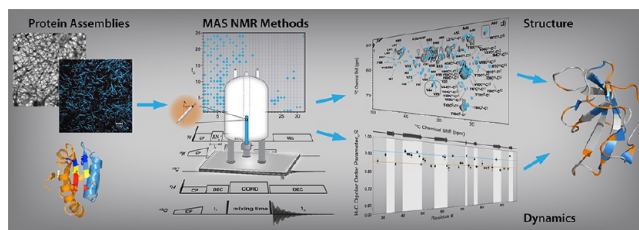
Probing Structure and Dynamics of Protein Assemblies by Magic Angle Spinning NMR Spectroscopy

SI YAN,[†] CHRISTOPHER L. SUITER,^{†,‡} GUANGJIN HOU,^{†,‡}
HUILAN ZHANG,[†] AND TATYANA POLENOVA^{*,†,‡}

[†]Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, United States, and [‡]Pittsburgh Center for HIV Protein Interactions, University of Pittsburgh School of Medicine, 1051 Biomedical Science Tower 3, 3501 Fifth Avenue, Pittsburgh, Pennsylvania 15261, United States

RECEIVED ON NOVEMBER 9, 2012

CONSPECTUS



In living organisms, biological molecules often organize into multicomponent complexes. Such assemblies consist of various proteins and carry out essential functions, ranging from cell division, transport, and energy transduction to catalysis, signaling, and viral infectivity. To understand the biological functions of these assemblies, in both healthy and disease states, researchers need to study their three-dimensional architecture and molecular dynamics. To date, the large size, the lack of inherent long-range order, and insolubility have made atomic resolution studies of many protein assemblies challenging or impractical using traditional structural biology methods such as X-ray diffraction and solution NMR spectroscopy. In the past 10 years, we have focused our work on the development and application of magic angle spinning solid-state NMR (MAS NMR) methods to characterize large protein assemblies at atomic-level resolution.

In this Account, we discuss the rapid progress in the field of MAS NMR spectroscopy, citing work from our laboratory and others on methodological developments that have facilitated the in-depth analysis of biologically important protein assemblies. We emphasize techniques that yield enhanced sensitivity and resolution, such as fast MAS (spinning frequencies of 40 kHz and above) and nonuniform sampling protocols for data acquisition and processing. We also discuss the experiments for gaining distance restraints and for recoupling anisotropic tensorial interactions under fast MAS conditions. We give an overview of sample preparation approaches when working with protein assemblies.

Following the overview of contemporary MAS NMR methods, we present case studies into the structure and dynamics of two classes of biological systems under investigation in our laboratory. We will first turn our attention to cytoskeletal microtubule motor proteins including mammalian dynactin and dynein light chain 8. We will then discuss protein assemblies from the HIV-1 retrovirus.

1. Introduction

The cell is a crowded environment. The most essential cellular functions are performed by assemblies of biological molecules (e.g., proteins, nucleic acids, and small biomolecules) working in concert. Such assemblies consisting of two or more interacting partners often undergo changes in conformation and/or molecular motions upon binding to

each other, and knowledge of their molecular architectures and mobility at atomic resolution is critical to our understanding of their biological functions in the healthy cell/organism and in the disease-associated states. While several structural biology techniques yield atomic-level structural information (X-ray diffraction and solution NMR spectroscopy), these methods are limited to systems that can be

crystallized or are soluble. Solid-state NMR (SSNMR) spectroscopy does not have limitations with respect to molecular size, solubility, and long-range order and is thus an excellent technique for studying large macromolecular assemblies.

A wide range of sample conditions amenable to SSNMR characterization and the uniquely high information content of SSNMR experiments make the method particularly attractive. Three-dimensional (3D) architectures, molecular motions on time scales ranging from picoseconds to seconds and longer, interactions between macromolecules, and a wide range of their binding partners including small molecules, can be inferred from the measurements, all with unprecedented site specificity and atomic-level resolution. Until recently, SSNMR studies of large biomolecules have been impaired by limited sensitivity and resolution. The rapid advances in all aspects of the technique, from hardware (e.g., high magnetic fields of 17.6 T and above, magic angle spinning (MAS) probes delivering rotation frequencies of 40–110 kHz, as well as the newest generation of digital radio frequency (rf) consoles), to novel pulse sequences and data acquisition and processing protocols, to advanced sample preparation approaches, have resulted in a surge of exciting investigations by multiple researchers into large macromolecular assemblies.

2. Recent Methodological Advances for MAS NMR Studies of Protein Assemblies

Contemporary SSNMR spectroscopy is a powerful tool for studying molecular structure and dynamics of protein assemblies at atomic resolution. In large biological systems, sensitivity and resolution are often a challenge, and many SSNMR laboratories including ours focused major efforts in development of sensitivity and resolution enhancement techniques. High magnetic fields (17.6 T and above) have proven necessary in studies of protein assemblies, to achieve the required sensitivity and resolution. Often, additional signal enhancement is needed, and it can be attained (without compromising the resolution) by nonuniform sampling (NUS),^{1–3} paramagnetic-relaxation-assisted condensed data collection (PACC),^{4,5} and a combination of these two methods (NUS-PACC).⁶

At high magnetic fields and in conjunction with MAS frequencies of 10–60 kHz now routinely available, multi-dimensional dipolar-based (through space) and scalar-based (through bond) correlation experiments remain the cornerstone of experimental protocols for resonance assignments and distance restraints in protein assemblies.⁷ Anisotropic tensorial interactions (such as chemical shift and dipolar)

contain indispensable structural and dynamics information, and to reintroduce those under MAS a variety of rf field pulse schemes have been developed recently.⁷ In this section, we review the current MAS NMR methods for studying protein assemblies that work under a wide range of spinning frequencies.

2.1. Through-Space Correlation Spectroscopy. Dipolar correlations are necessary for gaining distance restraints that form the basis for resonance assignments and 3D protein structure determination. In the past two decades, many approaches for dipolar correlation spectroscopy in solids have been developed that rely both on direct polarization transfer between heteronuclei (¹³C, ¹⁵N) and on proton-mediated or proton-driven polarization transfer (reviewed in ref 7). Most of these methods were established for MAS frequencies of 10–30 kHz. In the recent years, another promising approach has emerged- the application of fast MAS frequencies ($\omega_r = 40\text{--}110$ kHz) to enhance spectral resolution and sensitivity (for ¹H and, in some cases, heteronuclei detection).^{8–10} Under these conditions, the conventional spin diffusion experiments (such as DARR and PDS) and many of the sequences for dipolar and chemical shift recoupling do not work. Therefore, we pursued the development of methods that overcome the limitations of the existing techniques. We introduced a family of rotor-synchronized $R2_n^V$ symmetry sequences for spin diffusion (RDSD).⁸ The R_1^1 , R_2^1 , and R_2^2 recoupling efficiency at ω_r of 40 kHz is comparable with or better than that of DARR at ω_r of 10–20 kHz. One drawback of this approach is the dependence of the polarization transfer efficiency on the isotropic chemical shift difference, yielding efficient recoupling for only subsets of correlations in the spectra, which are different for each of the four $R2_n^V$ sequences.⁸ Subsequently, we have demonstrated that combined supercycled $R2_n^V$ -driven (CORD) sequences are the best suited for ¹³C–¹³C spin diffusion experiments under fast MAS as they yield efficient broadband polarization transfer and uniform distribution of cross peak intensities across the entire correlation spectrum¹¹ (see Figure 1). CORD sequences are useful for both resonance assignments and acquisition of long-range distance restraints for protein structure determination.

In protein assemblies, knowledge of structure and dynamics of intermolecular interfaces formed by the binding partners is also essential, and our laboratory employs differential isotopic labeling that permits to selectively illuminate through-interface contacts. Using 1–73(U–¹³C,¹⁵N)/74–108-(U–¹⁵N) *E. coli* thioredoxin reassembly, we introduced a family of 2D experiments to detect through-interface

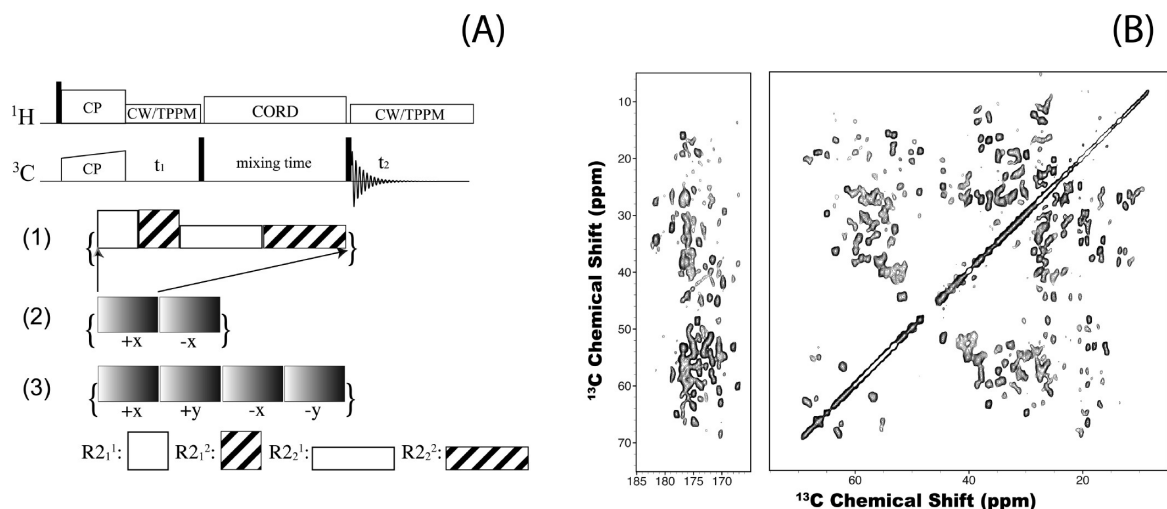


FIGURE 1. (A) 2D ^{13}C – ^{13}C CORD sequences: (1) basic CORD; (2) CORD_{XIX} ; (3) CORD_{XY4} . These irradiation schemes are composites of rotor-synchronized $\text{R}2_n^V$ -symmetry sequences, $\text{R}2_1^1$ ($\omega_{\text{rf}} = \omega_r$), $\text{R}2_1^2$ ($\omega_{\text{rf}} = \omega_r$), $\text{R}2_2^1$ ($\omega_{\text{rf}} = 1/2 \omega_r$), and $\text{R}2_2^2$ ($\omega_{\text{rf}} = 1/2 \omega_r$). Each $\text{R}2_n^V$ element consists of two π pulses per n rotor periods. (B) 2D ^{13}C – ^{13}C CORD_{XY4} spectra of $\text{U-}^{13}\text{C}, ^{15}\text{N}$ -LC8 ($\omega_r = 40$ kHz). Polarization transfer is uniformly efficient in both aliphatic and carbonyl regions of the spectrum.¹¹

heteronuclear $^{13}\text{C}/^{15}\text{N}$ or $^{13}\text{C}/^1\text{H}$ dipolar couplings.¹² These experiments are based on heteronuclear $^{13}\text{C}/^{15}\text{N}$ or $^{13}\text{C}/^1\text{H}$ dipolar dephasing of the signals belonging to the $\text{U-}^{13}\text{C}, ^{15}\text{N}$ -enriched molecule followed or preceded by either $^{15}\text{N-}^{13}\text{C}$ long-range magnetization transfer across the intermolecular interfaces or by $^1\text{H-}^{15}\text{N}$ or $^{15}\text{N-}^{15}\text{N}$ magnetization transfer within the ^{15}N -enriched molecule.

As illustrated in Figure 2, the $^{15}\text{N-}^{13}\text{C}$ REDOR-PAINCP experiment yields long-range $^{15}\text{N-}^{13}\text{C}$ correlations arising exclusively from the interfaces formed by the pair of differentially enriched complementary fragments of thioredoxin. In $^{15}\text{N-}^{15}\text{N}$ PDS-REDOR and $^1\text{H-}^{15}\text{N}$ HETCOR-REDOR experiments, correlations are observed corresponding solely to the 74–108($\text{U-}^{15}\text{N}$) thioredoxin fragment, while those associated with the 1–73($\text{U-}^{13}\text{C}, ^{15}\text{N}$) fragment are eliminated by $^{13}\text{C}/^{15}\text{N}$ REDOR filter.¹² The $^1\text{H-}(^{13}\text{C})\text{-}^{15}\text{N}$ REDOR-HETCOR experiment additionally highlights the residues situated at the interfaces between the two complementary fragments of reassembled thioredoxin.¹² This family of experiments is applicable to a broad range of macromolecular assemblies including (and particularly beneficial to) large systems.

2.2. Through-Bond Correlation Spectroscopy. Through-bond, scalar-coupling-driven correlation spectroscopy is another promising approach for structural investigations of protein assemblies.¹³ Scalar correlations offer information complementary to dipolar couplings. Scalar coupling-based experiments are less sensitive to the molecular motions than their dipolar-based counterparts. Several approaches exist for scalar-based spectroscopy in solids.^{14–17}

For resonance assignments, constant-time J -based spectroscopy yields outstanding resolution, which is essential when working with large proteins and protein assemblies.¹⁸ The sensitivity of these experiments is *on par* with or better than that in the dipolar-based transfers. J -based experiments are compatible with in-phase/antiphase (IPAP) selection in the direct dimension, yielding pure-phase spectra and further resolution enhancements.¹⁵ The performance of scalar-based polarization transfers is dramatically enhanced as MAS frequencies and decoupling field strengths are increased, and we anticipate that this approach will become widespread under fast-MAS conditions.

2.3. Recoupling of Anisotropic Spin Interactions. Magic angle spinning is essential for recording high-resolution SSNMR spectra. At the same time, MAS suppresses valuable information about molecular structure and dynamics, contained in the orientational dependence of the anisotropic spin interactions. Therefore, reintroduction of such interactions under MAS by suitable rf pulse sequences (termed recoupling) has been an active area of research since the 1990s.⁷ An elegant approach for the design of efficient recoupling sequences was established by Levitt and co-workers, who discovered symmetry theorems defining the relationships between the various degrees of freedom (spin, space, rotation, and radiofrequency),¹⁹ which in turn serve as a guide in selecting the desired terms of the Hamiltonian while discarding or minimizing the unwanted interactions. This approach has been used by multiple groups including ours for recoupling of chemical shift and dipolar interactions.

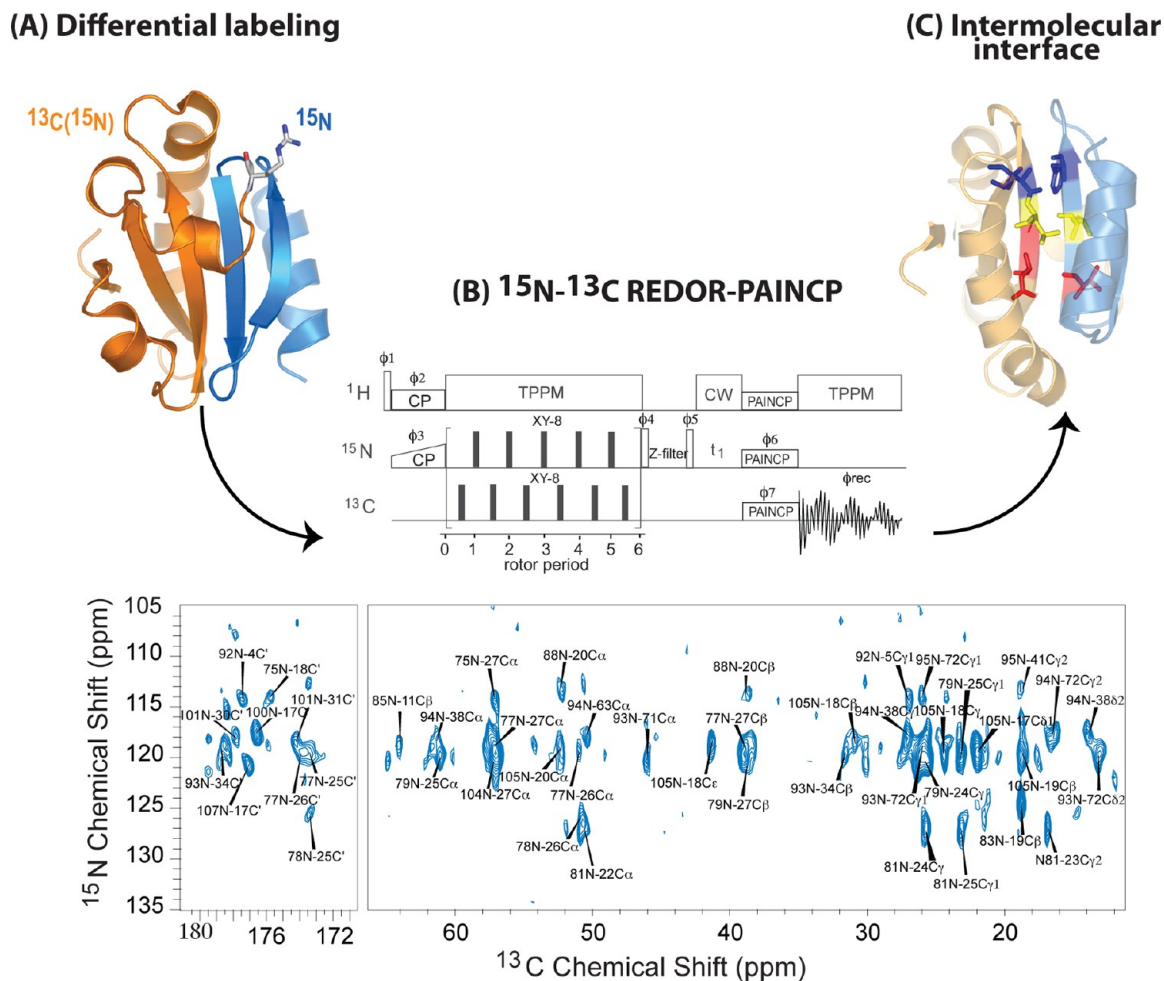


FIGURE 2. Experimental procedure for studying intermolecular interfaces in protein assemblies as illustrated with 1–73-(U- ^{13}C , ^{15}N)/74–108-(U- ^{15}N) thioredoxin reassembly. (A) Differential labeling: the N-terminal fragment of thioredoxin (residues 1–73) is U- ^{13}C , ^{15}N labeled; the C-terminal fragment (residues 74–108) contains U- ^{15}N labels. These two fragments assemble spontaneously in solution to form a noncovalent complex with the 3D structure of intact thioredoxin. (B) ^{15}N - ^{13}C REDOR-PAINCP sequence (top) and the corresponding 2D ^{15}N - ^{13}C correlation spectrum (bottom). The cross-peaks are correlations between residues comprising the intermolecular interface, as illustrated in (C). Adapted with permission from ref 12. Copyright 2008 American Chemical Society.

We explored the applications of RN_n^V -symmetry sequences for the recoupling of $^{13}\text{C}/^{15}\text{N}$ chemical shift^{20,21} and ^1H - $^{13}\text{C}(^{15}\text{N})$ dipolar^{22,23} anisotropies. In proteins, the CSA and dipolar tensors yield insights into structure and dynamics unavailable from the isotropic parameters. For the CSA recoupling, we have demonstrated that with the appropriately designed γ -encoded RN_n^V symmetry sequences either the first-rank or the second-rank spatial components of $^{13}\text{C}/^{15}\text{N}$ CSA interaction can be efficiently reintroduced.²⁰ These γ -encoded RNCSA symmetry schemes are suitable for CSA recoupling under a wide range of MAS frequencies, including 40 kHz and above, and perform well in both isotopically enriched and natural-abundance systems²⁰ (see Figure 3). RN_n^V -symmetry based CSA recoupling sequences are advantageous due to their tunability to

experimental conditions (rf field and MAS frequencies), tolerance to experimental imperfections (rf mis-set), and ability to recouple large CSA's (such as for carbonyl and aromatic carbons).

We have examined the performance of RN_n^V -symmetry sequences for the recoupling of heteronuclear (^1H - ^{13}C and ^1H - ^{15}N) dipolar interaction. As shown in Figure 4, these sequences display excellent selectivity for the recoupling of the first-order heteronuclear dipolar average Hamiltonian terms while the homonuclear dipolar Hamiltonian terms are suppressed.²² Most importantly, these experiments work very well under MAS frequencies of 40 kHz and above, and the high spectral resolution attained under these conditions makes them particularly well suited for studies of uniformly, extensively, and sparsely enriched proteins and

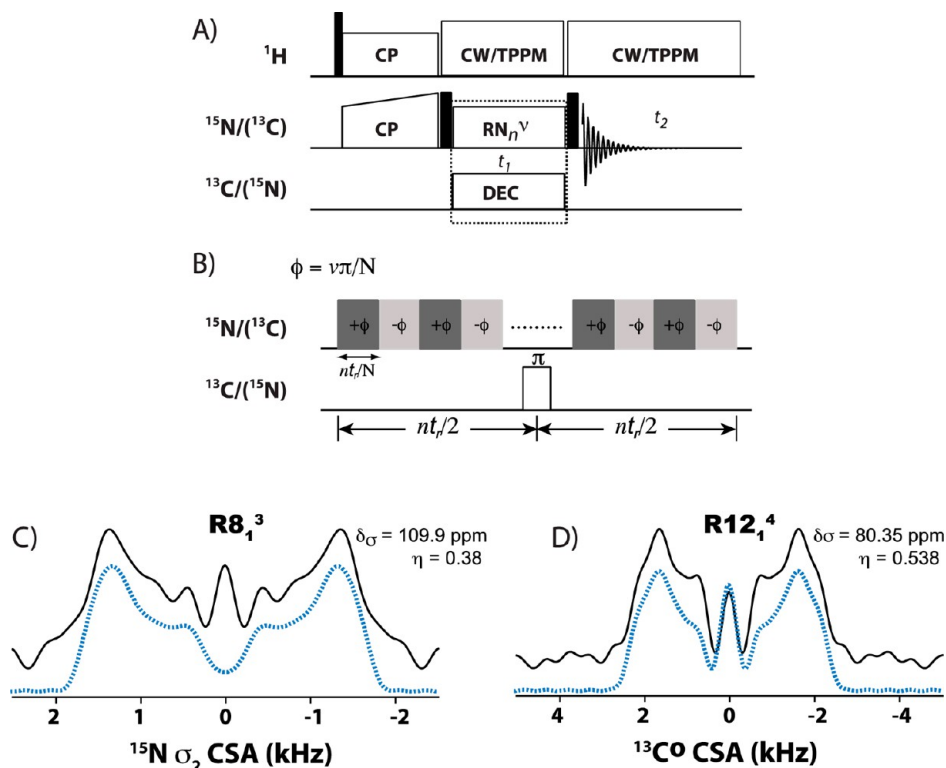


FIGURE 3. (A) General pulse scheme for R-symmetry-based recoupling of CSA interactions. (B) Rotor-synchronized RN_n^v -symmetry rf pulses are applied on heteronuclei during t_1 evolution time to reintroduce CSA interactions under MAS conditions. RN_n^v sequences consist of N π -pulses over n rotor periods; $\omega_{\text{rf}} = (N/2n) \cdot \omega_r$. (C) Experimental (black solid line) and simulated (blue dotted line) $^{15}\text{N} \sigma_1$ -CSA R8_1^3 lineshapes of Y169 residue in U- ^{13}C , ^{15}N -Tyr HIV-1 C-terminal domain (CTD) of CA protein. (D) Experimental (black solid line) and simulated (blue dotted line) $^{13}\text{C} \sigma_2$ -CSA R12_1^4 lineshapes of Y169 in U- ^{13}C , ^{15}N -Tyr HIV-1 CTD of CA protein. Adapted with permission from ref 20.

protein assemblies, as we have demonstrated for the CAP-Gly domain of mammalian dynactin²² (see Figure 4).

Finally, by correlating two anisotropic interactions, one can obtain the relative orientation between the two corresponding tensors, and this in turn informs on the bond and dihedral angles.⁷ We have demonstrated that using γ -encoded R-symmetry sequences in the context of a 3D experiment, where the combined CSA and heteronuclear dipolar recoupling are recorded, permits determination of the relative orientations between the ^1H – ^{15}N dipolar and ^{15}N CSA tensors.²¹ These experiments yield the relative tensor orientations for multiple sites and work well in U- ^{13}C , ^{15}N -labeled proteins and assemblies.

2.4. Nonuniform Sampling. We and others have explored the utility of nonuniform sampling for efficient data collection in multidimensional SSNMR spectroscopy.^{1,2} Rigorous analysis of the time-domain signals by Rovnyak et al. established that inherent gains of up to 2.5-fold are possible in each indirect dimension with appropriate NUS schedules, provided exponentially decaying signals,³ and that the resolution is maximized by sampling out to evolution times of $\pi \cdot T_2^*$. SSNMR can take particular advantage of these

sensitivity gains without compromising the resolution due to relatively fast spin–spin relaxation permitting sampling of data points to the required evolution times. Indeed, as we have demonstrated recently, sensitivity gains of 1.5–2.0-fold in each indirect dimension can be readily attained in multidimensional NUS-NMR experiments, compared to the uniformly sampled data sets acquired within the same experiment time.¹

To assess the inherent sensitivity gains of the frequency-domain signals in NUS experiments, we have introduced maximum entropy interpolation (MINT) that relies on the maximum entropy algorithm in the limit where the reconstructed spectra are tightly constrained to the raw data. This protocol ensures highly linear transformation between the time- and frequency-domain signals allowing for the direct comparison of spectral features with those in the data sets acquired by uniform sampling and processed by fast Fourier transform (FFT).¹

In Figure 5, we illustrate the application of NUS/MINT to 1–73-(U- ^{13}C , ^{15}N)/74–108-(U- ^{15}N) thioredoxin reassembly. The sensitivity enhancements from NUS are illustrated in the one-dimensional traces of the pair of 2D NCACX

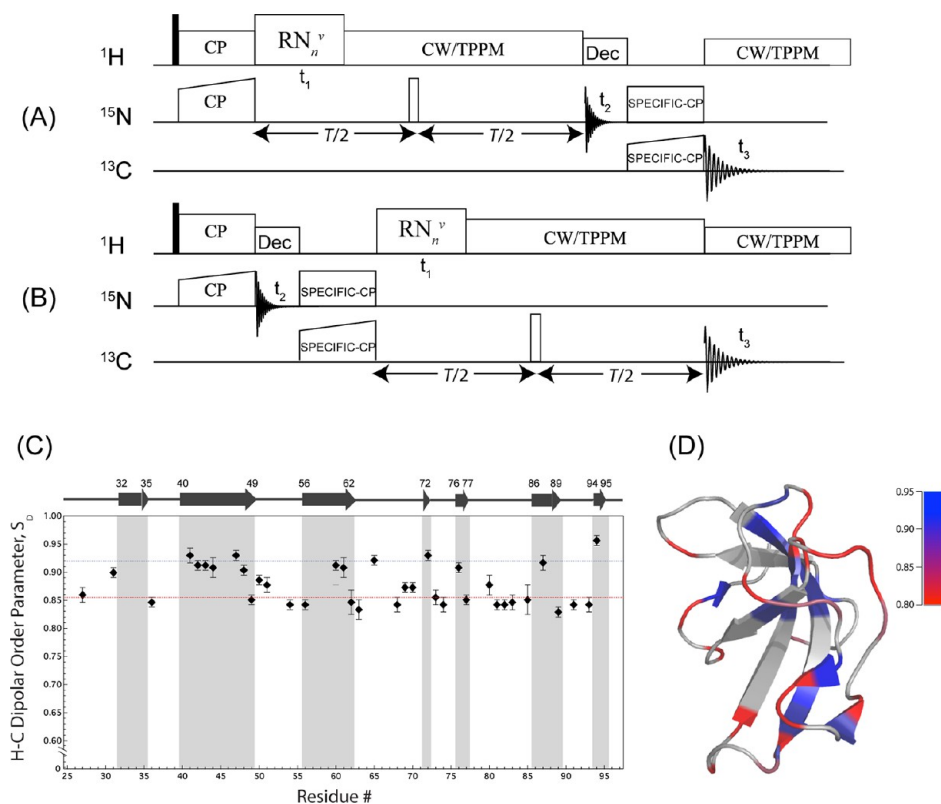


FIGURE 4. (A, B) Three-dimensional DIPSHIFT sequences for ^1H - ^{15}N and ^1H - ^{13}C heteronuclear dipolar recoupling experiments under MAS frequencies of 40 kHz and above. (C) Experimental H-C α dipolar order parameters S_D (R16 $_3$ -based DIPSHIFT) plotted as a function of the residue number for the sparsely- ^{13}C / ^{15}N enriched CAP-Gly. The blue and red dotted lines correspond to S_D of 0.919 and 0.854, which are the average values for the residues comprising β -sheets and loops/turns, respectively. (D) The nanosecond-to-microsecond time scale backbone dynamics of CAP-Gly mapped onto its tertiary structure, as captured by S_D . Blue, $S_D = 0.95$; red, $S_D = 0.80$; the values between these limiting order parameters were interpolated continuously. Reproduced with permission from ref 22. Copyright 2011 American Chemical Society.

spectra (NUS and US) acquired with the same experiment time. A 3D NUS/MINT NCACX spectrum is also shown. The experiment time was 4 days, and the corresponding US/FFT spectrum would require 16 days of experiment time on this sample and would be out of reach for less sensitive samples. NUS/MINT appears to be a promising venue for SSNMR studies of large protein assemblies.

2.5. Sample Preparation and Characterization. Sample preparation is an important practical aspect in studies of large biomolecular assemblies. While SSNMR spectroscopy does not have inherent limitations with respect to molecular size or sample conditions (long-range order, crystallinity, solubility, etc.), preparation of high-quality samples is the most essential determinant of their successful characterization.

Generating pure isotopically labeled protein in amounts sufficient for SSNMR is often a challenge, but rapid progress has been made recently in recombinant protein expression (using *E. coli* or mammalian or insect cells) as well as cell-free protein production.²⁴ While uniform labeling with ^{13}C and ^{15}N remains the most ubiquitous and information-rich

protocol, other schemes have emerged as well, such as sparse and amino-acid-specific labeling. The various labeling protocols for biomolecular SSNMR have been reviewed recently.²⁵

We have employed differential labeling to study interfaces formed by interacting proteins in protein assemblies.^{12,26,27} We have demonstrated that using a pair of protein molecules to prepare an assembly, one U- ^{13}C , ^{15}N and another U- ^{15}N , one can gain detailed structural information on the U- ^{13}C , ^{15}N molecule and at the same time define the intermolecular interface by examining through-interface correlations in specially designed experiments (see section 2.1 and Figure 2). These experiments were established on a 108-residue *E. coli* thioredoxin reassembly formed by reconstitution of the complementary 1-73/74-108 fragments; these assemble spontaneously to form native thioredoxin structure. Using this procedure, we have generated a pair of thioredoxin reassemblies, 1-73(U- ^{15}N)/74-108(U- ^{13}C , ^{15}N) and 1-73(U- ^{13}C , ^{15}N)/74-108(U- ^{15}N).^{12,23,26,27}

Once the suitable isotopic labeling protocols are identified, and the protein or protein assembly of interest is

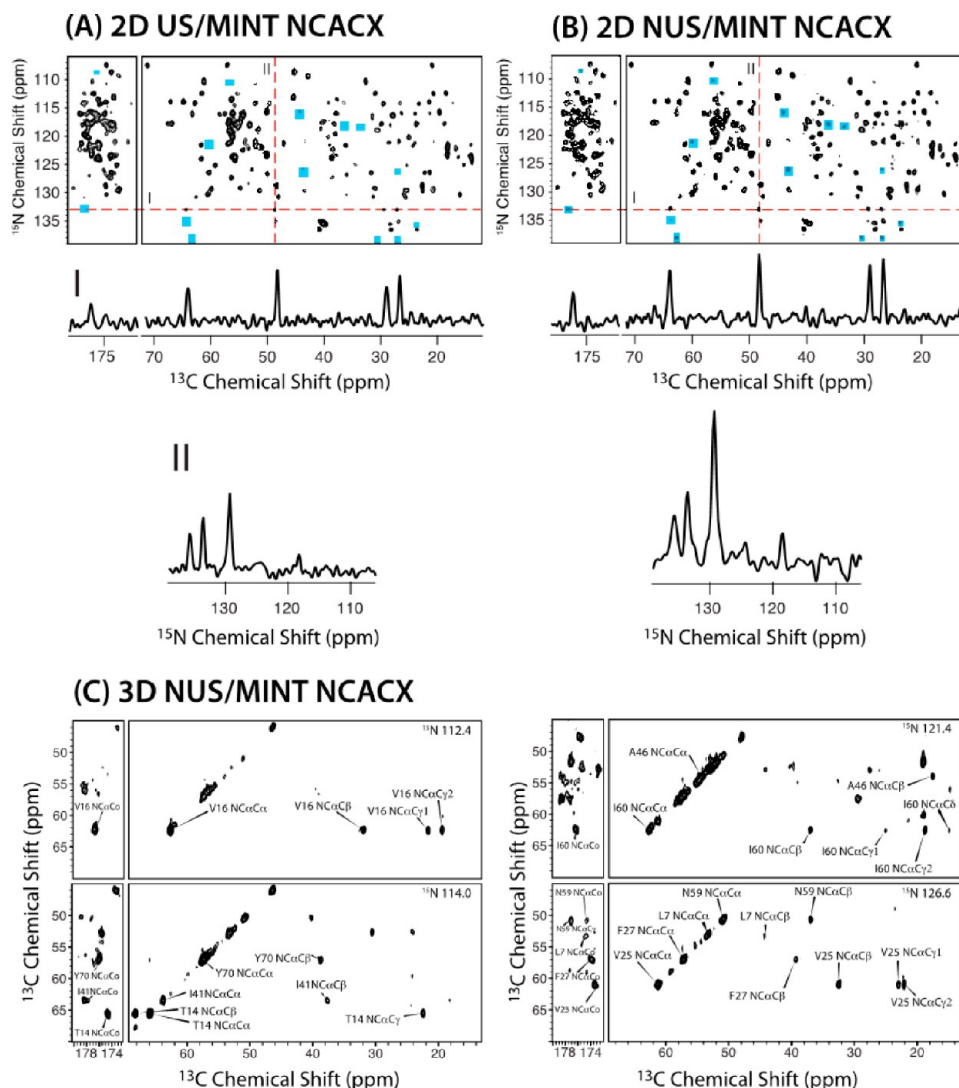


FIGURE 5. 2D NCA CX spectra of 1–73-(U- ^{13}C , ^{15}N)/74–108-(U- ^{15}N) thioredoxin reassembly: (A) US/MINT and (B) NUS/MINT. I and II: lineshapes extracted from the direct and indirect dimensions. Significant increase in sensitivity is observed for the NUS data set. New peaks in NUS spectra are indicated with blue boxes. (C) Representative 2D planes from a 3D NCA CX NUS/MINT experiment. The indirect-dimensions data points were sampled nonuniformly (25% points collected), and the experiment time is 4 days. The corresponding US experiment would be time prohibitive. Adapted with permission from ref 1. Copyright 2012 American Chemical Society.

purified, functional assays need to be conducted and/or sample morphology needs to be examined. In our work with microtubule-associated proteins and HIV-1 CA assemblies, we use a number of biophysical techniques to ensure correct sample morphology and high conformational homogeneity.^{28–31} Among other methods, we employ transmission electron microscopy (TEM), cryo-scanning electron microscopy (cryoSEM), and confocal microscopy to confirm that desired sample morphology is formed. It is beneficial to perform the morphology characterization both before and after the MAS experiments, to ensure that the samples are intact under magic angle spinning.³⁰

2.6. Emerging Techniques. With the ever-increasing size and complexity of biological systems under investigation, sensitivity and resolution remain the two cornerstones of biological SSNMR. In addition to the approaches outlined above which are based on high magnetic fields, fast MAS frequencies, and nonuniform sampling, there are several recent promising developments that lie at the intersection of two or more fields and deserve attention.

One example is the FROSTY protocol³² and its subsequent application to MAS NMR of sedimented proteins.³³ These methods are based on transient protein sedimentation from solution to the MAS rotor walls upon spinning and permit characterization of large-size proteins and protein

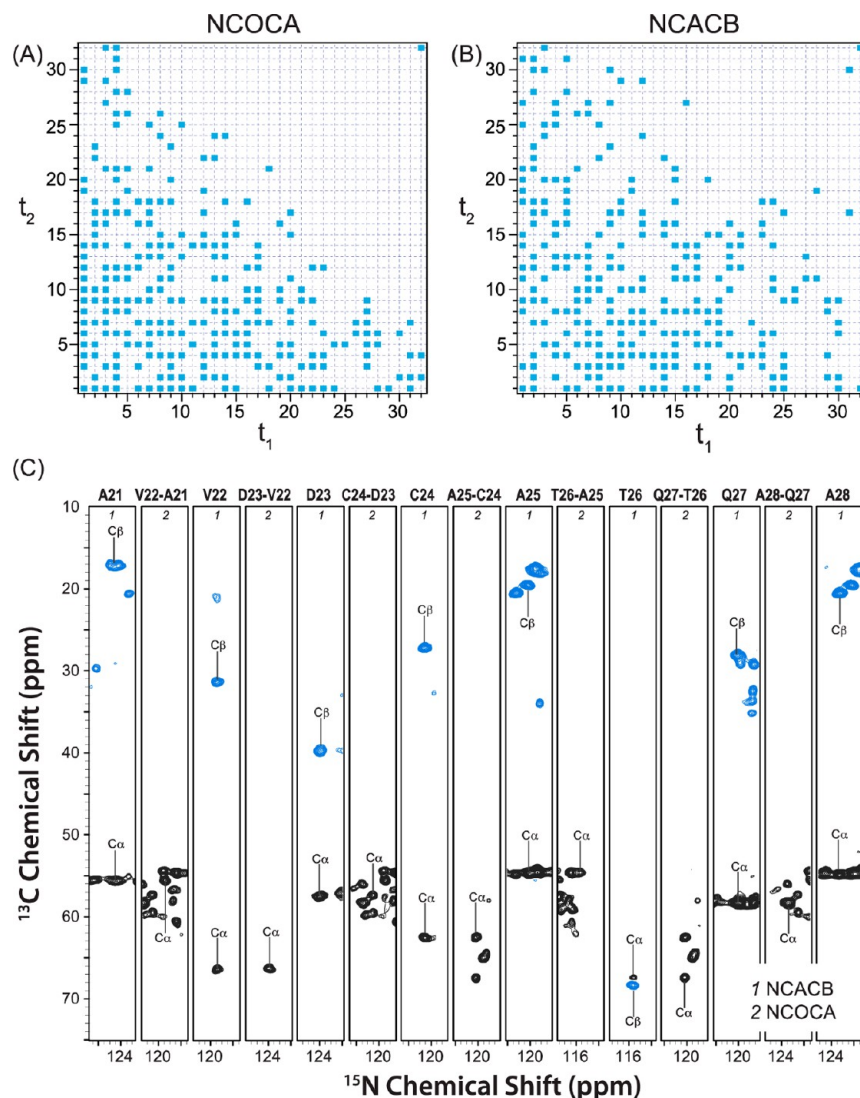


FIGURE 6. (A, B) Sampling schedule for NUS-PACC 3D (A) NCOCA and (B) NCACB experiments conducted on the $\text{U-}^{13}\text{C},^{15}\text{N}$ -LC8 protein doped with 5 mM Cu(II)-EDTA . (C) Backbone walk for the A21 – A28 stretch of residues in LC8 using three-dimensional NUS-PACC NCACB and NCOCA experiments. The total experiment times are 27 and 9 h for the NCACB and NCOCA experiments, respectively. Reproduced with permission from ref 6. Copyright 2012 American Chemical Society.

complexes without the need to precipitate them out of solution. Another direction is the use of paramagnetic probes³⁴ for assessing long-range distance information in proteins (20–30 Å), through paramagnetic relaxation enhancements³⁵ and paramagnetic pseudocontact shifts,³⁶ and speeding up SSNMR data collection by taking advantage of dramatically decreased spin–lattice relaxation times in the presence of paramagnetic dopant under fast-MAS (PACC).^{4,5} We have recently demonstrated that combining PACC with NUS yields further dramatic time savings in multidimensional NMR experiments permitting data collection in a fraction of time it takes to acquire a conventional data set,⁶ as illustrated in Figure 6.

3. Structure and Dynamics of Protein Assemblies by SSNMR: Case Studies

From the above discussion, the reader can appreciate the power and potential that contemporary SSNMR offers for atomic-resolution characterization of large biologically important protein assemblies. In this section, we provide an account of the recent studies where SSNMR offered unique insights into the structural biology of two classes of systems, cytoskeleton-associated and HIV-1 protein assemblies.

3.1. Cytoskeleton-Associated Protein Assemblies. Cytoskeletal structures and their associated proteins play fundamental roles in eukaryotic cells.³⁷ However, the interactions between the cytoskeletal structures (microtubules, actin, and intermediate filaments) and their associated

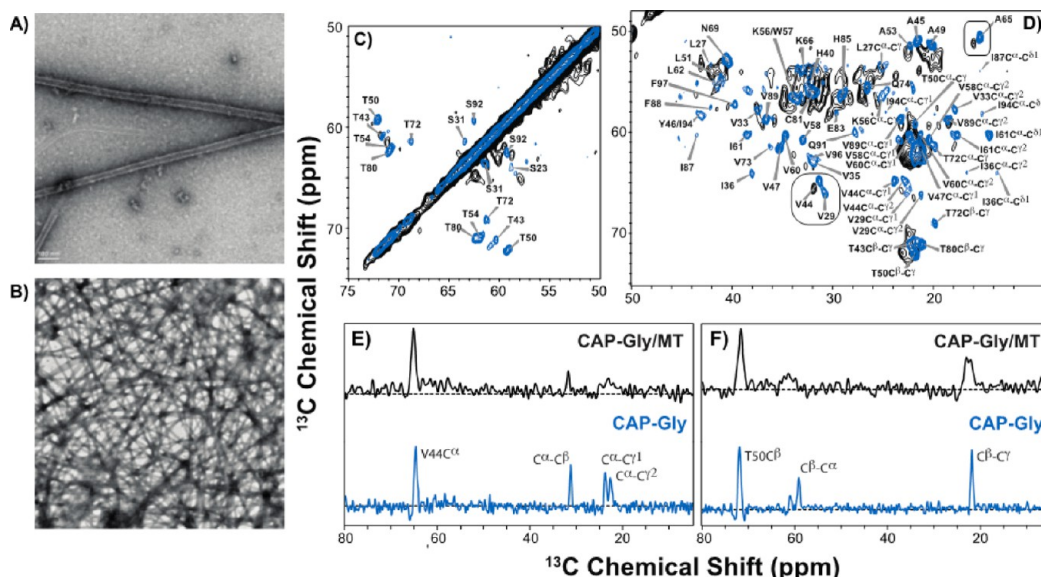


FIGURE 7. (A, B) Negatively stained TEM images of CAP-Gly/microtubule complex at different magnification levels. (C, D) 21.1 T DARR spectra of CAP-Gly/MT complex (black) overlaid with the spectra of the free CAP-Gly (blue): expansions of the aliphatic regions illustrating the chemical shift differences in CAP-Gly upon binding to microtubules. (E, F) Representative 1D traces drawn through the aliphatic regions of DARR spectra and illustrating the cross peaks for V44 (E) and T50 (F). Blue, free CAP-Gly; black, CAP-Gly/MT complex. Panels (C)–(F) are adapted with permission from ref 29. Copyright 2009 American Chemical Society.

proteins still remain poorly understood at the atomic level, due to the insolubility and lack of long-range order of cytoskeleton assemblies.

The focus of our laboratory is investigation of microtubule-associated motor proteins, dynein, dynactin, and kinesin, and their assemblies with microtubules by MAS NMR spectroscopy. Microtubules (MTs) and microtubule-associated proteins (MAPs) perform essential physiological functions, including cell migration, mitosis, polarization and differentiation, and vesicle and organelle transport.³⁸ The malfunctions of MAPs are associated with neurological disorders.³⁹ Understanding of the disease pathology requires atomic-level knowledge of the structure and interactions between MAPs and MTs.

We investigated the CAP-Gly microtubule-binding domain of the mammalian dynactin in its healthy state and in pathogenic (Perry's Syndrome associated) mutations, by a combination of SSNMR, solution NMR, and biophysical techniques.^{22,29,40} We discovered that in the free state, CAP-Gly displays unusual dynamic properties associated with the high loop content, as illustrated in Figure 4.²² In the Perry's Syndrome mutants, CAP-Gly exhibits reduced stability and a significant number of chemical shift perturbations while being well folded. Most strikingly and contrary to the original hypotheses in the literature, the binding affinity to the MTs is not decreased in the Perry's mutants with respect to the wild type protein; at the same time, binding to

an MT plus-end tracking protein EB1 is abrogated.⁴⁰ In line with these observations is our discovery that, when assembled on the microtubules, CAP-Gly exhibits multiple chemical shift perturbations, indicating that its conformation changes considerably upon binding.²⁹ The results from these studies are summarized in Figure 7.

We are studying another microtubule-associated protein, dynein light chain 8, LC8.^{41,42} LC8, an 89-residue protein, is an integral subunit of cytoplasmic dynein, a multisubunit microtubule-based retrograde motor. It is found in many other protein complexes, and, through its interactions with diverse binding partners, LC8 has a variety of dynein-dependent and dynein-independent functions. Using a combination of NMR spectroscopy, X-ray diffraction, and other biophysical techniques, the binding interface between LC8 and PAK1 peptide was characterized and a novel sequence identified that expands the LC8 interaction repertoire.⁴² We have assigned the resonances of LC8 in the solid state,⁴¹ and demonstrated using LC8 that NUS-PACC approach yields dramatic time savings that will enable rapid data collection in LC8 assemblies with its binding partners and in a wide variety of protein assemblies,⁶ as shown in Figure 6.

3.2. HIV-1 and Other Viruses. Viruses and assemblies of viral proteins and nucleic acids are of enormous biological importance and interest. Two systems have been under intense scrutiny during the past several years: the major coat protein of intact Pf1 filamentous phage whose structure

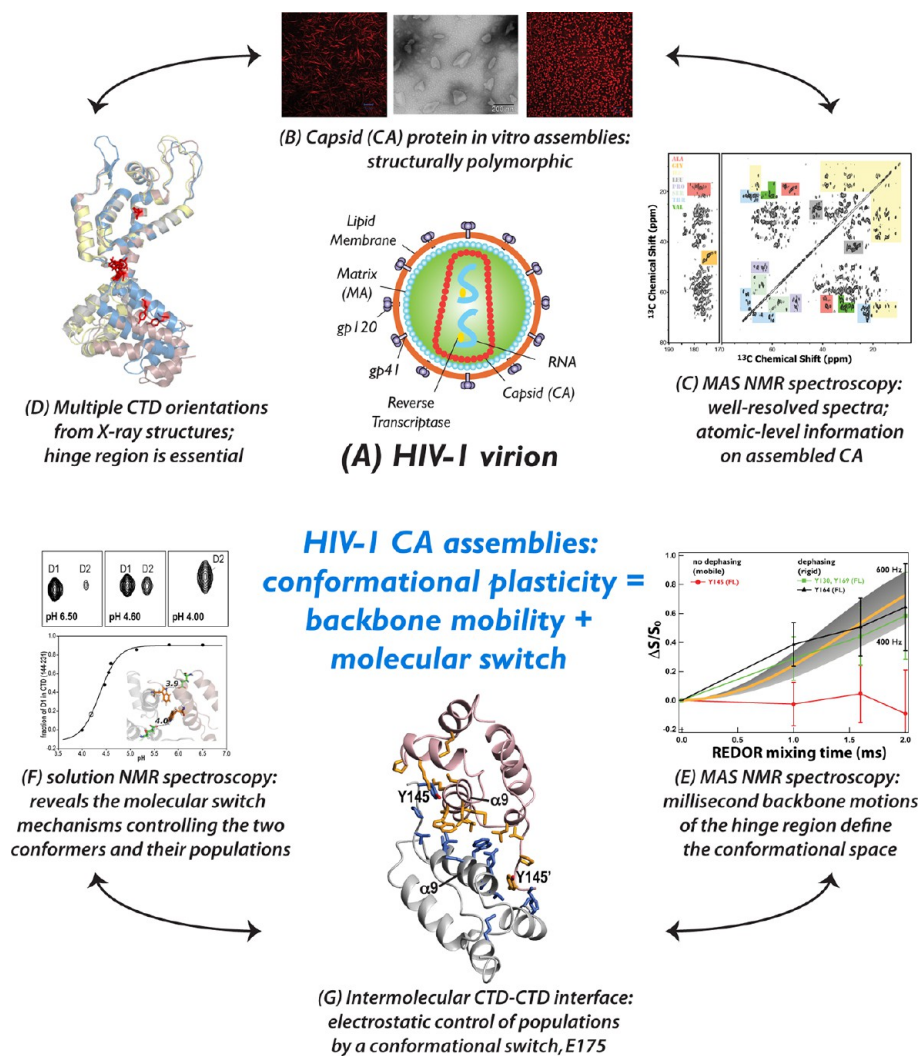


FIGURE 8. Summary of structural and dynamics studies HIV-1 CA assemblies of conical morphologies. (A) Schematic representation of the HIV-1 virion. (B) Morphologies of the in vitro assemblies of HIV-1 CA protein: tubes (left, confocal), cones (center, negatively stained TEM), and spheres (right, confocal). (C) 21.1 T DARR spectra of conical assemblies of U- ^{13}C , ^{15}N CA. (D) Superposition of several X-ray structures of unassembled CA revealing the different orientations of the C-terminal domain (CTD). (E) ^{13}C - ^{15}N REDOR dephasing curves reveal millisecond time scale motions in the hinge region (Y145) connecting the N- and C-terminal domains of CA, as probed through the tyrosine residues in U- ^{13}C , ^{15}N -Tyr-CA assembled into cones. These millisecond motions provide the conformational flexibility required for the assembly of CA into hexamers and pentamers and into the capsid cone surface. (F) pH dependence of the W184 H_{61} resonances of the two predominant conformers probed through HSQC spectra of the CA CTD dimer. (G) The intermolecular CTD-CTD interface structure. The solution NMR results reveal that the actual number of conformers accessible for CA assembly into varied morphologies is finite, and their relative populations in solution appear associated with an electrostatic interaction between the W184 and E175 side chains. Parts of the figure are reproduced with permission from refs 30 and 31. Copyright 2010 and 2012 American Chemical Society.

and dynamics were examined by MAS NMR^{43,44} and the HIV-1 viral capsid.^{30,31,45}

Our group investigates structure and dynamics of HIV-1 CA protein assemblies.^{8,20,30,31} HIV-1 capsid is a fullerene conelike structure comprising 1500 copies of a 231-residue CA protein, which encloses the viral RNA and a small complement of viral proteins.⁴⁶ The early stages of the HIV-1 infection are accompanied by the capsid disassembly (uncoating), a tightly spatially and temporally controlled

process, to release its contents into the cytoplasm of the host cell for subsequent virus replication. Formation of the conical structure requires pleiomorphic capsid with pentameric and hexameric oligomers coexisting in the same assembly (for recent review, see ref 46). In vivo and in vitro, CA protein assembles into various morphologies, such as cones, spheres, and tubes. We examined the molecular mechanism of the polymorphism and structural plasticity responsible for the pleiomorphic capsid assemblies.

Assignment of two-thirds of the residues in the CA assemblies of conical morphology was possible from the high-quality 21.1 T SSNMR spectra, as illustrated in Figure 8.³⁰ Through the measurements of the ^{13}C – ^{15}N and ^1H – ^{15}N dipolar couplings in the U– ^{13}C , ^{15}N -Tyr-labeled CA conical assembly, we have identified the presence of slow, millisecond time scale motions in the flexible hinge region linking the N- and the C-terminal domains of CA, which open up the conformational space and permit formation of multiple, varied conformers, as shown in Figure 8.³¹ Solution NMR spectroscopy revealed a molecular switch mechanism controlling the populations of the two conformers (one major, one minor) that coexist in solution and are available for the capsid assembly into cones.³¹ Studies are under way to determine the full 3D structure and dynamics of the CA assemblies and assemblies of Gag maturation intermediates, and to elucidate their interactions with various HIV-1 inhibitors.

4. Conclusions and Future Outlook

Solid-state MAS NMR spectroscopy has emerged as an essential technique for structure and dynamics characterization of protein assemblies. We hope that we conveyed to the reader our excitement and the tremendous potential of the field to provide atomic-resolution information inaccessible from other structural biology and biophysics methods currently available to the research community. We anticipate that synergistic collaborations between SSNMR spectroscopists and scientists in the fields of cell biology and virology as well as the use of hybrid methodologies will open new opportunities and new systems leading to exciting discoveries into the biological functions and mechanisms of complex cellular machineries.

The various aspects of this work were supported by the National Science Foundation (NSF-CAREER CHE-0237612) and the National Institutes of Health (R01GM085396, P50GM082251 P30GM103519, and P30RR031160). The 21.1 T spectra were acquired at the Environmental Molecular Sciences Laboratory. We thank our co-workers and collaborators who have contributed to the studies presented here.

BIOGRAPHICAL INFORMATION

Si Yan received her B.S. degree from the University of Science and Technology of China in 2008. She is a Ph.D. candidate at the University of Delaware. Her research focuses on microtubule-associated protein assemblies.

Christopher L. Suiter received his B.A. degree from West Virginia University in 2009. He is a Ph.D. candidate at the University of Delaware. His research focuses on HIV-1 protein assemblies.

Guangjin Hou is an NMR Spectroscopist at the University of Delaware. He received his M.S. degree from Huazhong University of Science and Technology in 2004 and his Ph.D. degree from the Chinese Academy of Sciences in 2007. After the postdoctoral appointments at the Max-Planck Institute for Polymer Research (Mainz, Germany) and University of Delaware, he joined the staff at the University of Delaware in 2012. His research focuses on developments of SSNMR methods and on HIV-1 protein assemblies.

Huilan Zhang received her B.S. degree from Central China Normal University in 2001 and Ph.D. from Huazhong University of Science and Technology in 2007. After a postdoctoral appointment at Leibniz-Institute for Polymer Research (Dresden, Germany), she joined the University of Delaware as a volunteer scientist. Her research focuses on HIV-1 protein assemblies.

Tatyana Polenova is a Professor of Chemistry and Biochemistry at the University of Delaware. She received her undergraduate degree with honors from Moscow State University (Moscow, Russia) and Ph.D. at Columbia University. After a faculty position at the City University of New York, she joined the faculty at the University of Delaware in 2003. Professor Polenova's work focuses on structural biology and biophysics of cytoskeleton and HIV-1 protein assemblies and on SSNMR spectroscopy.

FOOTNOTES

*To whom correspondence should be addressed. E-mail: tpolenov@udel.edu. Telephone: 302-831-1968. Fax: 302-831-6335
The authors declare no competing financial interest.

REFERENCES

- Paramasivam, S.; Suiter, C. L.; Hou, G. J.; Sun, S. J.; Palmer, M.; Hoch, J. C.; Rovnyak, D.; Polenova, T. Enhanced Sensitivity by Nonuniform Sampling Enables Multidimensional MAS NMR Spectroscopy of Protein Assemblies. *J. Phys. Chem. B* **2012**, *116*, 7416–7427.
- Matsuki, Y.; Eddy, M. T.; Griffin, R. G.; Herzfeld, J. Rapid Three-Dimensional MAS NMR Spectroscopy at Critical Sensitivity. *Angew. Chem., Int. Ed.* **2010**, *49*, 9215–9218.
- Rovnyak, D.; Sarcone, M.; Jiang, Z. Sensitivity Enhancement for Maximally Resolved Two-Dimensional NMR by Nonuniform Sampling. *Magn. Reson. Chem.* **2011**, *49*, 483–491.
- Wickramasinghe, N. P.; Parthasarathy, S.; Jones, C. R.; Bhardwaj, C.; Long, F.; Kotecha, M.; Mehboob, S.; Fung, L. W. M.; Past, J.; Samoson, A.; Ishii, Y. Nanomole-Scale Protein Solid-State NMR by Breaking Intrinsic ^1H T₁ Boundaries. *Nat. Methods* **2009**, *6*, 215–218.
- Laage, S.; Sachleben, J. R.; Steuernagel, S.; Pierattelli, R.; Pintacuda, G.; Emsley, L. Fast Acquisition of Multi-Dimensional Spectra in Solid-State NMR Enabled by Ultra-Fast MAS. *J. Magn. Reson.* **2009**, *196*, 133–141.
- Sun, S.; Yan, S.; Guo, C.; Li, M.; Hoch, J. C.; Williams, J. C.; Polenova, T. A Timesaving Strategy for MAS NMR Spectroscopy by Combining Non-Uniform Sampling and Paramagnetic Relaxation Assisted Condensed Data Collection. *J. Phys. Chem. B* **2012**, *116*, 13585–13596.
- Solid-State NMR Studies of Biopolymers*; McDermott, A., Polenova, T., Eds.; John Wiley & Sons Ltd.: 2010; p 572.
- Hou, G. J.; Yan, S.; Sun, S. J.; Han, Y.; Byeon, I. J. L.; Ahn, J.; Concel, J.; Samoson, A.; Gronenborn, A. M.; Polenova, T. Spin Diffusion Driven by R-Symmetry Sequences: Applications to Homonuclear Correlation Spectroscopy in MAS NMR of Biological and Organic Solids. *J. Am. Chem. Soc.* **2011**, *133*, 3943–3953.
- Ernst, M.; Meier, M. A.; Tuherm, T.; Samoson, A.; Meier, B. H. Low-Power High-Resolution Solid-State NMR of Peptides and Proteins. *J. Am. Chem. Soc.* **2004**, *126*, 4764–4765.
- Zhou, D. H.; Shah, G.; Cosmos, M.; Mullen, C.; Sandoz, D.; Rienstra, C. M. Proton-Detected Solid-State NMR Spectroscopy of Fully Protonated Proteins at 40 kHz Magic-Angle Spinning. *J. Am. Chem. Soc.* **2007**, *129*, 11791–11801.

- 11 Hou, G. Broadband Homonuclear Correlation Spectroscopy Driven by Combined R2nv Sequences under Fast Magic Angle Spinning for NMR Structural Analysis of Organic and Biological Solids. In revision.
- 12 Yang, J.; Tasayco, M. L.; Polenova, T. Magic Angle Spinning NMR Experiments for Structural Studies of Differentially Enriched Protein Interfaces and Protein Assemblies. *J. Am. Chem. Soc.* **2008**, *130*, 5798–5807.
- 13 Lesage, A.; Auger, C.; Caldarelli, S.; Emsley, L. Determination of Through-Bond Carbon-Carbon Connectivities in Solid-State NMR Using the INADEQUATE Experiment. *J. Am. Chem. Soc.* **1997**, *119*, 7867–7868.
- 14 Chen, L.; Kaiser, J. M.; Polenova, T.; Yang, J.; Rienstra, C. M.; Mueller, L. J. Backbone Assignments in Solid-State Proteins Using J-Based 3D Heteronuclear Correlation Spectroscopy. *J. Am. Chem. Soc.* **2007**, *129*, 10650–10651.
- 15 Chen, L. L.; Kaiser, J. M.; Lai, J. F.; Polenova, T.; Yang, J.; Rienstra, C. M.; Mueller, L. J. J-based 2D Homonuclear and Heteronuclear Correlation in Solid-State Proteins. *Magn. Reson. Chem.* **2007**, *45*, S84–S92.
- 16 Tian, Y.; Chen, L. L.; Niks, D.; Kaiser, J. M.; Lai, J. F.; Rienstra, C. M.; Dunn, M. F.; Mueller, L. J. J-Based 3D Sidechain Correlation in Solid-State Proteins. *Phys. Chem. Chem. Phys.* **2009**, *11*, 7078–7086.
- 17 Bertini, I.; Emsley, L.; Felli, I. C.; Laage, S.; Lesage, A.; Lewandowski, J. R.; Marchetti, A.; Pierattelli, R.; Pintacuda, G. High-Resolution and Sensitivity Through-Bond Correlations in Ultra-Fast Magic Angle Spinning (MAS) Solid-State NMR. *Chem. Sci* **2011**, *2*, 345–348.
- 18 Chen, L. L.; Olsen, R. A.; Elliott, D. W.; Boettcher, J. M.; Zhou, D. H. H.; Rienstra, C. M.; Mueller, L. J. Constant-Time Through-Bond ^{13}C Correlation Spectroscopy for Assigning Protein Resonances with Solid-State NMR Spectroscopy. *J. Am. Chem. Soc.* **2006**, *128*, 9992–9993.
- 19 Levitt, M. H. Symmetry-Based Pulse Sequences in Magic-Angle Spinning Solid-State NMR. In *Encyclopedia of Magnetic Resonance*, 2007.
- 20 Hou, G.; Byeon, I. J.; Ahn, J.; Gronenborn, A. M.; Polenova, T. Recoupling of Chemical Shift Anisotropy by R-Symmetry Sequences in Magic Angle Spinning NMR Spectroscopy. *J. Chem. Phys.* **2012**, *137*, 134201.
- 21 Hou, G.; Paramasivam, S.; Byeon, I. J. L.; Gronenborn, A. M.; Polenova, T. Determination of Relative Tensor Orientations by Gamma-Encoded Chemical Shift Anisotropy/Heteronuclear Dipolar Coupling 3D NMR Spectroscopy in Biological Solids. *Phys. Chem. Chem. Phys.* **2010**, *12*, 14873–14883.
- 22 Hou, G. J.; Byeon, I. J. L.; Ahn, J.; Gronenborn, A. M.; Polenova, T. ^1H - ^{13}C / ^1H - ^{15}N Heteronuclear Dipolar Recoupling by R-Symmetry Sequences Under Fast Magic Angle Spinning for Dynamics Analysis of Biological and Organic Solids. *J. Am. Chem. Soc.* **2011**, *133*, 18646–18655.
- 23 Yang, J.; Tasayco, M. L.; Polenova, T. Dynamics of Reassembled Thioredoxin Studied by Magic Angle Spinning NMR: Snapshots from Different Time Scales. *J. Am. Chem. Soc.* **2009**, *131*, 13690–13702.
- 24 Atreya, H. S. *Isotope Labeling in Biomolecular NMR*; Springer Berlin Heidelberg: New York, 2012; Vol. 992.
- 25 Renault, M.; Pawsey, S.; Bos, M. P.; Koers, E. J.; Nand, D.; Tommassen-van Boxtel, R.; Rosay, M.; Tommassen, J.; Maas, W. E.; Baldus, M. Solid-State NMR Spectroscopy on Cellular Preparations Enhanced by Dynamic Nuclear Polarization. *Angew. Chem., Int. Ed.* **2012**, *51*, 2998–3001.
- 26 Marulanda, D.; Tasayco, M. L.; McDermott, A.; Cataldi, M.; Arriaran, V.; Polenova, T. Magic Angle Spinning Solid-State NMR Spectroscopy for Structural Studies of Protein Interfaces. Resonance Assignments of Differentially Enriched *Escherichia coli* Thioredoxin Reassembled by Fragment Complementation. *J. Am. Chem. Soc.* **2004**, *126*, 16608–16620.
- 27 Yang, J.; Paramasivam, S.; Marulanda, D.; Cataldi, M.; Tasayco, M. L.; Polenova, T. Magic Angle Spinning NMR Spectroscopy of Thioredoxin Reassemblies. *Magn. Reson. Chem.* **2007**, *45*, S73–S83.
- 28 Renault, M.; Culkemane, A.; Baldus, M. Solid-State NMR Spectroscopy on Complex Biomolecules. *Angew. Chem., Int. Ed.* **2010**, *49*, 8346–8357.
- 29 Sun, S. J.; Siglin, A.; Williams, J. C.; Polenova, T. Solid-State and Solution NMR Studies of the CAP-Gly Domain of Mammalian Dynactin and Its Interaction with Microtubules. *J. Am. Chem. Soc.* **2009**, *131*, 10113–10126.
- 30 Han, Y.; Ahn, J.; Concel, J.; Byeon, I. J. L.; Gronenborn, A. M.; Yang, J.; Polenova, T. Solid-State NMR Studies of HIV-1 Capsid Protein Assemblies. *J. Am. Chem. Soc.* **2010**, *132*, 1976–1987.
- 31 Byeon, I. J. L.; Hou, G. J.; Han, Y.; Suiter, C. L.; Ahn, J.; Jung, J.; Byeon, C. H.; Gronenborn, A. M.; Polenova, T. Motions on the Millisecond Time Scale and Multiple Conformations of HIV-1 Capsid Protein: Implications for Structural Polymorphism of CA Assemblies. *J. Am. Chem. Soc.* **2012**, *134*, 6455–6466.
- 32 Mainz, A.; Jehle, S.; van Rossum, B. J.; Oschkinat, H.; Reif, B. Large Protein Complexes with Extreme Rotational Correlation Times Investigated in Solution by Magic-Angle-Spinning NMR Spectroscopy. *J. Am. Chem. Soc.* **2009**, *131*, 15968–15969.
- 33 Bertini, I.; Luchinat, C.; Parigi, G.; Ravera, E.; Reif, B.; Turano, P. Solid-State NMR of Proteins Sedimented by Ultracentrifugation. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 10396–10399.
- 34 Jaroniec, C. P. Solid-State Nuclear Magnetic Resonance Structural Studies of Proteins Using Paramagnetic Probes. *Solid State Nucl. Magn. Reson.* **2012**, *43–44*, 1–13.
- 35 Nadaud, P. S.; Helmus, J. J.; Hofer, N.; Jaroniec, C. P. Long-Range Structural Restraints in Spin-Labeled Proteins Probed by Solid-State Nuclear Magnetic Resonance Spectroscopy. *J. Am. Chem. Soc.* **2007**, *129*, 7502–7503.
- 36 Knight, M. J.; Felli, I. C.; Pierattelli, R.; Bertini, I.; Emsley, L.; Herrmann, T.; Pintacuda, G. Rapid Measurement of Pseudocontact Shifts in Metalloproteins by Proton-Detected Solid-State NMR Spectroscopy. *J. Am. Chem. Soc.* **2012**, *134*, 14730–14733.
- 37 Fulton, A. *The Cytoskeleton: Cellular Architecture and Choreography*; Chapman and Hall: 1984.
- 38 Vale, R. D. The molecular motor toolbox for intracellular transport. *Cell* **2003**, *112*, 467–480.
- 39 Gerdes, J. M.; Katsanis, N. Small Molecule Intervention in Microtubule-Associated Human Disease. *Hum. Mol. Genet.* **2005**, *14*, R291–R300.
- 40 Ahmed, S.; Sun, S.; Siglin, A. E.; Polenova, T.; Williams, J. C. Disease-Associated Mutations in the p150(Glued) Subunit Destabilize the CAP-Gly Domain. *Biochemistry* **2010**, *49*, 5083–5085.
- 41 Sun, S. J.; Butterworth, A. H.; Paramasivam, S.; Yan, S.; Lightcap, C. M.; Williams, J. C.; Polenova, T. Resonance Assignments and Secondary Structure Analysis of Dynein Light Chain 8 by Magic-Angle Spinning NMR Spectroscopy. *Can. J. Chem.* **2011**, *89*, 909–918.
- 42 Lightcap, C. M.; Sun, S.; Lear, J. D.; Rodeck, U.; Polenova, T.; Williams, J. C. Biochemical and Structural Characterization of the Pak1-LC8 Interaction. *J. Biol. Chem.* **2008**, *283*, 27314–27324.
- 43 Goldbourt, A.; Gross, B. J.; Day, L. A.; McDermott, A. E. Filamentous Phage Studied by Magic-Angle Spinning NMR: Resonance Assignment and Secondary Structure of the Coat Protein in Pf1. *J. Am. Chem. Soc.* **2007**, *129*, 2338–2344.
- 44 Lorieau, J. L.; Day, L. A.; McDermott, A. E. Conformational Dynamics of an Intact Virus: Order Parameters for the Coat Protein of Pf1 Bacteriophage. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 10366–10371.
- 45 Chen, B.; Tycko, R. Structural and Dynamical Characterization of Tubular HIV-1 Capsid Protein Assemblies by Solid State Nuclear Magnetic Resonance and Electron Microscopy. *Protein Sci.* **2010**, *19*, 716–730.
- 46 Ganser-Pornillos, B. K.; Yeager, M.; Sundquist, W. I. The Structural Biology of HIV Assembly. *Curr. Opin. Struct. Biol.* **2008**, *18*, 203–217.