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NMR of Macromolecular Assemblies and Machines at 1 GHz and Beyond: New Transformative Opportunities for Molecular Structural Biology

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1. Perspective

In the past two decades, the field of magnetic resonance has realized astounding advancements. One critical achievement toward progress in the fields of structural biology, materials science, and clinical imaging (to name just a few) has been the development of higher magnetic field strengths (1–4). Higher magnetic fields yield profound improvements in both sensitivity and resolution.

The greatest drawback of nuclear magnetic resonance (NMR) relative to other analytical methods is relatively low sensitivity. Increasing magnetic field strength is a compelling way to increase sensitivity, as the signal-to-noise ratio (SNR, i.e. sensitivity) scales approximately as the power of 3/2 with the magnetic field strength (5). The implications of improved sensitivity are numerous and include the capability to study increasingly large and complex biological systems (e.g. whole cells and viral particles), the expansion of applications to physiologically relevant nuclei beyond ¹H, ¹³C, and ¹⁵N, such as ¹⁷O, ²³Na, and ³⁵Cl, as well as practical considerations, for instance shorter experiment times for multi-dimensional experiments (6).

Increased magnetic fields also yield improvements in resolution:

$$\Delta\nu \propto \frac{1}{\gamma B_0}$$

where ν is the linewidth. As with improvements in sensitivity, increased resolution allows for the study of more complex systems and visualization of finer structural details.

Beyond significant gains in sensitivity and resolution, increased magnetic field strengths are particularly powerful for the study of quadrupolar nuclei ($I > 1/2$). Quadrupolar nuclei yield

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very broad spectra (on the order of MHz) due to the strong coupling (expressed by the coupling constant, C_Q) between the nuclear quadrupolar moment and the electric field gradient (EFG). At higher magnetic field strengths, the contributions of the second-order quadrupolar interaction are reduced, leading to narrower spectra (7).

Both NMR and clinical magnetic resonance imaging (MRI) benefit from increased magnetic field strengths. (For reviews of applications of high magnetic fields to MRI see references (8, 9).) Here we focus on advancements in technology and methodology as applied in the burgeoning field of NMR at field strengths above 1 GHz. We also discuss several recent applications of high magnetic fields to the study of biological systems.

1.1 Current State of the Art

Numerous technological gains have promoted the advancement of NMR to current levels, where field strengths above 1 GHz (23.5 T) have recently been achieved (Figure 1). Such advancements have included improvements in superconducting materials, cryo-cooling, and resistive coils. To date, NMR spectrometers up to 1 GHz have utilized low temperature superconducting (LTS) materials such as Nb_3Sn and NbTi . However, 1–1.1 GHz is believed to be the upper limit for magnets produced with only LTS materials due to limitations such as current density and temperature (1). Recent advancements have focused on the development of dual LTS/HTS (high temperature superconducting) magnets (1, 3, 10–13) wherein the inner HTS coil(s) are surrounded by outer LTS coil(s) (Figure 1). The development of HTS materials (14), such as Rare Earth BaCo oxides (REBCO, e.g. YBCO (15, 16)) and Bi-2223 ($\text{Bi}_2\text{Sr}_2\text{Ca}_2\text{Cu}_3\text{O}_{10-x}$ (17)), has faced challenges including mechanical properties of the materials and manufacturing (18, 19). HTS technology has very recently advanced to the stage where these materials can be used to generate reasonably stable and homogeneous magnetic fields for NMR. Beyond the development of HTS materials, challenges of the magnet technology have included jointing between the HTS and LTS coils (20) and field homogeneity, further discussed below. Furthermore, the addition of HTS coils creates a spatial challenge. A sufficiently wide bore is needed for biomolecular NMR measurements to be possible. (Bore size is also one of the critical limitations in applying MRI at higher magnetic fields.) One approach to overcoming this issue has been modifications in the geometry of LTS coils so that these take up less space (21).

To date, work on ultrahigh field LTS/HTS magnets for biomolecular NMR applications has focused on proof of concept, with the bulk of published work done on the 24 T magnet at the National Institute for Materials Science (NIMS) developed in collaboration with RIKEN, Kobe Steel, and JEOL (Japan) (1, 22). The magnet reached 1020 GHz in 2015 using a Bi-2223 inner HTS coil with outer NbTi and Nb_3Sn LTS coils. With this magnet, they have demonstrated the ability to acquire high-quality natural abundance spectra for insensitive nuclei such as ^{17}O (0.04% natural abundance (1, 23)). ^1H - ^1H - ^1H 3D data indicate the field homogeneity and stability achieved at 24 T (24). As discussed below, a significant advantage of magnetic field strengths over 1 GHz is in the measurement of chemical shift anisotropies (CSAs). The CSA scales with the B_0 field, thus at higher fields CSAs are larger and can be measured more accurately. This is particularly advantageous for small CSAs, such as for the

aliphatic protons. Pandey et al. demonstrated the measurement of ^1H CSAs for L-histidine with the dual LTS/HTS 24 T spectrometer (24).

Beyond superconducting coil technology, for successful operation of ultrahigh field spectrometers advancements have needed to be made with respect to other spectrometer components, concurrent with the development of LTS/HTS magnets. Magnetic field stability and homogeneity are critical to successful experiments. A significant issue in the use of HTS technology for NMR is the inhomogeneous magnetic fields generated by the HTS materials. To compensate for these inhomogeneities in the LTS/HTS systems requires the use of ferromagnetic shim coils in addition to the standard superconducting and room temperature shims used in LTS magnets (25, 26). Due to residual resistance in the HTS coils and further advancements still needed in HTS/LTS jointing, for stable operation of the 1.02 GHz NMR spectrometer at NIMS, the magnet needed be to run in external current or driven mode (27). Field fluctuations generated by the DC power supply had to be corrected with an external ^2H lock, not typically required for solid state NMR experiments (28). Further, use of a DC power supply required development of a new safety mechanism in case of power failure (29). Additional developments for the use of HTS materials in ultrahigh field magnets have included design of active quench protection (30) and advancements in cryo-cooling and refrigeration (31, 32).

The next stage in the evolution of increased magnetic field strengths for applications in NMR may be hybrid magnets, where an inner resistive coil is surrounded by an outer superconducting coil. NMR spectrometers currently in use utilize only superconducting coils, with 27 T the highest field achieved to date (the instrument setup for this system is not compatible with biomolecular NMR measurements due to the narrow bore size (33)). Challenges in the development of hybrid magnets for applications in NMR include field stability and homogeneity, cost of operation, and size considerations. The highest persistent magnetic field strength from a hybrid magnet to date is the 45 T spectrometer at the National High Magnetic Field Laboratory (NHMFL); however, due to the small bore size, applications are limited to condensed matter physics. The first-in-class hybrid magnet intended for applications including biomolecular NMR and chemistry is the 36 T Series Connected Hybrid (SCH) magnet being developed at NHMFL (34), which makes use of Florida-Bitter resistive coils (35) and Nb_3Sn superconducting coils. Designing the resistive and superconducting coils to be powered in series rather than in parallel was a significant achievement that greatly reduced the size, stability, and operating costs of the 36 T magnet relative to the 45 T hybrid magnet. An additional significant achievement of the 36 T magnet is the 40 mm bore size, large enough to accommodate the instrumentation needed for biomolecular NMR experiments. 1 GHz magnets currently produced by Bruker have a standard 54 mm bore. At present efforts are concentrated on achieving the high field homogeneity needed for NMR experiments.

As mentioned above, increased magnetic field strengths in NMR will have a major impact in a significant number of fields including structural biology, materials science, and imaging. NMR studies of inorganic materials will greatly benefit from increased magnetic field strengths as many inorganic nuclei have large quadrupolar coupling constants and/or low natural abundance. Nuclei of particular interest for inorganic applications that have been

shown to benefit from higher magnetic field strengths include ${}^6\text{Li}$ ($I = 1$, 7.6% natural abundance (NA) (36, 37)), ${}^{11}\text{B}$ ($I = 3/2$, 80.1% NA (36, 38)), ${}^{17}\text{O}$ ($I = 5/2$, 0.04% NA (38, 39)), ${}^{25}\text{Mg}$ ($I = 5/2$, 10.0% NA (36, 38)), ${}^{27}\text{Al}$ ($I = 5/2$, 100% NA (38)), ${}^{59}\text{Co}$ ($I = 7/2$, 100% NA (40, 41)), ${}^{73}\text{Ge}$ ($I = 9/2$, 7.7% NA (42)), and ${}^{127}\text{I}$ ($I = 5/2$, 100% NA (43, 44)). These elements are essential components of materials in many fields of inorganic chemistry research, including batteries and fuel cells, semiconductors, optical materials, metal organic frameworks (MOFs), catalysts, and glasses. NMR studies of these materials can yield valuable information often inaccessible by other methods including structure, oxidation state, hydrogen bonding environment, molecular motions, and effects of impurities.

Key applications in the field of structural biology that will benefit from development of ultrahigh magnetic fields include membrane proteins in native-like environments, intrinsically disordered proteins such as amyloids and other neurodegenerative proteins, as well as emergent properties associated with complex environments, such as whole cells and intact viral particles, and bodily fluids (in metabolomics). Increased field strengths will yield enhanced detection of low concentration species and increased chemical shift dispersion. In addition, the vast majority of structural biology work in NMR at present utilizes ${}^1\text{H}$, ${}^{13}\text{C}$, and ${}^{15}\text{N}$ nuclei. However, there are many additional NMR active nuclei with critical roles in biology for which work has been limited to date due to sensitivity- and resolution-related challenges, such as ${}^{35/37}\text{Cl}$ and ${}^{23}\text{Na}$. The group at NIMS in Japan has demonstrated measurements at 24 T on the spin $3/2$ nuclei ${}^{35}\text{Cl}$ and ${}^{37}\text{Cl}$ (44, 45), as well as ${}^{17}\text{O}$ (1, 23). One of the fields with greatest potential for growth with the advent of magnetic fields over 1GHz is ${}^{17}\text{O}$ NMR (39, 46), a critical element in biology which could serve as an exquisite probe for a variety of mechanistic and structural questions, including enzyme catalysis, pharmaceuticals analysis, protein structure and hydration (47, 48), as well as materials/MOF applications mentioned above. To date ${}^{17}\text{O}$ has not been extensively exploited due to its extremely low natural abundance and the often prohibitive cost of isotopic enrichment. In addition to expanding the repertoire of available nuclei, greater sensitivity will improve the ease of studying ${}^{13}\text{C}$ and ${}^{15}\text{N}$ at natural abundance (1% and 0.3% respectively). This is particularly advantageous for systems that cannot be readily isotopically labeled, as well as for *in vivo* studies. In the following sections we discuss several methods with potentially powerful applications in structural biology at ultrahigh magnetic fields, with regards to achieving the highest quality data, as well as methods to address pressing questions related to protein structure and function. We also highlight several recent notable applications of ultrahigh fields to the study of increasingly complex biological problems.

2. Methods for the Study of Biomolecules at Ultrahigh Magnetic Fields

Ultrahigh magnetic fields benefit the study of macromolecules both by solution NMR and solid-state NMR. At higher fields, the chemical shift dispersion increases linearly. As discussed above, increased field strengths improve both sensitivity and resolution. Narrower line widths facilitate the atomic resolution structural analysis of large biomolecules, ranging from soluble proteins, to protein assemblies, biological complexes, and membrane-embedded and disordered proteins.

2.1 Sensitivity and Resolution Enhancement

Detection Methods: Solid-State and Solution NMR—With increasing magnetic field strengths and concomitant improvements in sensitivity and resolution, additional detection methods have become available to both solution and solid-state NMR spectroscopists. To obtain maximum sensitivity it is preferable to detect on the highest γ nucleus. Given the large ^1H gyromagnetic ratio, for many biological systems, this means ^1H -detection. Another consequence of proton's high γ is the very strong ^1H - ^1H homonuclear dipolar coupling, which is on the order of 100 kHz:

$$D = -\frac{\mu_0 \gamma_1 \gamma_2 \hbar}{4\pi r_{12}^3}$$

where r_{12} is the distance between the two nuclei. In the absence of motional averaging, the strong ^1H - ^1H dipolar coupling causes severe line broadening. Molecular tumbling in solution averages the dipolar coupling, yielding narrow proton lines. Thus in solution NMR, ^1H -detection is the standard approach. However, in the solid state, ^1H lines are very broad. Therefore, magic angle spinning (MAS) NMR experiments traditionally have relied on the detection of ^{13}C and ^{15}N to obtain significantly higher resolution (49–51). However, this approach sacrifices sensitivity given the smaller gyromagnetic ratios of ^{13}C and ^{15}N . With heteronuclear detection in the solid state a large amount of isotopically labeled sample (typically 10–20 mg) and long data acquisition times are required to compensate for the low sensitivity (49, 52).

Given the resolution enhancement obtained at ultrahigh magnetic fields and the development of fast MAS probes (discussed below), it has become feasible to obtain high-sensitivity solution-like ^1H -detected spectra in the solid state. At MAS rates below ~ 100 kHz a degree of ^1H dilution is typically still required to eliminate broadening from residual ^1H - ^1H couplings (*vide infra*). A great deal of work toward the execution of ^1H detection in the solid state has focused on achieving the optimum level of deuteration for the best combination of sensitivity and resolution. Early work indicated that at moderate MAS rates (~ 10 – 30 kHz) relatively high levels of deuteration were required (53–55). Oschkinat and co-workers demonstrated that there is no difference in ^1H linewidths for samples with 10% or 100% $^1\text{H}/^2\text{H}$ back exchange at fast MAS rates (> 60 kHz) and 1 GHz magnetic field (56). Pintacuda and co-workers recently established for 2 proteins (GB1, 56 residues and AP205CP, 130 residues) that with 100 kHz MAS and ultrahigh fields, protein structure determination from ^1H -detected data sets of fully protonated proteins is feasible (57).

In recent years, heteronuclear detection has become increasingly utilized in solution NMR (58, 59). Heteronuclear chemical shifts are sensitive to different dynamics timescales than protons, allowing researchers to gain access to information that may be absent in ^1H -detected experiments. Heteronuclear detection eliminates issues associated with solvent exchange, an important consideration at or near physiological conditions (60). Furthermore, heteronuclear chemical shifts often have greater dispersion than ^1H shifts, an effect that becomes more pronounced at higher magnetic fields and is particularly advantageous for

disordered systems (61). Under certain conditions, heteronuclear detection may not result in loss of sensitivity compared to ^1H detection (62).

Fast Magic Angle Spinning—With significant advancements in probe and coil design, it is currently possible to spin samples at rates up to ~ 110 kHz. Fast MAS is a powerful tool for proton detection in biomolecular systems, which in conjunction with ultrahigh fields, leads to further improvement in spectral resolution. Fast magic angle spinning is able to more efficiently average and at upwards of 100 kHz effectively eliminate the strong ^1H - ^1H homonuclear dipolar coupling. This leads to substantially narrowed ^1H linewidths, making ^1H -detected experiments feasible in the solid state and with recent advances, eliminating requirements for deuteration. Critically, sensitivity losses due to the decreased sample amounts necessitated by the small rotor sizes are compensated by the increased sensitivity of ^1H detection and high magnetic fields (63). Reinstra and co-workers first demonstrated proton-detected spectra of fully protonated GB1 at 40 kHz MAS and a 750 MHz magnetic field strength (64). Subsequently, Marchetti et al. reported the resolution enhancement achieved by increasing the magnetic field strength to 1 GHz with 60 kHz MAS, illustrated by dipolar-based ^1H - ^{15}N CP-HSQC spectra of fully protonated single-stranded-DNA binding (SSB) protein from *E. Coli*. They also determined the unambiguous assignments of this medium-sized protein (18 kDa per monomer) with a set of 2D and 3D proton-detected ^1H - ^{15}N , ^1H - ^{13}C correlation experiments (65). Weingarth and co-workers demonstrated a labeling scheme dubbed inverse fractional deuteration (iFD) to obtain high-resolution ^1H -detected spectra for non-exchangeable protonation sites, such as membrane proteins (66). Recently, the combination of fast MAS (100 kHz) and proton detection at ultrahigh field was applied to a range of structurally diverse, fully protonated proteins including GB1, the bacteriophage coat protein AP205CP, and amyloid-forming HET-s (57, 67). The introduction of fast MAS permits the exploitation of fully protonated proteins for structural and dynamics investigations of biological macromolecules with ^1H detection. Working with protonated proteins is preferred, not only for cost and time savings, but also for the ability to access ^1H - ^1H distances, which can provide additional long-range information among side-chains to improve accuracy of protein structure determination.

Non-Uniform Sampling—One approach for resolution or sensitivity enhancement is non-uniform sampling (NUS). Traditionally, in uniformly sampled data sets, points acquired in indirect dimensions are evenly spaced with respect to time. In NUS applications, during acquisition, acquired points are distributed non-linearly according to a chosen sampling scheme. Rather than discrete Fourier transform, NUS data sets are processed using reconstruction algorithms (68). Non-uniform sampling using random schedules weighted by a decaying function results in bona fide time domain sensitivity enhancements by reducing the number of points acquired, thereby allowing for more transients to be acquired in a given time frame (69–72). NUS can also be used to obtain resolution enhancement by acquiring out to a longer acquisition time without increasing the number of points. This is a significant advantage given the enhanced resolution (and thus longer FIDs) obtained at higher magnetic fields (73).

With respect to high magnetic field applications in solution NMR, NUS is particularly valuable for multi-dimensional heteronuclear-detected experiments and challenging applications such as metabolomics (74), disordered proteins (75–77), and in cell NMR (78), with higher dimensionality experiments offering improved resolution. Maximum entropy reconstruction algorithms have been demonstrated to be robust for these applications (79). Several important applications of NUS in the solid state relevant for ultrahigh field experiments have also been demonstrated including quadrupolar nuclei (^{17}O MQMAS) (80), protein assemblies (70, 81), and high-dimensionality (e.g. 4D) proton-detected experiments (81–83) to obtain inter-residue ^1H - ^1H distances (methyl-methyl or amide-amide) for structure determination.

2.2 Resonance Assignment Strategies

To determine structures and examine dynamics of biological macromolecules by NMR, the first, essential step is unequivocal resonance assignments of the individual backbone and sidechain atoms in the target protein. Resonance assignments are determined by a set of 2D and 3D (and sometimes higher dimensionality) experiments that provide intra- and inter-residue backbone and side-chain correlations. To complete assignments and overcome challenges of spectral resolution associated with large proteins and complex biological systems, several strategies have been developed. In conjunction with ultrahigh fields, choice of appropriate labeling schemes for sample preparation and novel pulse sequences further contribute to obtaining high-resolution spectra for accurate and rapid resonance assignments.

Labeling Schemes—Many isotope labeling schemes exist to achieve spectral simplification as well as reduced linewidths due to decreased spin diffusion/transverse relaxation. In MAS NMR, precise structure calculations typically require ^{13}C - ^{13}C distance restraints and torsion angles. There are a number of sparse/extensive (as opposed to uniform) ^{13}C labeling schemes, such as [1,3- ^{13}C]-glycerol, [2- ^{13}C]-glycerol, [1,6- ^{13}C]-glucose, and [2- ^{13}C]-glucose metabolic precursors (52, 84–86) used to obtain distance restraints of 2–7 Å (52, 87). In addition, partial deuteration labeling schemes such as RAP, ILV, and SAIL are suitable to reduce homonuclear dipolar couplings and transverse relaxation, and resolve aliphatic ^1H - ^{13}C correlations with high resolution and sensitivity in both solution and solid state NMR (53, 55). To obtain increased resolution enhancement of aliphatic protons for tertiary structure determination, Reif and co-workers introduced Reduced Adjoining Protonation (RAP, (88)), in which side chain protonation is diluted by expressing the protein with ^2H -glucose and a low $\text{H}_2\text{O}/\text{D}_2\text{O}$ ratio (5–15% and 85–95% respectively) to obtain aliphatic ^1H linewidths ~ 25Hz. In this approach, re-protonation at exchangeable sites is not needed. ILV labeling schemes, introduced by Kay and co-workers for selective, stereospecific protonation of methyl groups in Ile, Leu, and Val (89–91) have been extended to Ala, Thr, and Met (for review see (92, 93)). This labeling scheme allows for the measurement of more ^1H - ^1H NOEs in a deuterated protein and for the study of larger proteins by solution NMR. A cell-free protocol that has been used is stereo-array isotope labeling (SAIL) of methyl, methylene, and aromatic groups with a combination of $^1\text{H}/^2\text{H}/^{12}\text{C}/^{13}\text{C}$ patterns (94). The cell free expression protocol prevents isotope scrambling and like ILV-labeling yields narrower lines than uniform ^2H , ^{13}C , ^{15}N -labeling. All of these labeling schemes are fully applicable for experiments at high magnetic fields.

Pulse Sequence Design—Just a few decades ago, solution NMR studies of biological systems were limited by the size of the target protein (~ 30 kDa). Since the introduction of Transverse Relaxation Optimized Spectroscopy (TROSY), solution NMR can be applied to analyze targets with molecular weights of up to 100 kDa in solution (95, 96). At high fields, the transverse relaxation (T_2) is dominated by the chemical shift anisotropy (CSA) and dipole-dipole coupling (DD). The TROSY pulse sequence suppresses T_2 by employing constructive interference between the CSA and dipolar coupling (96). Thus, the sensitivity enhancement yielded by the TROSY sequence is more pronounced at higher magnetic fields, with the optimal field strength for ^1H -detected TROSY being 1 GHz (97, 98).

While ^1H -detection has been the method of choice in solution NMR, ^{15}N - and ^{13}C -detected pulse sequences have been developed and make use of increased magnetic fields to achieve higher resolution and site-specific information. Takeuchi et al reported a modified TROSY sequence selective for amide protons (TROSY $^{15}\text{N}_\text{H}$), which benefits from the slow T_2 and overcomes the low sensitivity of ^{15}N (62, 99). Theoretical simulations indicate maximum sensitivity of TROSY $^{15}\text{N}_\text{H}$ at a strength field of 1.2 GHz, and the narrowest line width at 900 MHz (Fig. 2(A), (99)), allowing solution NMR spectroscopists to take advantage of higher field strengths where ^1H -detected TROSY suffers from reduced resolution. With this approach, deuterated samples are not necessary and high-resolution spectra can be obtained using a ^{15}N -detected TROSY-HSQC experiment (Fig. 2 B,C). Recently, Yoshimura et. al. demonstrated a ^{13}C -detected NMR experiment for probing arginine side chain $^{15}\text{N}^\eta/\epsilon\text{--}^{13}\text{C}^\zeta$ correlations at high magnetic field strengths, which facilitates determination of the arginine ionization states indicating conformational changes in the target protein (100). This method also makes use of J-based cross polarization, a method that has shown particular promise for disordered proteins as well (101, 102).

In the solid state, the development of proton detection in conjunction with fast MAS and ultrahigh fields enables the use of solution-state derived pulse sequences including 3D ^1H -detected pulse sequences for resonance assignments. ^1H -detected CP-HSQC experiments (103) have been utilized to build 3D ^1H - ^{13}C - ^{15}N correlation experiments, for example the inter-residue (H)CONH and intra-residue (H)CANH experiments (103), with additional ^{13}C - ^{13}C correlation dimensions added for increased information content, e.g. DREAM (dipolar recoupling enhanced by amplitude modulation (104, 105)). At spinning speeds above 40 kHz, dipolar magnetization transfers become less efficient. By employing ^{13}C - ^{13}C J-based coherence transfer blocks, multiple ^{13}C - ^{13}C transfers are achieved in additional experiments for complete backbone resonance assignments: (H)CO(CA)NH, (H)(CO)CA(CO)NH, (H)(CA)CB(CA)NH, and (H)(CA)CB(CACO)NH (63, 106)). For RAP labeled samples, a pair of 3D (H)CCH and HCC(H) experiments were developed by Asami and Reif (Figure 3 (107)) for assigning $^1\text{H}\alpha$ resonances based on correlations with $^{13}\text{C}\alpha$ and $^{13}\text{CO}/\text{C}\beta$. High resolution was achieved with ^{13}CO and $^{13}\text{C}\beta$ homonuclear scalar decoupling during the $^{13}\text{C}\alpha$ evolution period (108). An extension of these sequences, (HxCx)C γ CxHx experiments, can also be applied for assignments of ^{13}C -ILV-methyl labeled protonated samples (109).

2.3 Methods for Structural and Dynamics Studies of Biomolecules

Many methods for the structural and dynamics characterization of biomolecules benefit from applications at high and ultrahigh magnetic fields. Certain methods, such as measurement of ^1H CSAs, benefit directly from the higher fields as a result of changes in field-dependent nuclear properties. Other methods become easier to execute at higher fields due to inherent improvements in sensitivity and resolution, such as applications of relaxation and paramagnetism in the solid state. Here we highlight several methods that have been successfully applied at high and ultrahigh magnetic fields. Given the detailed structural and dynamics information these methods can provide, they will be essential components of ongoing and future work at ultrahigh magnetic fields.

^1H - ^1H Distance Restraints—Distance restraints are a principal input for structure determination by NMR. In solution NMR structure determination, NOE-derived ^1H - ^1H distances are a key restraint for structure determination, with an upper limit of ~ 5 Å. Until recently, distance restraints in the solid state were largely limited to heteroatoms such as ^{13}C - ^{13}C and ^{15}N - ^{13}C distances, which can typically probe distances up to ~ 7 Å. High magnetic fields in conjunction with fast MAS and ^1H detection presents the opportunity to obtain accurate, high-resolution ^1H - ^1H distance restraints. ^1H - ^1H restraints can access longer distances (up to ~ 13 Å) and correlations not readily detected otherwise, such as inter-residue sidechain methyl-methyl distances with high-quality multi-dimensional ^1H - ^{15}N and ^1H - ^{13}C spectra and have proven valuable for tertiary structure determination (81). Early work on small molecules and model proteins utilized moderate MAS rates with heteronuclear detection (110, 111). Increased MAS rates and the use of deuteration schemes enabled the application of proton-detected ^1H - ^1H distance restraints for structure determination (88, 103, 112). While previously extensive deuteration and/or selective ^{13}C labeling (e.g. RAP or ILV) had been required, it was recently demonstrated that ^1H - ^1H restraints of up to ~ 5.5 Å can be obtained for uniformly ^1H , ^{13}C , ^{15}N -labeled samples at 1 GHz field with ~ 100 kHz MAS (57). Numerous schemes for acquiring proton-detected ^1H - ^1H distance restraints at ultrahigh fields with fast MAS have been proposed. 3D (H)XHH with H-H RFDR mixing (113) has been used in structure determination of SOD (114), GB1 (57), and AP205 (57), with higher dimensionality experiments providing enhanced resolution and unambiguous assignments (112). Meier and co-workers applied DREAM (Dipolar Recoupling Enhanced by Amplitude Modulation) ^1H - ^1H recoupling to obtain methyl-methyl contacts (82), and was applied for structure determination of M2 (115) and the cytoskeletal assembly BacA (81). Several groups have applied non-uniform sampling for 4D ^1H - ^1H correlation experiments (81–83). Based on the success of these solid state NMR methods for obtaining ^1H - ^1H distance restraints applied to small- and medium-sized proteins, measurements of ^1H - ^1H distance restraints are very promising for applications to complex biological systems at ultrahigh magnetic fields.

Measurement of ^1H Chemical Shift Anisotropy—Hydrogen bonding in proteins is a key determinant of secondary and tertiary structure, and thus is a very valuable parameter for structure determination. The proton chemical shift tensor, and especially its anisotropic component, is highly sensitive to the hydrogen bonding environment (116, 117). Further, the ^1H CSA is sensitive to dynamics on the submillisecond to microsecond timescales. In

^{15}N CSA cross correlation was not sufficiently suppressed in the initial decay affecting accuracy of the R_1 measurements. The aforementioned cross correlated CSA-dipolar relaxation interference is a critical component of many solution NMR methods including the TROSY and nuclear Overhauser (NOE) techniques and CSA measurements. TROSY line narrowing is dependent on the CSA/DD cross-correlated relaxation, and hence the CSA magnitude and B_0 field (96). ^{15}N -detected TROSY takes advantage of the cross-correlated relaxation rate at high magnetic fields (900 MHz-1.2 GHz) to achieve comparable resolution and sensitivity to ^1H -detected TROSY (99), allowing solution NMR spectroscopists to exploit higher magnetic fields without losing sensitivity or resolution due to unfavorable relaxation effects.

Paramagnetic effects provide a great deal of long-range structural information for proteins that contain paramagnetic centers, which may be endogenous or synthetically incorporated. In close proximity to a paramagnetic center, four effects exist: paramagnetic relaxation enhancement (PRE), hyperfine shift (which includes the contact and pseudocontact shifts (PCS)), residual dipolar coupling (RDC), and cross-correlated relaxation (CCR) effects (135). Paramagnetic centers used in NMR applications include nitroxide radicals, Mn^{2+} , Gd^{3+} , Cu^{2+} , Co^{2+} , and lanthanides (136). Paramagnetic centers provide angular and distance information to facilitate the determination and further refinement of protein structures, with the distance and orientation dependence of PREs, PCSs, and RDCs used as restraints in NMR structure calculations (137). Paramagnetic centers have been used to examine many biological systems, such as metalloproteins, cysteine-containing targets attached with a metal tag, and biomolecules modified with a nitroxide spin-label (138–142). Measuring site-specific PREs requires examination of relaxation decays dependent on the proximity of the paramagnetic center while PCSs are determined by the difference in chemical shifts between a diamagnetic and a paramagnetic species of the target protein (143).

With remarkable progress in proton detection and fast MAS at high magnetic fields, directly detected ^1H spectra offer high sensitivity and resolution for rapid, quantitative measurements of PREs and pseudocontact shifts in MAS NMR. A further advantage of solid state NMR studies of paramagnetic systems is the absence of Curie relaxation, which leads to line broadening in solution, and very fast MAS allows for the observation of signals close to the paramagnetic center (142). ^1H -detected, fast MAS PRE and PCS measurements of the metalloenzyme superoxide dismutase (SOD) doped with paramagnetic Cu^{2+} or Co^{2+} demonstrate the potential power of ultrahigh fields for the study of paramagnetic systems (128, 143, 144). The different properties of these two paramagnetic centers made one ideal for PRE measurements (Cu^{2+}) and the other better suited for PCS measurements (Co^{2+} , Fig 4(B-D)). With the high-resolution ^{15}N - ^1H CP-HSQC spectra, PREs were obtained from ^{15}N and ^{13}CO R_1 relaxation rates for distances of up to ~ 20 Å (Fig 4(A)). High sensitivity enabled the use of 3D experiments for unambiguous assignment of the PCSs. These paramagnetic restraints were combined with ^1H - ^1H distances to subsequently determine a well-defined backbone geometry and metal binding sites in human SOD (Fig 4(E-H)). It has also been demonstrated that ^1H T_2 relaxation rates can be used to obtain distance restraints of up to 32 Å (145). With access to spectrometers at and beyond 1 GHz, site-specific PRE and PCS measurements will be a significant contribution to structural and dynamics

investigations of complex biological environments and high molecular weight macromolecules.

Quadrupolar Nuclei—Quadrupolar nuclei (spin $I > 1/2$) account for a significant number of NMR active nuclei and these elements, including Li, B, O, Na, Al, Cl, and Ca are essential components of biology, materials science, and pharmaceuticals. NMR studies of quadrupolar nuclei can yield information such as the chemical and hydrogen bonding environment and molecular motions. The study of quadrupolar nuclei with NMR has been a challenge due to line broadening induced by the strong quadrupolar couplings and the very broad (MHz) spectra limiting sensitivity and making it difficult to obtain uniform excitation. Higher magnetic fields are beneficial in the study of quadrupolar nuclei because the second order quadrupolar coupling, which typically cannot be eliminated by MAS alone, becomes smaller, hence observed central transition lines are narrower. Improved sensitivity is also beneficial, particularly for low- γ nuclei.

Early methods for the study of quadrupolar nuclei in the solid state included double frequency sweeps (DFS), rotor-assisted population transfer (RAPT), hyperbolic secant (HS) pulses, and double rotation (DOR) (for review see (146) and references therein). A significant advancement in the study of quadrupolar nuclei in the solid state was the development of multiple quantum magic angle spinning (MQMAS (147)), which removes the quadrupolar broadening, allows for the resolution of different species by retaining the isotropic shift, and enables quadrupole to spin-1/2 correlations (148). Though sensitivity can be limited, the method is more robust and easier to implement than others (149, 150). When very broad quadrupolar lines are present, static methods can be a better choice than MAS. Static methods for ultra-wideline measurement of quadrupolar nuclei include quadrupolar echo and QCMPG (quadrupolar Carr-Purcell-Meiboom-Gill (151, 152)) which uses refocusing pulses during acquisition to record the spin echo and recapitulates the NMR lineshape. Typically several frequency steps (i.e. several spectra at different transmitter frequencies) are required to record the full spectrum. Alternatively, Schurko and co-workers have demonstrated the use of adiabatic pulses to excite a broader region of the spectrum, dubbed adiabatic WURST (wide-band uniform-rate smooth truncation (153)) in combination with QCPMG to obtain broadband excitation and high sensitivity (154, 155).

Quadrupolar relaxation can be exploited for solution NMR structure and dynamics studies of quadrupolar nuclei (156, 157). One approach that exploits quadrupolar relaxation and has been successful for the study of quadrupolar nuclei in the context of biological macromolecules (including ^{17}O , ^{27}Al , and ^{51}V) is quadrupole central transition (QCT) NMR (48, 158–160), which utilizes relaxation properties of the central transition at high magnetic fields (i.e. the slow tumbling regime) to obtain narrow lines. The combination of high magnetic fields and narrow lines allows for the observation of fine structural details and applications to larger biological systems (158), as demonstrated in ^{17}O QCT experiments to study the catalytic mechanism of the enzyme Tryptophan synthase (48).

3. Applications of Ultrahigh Fields to the Study of Biological Systems

Ultrahigh field NMR spectroscopy is a powerful tool to investigate the structural and dynamics properties of macromolecules and their physiological relevance. In the past decade, numerous studies have convincingly demonstrated that increased magnetic fields bring improved resolution and sensitivity, enabling applications in both solution and solid states, including protein-ligand binding interactions (161, 162), and structure determination of soluble macromolecules (163, 164), cellular and viral assemblies (165), intact virus particles (166), membrane proteins (167), and amyloids (168, 169). Ultrahigh field NMR has demonstrated promising high quality results for future atomic-resolution studies of these highly challenging macromolecule targets and others, such as intrinsically disordered proteins (IDPs, (61, 170)) and whole cells (171). Here, we highlight several remarkable studies benefited by ultrahigh field NMR techniques to solve interesting biological problems, as well as the current state-of-the-art in emerging fields that will benefit greatly from these technological developments.

The development of proton detection techniques under fast MAS frequencies at ultrahigh fields significantly expands the application of MAS NMR to biological complexes and large proteins. In recent years, the Pintacuda group has developed a rapid proton detection protocol (discussed in section 2.2) and applied it to numerous proteins in a range of assembly states, including the metalloenzyme SOD, microcrystalline SH3, β 2m, sedimented nucleocapsids of AP205, the membrane protein M2 channel, and OmpG (63).

Structure determination using proton detection in fully protonated, uniform ^{13}C , ^{15}N -labeled AP205CP was achieved with MAS frequencies of 100–111 kHz at 1 GHz magnetic field (57). AP205CP is a dimeric protein comprising the 2.5-MDa viral capsid of a single-stranded RNA bacteriophage. As shown in Figure 5, a series of remarkably high-resolution ^1H -detected spectra including ^{13}C - ^1H , ^{15}N - ^1H CP-HSQC and (H)CCH experiments were used to obtain unambiguous assignments of about 78% of backbone protons and 65% of all proton resonances. In 3D (H)CCH experiments, ^{13}C resonances of the aliphatic side chains were correlated to their protons, providing proton side chain information. 3D (H)CHH_{RFDR} spectra were acquired to record the inter-molecular ^1H - ^1H contacts for the final structure calculation (Fig 5C). Importantly, 104 inter-monomer contacts were detected, which served to define the dimer interface. The AP205CP dimer structure was determined by this approach with 0.5 mg of protein, less than 2 weeks of instrument time, and rapid analysis of the high-quality data. Further, together with this MAS NMR structure of AP205CP, an atomic-resolution model of the AP205 virus like particle (VLP) was proposed based on the crystal structure of the protein dimer and the cryo-EM (cryo-electron microscopy) structure of the assembled particle (172). Significantly, this somewhat heterogeneous sample exhibited broader ^1H linewidths than GB1 (0.15–0.2 ppm vs 0.1 ppm for $^1\text{H}^{\text{N}}$), but a high resolution structure could still be determined, suggesting the applicability of the approach to other challenging systems.

Andreas et al. reported the MAS NMR structure of a drug-resistant mutation of Influenza A M2_{18–60} in lipid bilayers (S31N mutant) (115). M2 is a tetrameric transmembrane protein that triggers membrane fusion by transporting protons at low pH during viral replication

(173, 174). Understanding the structure and H^+ transport mechanism of this drug-resistant mutant is essential for the development of novel influenza inhibitors. To obtain long-range distance restraints, several samples were prepared with different isotopic labeling schemes for various measurements, including uniform $^{13}C/^{15}N$ labeling, extensive 1,6- ^{13}C glucose labeling, labeling by residue type, deuteration and ILV-methyl labeling. Using MAS NMR at high and ultrahigh fields (750 MHz – 1GHz), well-resolved 2D and 3D ^{15}N - ^{13}C , ^{13}C - ^{13}C , and a series of 1H -detected spectra (63) were utilized for resonance assignments of M2. Many residues in the spectra exhibited peak doubling, which indicated M2_{18–60} forms a two-fold symmetric dimer of dimers (Fig 6A). In addition to ^{13}C - ^{13}C and ^{15}N - ^{13}C long range correlations, 1H -detected 3D and 4D experiments were performed in order to measure inter-helical distances necessary for tetramer structure determination. 3D and 4D variations of (H)CHH (Fig 6 B-F) and (H)NHH experiments provided intermolecular contacts including methyl-methyl contacts and H37-W41 imidazole-indole contacts (Fig 6G). The M2 structure was determined with a total of 283 structural constraints. Different side chain conformations were observed for the gating and pH-sensing residues W41 and H37, indicating that two of the four helices exhibited an “indole in” conformation in one dimer and an “indole out” conformation in the other (Fig 6 H-J). This novel structure suggests that W41 and H37 are likely important in the H^+ transport process. Furthermore, the conformation at the S31N mutation lends insight into the mechanism of drug resistance. Through this work, a protocol was established for structure determination of membrane proteins and other challenging protein targets at ultrahigh fields.

Barbet-Massin et al. demonstrated the potential to investigate large and complex assemblies with fast MAS at ultrahigh fields (175). They characterized the structure and dynamics of a native-like Measles virus (MeV) nucleocapsid. The MeV nucleocapsid consists of a macromolecular assembly of a 525 residue protein, which has 2 domains: an ordered N_{CORE} domain (residues 1–400) and a disordered N_{TAIL} domain (residues 401–525). CP and J-based ^{15}N - 1H HSQC experiments were used to observe the rigid N_{CORE} and dynamic N_{TAIL} domains respectively. Bulk ^{15}N $T_{1\rho}$ measurements indicated differences in local dynamics between N_{CORE} of intact nucleocapsids and cleaved N_{CORE} domains (where N_{TAIL} is removed by trypsin digestion). An additional advantage of 1H -detection at ultrahigh fields is the ability to characterize hydration, solvent accessibility, and inter-molecular packing, which in this work detailed the increased disorder of the intact nucleocapsids relative to the cleaved N_{CORE} domain. These studies further demonstrated that sample preparation by sedimentation protocols yields narrow proton linewidths under the experimental conditions utilized (i.e. 1 GHz field and 60 kHz MAS), paving the way for future studies of large, heterogeneous systems such as intact viral particles.

The development of ultrahigh field NMR spectrometers open further opportunities for the study of emerging phenomena such as whole cells, intact viral particles, and complex biomaterials. These samples are often very insensitive (low natural abundance, low γ nuclei, or isotopically labeled component is a small percentage of the total sample mass), and, given the complexity of *in vivo* systems, spectra are often crowded and suffer from line broadening. Furthermore, many nuclei of interest such as ^{43}Ca are quadrupolar. These challenges can be overcome by the use of ultrahigh field spectrometers, and take advantage of the numerous methodological advances described above.

Whole Cell NMR

Whole cell NMR enables the study of biological systems under physiological conditions, such as *in vivo* cell density and effects of binding interactions and other elements of the cellular environment (176). Challenges to whole cell NMR include sample heterogeneity and cell integrity over the relatively long acquisition times of NMR experiments. To date only a few examples of *in vivo* protein structure determination have been presented. The first in-cell structure determination by NMR was of a 66 residue heavy metal binding protein overexpressed in *E. coli* using non-uniform sampling to efficiently acquire data before sample degradation (78). The same protocol was subsequently applied to structure determination of GB1 at 250 μ M concentration, closer to physiological cellular concentrations (177). Another recent study presented structure determination of GB1 with a paramagnetic tag for PCS structural restraints in eukaryotic cells (178). Enhanced sensitivity from ultrahigh magnetic fields will likely increase the efficiency of *in vivo* structure determination of low concentration proteins. While remarkable results have been accomplished with respect to *in vivo* studies of disordered proteins (179), ultrahigh fields in combination with ^{13}C detection methods (60, 102) will further benefit this area of research. NMR of IDPs suffers from severe sensitivity and resolution challenges due to dynamics, solvent exchange, and the lack of an ordered secondary structure. Lippens and co-workers presented a method for combined J- and CP-based correlation with ^{13}CO detection at 900 MHz for characterization of disordered α -synuclein in *E. coli* cells (102). Whole cell solid-state NMR studies have covered a wide range of systems including bacteria and biofilms to address many significant questions including perturbations in bacterial cell walls induced by drug binding (180, 181), cellular composition (182) and metabolomics (183), as well as monitoring of protein expression and cellular processes (184–186). These studies are essential to address pressing issues such as antibiotic resistance (187–189). In cell MAS NMR studies typically rely on ^{13}C detection and due to the complex, heterogeneous environments resolution is a hindrance to obtaining atomic level information. The expanded use of ultrahigh magnetic fields, associated gains in sensitivity and resolution, and continued methods developments will significantly advance whole cell structural and dynamics studies by NMR.

Biomaterials is a broad discipline with both technological and clinical applications. NMR is a potentially powerful method for characterizing the structure and dynamics of biomaterials. Recent advances in NMR including ultrahigh field spectrometers will further expand the role of NMR in the study of biomaterials. There is great interest in furthering our understanding of the effects of disease and aging on human bone. MAS NMR at ultrahigh fields is uniquely suited to characterize the high-resolution structural features of bone for both high-resolution studies of ^1H nuclei, as well as improved resolution for studies of quadrupolar nuclei that form essential components of bone such as ^{23}Na and ^{43}Ca . Ramamoorthy and co-workers demonstrated the first ^1H - ^1H correlation spectra of bone at fast MAS (up to 110 kHz (190)) and extracted relaxation parameters to characterize the water distribution in bone. ^{43}Ca (0.14% natural abundance) studies of hydroxyapatite, the primary component of bone, detected 2 different calcium coordination environments not observed at lower B_0 fields, and determined the NMR parameters (δ_{iso} , C_Q , η_Q) of the two local environments (191) which were subsequently observed in bovine cortical bone at 833 MHz (192). In addition to ^{43}Ca

NMR, Laurencin et al. used ^{23}Na of equine bone and bovine tooth to identify two sodium sites in those materials (193). For these studies, high magnetic fields (800 MHz) were valuable to overcome challenges associated with quadrupolar nuclei. Biosilica materials, such as enzymes encapsulated in or immobilized on an inorganic matrix of silica, are of great interest for their applications in biotechnology and chemical catalysis (194, 195). Catalytic activity of biosilica-associated enzymes requires conservation of active site structure upon association with the silica network. Stability of the catalytic complex is also essential. It has recently been demonstrated that MAS NMR at ultrahigh fields is a potentially powerful means to characterizing protein structure in these biosilica materials (196). Luchinat and co-workers showed sufficient resolution can be achieved at fast MAS and high fields (850 MHz) for ^1H -detected studies of biosilica-encapsulated proteins (190). Another application of biosilica materials, diatoms are unicellular algae with a cell wall composed of silica. Structural features of the diatom cell wall are of interest for a wide range of applications including biotechnology, catalysis, and nanotechnology (197). ^{29}Si NMR is a common approach for studies of both whole cell and isolated cell wall components of diatoms (see (198) for review). While to our knowledge, ultrahigh field studies have not been reported to date, this is another active field of biosilica research that may benefit from greater magnetic field strengths and associated advances.

4. Conclusions

Recent advancements in superconductor technology have made magnetic field strengths over 1 GHz accessible for NMR studies of biomolecular systems. Significant challenges such as field inhomogeneity have needed to be overcome for these instruments to provide atomic level information. Ultrahigh field spectrometers will have a significant impact in a wide range of disciplines including biology and materials science. Numerous methods have been developed that will further enhance the resolution and sensitivity gains that are realized as increased magnetic field strengths. The pioneering applications highlighted in this work indicate the promise of experiments at ultrahigh fields of 1 GHz and beyond in for a variety of complex biomolecules and biomaterials.

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Fig. 1.

(a) Advancements in magnetic field strengths for NMR over the last few decades. Yellow indicates LTS magnets while green indicates dual LTS/HTS magnets. The dashed line indicates a frequency of 1 GHz (23.5 T). (b) General schematic of dual LTS/HTS magnet including shim coils and superconducting magnet coils. The precise number of LTS and HTS coils may vary. Purple represents superconducting shim coils, while blue represents outer NbTi LTS coils, orange represents Nb₃Sn inner LTS coils, and green represents HTS coils. Within the magnet bore, maroon (outer) and pink (inner) bars represent the ferromagnetic and room temperature shim coils respectively.

Fig. 2.

(a) Simulated plots of TROSY $^{15}\text{N}_\text{H}$ relaxation rates and relative peak heights as a function of magnetic field strength. (b) Pulse sequences for the ^1H - (right) and ^{15}N - (left) detected 2D TROSY-HSQC experiments. (c) Left: ^{15}N -detected TROSY-HSQC, right: ^1H -detected TROSY-HSQC. (d) Top: ^{15}N projection of the 2D ^{15}N -detected TROSY-HSQC (left) and ^1H -detected TROSY-HSQC (right). Bottom: Selected ^{15}N cross sections of the 2D ^{15}N -detected TROSY-HSQC (left) and ^1H -detected TROSY-HSQC (right). Reprinted with permission from Takeuchi, K et al., *J. Biomol. NMR.*, **2015**, *63* (4), 323–331. Copyright 2015 Springer.

Fig. 3.

(a) Pulse sequences of 3D (H)CCH and HCC(H) experiments for RAP-labeled proteins with (b) schematic of magnetization transfers at each step of the pulse sequence. Adapted with permission from Asami, S et al., *J. Biomol. NMR.*, **2012**, *52* (1), 31–39. Copyright 2012 Springer.

Fig. 4. Paramagnetic effects in $\text{Cu}^{2+}/\text{Co}^{2+}$ loaded SOD by MAS NMR. (a) ^{15}N and ^{13}CO R_1 and $R_{1\rho}$ relaxation rates for Cu^{+} - (red, diamagnetic) and Cu^{2+} - (blue, paramagnetic) bound SOD (144). (b) CP-HSQC spectra of Zn^{2+} - (black, diamagnetic) and Co^{2+} - (purple, paramagnetic) bound SOD (144). (c) Left: anisotropic susceptibility tensor for Co^{2+} with respect to SOD structure. Right: Identification and assignment of PCS from 3D (H)CONH experiment, color scheme as in (b). (d) Expansions of select PCSs from 3D (H)CONH experiment, color scheme as in (b). (e-h) Structure refinement of SOD (143). (e) No paramagnetic restraints, (f) with PRE restraints, (g) with PCS restraints, (h) with PRE and PCS restraints. (a) Reprinted with permission from Knight, MJ et al., *Proc. Nat. Acad. Sci.*, **2012**, 46(9), 2108–2116. (b-d) Reprinted with permission from Knight, MJ et al., *J. Am. Chem. Soc.*, **2012**, 134(36), 14730–14733. Copyright 2012 American Chemical Society. (e-h) Reprinted with permission from Knight, MJ et al., *Acc. Chem. Res.*, **2013**, 46(9), 2108–2116. Copyright 2013 American Chemical Society.

Fig. 5. Structure determination of the viral protein AP205CP assemblies by MAS NMR at ultrahigh field. (a) (left) A superposition of ^{15}N - ^1H CP-HSQC spectra of (red) a fully protonated sample at 100 kHz and (black) a perdeuterated, 100% N^{H} sample at 60 kHz MAS frequency. (right) Expansions of ^{13}C - ^1H CP-HSQC spectra of AP205CP methyl (top) and $\text{H}\alpha$ - $\text{C}\alpha$ (bottom) regions at 100 kHz MAS and 1 GHz field. (b) ^{13}C - ^{13}C correlation spectrum (top) and select strips of a 3D (H)CCH spectrum of AP205CP (bottom). (c) Selected strips of a 3D (H)CHH spectrum for determination of distance restraints. The intermolecular peaks are underlined. (d) Expansion of the AP205CP dimer structure showing the restraints extracted from (c). Adapted with permission from Andreas, LB et al., *Proc. Nat. Acad. Sci.*, **2016**, *113* (33), 9187–9192.

Fig. 6.

Structural and mechanistic studies of the membrane protein Influenza A M2 by MAS NMR at ultrahigh field. (a) Assigned ^{15}N - ^1H CP-HSQC spectrum recorded at 60 kHz MAS and 1 GHz field. Peak doubling labeled in black and blue indicates the different conformations for each subunit of the dimer. (b) Methyl region of a 2D ^{13}C - ^1H J-based spectrum of ^{13}C , $^2\text{H}_2$, ^1H -ILV labeled M2 and (c-f) selected strips of a 4D HCHHCH spectrum. (g) H37'-W41 inter-residue cross peaks from an (H)NHH_{RFDR} experiment. (h) Positions of H37 and W41 in the M2 dimer of dimers. (i) M2 pore surface indicating water accessibility/pore width: red: $< 1 \text{ H}_2\text{O}$, green = $1 \text{ H}_2\text{O}$, and blue $> 1 \text{ H}_2\text{O}$. (j) C-terminal H37 and W41 adopts two different conformations in the dimer of dimers. Adapted with permission from Andreas, LB et al., *J. Am. Chem. Soc.*, **2015**, *137*(47), 14877–14886. Copyright 2015 American Chemical Society.