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Magic Angle Spinning NMR of Viruses

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Abstract

Viruses, relatively simple pathogens, are able to replicate in many living organisms and to adapt to various environments. Conventional atomic-resolution structural biology techniques, X-ray crystallography and solution NMR spectroscopy provided abundant information on the structures of individual proteins and nucleic acids comprising viruses; however, viral assemblies are not amenable to analysis by these techniques because of their large size, insolubility, and inherent lack of long-range order. In this article, we review the recent advances in magic angle spinning NMR spectroscopy that enabled atomic-resolution analysis of structure and dynamics of large viral systems and give examples of several exciting case studies.

Keywords

viruses; magic angle spinning; HIV-AIDS; CA capsid protein; bacteriophage; influenza

1. Introduction

Viruses are relatively simple pathogens that are intimately linked with all forms of life. They are adept at replicating in bacteria, archaea, protists, fungi, plants, and animals while adapting to a variety of environmental conditions, some of which are extremely abrasive [1–5]. Once a virus penetrates a host cell and releases its genetic material, it is wholly reliant on the infected host to carry out its viral life cycle. Viruses seize control of their host cell machinery to replicate and assemble new virus particles for release and propagation. This leads to infections that can have a significantly negative impact on the health of the host cell and eventually lead to cell death [6].

In contrast to the generally negative perception of viruses, they do possess many potentially beneficial applications [7, 8]. Although viruses and living beings share a close relationship, there are still many questions regarding their structure and function. There are a variety of different methods for studying viruses at different levels of spatial and temporal resolution, including: transmission electron microscopy (TEM) [9], cryo-electron microscopy (cryo-EM) [10], cryo-electron tomography (cryo-ET) [11], X-ray crystallography [12], solution-state NMR [13], atomic force microscopy (AFM) [14], mass spectrometry (MS) [15], circular dichroism (CD) [16], optical tweezers [17], molecular dynamics (MD) [18], and solid-state NMR [19–21]. Of these, only X-ray crystallography, solution-state NMR, and solid-state NMR yield atomic level resolution, with recent exciting advances in cryo-EM bringing the resolution close to atomic for that technique as well [22].

X-ray crystallography is limited to samples that can be crystallized. Solution NMR requires that samples be soluble and is limited to relatively small systems. In contrast, solid-state NMR (SSNMR) has no requirement with respect to long-range order, solubility, or the molecular weight of the system under analysis. SSNMR has therefore found an important role in the structural biology of viruses. The rich information content of the experiments, including insights into structure and dynamics as well as interactions with host cell factors and small-molecule inhibitors, coupled with a wide range of sample conditions amenable for characterization, and the high-interest in studying viral systems has led to many exciting studies on viruses.

One of the most powerful and commonly used SSNMR techniques for studying viral systems is magic angle spinning (MAS) [23, 24]. Under MAS conditions, high-resolution NMR spectra of viruses and their constituent macromolecules (proteins, nucleic acids, and their assemblies) can be collected, and site-specific resonance assignments can be attained for many such systems through 2D and 3D correlation experiments. With chemical shift assignments in hand, additional MAS experiments are performed to acquire the distance and orientational information necessary to build atomic-level three-dimensional structures, to gain insight into dynamic processes on a variety of timescales, and reveal interactions with lipids or other molecules that bind to the macromolecules constituting the virus. The recent advances in fast MAS technologies allowing for sample spinning at frequencies of 40 – 110 kHz have enabled atomic-resolution studies of challenging virus systems which would have been considered intractable only several years ago. Microscopic images of some viral systems that have been studied by MAS NMR are shown in Fig. 1.

In this article, we review the recent advances in SSNMR methodologies as applied to viral systems and give examples of several exciting case studies. We focus our attention mainly on systems which were studied by MAS NMR. We note that there have been beautiful studies conducted on viral systems using static SSNMR, these are beyond the scope of this review and have been discussed in depth elsewhere [1, 4, 25–53].

2. Recent Methodological Advances for MAS NMR Studies of Viruses

Sensitivity and resolution are significant challenges to the study of large biomolecules by MAS NMR. Advances in hardware, NMR methodology, and sample preparation are

continually improving the attainable sensitivity and resolution, permitting analysis of increasingly complex biological systems, including entire viruses and assemblies of their constituent molecules. Key breakthroughs have included the use of extensive and selective labeling including sample deuteration, magnetic fields of up to 1 GHz, development of MAS NMR coils with reduced electric-field penetration into the sample (“low-E”, “EFree, and “scroll coil”) [54–59], fast MAS enabling proton detection, dynamic nuclear polarization (DNP) sensitivity enhancements, as well as nonuniform sampling. In this section we will discuss recent methodological developments in biological MAS NMR and their application to viral systems.

2.1 Sample Preparation

One of the key steps in obtaining useful, interpretable, and relevant information about viruses and their constituent macromolecules and assemblies is the preparation of high-quality samples. In the subsequent discussion we will focus mainly on preparation of samples of proteins and protein assemblies. There are intense developments in the sample preparation of viral DNA and RNA systems [60–62], but these will not be addressed here in any detail.

Protein expression and purification as well as peptide synthesis techniques are crucial to the success of many structural biology methods, including MAS NMR. One key ingredient in the preparation of structurally (morphologically) and functionally relevant viral protein assemblies is to ensure their assembly and/or reconstitution in biologically relevant conditions. Another key consideration is the choice of isotopic labeling scheme to obtain the desired information content with the minimal number of samples. Various procedures have been implemented to prepare viral systems for MAS NMR analysis, and some examples are considered below.

Intact or Assembled Viral Systems: Several of the viral systems studied by MAS NMR are either intact viruses or assemblies of proteins that are prepared for MAS NMR by centrifugation, lyophilization, precipitation [19–21, 63–73], crystallization [74], or sedimentation [75]. Some of these systems are difficult or impossible to crystallize and have molecular weights that are not compatible with solution NMR.

Membrane Reconstitution: For membrane proteins and peptides, the choice of the lipid for reconstitution plays a major role in the physical properties of the system in question. The structure and dynamics of a given protein or peptide can differ dramatically depending on the choice of lipid [43, 44, 76–80].

Uniform $^{13}\text{C}/^{15}\text{N}$ Isotopic Labeling: Uniform $^{13}\text{C}/^{15}\text{N}$ isotopic labeling is the most common [81] labeling strategy for the MAS NMR study of viruses and viral proteins. ^{13}C and ^{15}N labels are incorporated into the growth media during protein expression as U- $^{13}\text{C}_6$ glucose and $^{15}\text{NH}_4\text{Cl}$ as the sole carbon and nitrogen sources, respectively. If synthetic peptides are investigated, ^{13}C , ^{15}N labeled amino acids could be used during the solid-state synthesis [51]; this approach is limited to relatively short peptides due to high costs. An alternative approach is an expression of U- ^{13}C , ^{15}N fusion protein in *E. coli*, followed by a

cleavage of the tag to release the short peptide of interest [81]. Uniform labeling is an attractive option as it provides the richest information content for a given sample. The major drawback is spectral overlap, particularly in large molecules, that may complicate or even preclude resonance assignments for a significant fraction of residues. Spectral overlap can be alleviated with the increased-dimensionality experiments (3D and 4D) albeit the experimental time increases dramatically. A common alternative is selective, extensive, and sparse amino acid labeling considered below.

Selective Amino Acid Labeling: Selective amino acid labeling is used both in recombinant protein expression [70, 82, 83] and in peptide synthesis [43, 44, 77, 84–86]. The choice of amino acid(s) to be incorporated into a system under analysis is dictated by the desired information content as well as by which amino acids can be incorporated recombinantly without scrambling. In a recent study from the authors' lab, tyrosine residues were used as a probe of internal dynamics in HIV-1 CA capsid protein assemblies [70]. ^{13}C , ^{15}N -Tyrosine was added to the growth media, which contained $^{14}\text{NH}_4\text{Cl}$ and $^{12}\text{C}_6$ -glucose, before induction by IPTG. In this particular example, CA protein contains only four tyrosine residues, which are located in α -helical regions of the N- and C-terminal domains (NTD and CTD, respectively) and in a hinge region connecting these domains [70]. Tyrosine therefore was an ideal probe of the hinge region dynamics occurring on micro- to millisecond timescales, with the three helical tyrosine residues serving as internal controls. In another example, Yu and Schaefer prepared a selectively L- $[\epsilon\text{-}^{15}\text{N}]$ lysine sample by adding L- $[\epsilon\text{-}^{15}\text{N}]$ lysine to modified M9 medium during growth for use in REDOR studies of bacteriophage T4 [83]. Morag, Abramov, and Goldbourt recently employed a similar concept where uniform ^{13}C , ^{15}N labels were selected for by the use of U- $^{13}\text{C}_6$ glucose and $^{15}\text{NH}_4\text{Cl}$ but unlabeled aromatic amino acids (Trp, Phe, Tyr) were also added to the growth media. This blocked the production of the aromatic amino acids, making them NMR silent, which allowed for the observation of DNA signals displaying chemical shifts in the region where the protein aromatic signals would have been present if labeled [64].

Even more versatility can be achieved by using solid-state peptide synthesis, where a judicious combination of unlabeled and ^{13}C , ^{15}N labeled amino acids can produce a labeling pattern that highlights a region of the sequence with high biological significance [87, 88]. The Hong lab has used this approach to label selectively different regions of the M2 transmembrane peptide from the Influenza A virus. Additionally, they have incorporated specialized labels to measure intermolecular distances, such as 4- ^{19}F -Phe probes [89].

Sparse and Extensive Labeling: The benefits of sparse and extensive labeling are two-fold. First, the reduction of labeled sites reduces the complexity of the spectra making interpretation easier. Second, resolution is improved in the remaining signals due to the removal of one-bond J couplings. Sparse/extensive labeling can be achieved in several different ways. Choosing $[1,3\text{-}^{13}\text{C}]$ - or $[2\text{-}^{13}\text{C}]$ -glycerol as the sole carbon source gives rise to distinct and characteristic labeling patterns for each type of label resulting from the metabolic pathways of amino acid production from glycerol in *E. coli*. Different characteristic patterns are obtained when $[1\text{-}^{13}\text{C}]$ or $[2\text{-}^{13}\text{C}]$ -glucose is used as the sole carbon source [72, 90]. Goldbourt, Day, and McDermott have explored the Entner-

Doudoroff (ED) pathway for sparse labeling in the Pf1 bacteriophage. Using 1-¹³C glucose as the sole carbon source, they prepared samples extensively labeled in the backbone carbonyl region while aliphatic and aromatic carbons are almost entirely suppressed [69].

Deuteration: Deuteration of proteins to varying degrees has been applied to several viral assemblies including Pf1 [91], AP205, and the M2 proton conduction channel [74, 75]. Perdeuteration improves resolution by diluting the ¹H spins, thus eliminating ¹H-¹H couplings [92, 93]. During the expression of perdeuterated proteins, ²H₂O is used in place of ¹H₂O, and [²H, ¹³C]-glucose is used as the sole carbon source. Once the protein is prepared, protons can be reintroduced at exchangeable sites by back exchange against buffers containing the desired ratio of H₂O/D₂O buffers [94–97].

2.2 Resonance Assignments

Resonance assignments are required for subsequent site-specific structural and dynamic analysis by NMR. For small peptides derived from viral proteins, resonance assignments can be achieved by 2D correlation experiments. For large viral proteins, 3D spectra are often required. As discussed above, isotopic dilution through amino-acid selective, sparse, or extensive labeling can be employed for spectral simplification and to facilitate resonance assignments [69, 70, 72]. For ¹³C-detected data sets, assignments of viral proteins follow a well-established protocol. 2D ¹³C-¹³C correlation spectra provide valuable information about amino acid types and side chain correlations, enabling spin-system assignments. Backbone walks can be achieved in viral proteins by combining three types of 3D experiments: NCACX, NCOCX, and CANCO [19–21, 69, 98]. Recently, Pintacuda and colleagues have reported a resonance assignment protocol based on proton-detected solid-state NMR at 60 kHz MAS, demonstrated on the *Acinetobacter phage* 205 (AP205) and the M2 protein of influenza A reconstituted in a lipid bilayer [74, 75]. The 3D (H)(CO)CA(CO)NH experiment with CO-CA-CO out-and-back scalar transfer provides inter-residue correlations, while the 3D (H)(CA)CB(CA)NH experiment with CA-CB-CA out-and-back scalar transfer provides intra-residue correlations. Additional experiments included (H)CONH, (H)CANH, (H)CO(CA)NH, and (H)(CA)CB(CACO)NH. Once the full sequence of six experiments was executed, the MATCH program [99] was used to perform residue-specific backbone assignments automatically. For AP205, one week of experiment time was necessary to collect the suite of six experiments, which resulted in assignments for 94 of the 130 (72%) total residues. For M2, two weeks of experiment time resulted in 44 assignments using MATCH.

Compared to proteins, nucleic acids have been less studied by MAS NMR. Currently, there are no well-developed general MAS NMR assignment protocols for nucleic acids. Corresponding signals in nucleotides usually have very similar chemical shifts, yielding highly congested spectra and making assignments difficult. Moreover, inter-nucleotide correlations are hard to obtain due to the phosphate linkage and consequently long ¹³C-¹³C/¹³C-¹⁵N distances. Despite these challenges, several reports have presented assignment approaches for single-stranded DNA in bacteriophages [64, 65, 67]. Resonance assignments of nucleic acids based on 2D ¹³C-¹³C correlation spectra usually start with nuclei whose chemical shifts are well resolved [100]. Subsequently, nucleotide spin systems

can be assigned based on the previously assigned peaks and their intra-nucleotide cross-peaks, similar to the approach used for side chain assignments for proteins. Assignments of inter-nucleotide correlations can then be inferred from unique cross-peaks [64].

2.3 High Magnetic Fields

The recent development of magnetic fields of 17.6 – 28.1 T has been critical for analysis of large biomolecular systems by MAS NMR, including viruses and assemblies of their constituent macromolecules. The work from the authors' group on HIV-1 CA protein assemblies has underscored the importance of high fields (17.6 – 21.1 T) to attain the requisite sensitivity and resolution [20, 21]. Pintacuda and co-workers have used magnetic fields of 23.5 T in conjunction with fast MAS and ^1H detection (discussed below) to study the measles virus (MeV) nucleocapsid, M2, and AP205 bacteriophage, and demonstrated that the combination of these three technologies produced outstanding-quality data with a fraction of sample required for conventional experiments [75, 101]. High magnetic fields are envisioned to be “a must” for atomic-resolution analysis of multicomponent assemblies of viral macromolecules and of intact viruses.

2.4 Fast Magic Angle Spinning

With the advent of probes capable of spinning at MAS frequencies of 40 – 110 kHz, developments of fast MAS experiments and their applications are rapidly gaining momentum as fast MAS offers dramatically enhanced sensitivity and resolution. With MAS frequencies of 40 kHz and above, ^1H detection is readily attainable due to the efficient suppression of ^1H - ^1H homonuclear dipolar couplings, resulting in sharp proton lines, particularly at MAS frequencies above 60 kHz. This also results in greatly improved sensitivity, especially in challenging systems such as viral assemblies and membrane proteins. ^1H detection can be performed both in fully protonated and perdeuterated samples [92, 95, 102, 103]. At MAS frequencies of 40–110 kHz, many of the canonical dipolar and chemical shift anisotropy (CSA) recoupling experiments fail. Therefore, much emphasis in the field has been placed on the development of effective dipolar and CSA recoupling schemes. Some of the contemporary techniques were reviewed by the authors recently [104].

2.4.1 Spin Diffusion Experiments—Work in the authors' laboratory employs homonuclear ^{13}C - ^{13}C R-symmetry based spin diffusion sequences for correlation spectroscopy, particularly at MAS frequencies of 40 kHz and above where conventional PDSD and DARR experiments fail [71]. In such cases, the R2-symmetry [105] and CORD [106] experiments were demonstrated to work efficiently in a broad range of systems, including HIV-1 CA protein assemblies. Most recently, a combined RFDR-CORD experiment was developed that exhibits superior performance to both RFDR and CORD methods at fast MAS (40–60 kHz), results in fully broadbanded ^{13}C - ^{13}C correlation spectra with high cross peak intensities, and yields long-range cross peaks with monotonous dependence of polarization transfer rates on distances [107]. This recent experiment is anticipated to be advantageous for structure determination of viral assemblies.

2.4.2 Dipolar Recoupling Experiments—At fast MAS frequencies (40 kHz and above), ^1H - ^{13}C and ^1H - ^{15}N heteronuclear recoupling is very efficient by R-symmetry sequences [105]. $\text{R}16_3^2$ -symmetry based ^1H - ^{13}C and ^1H - ^{15}N DIPSHIFT experiments were demonstrated in fully protonated conical assemblies of U- ^{13}C , ^{15}N tyrosine labeled HIV-1 CA protein assemblies. These experiments revealed that the tyrosine residues in CA are rigid on the timescales of nano- to microseconds, including the hinge residue Y145, which undergoes motions on much slower, micro- to millisecond timescales [70]. Most recently, an improved R-symmetry based experiment dubbed PARS was developed, where the accuracy of the measurements of heteronuclear dipolar couplings is greatly improved compared to the conventional DIPSHIFT [108]. This is accomplished by supercycling the R-symmetry elements in the sequence, which eliminates residual second-order interactions, such as ^1H CSA, that introduce non-negligible error in the determination of heteronuclear dipolar couplings [109, 110].

It is important to note that these R-symmetry dipolar recoupling sequences are well-suited for moderate MAS frequencies as well, and the present authors employ them extensively for probing nano- to microsecond timescale mobility in a variety of systems, including HIV-1 protein assemblies [104].

2.4.3 Heteronuclear CSA recoupling—R-sequences of appropriate symmetry are useful for efficient recoupling of the heteronuclear chemical shift anisotropy (CSA), as was demonstrated originally by Zhao et al. [111]. R-symmetry sequences were employed under fast and moderate MAS conditions to recouple ^{15}N and ^{13}C CSAs in the context of 2D and 3D experiments in various systems, including HIV-1 CA protein assemblies [70, 104, 105, 109, 112]. Measurement of heteronuclear CSA tensors is essential in viral systems as they provide a probe of hydrogen bonding environments and dynamics [109, 113].

2.4.4 Proton Detection—Proton detection is particularly advantageous for mass-limited samples, such as intact viral assemblies, which can be difficult to obtain in large quantities. ^1H detection has found broad applications in MAS NMR including resonance assignments [114–117], structure determination [118, 119] including ^1H - ^1H distance restraints [120], studies of protein dynamics [93, 121–124], and protein-RNA interactions [125].

Opella and coworkers recently demonstrated the application of ^1H detection for the measurement of ^1H - ^{15}N dipolar couplings in filamentous Pf1 at 50 kHz MAS [91]. High sensitivity was attained with ~0.5 mg perdeuterated protein, and the resolution was high with both full and partial amide proton back-exchange. Pintacuda and co-workers utilized ^1H detection for studies of both fully protonated and amide ^1H back-exchanged AP205 bacteriophage capsids, measles virus nucleocapsid, as well as M2, as shown in Fig. 2 [74, 75, 101]. A combination of deuteration, fast MAS, and high magnetic fields was required in all these studies. Importantly, the high-quality spectra enabled the use of automated assignment protocols permitting the process to be streamlined [99], relative to the time-intensive manual assignment that is generally required in MAS NMR.

2.5 Nonuniform Sampling

Nonuniform sampling (NUS) has been routinely applied in solution state NMR [126–130]. Typically NUS has been applied to streamline the data collection process in cases where sensitivity is not a limiting factor. However, in the case of sensitivity-limited MAS NMR experiments on complex biological systems, NUS can be exploited to attain *bona fide* sensitivity gains in the time domain, provided appropriate sampling schedules, as was derived analytically by Rovnyak [131]. More recently, Rovnyak and colleagues demonstrated experimentally and analytically that 2-fold sensitivity enhancements can be achieved in each indirect dimension using random, exponentially-weighted nonuniform sampling schedules [131, 132]. Processing NUS data requires specialized, typically nonlinear, reconstruction algorithms. Examples of these include maximum entropy (MaxEnt) [133–135], and its linear regime implementation dubbed maximum entropy interpolation (MINT) [136], forward and fast forward maximum entropy [137–140], GFT [141, 142], covariance NMR [143, 144], and SIFT [145, 146]. There are multiple acquisition schedules and data reconstruction algorithms, detailed elsewhere [133, 147–150].

While NUS has been used extensively in solution NMR, its applications to MAS NMR are only now gaining momentum, and the advantages of this approach as well as the development of standard protocol for NUS data collection and processing are not yet fully realized. To date, in addition to the authors' work on thioredoxin and LC8, [132, 136, 151], NUS and variations of the technique have been applied to several other biological systems in the context of MAS NMR, including GB1 [145, 152] ubiquitin [118], SH3 [44], A β fibril [153], proteorhodopsin [152], a truncated MerF construct [154], and magnetically-aligned, phospholipid-embedded Pf1 [144, 154]. It is anticipated that the application of nonuniform sampling to complex viral systems will be a widely used approach for gaining sensitivity or reducing the experiment time in multidimensional MAS NMR experiments.

2.6 Dynamic Nuclear Polarization

Dynamic nuclear polarization (DNP) can provide drastic sensitivity enhancement, theoretically of up to 660 fold compared to the conventional NMR spectroscopy. In DNP experiments, polarization of unpaired electrons is transferred to nuclear spins by microwave irradiation at the EPR frequency, leading to large signal enhancements. These require introduction of radicals or biradicals, such as TEMPO or TOTAPOL, into the system [155, 156]. If an endogenous radical is present in the sample, its unpaired electron can also be used as a polarization source, albeit with much lower efficiency [157–159]. Key to the study of viruses and other complex biological systems with DNP in conjunction with MAS NMR have been developments such as high magnetic fields, high frequency microwave sources, DNP probes, and new radical polarizing agents [160]. Detailed review of DNP theory and practical aspects is beyond the scope of this article, and below only relevant applications to viral systems are discussed very briefly.

DNP studies of fd bacteriophage demonstrated that polarization from TEMPO can be distributed to the encapsulated DNA, despite the absence of the direct contact with the radicals in the solvent [161]. DNP sensitivity enhancement enabled the observation of the significantly weaker DNA peaks inside the capsid of Pf1 as compared to the coat protein

resonances. This allowed for nucleotide-type assignment of the DNA. From this, McDermott and co-workers concluded that the DNA of Pf1 is highly extended and twisted as compared to other bacteriophages [67]. With cryogenic DNP, Griffin and co-workers were able to observe the external binding site(s) of rimantadine with the influenza A M2 proton transporter and measure the protein-ligand distance with an accuracy of ± 0.2 Å. The application of DNP was particularly powerful for the observation of this weak (mM) protein-ligand interaction [162]. Overall, DNP is rapidly becoming an important technique for the study of large viral and other protein assemblies and even whole cells [163, 164].

3. Structure and Dynamics of Viral Systems with MAS NMR

In this section, we discuss applications of MAS NMR to both proteins and nucleic acids of numerous viral systems, including the retrovirus HIV-1, bacteriophages, and lipid membrane enveloped viruses.

3.1 HIV-1

Human Immunodeficiency Virus Type 1 (HIV-1), the causative agent of an acquired immunodeficiency syndrome (AIDS), is a retrovirus carrying its genetic information in the form of single-stranded RNA. Once inside the cytoplasm of the host cell, viral RNA is released from the capsid core, and reverse transcribed into DNA, and integrated into the host cell's genome. HIV-1 consists of an outer envelope composed of lipids and glycoprotein, a viral capsid, a dimer RNA, and viral enzymes including protease, reverse transcriptase, and integrase [165]. MAS NMR spectroscopy has emerged as a popular method to study HIV-1. In this section, we review the highlights of MAS NMR studies of HIV-1.

3.1.1 HIV-1 Capsid Protein Assemblies—HIV-1 encodes a polyprotein called Gag, which directs the packaging of viral genome and assembles into immature, noninfectious virions [166]. In the maturation process, the Gag polyprotein is cleaved at five positions into its constituent domains, forming a conical capsid core to enclose the viral genome and proteins, as illustrated in Fig. 3A [21, 167–169]. The HIV-1 capsid contains approximately 1200 copies of 25.6 kDa capsid protein (CA), and in an idealized model can be thought as a ‘fullerene cone’, composed of a hexameric lattice, with 12 pentameric CA subunits incorporated to complete the closed structure [170, 171]. Recently, a pseudoatomic structure was solved of a CA capsid, using a hybrid approach including all-atom molecular dynamics simulations, cryo-EM, and solution NMR spectroscopy [172]. The architecture of the HIV-1 capsid is shown in Fig. 3B [21, 170, 172]. *In vitro*, CA assemblies can exhibit conical, tubular, spherical, or donut-like morphologies under different assembly conditions [170, 173–175].

Work from the authors' laboratory established sample preparation protocols for generating conical, spherical, and tubular assemblies of CA, which are particularly suitable for high-resolution structural analysis by MAS NMR spectroscopy [20]. Among these three kinds of CA assemblies of different morphologies, tubular assemblies of CA yielded so far the best quality of MAS NMR spectra with remarkably high resolution, as shown in Fig. 4, while conical and spherical assemblies of CA also exhibit reasonably high resolution, permitting the determination of structure and dynamics at atomic level [20, 21]. Comparison between

the spectra of conical and spherical assemblies showed no significant differences, indicating that the structure of CA is very similar in different morphologies. Using data sets acquired at 21.1 T, resonance assignments were completed for 66% of CA protein in conical assemblies [20]. The hinge region between the N-terminal domain and C-terminal domain was found to be flexible on the millisecond timescales as probed through ^{13}C - ^{15}N and ^1H - ^{15}N dipolar coupling measurements in R-based DIPSHIFT experiments discussed above [70]. These hinge motions were proposed to allow the protein to access multiple conformers, permitting the formation of different assembly morphologies.

The authors also addressed HIV-1 maturation process. They have examined the Gag cleavage intermediate CA-SP1 assemblies. As the final step in the maturation process, the cleavage of CA-SP1 triggers the formation of the conical capsid core. The detailed mechanism of this process is not fully understood, including the conformation of SP1 peptide in CA-SP1, which has been a subject of debate [176–178]. To investigate the conformation and dynamics of CA-SP1 assemblies, dipolar- and scalar-based correlation experiments were employed; the measurements revealed that the SP1 peptide adopts a dynamic random coil conformation in CA-SP1 assemblies [21]. Furthermore, analysis of two variants of the CA protein with slightly different primary sequences indicated that sequence variations at distal sites induce conformational plasticity in the SP1 region, in the Cyclophilin A-binding loop, and in the loop preceding helix 8.

Chen and Tycko prepared tubular CA assemblies, observed by dark-field transmission electron microscopy to be mixtures of single- and multiwall tubes, which yielded congested MAS NMR spectra [73]. Subsequently, Bayro et al. improved the sample preparation and acquired well-resolved homo- and heteronuclear correlation spectra [72]. Using the resonance assignment program MCASSIGN2 [179], along with traditional, manual spectral analysis, Bayro et al. assigned about 69% of the residues. They also suggested that the structures of the N-terminal domain and C-terminal domain of CA protein in tubular assemblies remain largely the same as observed in solution, and consistent with the observations from the authors' laboratory [20, 21]. From the resonance assignments and additional two-dimensional ^1H T_2 -filtered NCA experiments, Bayro et al. distinguished regions that are well-ordered, regions that are dynamically or statically disordered, and regions that are partially mobile. They also carried out ^{15}N - ^{15}N backbone recoupling (^{15}N -BARE) experiments to measure distances between sequential amides, thus probing the backbone conformation, as shown in Fig. 5 [72, 180]. By comparing the experimental decay curves obtained in tubular assemblies with those calculated from solution NMR and crystal structures, local conformational changes were detected, including the 3^{10} -helix in the C-terminal domain. Structure calculations using resonance assignments and ^{15}N - ^{15}N couplings as restraints via the Xplor-NIH program revealed multiple structural changes upon tube formation [72].

3.1.2 HIV-1 Rev Protein Assemblies—The 13 kDa HIV-1 Rev protein binds to the Rev response element (RRE) on incompletely spliced viral mRNAs and enables their transport from the nucleus to the cytoplasm [181, 182]. As Rev is prone to aggregate and fibrillize, no high-resolution structural information on full-length Rev had been reported until Blanco et al. applied MAS NMR spectroscopy to the system [183]. In this work, the Tycko group

examined Rev filaments in frozen solution with double-quantum chemical shift anisotropy (DQCSA) and constant-time double-quantum-filtered dipolar recoupling (CTDQFD) experiments, which provided site-specific information on backbone torsion angles, supporting the helix-loop-helix model in the N-terminal half of Rev. In a follow-up study, Havlin et al. reported new MAS NMR data on both free Rev and Rev in complex with a 45-base RNA molecule, using selectively ^{13}C labeled samples at all Ala, Val, and Ile positions [82]. Analysis of two-dimensional ^{13}C - ^{13}C correlation spectra suggested that along with the helical structure in the N-terminal half of Rev detected in the previous study, the C-terminal half of Rev also adopts a partially helical structure. Moreover, spectral comparisons between free Rev and Rev/RNA co-assemblies showed no significant differences, indicating that Rev protein remains in the same conformation upon RNA binding [82].

3.1.3 HIV-1 Membrane-Associated Proteins—HIV-1 membrane-associated proteins include glycoprotein gp120 and gp41, the trans-activator of transcription Tat, and viral protein Vpu. While we focus on MAS NMR studies of these proteins in this section, we refer our readers to references [29–41] for studies using oriented sample solid-state NMR.

The complex of HIV-1 gp120/gp41 facilitates the fusion of viral and host cell membranes in the first step of HIV infection [184, 185]. Derived from a ~20-residue N-terminal domain of gp41, HIV-1 fusion peptide (HFP) has the ability to bind to the host cell membrane and catalyze the fusion process. A series of MAS NMR studies from the Weliky group, including rotational-echo double resonance (REDOR) measurements and two-dimensional ^{13}C - ^{13}C correlation experiments, revealed that HFP largely adopts an antiparallel β -sheet conformation in cholesterol-containing membrane mimics [80, 85, 186]. In addition, ^{31}P relaxation measurements showed increased membrane curvature in the presence of HFP [187]. Qiang et al. synthesized several HFP constructs and examined their fusion rates. They discovered that the smallest oligomer for efficient fusion catalysis is HFP_{tr} [78, 188]. In order to probe the membrane location of HFP, the authors measured the distance between the ^{13}CO of HFP and ^{31}P of the lipid using REDOR experiments, and revealed a positive correlation between the depth of membrane insertion of HFP and their fusogenicities [78, 189]. A later study enabled the measurement of site-specific membrane locations of HFP by examining the distance between specifically ^{13}C labeled HFP and specifically ^2H labeled lipids using ^{13}C - ^2H REDOR experiments [190]. In order to probe the structure of the fusion peptide in large gp41 constructs, Sackett et al. built the N70 construct that models a pre-hairpin intermediate, and the FP-Hairpin construct that models the final fusion state with six-helix bundle structure [191, 192]. MAS NMR probed the secondary structure of the fusion peptide in these large gp41 constructs, and molecules with either α -helical or predominantly antiparallel β -sheet conformation were observed in both [79, 192]. Another study from the Weliky group introduced an approach to examine lyophilized whole cells containing an expressed 154-residue gp41 fragment using MAS NMR, and detected helical structure at the C-terminal of this protein construct by REDOR experiments [193]. In addition to the studies on fusion peptides, MAS NMR was also applied to investigate the V3 loop in gp120 and its interaction with antibody fragments [194, 195].

The HIV-1 Tat protein contains a transduction domain, which is known as the cell-penetrating peptide [196]. Su et al. applied MAS NMR to study the conformation and

dynamics of a Tat peptide (residues 48–60) in anionic lipid bilayers [197]. Chemical shifts obtained from ^{13}C - ^{13}C and ^1H - ^{13}C correlation experiments suggested a random coil structure for the Tat peptide. The authors also conducted DIPSHIFT experiments to measure ^{13}C - ^1H and ^{15}N - ^1H dipolar couplings whose corresponding order parameters had low values, indicating large-amplitude motions of Tat peptide in membrane lipids. ^{31}P spectra of oriented membrane lipids measured with increasing amount of Tat peptide revealed that the membrane remains intact upon the insertion of Tat peptide. ^1H spin diffusion measurements suggested that Tat peptide is not deeply inserted into the membrane lipids. In addition, site-specific distances between Tat peptide and the membrane surface were measured by ^{13}C - ^{31}P REDOR experiments, which provided evidence for a salt bridge interaction between the Arg residue of Tat peptide and the phosphate group in the membrane.

HIV-1 Vpu is an 81-residue accessory protein containing an N-terminal transmembrane (TM) domain [198]. Sharp et al. employed MAS NMR to examine the structure and dynamics of a Vpu peptide, comprising N-terminal residues 1–40, in phospholipid bilayers [86]. ^{15}N and ^{13}C chemical shifts were obtained based on two-dimensional ^{13}C - ^{13}C and ^{15}N - ^{13}C spectra acquired on selectively labeled samples. Spectral analysis suggested helical secondary structure and rigid-body dynamics for residues 3–27. In addition, site-specific information about solvent exposure was provided by H-D exchange experiments. Later, Do et al. characterized full-length Vpu in POPC bilayers using MAS NMR [199]. Partial resonance assignments for ^{13}C were obtained by examining uniformly labeled protein, supporting the α -helical conformation in the TM domain. Furthermore, two- and three-dimensional proton spin diffusion experiments confirmed the correct insertion of TM domain of Vpu into the lipid bilayers and the close proximity between water and cytoplasmic domain of Vpu.

3.1.4 HIV-1 TAR RNA—The HIV-1 transactivation response (TAR) RNA is known to bind with the viral regulatory protein Tat via a TAR hairpin and facilitates viral replication [200]. In order to probe the interactions between TAR RNA and Tat peptides, the Drobny group utilized REDOR experiments to obtain distance information. A lyophilized sample of a 29-nucleotide TAR RNA construct, comprising the Tat-binding site, was examined using ^{31}P - ^{19}F REDOR measurements, with and without the presence of a Tat-derived peptide. A nearly 4 Å decrease of distance between the $2'\text{F}$ label at U23 and the phosphorothioate at A27 revealed a significant conformational change upon peptide binding [201]. In a later study, TAR RNA with 5-fluorouridine incorporated at U23 in complex with a Tat-derived peptide with one crucial arginine labeled with ^{13}C and ^{15}N , was examined using ^{13}C / ^{15}N - ^{19}F REDOR experiments, providing intermolecular distance information between RNA and the peptide [202]. In addition, Olsen et al. also performed site-specific deuterium solid-state MAS NMR experiments to probe the dynamics of TAR, and discovered motions on the μs -ns timescale, which could not be detected by solution NMR [203]. Motional models of residues on the TAR binding interface were established on the basis of deuterium MAS NMR data, which indicates a conformational capture mechanism for the recognition of Tat [204]. The authors also probed the dynamics of TAR RNA as a function of hydration by measuring deuterium lineshapes and relaxation times, and observed

sudden dynamics changes when hydration levels were coincident with the initial bulk hydration [205]. Furthermore, the Drobny group presented a sample preparation protocol using polyethylene glycol precipitation, which improved the ^{13}C line widths of TAR RNA spectra from > 6 ppm to ~ 1 ppm [60].

3.2 Bacteriophages

Bacteriophages are a class of viruses that target and destroy bacterial cells. Their general structure consists of a single- or double-stranded DNA or RNA genome surrounded by an assembly of coat protein subunits. This diverse class of viruses exists in a variety of shapes and sizes, but can be divided into 3 general structural groups: filamentous, icosahedral with tail, and icosahedral without tail. Filamentous bacteriophages, the class that includes Pf1, fd, and M13, possess a rod-like structure, 800–2000 nm in length, with several thousand copies of an α -helical coat protein in a helical array around the ssDNA [206]. *Caudovirales*, such as T4 and T7, are tailed bacteriophages with DNA stored inside an icosahedral capsid, with a tail to attach to the host cell [207]. They range from approximately 100 to 500 nm in length.

There is a broad range of scientific interest in bacteriophages [208], including applications in nanotechnology [209–212], phage display and targeted gene therapy [213–215], as well as DNA cloning and sequencing [216–218]. Bacteriophages are also widely used as alignment media for residual dipolar coupling measurements in solution-state NMR [219, 220]. Structural analysis of bacteriophages can lend valuable insight into applications of bacteriophages in these fields. MAS NMR studies of bacteriophages have provided insight into the structure and dynamics of bacteriophage coat proteins [19, 68, 221], as well as nucleic acid conformation [67], and interactions between coat protein sites and nucleic acids [64–66]. These studies have also contributed to a growing knowledge base of the relationship between DNA chemical shifts and conformation.

3.2.1 Filamentous Bacteriophages—Filamentous bacteriophages that have been characterized by MAS NMR include Pf1, fd, and M13. Pf1 has been studied through a wide range of other spectroscopic and computational methods and has become a model system for the study of filamentous bacteriophages. McDermott and co-workers presented site-specific resonance assignments of the coat protein of intact, PEG-precipitated Pf1 bacteriophage with 2- and 3-dimensional ^{13}C and ^{15}N correlation spectra, as illustrated in Fig. 6 [19]. This was the first study to assign the coat protein of an intact virus with MAS NMR. They were able to assign 92% of the 46-residue protein in its high temperature form, despite significant helical content and resulting spectral overlap. A single observed peak for most sites of the capsid protein indicated that the asymmetric unit was a single copy of the coat protein, rather than a trimer of slightly differing conformers as proposed in a prior study [222]. Based upon the well-known correlation between protein secondary structure and ^{13}C chemical shifts [223], they determined that the Pf1 coat protein is unstructured at the N-terminus, and a continuous α helix from residues 6–46, in agreement with structural models derived from static SSNMR and X-ray fiber diffraction [27, 222]. Further studies by Lorieau et al. probed the site-specific dynamics of the intact Pf1 coat protein with ^1H - ^{13}C dipolar order parameters [221]. They demonstrated that while the protein backbone is very rigid,

there are several amino acid sidechains that exhibit conformational mobility. These include solvent-exposed residues, and notably, Arg 44 and Lys 45, which interact with the viral DNA inside the capsid. The observed sub-microsecond dynamics of these 2 residues may be an important feature of capsid-DNA interactions in Pf1.

Pf1 undergoes a 2-state structural transition near 10°C. Goldbourn et al. probed the effects of temperature on Pf1 coat protein structure with ^{13}C - ^{13}C DARR spectra at high and low temperature [68]. Several reversible chemical shift changes as a function of sample temperature were observed, notably among sidechain sites of hydrophobic amino acids. A total of 14 amino acids were observed to have chemical shift changes. Many of the resonances with significant chemical shift perturbations were located near the C-terminus of the coat protein. At longer mixing times (200 ms), some intersubunit contacts could be observed. Their results suggest that the observed chemical shift changes occur due to changes in intersubunit hydrophobic interactions rather than changes in backbone structure or solvent exposure, in agreement with previous studies by Marvin and co-workers [224].

Sergeyev et al. applied DNP to the study of DNA conformation in Pf1 [67]. DNP signal enhancement of the otherwise weak DNA resonances was key for resonance identification and assignment (Fig. 7). It is to be noted that under DNP conditions, significant line broadening was observed, as evident in the 2D contour plots shown in Fig. 7. Nevertheless, Sergeyev et al. were able to obtain nucleotide-type assignment of the Pf1 DNA base region with ^{13}C - ^{13}C DARR spectra (Fig. 7). Ribose ^{13}C chemical shifts have been shown to be sensitive reporters of nucleic acid conformation [225, 226]. The downfield-shifted SC3' and SC5' resonances indicated that the DNA is in a C2'-endo/gauche conformation. Many observed ^{13}C and ^{15}N chemical shifts in the DNA base region lie outside the range of shifts previously reported in the BioMagResBank (BMRB) [100]. This chemical shift behavior indicates an unusual DNA conformation where Watson-Crick base pairing is absent, but base-stacking is still observed, in agreement with studies concluding that Pf1 has an unusually extended and twisted DNA conformation [227].

Purusottam et al. characterized water-protein interactions of Pf1 using 2D ^1H - ^{15}N dipolar-edited medium- and long-distance (MELODI) HETCOR experiments [228]. ^{13}C and ^{15}N dipolar dephasing was utilized to reduce polarization arising from aliphatic and amino protons, such that cross peaks at 4.78 ppm in the proton dimension predominantly arise from water-protein contacts. This dephasing made it possible to work with 100% protonated samples. ^1H - ^{15}N cross peaks were observed between hydration waters and 7 protein residues, including the dynamic Arg 44. They propose that the capsid-DNA interaction at this site is water-mediated, and hydration waters may play a role in sequence-specific recognition of DNA.

The McDermott group has recently published studies of Pf1 hydration as well [229]. Using ^1H - ^{13}C HETCOR measurements, water-protein cross peaks of 25 amino acids were observed, including 6 residues in the virion core. Water-DNA contacts were also observed. These results demonstrate that the capsid interior is water-accessible and support the hypothesis that hydration waters have an important role in mediating protein-DNA interactions.

The Goldbourn lab has examined structural features of both the capsid protein and ssDNA of the fd bacteriophage, as well as interactions between the two [64–66]. Fd and other Ff class bacteriophages including M13 have important applications in phage display and nanotechnology [230]. Previously, structural inhomogeneity had limited high-resolution characterization of the wild-type fd capsid [231, 232]. Abramov et al. obtained high-resolution spectra of intact phage particles composed of the wild type capsid, allowing for almost complete site-specific assignment of the capsid protein, as well as qualitative assignment of DNA signals [65]. Characteristic chemical shifts indicated the capsid is predominantly α -helical with a mobile N-terminus and slight curvature. Interestingly, they observed no evidence of structural inhomogeneity. Downfield-shifted C3' and C5' ^{13}C peaks of DNA suggest the nucleic acid is in a C2'-endo or C3'-exo sugar pucker conformation, in contrast to previous studies [233, 234]. Subsequent work probed interactions between the protein capsid and nucleic acid [64]. Utilizing ^{13}C - ^{13}C and PDSD-mediated ^{31}P - ^{13}C correlations, they demonstrated that the primary protein-DNA interaction in fd is an electrostatic interaction between lysine sidechains and the DNA phosphate backbone. A key feature of this study was the use of natural abundance aromatic amino acids to avoid spectral overlap with DNA ^{13}C resonances. They demonstrated that even at moderate spinning frequencies (12 kHz), efficient polarization transfer within DNA nucleotides can be obtained with ^{13}C - ^{13}C COrbined R $_2$ $^{\nu}$ -Driven (CORD) experiments [106], particularly important given the scarcity of protons within the base region of nucleic acids. Using the unique chemical shifts of some nucleic acid sites (i.e. deoxythymidine C7), and nucleotide chemical shifts reported in the BMRB and B-DNA chemical shifts compiled by Sergeyev et al. [67] for reference, almost complete nucleotide-type assignment of DNA base resonances was possible. Internucleotide correlations indicative of base pairing or stacking were also observed. Capsid-DNA interactions were observed for the sugar region and base region, as well as the phosphate backbone of the DNA, based upon cross peaks in ^{13}C - ^{13}C and ^{13}C - ^{31}P correlation spectra. ^{13}C - ^{13}C correlations of note included DNA sugar and base to lysine sidechain cross peaks near the C terminus. Proton-mediated ^{31}P - ^{13}C correlations (Fig. 8) also indicate interactions of the phosphate backbone with Lys residues as well as other amino acid residues near the C terminus, elucidating the capsid-DNA interface.

In further studies, Morag et al. characterized the structure of the coat protein in infectious M13 particles [66], which differs from fd by only 1 amino acid residue (fd's negatively charged Asp12 is Asn in M13). In addition to the traditional experiments for resonance assignment, J-based z-filter refocused INADEQUATE was applied to M13, allowing assignment of the mobile N-terminus and confirmation of additional assignments. The general chemical shift agreement between fd and M13 indicates overall structural similarity, as well as similar hydrophobic packing and mobility. Amino acids for which chemical shift differences were observed are in the region of residue Asx12, including Lys8 which is involved in intra- and inter-subunit electrostatic interactions, leading to changes in local conformation, packing, and rigidity between fd and M13 in this region. Very recently the Goldbourn lab published a structural model of M13 using structural restraints derived from MAS NMR data and Rosetta for model building [235]. The structure demonstrates the important role of hydrophobic residues for capsid stabilization. This work reports the first MAS NMR structure of an intact viral capsid.

Opella and co-workers have extensively characterized Pf1 and fd with static SSNMR, including structure determination of the Pf1 and fd coat proteins as both an intact viral particle and in a membrane-bound form [25–28, 236]. They have utilized methods such as PISEMA to characterize magnetically aligned samples [237, 238]. MAS and static NMR studies of the M13 coat protein embedded in a phospholipid bilayer have also been performed [239, 240]. This work is beyond the scope of the current review.

3.2.2 Tailed Bacteriophages—Tailed bacteriophages (*Caudovirales*) such as T4 and T7 have a highly condensed genome within the viral capsid [241]. The mechanism of genome packaging and structure of the DNA within the tightly packaged capsid are of particular interest. Abramov et al. characterized the structure of double-stranded DNA in bacteriophage T7, including development of a large-scale sample preparation protocol [63]. ^{13}C - ^{13}C dipolar-assisted rotational resonance (DARR) and ^{15}N - ^{13}C transferred echo double resonance (TEDOR) experiments were used for complete ^{13}C and near-complete ^{15}N nucleotide-type assignment of the 40 kbp DNA. As compared to other phages, DNA resonances in T7 are more readily detected given that the nucleic acid comprises > 50% of the virion's mass. ^{13}C and ^{15}N chemical shift reporters in both the sugar and base regions indicated B-form DNA and the presence of expected Watson-Crick base pairing. This work is an additional contribution to the somewhat limited database of ^{13}C and ^{15}N chemical shifts for nucleic acids, particularly for native systems.

Schaefer and co-workers characterized DNA packaging in bacteriophage T4 with rotational echo double resonance (REDOR) dephasing experiments [83]. ^{31}P - ^{15}N REDOR dephasing of thymine and guanosine N1 coupled to all neighboring backbone phosphorous atoms indicated that DNA packaged inside the phage is B form. The observation of REDOR dephasing for ϵ -lysyl and polyamines also suggested a role for these protein residues in DNA backbone charge stabilization.

3.3 Lipid Membrane Enveloped Viruses

Enveloped viruses contain at the very least the viral genome, a capsid shell composed of proteins, and a lipid membrane [8]. Additional proteins and molecules can also be present. For instance, glycoproteins can decorate the lipid membrane helping the virus attach to a host cell. The lipid membranes that make up the viral envelope are generally derived from lipids present within the host cell.

Many of the viruses that pose major human health concerns are enveloped. These include: herpes simplex, hepatitis B & C, rubella, influenza A, B, & C, measles, mumps, rabies, and Ebola. Several groups have invested time into understanding the structure, dynamics, and function of enveloped viruses. MAS NMR studies of influenza A, parainfluenza 5, and measles viruses, which have been studied by SSNMR, are described below.

3.3.1 Influenza A—One of the most extensively studied virus related systems by solid-state NMR is the transmembrane peptide of the M2 protein of Influenza A (M2TMP). The M2 protein of influenza acts as an ion channel and is responsible for the conduction of protons to the interior of the virion. This lowering of pH promotes uncoating and is critical for the viability of the virus. Antiviral drugs based on adamantane derivatives such as

amantadine and rimantadine, which were once effective at binding M2 and inhibiting viral replication, have since found a reduction in clinical use due to many influenza A strains displaying drug resistance.

Beginning in the late 1990s, the laboratory of Tim Cross was the first to begin intense investigation of the structural features of M2TMP using oriented sample SSNMR. These groundbreaking studies, conducted on synthesized peptides containing selective amino acid labels and reconstituted in lipids, served as the foundation for many of the studies that have followed. The original experiments resulted in the first SSNMR evidence for symmetry, orientation, and helical packing in the M2TMP tetrameric bundle [44, 242, 243]. In subsequent studies, Cross and co-workers developed the first high-resolution structure of the backbone for the M2TMP monomer as determined using orientational restraints from PISEMA spectra [51]. They were also the first to report on interhelical distances in M2TMP. Using a peptide labeled with ^{13}C , ^{15}N Trp and His amino acids, REDOR measurements were conducted under MAS NMR spectroscopy which yielded a maximum distance of 3.9 Angstroms between the $^{15}\text{N}\pi$ site of His37 and the $^{13}\text{C}\gamma$ site of Trp41 [45]. In 2003, they published structural and dynamic results characterizing the full-length M2 protein using PISEMA and hydrogen/deuterium exchange experiments [48]. Recently, Cross and coworkers have continued to contribute to the understanding of the influenza A M2 protein with studies including investigations on the role of histidine side chains in proton conduction by monitoring His37 sites using ^{15}N CPMAS experiments [244], the backbone structure of the amantadine blocked proton channel [245], dynamics of residues in the proton channel [246], the role of W41 in proton conduction [52], structural investigations of full length M2 in synthetic bilayers and *E. coli* membranes [247], and the quaternary structure of the transmembrane bundle [248]. To explore the quaternary structure, 2D ^{13}C - ^{13}C as well as 3D NCOCX and NCACX MAS correlation experiments were conducted on a M2 construct expressed in *E. coli*. Using these correlation data, isotropic chemical shifts were assigned to residues in regions of the spectra with high resolution. Additional 2D ^{13}C - ^{13}C correlation experiments were collected with different mixing times, as shown in Fig. 9, to obtain distance restraints for the quaternary structure [248].

In the past decade, Hong and co-workers have investigated a variety of structural, functional, and dynamical features for the M2TMP with MAS NMR. An early study examined the quaternary structure of the M2TMP bound to DMPC bilayers [89]. Peptides were synthesized with an A30F mutation allowing for a 4- ^{19}F label to be incorporated. ^{19}F - ^{19}F interhelical distances between nearest neighbor Phe30 residues were measured to be between 7.9 and 9.5 Å using a CODEX experiment. Follow up studies examined uniaxial rotational diffusion [76, 249], intermolecular distances, and side-chain conformations in M2TMP [249–251]. In 2008, the effects of amantadine binding on the conformation and dynamics of the transmembrane portion of the M2 channel of Influenza A were explored. The M2 peptides used in this study contained ^{13}C , ^{15}N labels at several of the amino acid sites that are intimately involved in the binding interaction with the drug, including residues lining the proton conduction channel, involved in helix-helix interactions, and those facing the lipid. ^{13}C CPMAS, ^{13}C - ^{13}C , and ^{15}N - ^{13}C correlation experiments were used to monitor chemical shift changes upon amantadine binding. ^{13}C T_2 relaxation

measurements were performed to monitor microsecond dynamics where T_2 was found to increase in amantadine bound samples [252]. Additional studies related to the structural and dynamic changes occurring upon amantadine binding [77, 253–255] and the derivation of the amantadine binding site [84] were conducted thereafter. One such study monitored the chemical shifts of several amino acids including Ser31. From 2D ^{15}N - ^{13}C correlation experiments, large chemical shift deviations were observed for this residue in the presence of amantadine. To ensure these shifts were caused by direct protein-drug interactions and not as a result of changes to the lipid environment, the authors prepared samples where amantadine was added to the buffers during sample preparation as well as added directly to the membrane pellet after reconstitution. The results of these experiments are summarized in Fig. 10 [253]. In the past few years, proton sensing, gating, and conduction [256, 257], membrane curvature [88], and the conformational analysis of full-length M2 protein [258] have been explored by the Hong laboratory.

Andreas, Griffin, and colleagues investigated an elongated construct of the M2 proton channel spanning residues 18–60 reconstituted in POPC and DPhPC lipids. ^{15}N - ^{13}C z-filtered TEDOR and ^{13}C - ^{13}C PDSO experiments were conducted in the presence and absence of rimantadine. Large chemical shift changes observed upon binding of rimantadine were indicative of an allosteric mechanism of inhibition [259]. The authors also note that the choice of lipid had very little effect on stability and structure in the case of the elongated construct. Follow up studies included studies of a S31N drug resistant mutant [260] and investigations of inhibitor binding using dynamic nuclear polarization [162]. Taken together, these studies have provided a wealth of information about the transmembrane peptide.

3.3.2 Parainfluenza 5 (PIV5)—Another interesting system that has recently been investigated with MAS NMR is parainfluenza virus 5 (PIV5) from the *Paramyxoviridae* family. This enveloped virus enters a host cell through a fusion between the viral membrane and the host cell membrane. Hong and co-workers have investigated structural and dynamics aspects of the fusion peptide from the F protein of PIV5. [261, 262].

Using selectively labeled amino acids, peptides corresponding to the F protein of PIV5 were synthesized and reconstituted in a variety of different lipids. A combination of 1D and 2D ^{13}C experiments were used to monitor the conformations of the peptide in different membranes. Dynamics were monitored based on temperature dependent ^{13}C CPMAS measurements and indicated mobility between 273 and 313 K. Peptide depth and hydration levels were monitored through ^{13}C - ^1H spin diffusion and ^1H - ^{31}P correlation experiments, respectively [261]. A follow up study provided additional insights into the peptide conformation through the use of ^{13}C CPMAS, 2D ^{15}N - ^{13}C , and 2D ^{13}C - ^{13}C correlation experiments of peptides in several different lipids. ^{31}P experiments provided evidence for membrane curvature and dehydration in DOPC/DOPG membranes. The data indicate that several different peptide conformations are present depending on the type of lipid that is used for reconstitution [262].

3.3.3 Measles Virus (MeV)—In a very recent report by Pintacuda and coworkers [101], the measles virus (MeV) from the *Paramyxoviridae* family was examined by MAS NMR. The MeV nucleocapsid is composed of two domains dubbed N_{CORE} and N_{TAIL} and no

atomic level structural information was present for the assembled nucleocapsids previously. The authors addressed both fully intact and cleaved MeV nucleocapsids using proton detection to monitor structural and dynamic features of the system. ^1H detected experiments were performed at 60 kHz throughout.

Dipolar based ^{15}N - ^1H correlation experiments were collected for sedimented intact MeV particles and displayed a significant number of signals from the N_{CORE} domain. Scalar based ^{15}N - ^1H correlation experiments were then collected on the sedimented intact MeV nucleocapsids as well as for MeV nucleocapsids resuspended in solution. From these data, signals from the N_{TAIL} domain were observed. Signals from the sedimented nucleocapsids and those that were resuspended in solution were found to be similar.

When comparing ^{15}N - ^1H dipolar based correlation experiments for sedimented nucleocapsids that were intact to those that were cleaved by trypsin digestion (thus removing the N_{TAIL} domain), it was observed that most of the signals were present in both cases without large chemical shift deviations, suggesting similar structures between the two constructs. Although similar structures were observed, T_1 measurements revealed that the local dynamics were significantly different. Additional experiments were conducted which included a spin diffusion step to transfer magnetization from water protons to the protons of the protein. This experiment was used to monitor solvent levels in the intact and cleaved nucleocapsids. The data indicated that the intact nucleocapsid was surrounded by an increased amount of interstitial water providing evidence for a less ordered structure than that observed for the cleaved MeV nucleocapsids.

4. Conclusions and Future Outlook

The work outlined in this review lays a foundation for the expansion of MAS NMR studies to a wide range of viral systems that cannot be investigated with atomic-level resolution under physiological conditions using other biophysical techniques. As demonstrated in these recent developments, contemporary MAS NMR has the ability to provide exquisite information on the structure and dynamics of such systems. With intense methodological developments in magnet technologies, probe hardware, and pulse sequences we anticipate that in the next several years we will witness MAS NMR evolving as a method for analysis of large complex multicomponent assemblies of macromolecules constituting various viruses and even intact virions, at atomic level detail.

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Highlights

- MAS NMR can be used to study complex viral assemblies and intact virus particles
- MAS NMR provides structural information with atomic level resolution
- Recent advances in methodology yield spectra with high sensitivity and resolution
- MAS NMR has probed the structure and dynamics of viral systems such as HIV and Pf1

Fig. 1.

Images of viral systems that contain individual protein domains, peptides, or entire assemblies that have been investigated by solid-state MAS NMR. (A) fd bacteriophage, (B) the T4 bacteriophage, (C) the T7 bacteriophage, (D) the Pf1 bacteriophage, (E) influenza A, (F) parainfluenza PIV5, (G) measles, (H) HIV-1. (A) Reprinted with permission from Wang *et al. J. Mol. Biol.* **2006**, 361 (2), pp 209–215. Copyright 2006 Elsevier. (B) Reprinted with permission from Danev *et al. J. Struct. Biol.* **2010**, 171 (2), pp 174–181. Copyright 2010 Elsevier. (C) Reprinted with permission from Serwer, *J. Ultra Mol. Struct. R.* **1977**, 58 (3), pp 235–243. Copyright 1977 Elsevier. (D) Reprinted with permission from Kneale *et al. J. Mol. Biol.* **1982**, 156 (2) pp 279–292. Copyright 1982 Elsevier. (E) From Centers for Disease Control and Prevention (CDC) Public Health Image Library (PHIL), content provided by CDC and Dr. F. A. Murphy. (F) Reprinted with permission from Terrier *et al. Virus Res.* **2009**, 142 (1) pp 200–203. Copyright Elsevier 2009. (G) From Centers for Disease Control and Prevention (CDC) Public Health Image Library (PHIL), content provided by CDC, Cynthia S. Goldsmith, and William Bellini, Ph. D. (H) From Centers for Disease Control and Prevention (CDC) Public Health Image Library (PHIL), content provided by CDC and Dr. Edwin P. Ewing.

Fig. 2.

Proton-detected ^{15}N - ^1H spectra of $[\text{U-}^{15}\text{N}, ^2\text{H}, ^{13}\text{C}, ^{15}\text{N}]$ -labeled proteins acquired at 1 GHz, 60 kHz MAS. (A) microcrystalline SH3, (B) microcrystalline $\beta 2\text{m}$, (C) sedimented nucleocapsids of AP205 bacteriophage, (D) M2 channel, and (E) OmpG. Reprinted with permission from Barbett-Massin et al, *J. Am. Chem. Soc.*, **2014**, 136 (35), pp 12489–12497. Copyright 2014 American Chemical Society.

Fig. 3.

(A) Domain organization of the Gag polyprotein and schematic illustration of the proteolytic cleavages during the HIV-1 maturation. The proteolytic cleavage sites are indicated by arrows. (B) Top: All-atom molecular dynamics derived model of mature HIV-1 capsid constructed on the basis of cryo-ET studies. The capsid is comprised of 216 CA hexamers (depicted in orange) and 12 CA pentamers (depicted in magenta), PDB ID 3J3Y. Bottom: the CA hexamer of hexamers (HOH) building block, viewed from the top (left) and from the side (right), PDB ID 3J34. Reprinted with permission from Han et al, *J. Am. Chem. Soc.*, **2013**, *135* (47), pp 17793–17803. Copyright 2013 American Chemical Society.

Fig. 4.

(A) 2D spectra of U- ^{13}C , ^{15}N tubular assemblies of HIV-1 CA-SP1 A92E acquired at 19.9 T and 4 °C: NCACX (top), NCOCX (middle right and bottom), and DARR (middle left and middle center). The 1D trace at 112.4 ppm along the ^{15}N dimension of the NCACX spectrum is shown on top. The DARR mixing time was 50 ms. Backbone walk for the stretch of residues R100-T110 is shown on the spectra. (B) Expansion of the 2D DARR spectrum around the region containing the $\text{C}_\alpha\text{-C}_\beta$ resonances of Ser and Thr residues.

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Fig. 5.

^{15}N -BARE signal decay curves for the indicated residues. These curves are measurements of sequential amide ^{15}N - ^{15}N distances, which are conformation dependent. Filled circles are experimental MAS NMR data of HIV-1 CA tubes. Broken and dotted lines are simulations based on a CA monomer structure from solution NMR (red dots, PDB code 2LF4), a crystal structure of cross-linked CA hexamers (short green dashes, PDB code 3MGE), and a solution NMR structure of CTD dimers (long blue dashes, PDB code 2KOD). Simulations based on the refined structure are shown in gray continuous lines ("Fit"). Reprinted with permission from Bayro et al, *J. Mol. Biol.*, **2014**, 426 (5), pp 1109–1127. Copyright 2014 Elsevier.

Fig. 6.

3D heteronuclear correlation spectra of Pf1 acquired at 750 MHz. (A) Strip plots for residues 8–15 at the indicated ^{15}N frequency with ^{13}CO chemical shifts on the horizontal axis, and $^{13}\text{C}_\alpha$ shifts on the vertical axis, from NCACX and NCOCX experiments, illustrating the sequential assignment. (B) The projection down the CO dimension of the NCOCX sequential experiment, showing regions corresponding to crosspeaks between the Gly/Ser ^{15}N shifts and the CO- C_α resonance of the preceding residue. (C) Projection down the ^{15}N dimension of the NCACX experiment, showing crosspeaks from many 2- and 3-bond transfers. The gray box indicates aliased peaks arising from N- C_β and N- C_γ transfers. Reprinted with permission from Goldbourn et al, *J. Am. Chem. Soc.*, **2007**, 129 (8), pp 2338–2344. Copyright 2007 American Chemical Society.

Fig. 7. DNP-enhanced ^{13}C - ^{13}C DARR spectra of Pf1, indicating the nucleotide-type assignments of the DNA. (A) dC/dT base resonances, (B) dA/dG base resonances, (C) sugar spin systems. Reprinted with permission from Sergeyev et al, *J. Am. Chem. Soc.*, **2011**, 133 (50), pp 20208–20217. Copyright 2011 American Chemical Society.

Fig. 8. 2D PHHC spectrum of YFW^{unlabeled}-[¹³C, ¹⁵N] fd bacteriophage. The spectrum shows crosspeaks between the phosphorous backbone linkage of the DNA with both DNA sugar and base sites, as well as protein capsid residues. Reprinted with permission from Morag et al, *J. Am. Chem. Soc.*, **2014**, 136 (6), pp 2292–2301. Copyright 2014 American Chemical Society.

Fig. 9.

^{13}C - ^{13}C DARR spectra for the M2 protein (spanning residues 22–62) from influenza A as expressed in *E. coli*. (A) Aliphatic to carbonyl correlations are shown for experiments with a short mixing time 9 ms (blue) and 50 ms (red). (B) Correlations between aliphatic and aromatic sidechains are shown with short mixing times of 9 ms (blue) and longer mixing times of 100 ms (red). (C) Aliphatic to aliphatic correlations are shown for shorter mixing times of 20 ms (blue) and longer mixing times of 50 ms (red). Reprinted with permission from Can *et al.*, *J. Am. Chem. Soc.*, **2012**, *134*, pp 9022–9025. Copyright 2012 American Chemical Society.

Fig. 10.

2D ^{15}N - ^{13}C correlation spectra for the selectively labeled (V28, S31, L36) M2 transmembrane peptide (spanning residues 22–46) reconstituted in DLPC lipids. The three spectra display chemical shift changes based on the amount of amantadine present (none, 10 mM, and 0.4 mg from top to bottom, respectively). (A) No amantadine is present and peak S31 is located at a chemical shift representing the unbound amino acid. (B) As amantadine is added to the buffer at a concentration of 10 mM nearly all of the intensity for S31 is displayed at a chemical shift representing the bound state. (C) This condition is based on the addition of amantadine directly to the reconstituted pellet at a ratio of 1:2 (pellet to drug)

and shows 70% of the signal intensity in the bound form. Reprinted with permission from Cady *et al.*, *J. Mol. Biol.*, **2009**, 385, pp 1127–1141. Copyright 2008 Elsevier.

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