**Solid-state NMR approaches to investigate large enzymes in complex with substrates and inhibitors**

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**Abstract**

Enzyme catalysis is omnipresent in the cell. The mechanisms by which highly evolved protein folds enable rapid and specific chemical transformation of substrates belong to the marvels of structural biology. Targeting of enzymes with inhibitors has immediate application in drug discovery, from chemotherapeutics over antibiotics to antivirals.

NMR spectroscopy combines multiple assets for the investigation of enzyme function. The non-invasive technique can probe enzyme structure and dynamics and map interactions with substrates, cofactors and inhibitors at the atomic level. With experiments performed at close to native conditions, catalytic transformations can be monitored in real time, giving access to kinetic parameters. The power of NMR in the solid state, in contrast to solution, lies in the absence of fundamental size limitations, which is crucial for enzymes that are either membrane-embedded or assemble into large soluble complexes exceeding hundreds of kilodaltons in molecular weight. Here we review recent progress in solid-state NMR methodology, which has taken big leaps in the past years due to steady improvements in hardware design, notably magic angle spinning, and connect it to parallel biochemical advances that enable isotope labelling of increasingly complex enzymes.

We first discuss general concepts and requirements of the method and then highlight the state-of-the-art in sample preparation, structure determination, dynamics and interaction studies. We focus on examples where solid-state NMR has been instrumental in elucidating enzyme mechanism, alone or in integrative studies.

**Underlined terms in bold print are explained in the Glossary.**

**Introduction**

Enzyme catalysis is required for the vast majority of biochemical reactions to attain rates compatible with the time scales of living organisms. Highly evolved three-dimensional protein structures confer the required specificity and efficiency. Yet equilibrium structures alone do not explain catalysis – the conformational transitions of both the enzyme and its substrate along the reaction pathway do. A detailed mechanistic understanding of enzyme catalysis is desirable, given that potent drug molecules are enzyme inhibitors: protease inhibitors combat viral infections, antibiotics such as penicillin target bacterial enzymes while chemotherapy agents are often inhibitors of human enzymes.

To investigate enzymes at the atomic level, nuclear magnetic resonance (NMR) spectroscopy combines several assets. The technique relies of the interaction of atomic nuclei that possess a magnetic moment with a strong constant magnetic field. The interactions are modulated and probed by a second weak oscillating magnetic field. The direct and non-invasive observation of nuclear spins enables atomic resolution at physiological conditions, on hydrated specimen at ambient temperature and even inside living cells. It reports on enzyme structure, interactions with cofactors or substrates, molecular motions as well as kinetic and thermodynamic properties.

While some enzymes consist of single polypeptide chains, many are large multi-component complexes. In the BRENDA enzyme database (1), polypeptide chains exceeding 100 kilodalton in molecular weight represent almost a quarter of entries and a majority of deposited enzymes engage in self-assembly (2). Many enzymes are membrane-associated in their native states. The power of solid-state NMR spectroscopy lies in the investigation of such large and immobilized targets. In this review, we highlight recent achievements and current challenges of this technique.

**Solid-state NMR: scope and requirements**

Proteins in solution undergo random Brownian motion, which leads to their stochastic reorientation. The time scale of this reorientation (**rotational correlation time**, nanoseconds for small proteins up to microseconds for large assemblies) determines the lifetime of nuclear spin signals, with the effect that as molecular weight increases, sensitivity of NMR experiments is lost and resonance lines broaden, leading to a loss of information extractable form the spectra. Even though techniques exist to lengthen the coherence lifetime (3), solution-state NMR of biomolecules exceeding 100 kilodaltons has limitations. This size is equivalent to one or more polypeptide chains with a combined length of approximately 1000 amino acids.

In solid-state NMR spectroscopy (ssNMR), on the other hand, the random reorientation of molecules is restricted, defined by rotational reorientation slowed down to hundreds of microseconds or longer, below the frequencies of strong **nuclear spin interactions** (see Glossary for examples). When these interactions are not motionally averaged, severe signal overlap ensues. Where spectral resolution of single nuclei is desired, *e.g.* in structural studies of enzymes, the specimen is filled into a cylindrical rotor and rotated at a rate of tens of kilohertz inside the NMR spectrometer (4, 5). This coherent imitation of Brownian motion refocuses certain orientation-dependent interactions of the nuclear spins (**magic-angle spinning**, MAS ssNMR, see Figure 1). Importantly, the effect is independent of molecular size. Hence, MAS ssNMR underlies no fundamental limit in the size of immobilized molecules that are accessible to investigation. The rapid sample rotation causes frictional heat yet external cooling still enables experiments all the way from cryogenic to physiological temperatures and above. Practical limits exist regarding the number of non-equivalent nuclear spins whose resonances are resolved in multi-dimensional experiments. Yet due to technical advancements, with regard to field strength of NMR magnets, probe design and achievable MAS rates, design of pulse sequences and, last but not least, sample preparation, the boundaries are being pushed towards more and more complex biomolecules.

Since MAS eliminates interactions of the nuclear spins that carry valuable information, a key concept is **recoupling** of specific interactions. The strong distance dependence of the **dipolar coupling** is exploited to determine distances to below Ångström resolution and thus probe arrangements of nuclei in space. A wide array of dipolar recoupling methods for different nuclear spin combinations have been developed, each geared towards specific applications (6). These experiments are typically referred to under acronyms and such ssNMR jargon is reviewed in Table 1.

The last few years have witnessed a rapid shift in the way experiments are designed: from detection of heteronuclei, typically carbon-13, to protons. Proton detection, which is routine in solution-state NMR, capitalizes on the high magnetic moments of these nuclei to achieve higher sensitivity. In ssNMR, the strong dipolar couplings between the protons were for a long time not sufficiently averaged by MAS, leading to line broadening and resolution loss. Partial deuteration dilutes the dense network of coupled protons and alleviates broadening (7) but it is costly and not compatible with all cell-based systems for recombinant protein production. Owing to commercial probes attaining MAS frequencies above 100 kHz, carbon and proton detection on fully protonated proteins can now compete in sensitivity and resolution – the latter on sub-milligram sample amounts, a reduction of sample requirements by two orders of magnitude (8-10)! As advances in probe design continue, proton detection under fast MAS will profoundly widen the size and complexity of enzymes amenable to ssNMR (11).



**Figure 1. Basic concepts of solid-state NMR spectroscopy.**

**A.** When the sample inside the NMR rotor is rapidly spun around an axis inclined at the **magic angle** with respect to the static magnetic field, certain anisotropic spin interactions are averaged out, including the **chemical shift anisotropy** and the **dipolar coupling**. **B.** The heteronuclear dipolar coupling between two spins in the presence of a strong magnetic field is given by the depicted (secular) Hamiltonian. It depends on the distance *r* between the spins, their gyromagnetic ratios 𝛾 and the angle *θ* between their connecting vector and the magnetic field. **C.** The anisotropy of the chemical shift gives rise to a powder pattern for samples with randomly oriented molecules (as opposed to aligned specimen). **Magic angle spinning** (MAS) averages out their orientations, leaving a signal at the isotropic chemical shift. **D.** The cross-polarization experiment accomplishes polarization transfer from abundant nuclei with high gyromagnetic ratio, typically protons, to rare nuclei with a lower gyromagnetic ratio, for example carbon-13. The transfer is mediated by the dipolar coupling between the nuclei and enabled by matching the amplitudes of the respective radiofrequency irradiation. The technique can be applied on static samples and under MAS. **E.** **Recoupling** enables the reintroduction of desired spin interactions under MAS. For example, the REDOR experiment (see Table 1) reintroduces a heteronuclear dipolar coupling, here between carbon-13 and nitrogen-15, by applying refocusing 180° pulses synchronized with the MAS rotor period. The size of the dipolar coupling encodes the distance between the spins, compare panel B.

**Preparation of enzymes for solid-state NMR**

In biological macromolecules, the nuclei of special interest include protons (1H) and deuterons (2D), carbon-13 (13C), nitrogen-15 (15N), oxygen-17 (17O), phosphorus (31P) and fluorine (19F), the latter being frequently incorporated in drug molecules. Solid-state NMR of quadrupolar nuclei, in particular oxygen-17 and deuterons, has its special challenges and opportunities, notably in motional studies, *e.g.* in observing water (12) and membranes (13).

Except hydrogen, fluorine and phosphorus, many NMR-active atoms need to be artificially enriched in the specimen by stable isotope labelling. For enzymes, uniform labelling with carbon-13, nitrogen-15 and deuterium is routinely achieved by means of recombinant protein biosynthesis in bacterial cells, exploiting labelled glycerol or glucose, ammonium salt and deuterium oxide (heavy water) as relatively affordable starting materials. In addition, a plethora of selective labelling schemes have been tailored to specific applications (14). To decongest crowded spectra of large enzymes, amino acid selective labelling is a straightforward biochemical approach (15) although metabolic scrambling complicates analysis. Segmental labelling of individual domains within multi-domain proteins, followed by chemical or enzyme-mediated ligation, is cleaner but requires optimization (16, 17).

A major impediment to carbon-detected ssNMR has been the requirement for tens of milligrams of purified enzyme per sample, with multiple labelling patterns required for typical projects. Progress in proton detection has pushed this requirement below one milligram, allowing researchers to tackle challenging targets accessible only in small quantities. In the realm of proton-detected NMR, uniform carbon-13 and nitrogen-15 labelling, combined with partial deuteration at MAS rates up to 60 kHz, or full protonation above 100 kHz, are commonly used. A plethora of selective methyl labelling schemes, originally established for solution-state NMR (18), can be readily transferred to the solid state (19). Such sparse labelling bears great potential to study enzyme structure and dynamics. Table 2 sums up typical experimental setups.

To produce functional enzymes from higher organisms, bacteria sometimes lack the molecular machinery for post-translational modifications, folding and insertion into membranes. In such cases, structural biologists resort to eukaryotic expression systems, e.g. yeast, insect or mammalian cells for heterologous expression. The necessity for isotope labelling has delayed their use in NMR but several research groups have now overcome this challenge using insect cells. This requires either the supplementation of expression media with labelled amino acids, which is economical only in a selective manner. Alternatively, labelled home-made yeast or algal extracts can serve as amino acid sources (20, 21) but the cost of such media still exceeds that of bacterial expression by more than tenfold, hopefully to decrease as the approach becomes mainstream. Cell-free protein synthesis, i.e. the process of translation in the absence of cells, is another promising route (22). It combines the strengths of eukaryotic machineries for protein modification and chaperoning with the flexibility to add of cofactors, lipids and isotope labelled building blocks into the reaction mixture (23).

Enzymes are subsequently purified and prepared in their catalytically active states. Recently concerns have been voiced that common detergents potentially destabilize or denature membrane proteins, in particular α-helical ones (24), underscoring the importance of native-like lipid bilayer environments for studies of membrane-associated enzymes. ssNMR is compatible with reconstitution into lipid bilayers membranes or nanodiscs, which are small lipid patches enclosed by protein belts composed of amphipathic helices. This tool has shown success in cryo-EM, solution state NMR (25) and MAS ssNMR (13, 26).

For ssNMR, biomolecules need to be restricted in their rotational freedom. Soluble protein complexes can nevertheless be investigated because centrifugal forces during MAS reach up to 107 gravitational equivalents, which leads to reversible sedimentation of complexes exceeding 100 kilodalton molecular weight, termed SedNMR (27, 28). When proteins pack densely at the rotor wall, concentrations of ~ 400 mg/mL are reached, such that half of space is occupied by protein and half by its hydrated environment. Optimal rotor filling is achieved by sedimentation *ex situ* in an ultracentrifuge using commercial or home-built filling tools for targets above 100 kDa (27, 29). For smaller proteins that do not oligomerize, precipitation or (micro)crystallization procedures are employed. Practical aspects of sample preparation have been comprehensively reviewed in (30). Figure 2 summarizes the typical pipeline to get enzymes into shape for MAS ssNMR studies.



**Figure 2. Preparation of enzymes for MAS ssNMR investigations.**

The flowchart highlights key steps in the production of enzymes for MAS ssNMR studies. To obtain enzymes with NMR active nuclei, recombinant expression is accomplished in bacterial cells staring from simple nitrogen-15 and carbon-13 containing building blocks (**A**). Alternatively, more costly labelled amino acids need to be supplemented into media for insect-cell expression or into the cell-free reaction mix (**B**). Following purification (**C**) and, if required, reconstitution into lipid membranes or nanodiscs (**D**), enzymes are transferred into the relevant functional state for the study, by adding substrates or mimics thereof, inhibitors, activators and cofactors, with isotope labelling if desired (**E**). Optimal rotor filling is accomplished using dedicated filling tools, often with the help of an ultracentrifuge (**F**). The rotor type determines the maximal MAS rate (compare Table 2), which in turn indicates the use of proton detection or heteronuclear detection for ssNMR experiments (**G**). For spectral analysis, structure calculation and molecular docking, dedicated software packages are available (**H**).

**Enzyme structure by MAS NMR**

First step and prerequisite for atomic-resolution NMR studies of enzymes is the sequential assignment, whereby the resonance frequencies of all observable nuclei are matched to their position in the primary sequence. The concept of sequential backbone walks, relying on amide proton and nitrogen plus C𝛼, Cβ and carbonyl atoms to establish connectivity, has been adapted from solution to ssNMR (31). Sets of three- and four-dimensional correlation experiments can be realized in carbon (32) or proton detected (33) fashion, the latter also as a combination of **dipolar** and **scalar** polarization transfers. The acquisition of such datasets can require up to a month of access on a high field magnet, their analysis even longer, making assignment a painstaking aspect in studies of larger proteins. Currently, polypeptide chains of up to 500 amino acids are being tackled. Two enzymes, the aminopeptidase TET2 (350 residues) (34) and the helicase DnaB (460 residues) (35), mark two of the largest assemblies, in terms of unit size, that have been assigned by a combination of proton and carbon detected ssNMR so far. A divide-and-conquer approach, in which domains were first assigned individually, proved successful in the case of DnaB. While complete assignment using a single uniformly labelled sample is desired, the use of multiple samples with complementary labelling schemes is often required, as exemplified on the 280-residue outer membrane protein G in lipid bilayers (15). In this work, the strengths of proton and carbon detection in accessing backbone and sidechain resonances were united, with multiple sparsely and reversely labelled samples. Automatic assignment algorithms, notably FLYA (36), have been adapted for ssNMR and demonstrated on a 370-residue model protein to enable assignment using a single sample (37). An alternative to backbone walks is the structure-based assignment of methyl groups using through-space distance information in combination with automatic assignment (38).

The power of solution-state NMR in protein structure determination relies on the nuclear Overhauser effect, which utilizes the strong distance dependence of the dipolar coupling between nuclei in an indirect, relaxation-mediated manner. In MAS ssNMR, the dipolar coupling is utilized in a direct, coherent manner to obtain distance information via recoupling. The sensitivity of the resonance frequency of each nucleus, reported as the chemical shift parameter, towards protein secondary and tertiary structure is exploited empirically in the form of database approaches for backbone torsion angles (39) and tertiary elements (40). Furthermore, automated structure calculation algorithms (41, 42), analysis software (43) and repositories for sharing data (44) are vital for the NMR community.

In establishing structure determination routines for MAS ssNMR, microcrystalline proteins and amyloid fibrils composed of small building blocks served as early model systems (45). While many fibrils are pathogenic, functional amyloids can be catalytically active, as exemplified by a zinc-binding amyloid that catalyses ester hydrolysis, for which the structure and coordination geometry of the active-site zinc ion were determined (46). Moving towards larger building blocks, recent highlights have been achieved on viral capsids (9, 47). For integral membrane proteins, proton detection under fast MAS shows great promise and has enabled a significant push in the size of targets (15, 48). Specifically in the field of soluble enzymes, the complex of carbonic anhydrase (30 kDa, 260 residues) and a small molecule inhibitor was investigated on fully protonated microcrystalline material under MAS above 100 kHz. With backbone and sidechains proton assignment at hand, proof-of-principle structure determination on a single sample was accomplished including the assessment of histidine protonation states (49). Another highlight in structure determination was achieved on the aforementioned TET2 aminopeptidase complex, which is composed of twelve identical subunits (40 kDa, 350 residues), giving an assembly size just below half a megadalton. The integration of ssNMR restraints and a cryo-electron microscopy (cryo-EM) density map in the medium-resolution range (4 Å) improved the resolution of the structure to 1 Å, more than would be currently feasible with either method alone. In contrast to the previous example, the authors utilized multiple samples and performed carbon and proton detected experiments, the latter relying on backbone amide as well as methyl groups for collecting distance restraints in partially deuterated samples at MAS rates below 60 kHz (34).

**Molecular interactions by MAS NMR**

The coordinates of small molecules, such as enzyme cofactors, substrates and inhibitors, are not always ambiguous from crystal or cryo-electron microscopy (cryo-EM) structures at intermediate resolution. The potential of NMR for deciphering molecular interactions is owed to the extraordinary sensitivity of the chemical shift towards structural changes that result from noncovalent interactions and thus report on binding events. Chemical shift perturbations (CSPs) can be easily determined by comparing spectra of the enzyme in the bound versus unbound states (50, 51). CSPs reflect protein conformational changes induced upon binding, the formation of hydrogen bonds and also through-space (de)shielding effects. Complex formation can be characterized by an exchange rate that reports on the rates of association and dissociation. The phenomena of slow and fast exchange, characterized by an exchange rate below or above the chemical shift difference between the bound and free states, determine the spectral observables (Figure 3A). In ssNMR, the slow exchange regime has been predominantly observed (35), possibly due to slower kinetics in the densely packed protein sediment compared to solution (52, 53).

CSP analysis is straightforward but reports on all structural rearrangements within the enzyme upon complex formation, some of which can be allosteric. Therefore, CSPs are often complemented by methods that directly probe spatial proximity. In MAS ssNNMR, heteronuclear polarization transfer (compare experiments in Table 1) can be performed in conjunction with selective isotope labelling to identify intermolecular contacts (Figure 3B), which can serve as distance restraints for docking software such as HADDOCK (54). This approach can map interfaces within protein multimers (55, 56) and between proteins and small molecules (57, 58). Large protein-ligand assemblies that lack long-range order and inter-particle homogeneity are not amenable to many methods but ssNMR. For example, the outer plasma membrane of bacteria is enclosed by peptidoglycan, a polymer from sugar and amino acids that is cross-linked by transpeptidases. MAS ssNMR was employed to obtain a high-resolution model of the complex between this enzyme and an intact peptidoglycan based on CSP data (59).

Phosphorus is an advantageous nucleus in biomolecular NMR due to its absence in unmodified polypeptide chains but 100% natural abundance in nucleotides and membrane phospholipids. Furthermore, its chemical shift dispersion is such that it can distinguish binding environments with high sensitivity (Figure 3C). The ATP-dependent helicase DnaB served as a model system to establish methods to monitor enzyme-substrate and enzyme-cofactor interactions. Multiple snapshots along the reaction cycle of this enzyme were mimicked by nucleotides and their analogues (35, 60), notably all prepared by straightforward co-sedimentation of enzyme, nucleotides and DNA substrate. In case of the ATP-binding cassette exporter MsbA (61) **dynamic nuclear polarization** (DNP)-enhanced MAS ssNMR helped compensate for the intrinsically low sensitivity of membrane proteins embedded in lipid bilayers and selective nitrogen-15 labelling of the nucleotide enabled the implementation of a spectroscopic filter to identify residues inside the binding pocket. Ligand-observed NMR against a spectroscopically inactive enzyme background is an elegant tool to monitor binding and to probe the ligand conformation in the bound and free states at atomic resolution. Similar to phosphorus, fluorine nuclei come with optimal NMR properties at natural abundance (62, 63).

If the effort of introducing isotope labels at specific sites via chemical synthesis is taken, details of ligand conformation become accessible. Tensor correlation methods can determine the relative orientation of two anisotropic interactions and thus of two chemical moieties in space, such as torsion angles deduced from the relative orientation of two **CSA** tensors (64).

The combination of crystallography and quantum mechanical modelling plus MAS ssNMR, dubbed NMR crystallography, established the carbanionic nature of the reaction intermediate as well as the charge distribution in the active site of the enzyme tryptophan synthase in complex with cofactor and substrate (65). The special ability of NMR to probe the locations of catalytically relevant protons could shed light on the tautomerization states of the vitamin B1(thiamine)-pyrophosphate cofactor in its enzyme-bound form (66). Finally, freeze-trapping transient reaction intermediates *ex situ*, followed by MAS ssNMR of the isotope-labelled ligands under **DNP** signal enhancement, enables fine time resolution of multiple intermediate stages for enzymes in which activity can be synchronized, *e.g.* by illumination (67).



**Figure 3. Techniques for probing enzyme-ligand binding.**

**A.** To identify the binding site of a ligand to an enzyme, chemical shift perturbations are a valuable initial analysis method. Slow exchange between apo and bound states often applies in the protein sediment and leads to the appearance of two distinct signal sets. Meanwhile, fast exchange is frequently encountered in solution, where it enables classical NMR titration experiments. **B.** While chemical shift perturbations can be allosteric, polarization transfer across the binding interface of differentially labelled partner molecules establishes reliable through-space restraints for molecular docking. Exemplary complementary isotope labelling patterns are shown alongside NMR experiments that enable the corresponding polarization transfer (explanations in Table 1). Perdeuteration of the target molecule enables selective transfer starting from protons of a source molecule. Protons are naturally abundant in the vast majority of ligands, which makes this approach broadly applicable where artificial isotope labelling via chemical synthesis is not feasible (58). Where available, fluorine-19 and phosphorus-31 are favourable nuclei that are NMR active, occur at 100% natural abundance, and provide sparse labels with high sensitivity (68). **C.** Ligands bound to a large immobilized enzyme complex can be detected in cross-polarization based experiments (Table 1). For example, the chemical shifts of phosphate groups in nucleotide substrates give insight into the reaction steps of ATPase enzymes (69). **D.** Precise conformational restraints can be obtained from tensor correlation methods, whereby the relative orientation of two anisotropic interactions is determined (64).

**Enzymes in action**

NMR spectroscopy has the special aptitude to characterize how proteins fluctuate around their equilibrium coordinates over picoseconds to seconds (70), which spans the time scales of motions that potentially relate to enzymatic activity. The overall molecular tumbling in solution partially occludes the spectroscopist’s view of internal motions. Hence, MAS ssNMR of immobilized enzymes is potentially more potent for the investigations of dynamics. Moreover, the technique can read out the amplitudes and time scales of local motions via additional observables, namely the dynamic averaging of anisotropic interactions, such as the **CSA**, **dipolar** and quadrupolar couplings, which are fully averaged by overall tumbling in solution. If proper care is taken to separate coherent from stochastic, motion-induced effects of spin interactions, ssNMR opens up new opportunities for enzyme dynamics studies, in particular under fast MAS (71). For example, in the above-mentioned study of carbonic anhydrase, a combination of relaxation dispersion measurements and molecular dynamics simulations could identify microsecond motions in the water network at the active site of the enzyme (72). Characterization of aromatic ring flipping in the TET2 peptidase, enabled by a novel isotope labelling scheme, revealed microsecond conformational fluctuations at the entry to the catalytic chamber (73).

With enzymes studied in equilibrium, it is not always evident whether their inherent motions are functionally relevant. Real-time NMR reveals how enzymes respond to off-equilibrium conditions. Processes such as (un)folding, substrate binding and turnover can be monitored over seconds to days (74, 75). Enzymes for which real-time MAS ssNMR has proven particularly fruitful are large multimeric ATPases (69) and membrane-bound kinases (76, 77). Dipolar-based experiments filter out substrate immobilized in the binding pocket and thus reveal the nucleotide occupancy of the enzyme while scalar-based or directly pulsed experiments quantify substrate turnover over time. For the human ATPase p97, an integrated approach uniting real-time NMR in solution and in the sediment identified a transient reaction intermediate that had escaped observation by other methods, the host-hydrolysis ADP.Pi state (69). This example illustrates how the overall architecture of an enzyme, delineated by cryo-EM or crystallography, can be complemented by NMR with fine molecular features in multiple states of the reaction cycle, which can be crucial for a deeper mechanistic understanding.

**Integrative approaches and emerging techniques**

In past few years, integrative and hybrid approaches uniting the strengths of solution and solid-state NMR, crystallography, cryo-EM and other biophysical techniques have almost become a necessity in the structural biology of large enzyme complexes.

Already the combination of multiple magnetic resonance techniques can yield complementary information: electron paramagnetic resonance was employed in the above mentioned studies of MsbA (61) and DnaB (78) to determine the coordination geometry of the metal ion cofactors at the active sites of the enzymes.

A recent demonstration of integrative biophysics was the study of caseinolytic protease (ClpP), a homomeric complex of fourteen subunits and potential target for antibiotics against pathogenic bacteria, which displayed unexpected activation by designed inhibitors, challenging drug development efforts. Crystallography, NMR in solution and in the sediment, molecular dynamics simulations and isothermal titration calorimetry unveiled an allosteric transition within the multimer induced by binding of inhibitors to single subunits (79). Similarly, it took the union of crystallography, NMR in solution and in the sediment as well as bioinformatics to unravel how subunits in a non-ribosomal peptide synthetase assemble dynamically into a complex to achieve catalysis (80).

Signal enhancement, in particular **DNP**, and high-speed MAS beyond 100 kHz are currently among the most active fields in method development for ssNMR (81) while in terms of biological applications, magnetic resonance in the cell is gaining momentum. Although predominantly performed on biomolecules in solution (82), MAS ssNMR has also proven useful for examining membrane proteins in cells (83, 84), notably under DNP enhancement (85, 86). In a proof-of-concept study, protein-specific signal enhancement has been demonstrated, whereby a radical-labelled ligand targets polarization transfer to the protein of interest (87). One could envisage that by joining radical moieties to substrates and inhibitors, investigations of enzymes even against a strong background from cellular components will become feasible, in the frozen state but also in solution (88).

The union of spectroscopic method development and advances in biochemical sample preparation is bound to shape the future of NMR as an integral building block in the structural biology of large enzyme complexes.

**Box : Perspectives**

* In studying the mechanisms of large or membrane-embedded enzymes, ssNMR under MAS can reveal fine structural details and motional processes in proteins, their substrates and inhibitors, at key stages of the enzymatic reaction pathway.
* Owing to advances in hardware development, in particular design of fast magic angle spinning probes and magnets operating at increasing field strengths, atomic-resolution studies on sub-milligram amounts of non-deuterated enzymes have become a reality.
* Affordable and accessible stable isotope labelling techniques in eukaryotic expression hosts will considerably widen the range of enzymes that are amenable to ssNMR investigations. Signal enhancement techniques will alleviate sensitivity issues while NMR in cell will shed light onto the physiologically relevant states of enzymes.

**Glossary: Key concepts in ssNMR**

**Rotational correlation time (τc).** The time it takes a molecule to rotate by one radian (~ 60°) by random motion. It depends on the temperature, the viscosity of the solvent and the shape and size of the molecule. In rough approximation, the correlation time scales with molecular weight. Typical values for small soluble proteins are a few nanoseconds, which extends to microseconds for large supramolecular assemblies.

**Magic angle (MA, 𝜃m).** Angle between the space diagonal of a unit cube with any axis (~ 54.7°). Points on the space diagonal have equal x,y, and z coordinates. When the principal axis of a nuclear spin interaction is oriented at the magic angle with respect to the external magnetic field, the interaction disappears if it depends on the second-order Legendre polynomial, $P\_{2}\left(\cos(θ\_{m})\right)=\frac{1}{2}\left(3cos^{2}θ\_{m}-1\right)=0$.

**Magic angle spinning (MAS).** Technique to mechanically average orientation-dependent (anisotropic coherent) interactions of nuclear spins with the external magnetic field and with each other, e.g. dipolar couplings among spins and the anisotropy of the chemical shift. This is achieved by rotating the specimen about an axis inclined at the magic angle with respect to the external field.

**Dipolar coupling.** The magnetic interaction between two nuclear spins through space, which scales with the spins’ magnetic dipole moments and inversely with the cube of their distance. The strength of the interaction is orientation-dependent and it has no isotropic component.

**Scalar coupling (J-coupling).** An interaction between chemically bonded spins mediated by binding electrons. Unlike the dipolar coupling, it has an isotropic component and persists even under fast stochastic motion or MAS.

**Chemical shift anisotropy (CSA).** The size of the chemical shift experienced by a nuclear spin depends on the orientation of its surrounding molecule with respect to the external magnetic field. It can be described by a matrix, or second-rank tensor, whose anisotropy, a measure of deviation from uniform strength in all dimensions, is reflected in the line width of NMR spectra recorded on static solids.

**Recoupling.** The application of radio-frequency irradiation in a manner that constructively interferes with MAS. This method enables the selective re-introduction of desired anisotropic interactions averaged out by MAS, notably the dipolar coupling or CSA, thereby giving access to molecular geometry in the form of inter-nuclear distances and angles.

**Decoupling.** The application of continuous or pulsed radiofrequency irradiation to average out spin-spin couplings, dipolar or scalar, in order to reduce the number and line width of NMR resonances and hence facilitate spectral analysis.

**Dynamic nuclear polarization (DNP).** A method to enhance the sensitivity of ssNMR experiments by transferring the larger equilibrium polarization of unpaired electron spins in a magnetic field to nuclear spins by means of microwave irradiation. Experiments are typically performed under cryogenic conditions at around 100 K in an amorphous matrix.

**Table 1. Solid-state NMR jargon.**

Overview of frequently employed building blocks for ssNMR experiments geared towards biomolecules.

|  |  |  |
| --- | --- | --- |
| **Name and abbreviation** | **Purpose and applications** | **Citations** |
| Cross polarizationCP | The primary method in ssNMR to transfer polarization from an abundant nucleus with high resonance frequency, typically 1H, to a rare nucleus with lower frequency such as 13C, 15N or 31P spins in order to improve the sensitivity of experiments.The transfer is mediated by the dipolar coupling between the spins and enabled by matching the amplitudes of the radiofrequency fields applied to both nuclei (Figure 1D). | (89) |
| Insensitive nuclei enhanced by polarization transferINEPT | Frequent building block in which magnetization is transferred via scalar couplings, typically from 1H to a heteronucleus such as 13C and 15N or back. This transfer requires no anisotropic spin interaction and is exploited in solution and ssNMR alike. | (90) |
| Radio-frequency driven recouplingRFDR | Homonuclear (referring to two nuclear spins of the same type) recoupling experiment in which a pulse train synchronized with the MAS frequency is applied to reintroduce the dipolar coupling. This element allows to identify nuclear spins that are close in space. The spectroscopic range of sight depends on the type and abundance of the spins and lies in the range of ~ 1-10 Å for 13C and 1H spins.This element is suited for assignment experiments but also to collect structural restraints, in particular long-range 1H-1H contacts in sparsely labelled samples. | (91)(49) |
| Homonuclear rotary resonanceHORROR/DREAM | Homonuclear recoupling experiment with similar applications as RFDR above. Here, the dipolar coupling is reintroduced under MAS by exploiting interference effects between continuous radiofrequency irradiation and the MAS frequency. | (92)(93)(8) |
| Proton-driven spin diffusionPDSDDipolar Assisted Rotational RecouplingDARR | A classic recoupling experiment that utilizes the incomplete elimination of homonuclear dipolar couplings under MAS in the presence of dense proton spin networks to promote polarization transfer between homonuclear spin pairs, typically 13C, in non-deuterated samples. In contrast to its designation, spin diffusion in the solid is not a stochastic but a coherent process.The mixing time, which ranges from tens to hundreds of milliseconds, determines the type of correlations obtained, which can be within amino acids but also further through space, providing distance information up to ~10 Å.Additional irradiation on the 1H spins at an amplitude matching the MAS frequency allows for more efficient polarization exchange, especially at high MAS rates and large chemical shift differences between the recoupled spins. | (94)(95, 96) |
| CHHCNHHC, NHHP, CHHP | Method to detect contacts between 1H spins with a spectral readout on heteronuclear spins. Polarization transfer between protons is achieved via a mixing time in the range of tens to hundreds of microseconds. This experiment is frequently employed for structure determination of 13C labelled proteins, in analogy to NOESY spectroscopy in solution.Heteronuclear equivalents can be used to detect intermolecular interfaces between proteins, ligands or nucleic acids if used in conjunction with a selective isotope labelling scheme (Figure 3B). | (97, 98) |
| Proton Assisted RecouplingPARProton Assisted Insensitive Nuclei Cross PolarizationPAIN | A recoupling experiment in which polarization transfer between two heteronuclei, typically two 13C nuclei, that are dipolar coupled to the same 1H spin is achieved, with the latter promoting polarization transfer. It is well-suited to obtain long-range contacts for structure determination due to its ability to identify nuclear spin pairs through space even in the presence of closer neighbours.PAIN is the heteronuclear equivalent of PAR, which can be used to detect intermolecular interfaces, as described above for NHHC. | (99)(100)(101) |
| Rotational echo double resonanceREDOR | A recoupling method in which a pulse train synchronized with the MAS frequency is applied to reintroduce the dipolar coupling between two heteronuclei. It is often employed for internuclear distance measurements (Figure 1E). | (102) |

**Table 2. Typical experimental setups for MAS ssNMR.**

Depending on the MAS rate, different detection modes and labelling schemes are beneficial. For a more detailed overview of rotor dimensions and properties see (30).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Outerrotordiameter[mm] | Active volumein coil[uL] | Max.proteinamount[mg] | Max. MAS rate[Hertz] | Typical detection mode | Typical isotope labelling | Availability |
| 3.2 | 30 |  24.000 | Carbon  | Uniform or sparse 13C,15N labelling with full protonation | CommercialBruker/JEOL |
| 1.9 | 10 |  42.000 | Carbon  | Uniform or sparse 13C,15N labelling with full protonation | CommercialBruker Biospin |
| Proton  | Perdeuteration of aliphatic carbon atoms (2H,13C,15N) with partial back exchange of amide protons |
| 1.3 | 2 |  65.000 | Proton  | Perdeuteration of aliphatic carbon atoms (2H,13C,15N) with full back exchange of amide protons | CommercialBruker Biopsin |
| 0.7 | 0.5 | 111.000 | Proton  | Uniform 13C,15N labelling with full (or partial) protonation | CommercialBruker/JEOL |

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**Competing interests**

The author declares no competing interests associated with this manuscript.

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