

Recent developments in solution nuclear magnetic resonance (NMR)-based molecular biology

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Abstract Visualizing post-translational modifications, conformations, and interaction surfaces of protein structures at atomic resolution underpins the development of novel therapeutics to combat disease. As computational resources expand, *in silico* calculations coupled with experimentally derived structures and functional assays have led to an explosion in structure-based drug design (SBDD) with several compounds in clinical trials. It is increasingly clear that “hidden” transition-state structures along activation trajectories can be harnessed to develop novel classes of allosteric inhibitors. The goal of this mini-review is to empower the clinical researcher with a general knowledge of the strengths and weaknesses of nuclear magnetic resonance (NMR) spectroscopy in molecular medicine. Although NMR can determine protein structures at atomic resolution, its unrivaled strength lies in sensing subtle changes in a nuclei’s chemical environment as a result of intrinsic conformational dynamics, solution conditions, and binding interactions. These can be recorded at atomic resolution, without explicit structure determination, and then incorporated with static structures or molecular dynamics simulations to produce a complete biological picture.

Keywords Integral membrane proteins · Conformational dynamics · Intrinsically disordered proteins · Low-gamma nuclei detection

Clinical significance—The awesome power of NMR

Visualizing post-translational modifications, conformations, and interaction surfaces of protein structures at atomic resolution underpins the development of novel therapeutics to combat disease. As computational resources expand, *in silico* calculations coupled with experimentally derived structures and functional assays have led to an explosion in structure-based drug design (SBDD) with several compounds in clinical trials. It is increasingly clear that “hidden” transition-state structures along activation trajectories can be harnessed to develop novel classes of allosteric inhibitors. The goal of this mini-review is to empower the clinical researcher with a general knowledge of the strengths and weaknesses of nuclear magnetic resonance (NMR) spectroscopy in molecular medicine. Although NMR can determine protein structures at atomic resolution, its unrivaled strength lies in sensing subtle changes in a nuclei’s chemical environment as a result of intrinsic conformational dynamics, solution conditions, and binding interactions. These can be recorded at atomic resolution, without explicit structure determination, and then incorporated with static structures or molecular dynamics simulations to produce a complete biological picture.

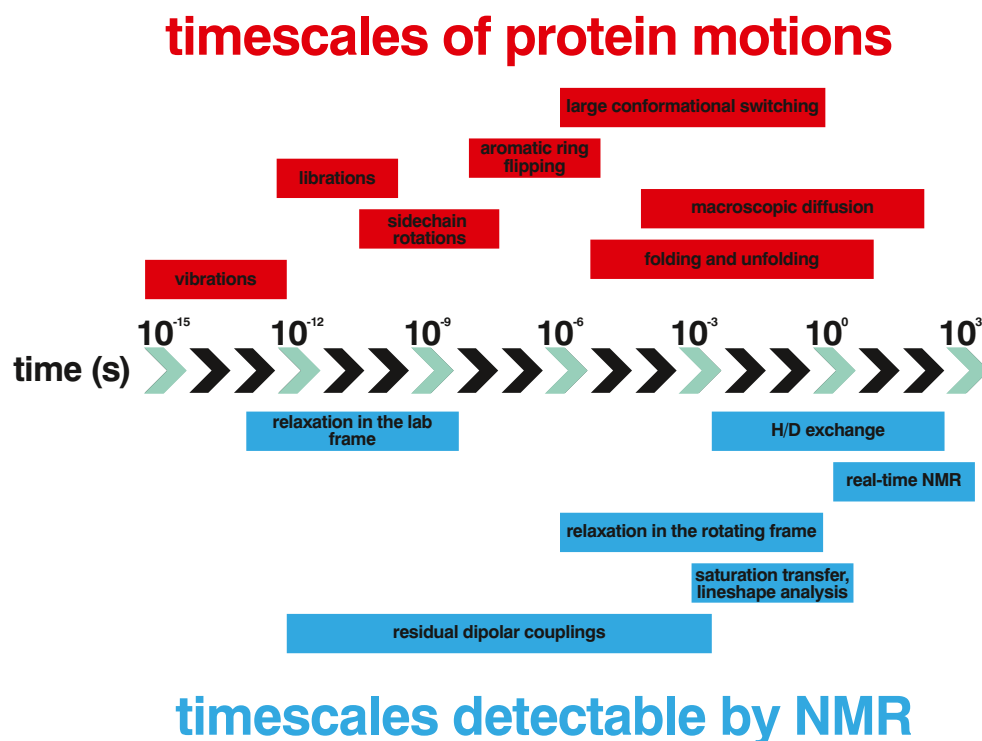
The niche of NMR in modern structural biology

Static structural representations subconsciously train scientists to envision a rigid protein architecture. However, macromolecules are exceptionally acrobatic possessing fast, local fluctuations and slow, concerted structural motions (Fig. 1). Therefore, a more accurate depiction is a series of structures where the kinetics, thermodynamics, and function of each conformation varies significantly within a statistical ensemble. Understanding how these dynamic molecules behave in

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Fig. 1 NMR is capable of monitoring molecular motions over 15 orders of magnitude at atomic resolution by probing a multitude of properties with a variety of experiments



solution with atomic resolution is essential for deciphering their roles in biological processes such as ligand binding and protein-protein interactions. A classic example is how oxygen accesses the cavity of myoglobin—whose crystal structures reveal no channels for the ligand to enter the heme-binding site [1]. NMR spectroscopy is the only technique available to probe these dynamics, at atomic resolution, on timescales ranging over 15 orders of magnitude. As illustrated in Fig. 1, there are NMR experiments to look at backbone vibrations, side-chain motions, secondary structure movements, domain rearrangements, and even folding all under physiological solution-state conditions [2–5]. This unique perspective from NMR spectroscopy lends major contributions to understanding the biological impact of inherently flexible systems such as intrinsically disordered proteins (IDPs) and integral membrane proteins (IMPs). It should be emphasized that this mini-review is written for the NMR novice. We aim to give an introduction for modern NMR applications to clinically relevant systems such as structure-based drug design, IDPs, and IMPs. Recommendations to in-depth reviews are found throughout the text for the interested researcher.

The business end of NMR: Chemical shifts

Spectroscopy concerns the interaction between light and matter. NMR spectroscopy probes nuclei that possess non-zero quantum spin angular momentum, or simply *spin*. Biomolecular NMR typically focuses on spin $\frac{1}{2}$ nuclei which produce easily

interpretable spectra. The primary atomic constituents of biomolecules carbon, nitrogen, and hydrogen, all possess a stable spin $\frac{1}{2}$ isotope (e.g., ^{13}C , ^{15}N , and ^1H). When placed in an external magnetic field, electromagnetically irradiated nuclei resonate at a frequency dictated by their chemical environment—what atoms it is bonded to or spatially nearby. In other words, the resonance frequency of each nuclei is modulated by its primary, secondary, tertiary, and quaternary structure. This unique chemical dependence enables structure determination as well as the ability to monitor changes in motion, and even map atom-specific changes upon ligand binding. For practical purposes, spectroscopists express resonance frequency in terms of *chemical shift* (units of parts per million; ppm)—which is the resonance frequency scaled by magnetic field strength. Chemical shifts are the most easily accessible NMR parameter that can be measured with high accuracy. As illustrated in Fig. 2a, the unique chemistry of each functional group causes them to occupy a particular region of the spectrum. Subsequently, each amino acid gives rise to distinct chemical shift ranges assuming the residue exists in isolation, or an unstructured conformation, known as a random coil chemical shift (Fig. 2b). These chemical shifts are further altered by the nuclei's oxidation state, orientation of covalent bonds, isotope of neighboring atoms, hydrogen bonding, and proximity to groups with high magnetic susceptibility such as carbonyl, aromatic, or paramagnetic ions (metals, lanthanides, etc.). In fact, a common technique involves intentional incorporation of paramagnetic ions to modulate the chemical shift for unique structure and dynamic information (as reviewed in [6]).

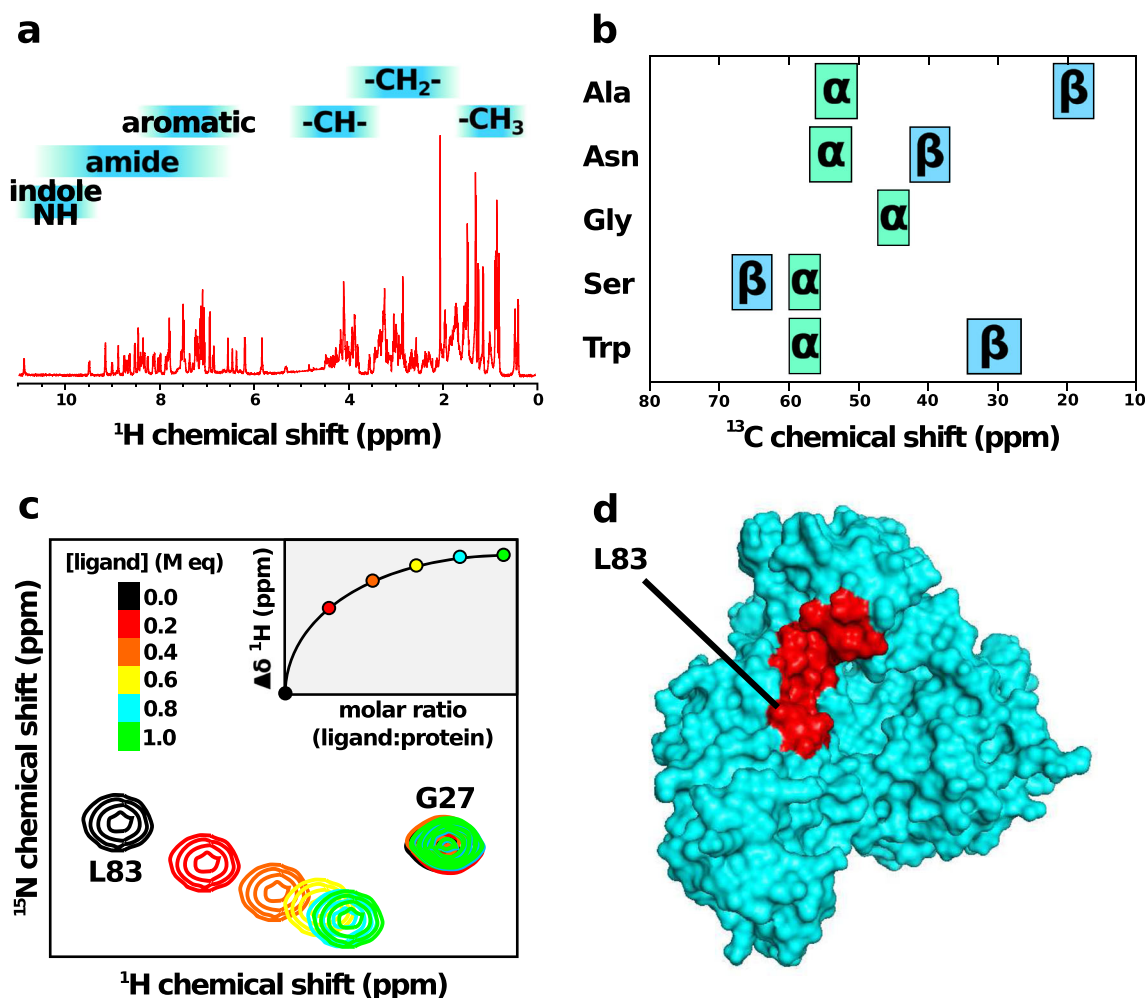


Fig. 2 NMR is highly sensitive to chemical environment based on primary, secondary, tertiary, and quaternary protein structure. **a** Each atom within a functional group possesses a distinct chemical shift range as illustrated for ^1H nuclei. **b** This translates to unique values for each atom on a per residue basis as illustrated for ^{13}C groups of select amino acids. A complete list of predicted chemical shift values is available online at the Biological Magnetic Resonance Data Bank [53]. **c** A series of two-dimensional spectra collected with increasing ligand

concentrations (colored from black to magenta); only L83 is specifically located at the binding interface as demonstrated by its large chemical shift perturbations. Inset: the complex affinity can be quantified by fitting changes in chemical shift, and/or peak intensity, as a function of ligand concentration to an appropriate binding equation [28]. **d** Finally, the residues that experience chemical shift changes can be highlighted on the surface of structure or homology model to map the binding interface

It was not until the early 1990s that the NMR structural database reached a sufficient sample size for “chemical shift homology” searches to predict secondary structures for sequence alignment, fold classification, protein visualization, and homology modeling. Qualitative secondary structure predictions first measured the difference between a residue’s chemical shift from the random coil value, known as the secondary chemical shift, and plotted it as a function of primary sequence. Initial estimates focused on how $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ secondary chemical shifts could discriminate the electronic distribution of beta-strand and alpha-helical regions [7] that formed the chemical shift index (CSI) method [8]. CSI then paved the way for more robust methods that utilize ^{15}N , $^1\text{H}^\alpha$, $^{13}\text{C}'$, $^{13}\text{C}^\alpha$, and $^{13}\text{C}^\beta$ chemical shifts in numerous web-based or downloadable programs to estimate secondary structure

based on chemical shifts, with DANGLE and PsiCSI reporting the highest fidelity [9, 10]. The random coil index (RCI), developed by the Wishart group, goes further by using chemical shifts alone to accurately quantify protein flexibility [11]. Without explicit knowledge of the 3D structure or additional NMR experiments, the chemical shift values of nuclei that sample multiple conformations on a sub-millisecond scale are population averaged. Comparison of these data with molecular dynamics simulations or crystallographic B-factors can quickly identify whether the B-factor is a result of static conformational heterogeneity or true conformational dynamics. Backbone chemical shift “homology searches” are also exploited to predict backbone ϕ and ψ dihedral angles within $\leq 30^\circ$ for at least 85% of sites; the most popular programs are PREDITOR, TALOS+, and DANGLE [10, 12, 13]. Stereospecific methyl labeling has

led to the development of a simple formula for side-chain rotamers of Val (χ_1), Leu (χ_2), Ile (χ_2) and calculations of further polar and aromatic residues should be possible with larger structural datasets [14, 15].

Current structure determination procedures rely primarily on cataloging short-range (typically ≤ 5 Å) ^1H - ^1H distance constraints to define the polypeptide architecture. The holy grail of NMR would be tertiary and quaternary structure calculations from chemical shifts alone. Since the year 2000, international structural genomics initiatives rapidly added datasets to the point that high-resolution structures can now be calculated solely from chemical shifts. The dominant methods, CHESSIRE, CS-ROSETTA, and CS23D, rely on backbone chemical shift assignments alone but can faithfully calculate model structures with backbone RMSD < 1.5 Å from reference structures [16–18]. These model structures are currently limited to ~ 110 amino acids but inclusion of side-chain chemical shifts, residual dipolar couplings (RDCs), and unassigned nuclear Overhauser effect (NOE) restraints has been successfully applied to proteins up to 200 amino acid proteins [19]. More recent iterations model oligomeric structures from chemical shift perturbations upon complex formation. Programs such as HADDOCK, CamDock, and CS-ROSETTA sequentially, or simultaneously, combine docking and structure determination algorithms to produce tertiary structures from inputs of the free-state structures [20–22].

Applications

Solution NMR in drug discovery

In the last two decades, drug discovery's focus has shifted to screening fragment molecules (typically containing less than 20 atoms) for development into high-affinity lead compounds. Initial fragments with optimal binding efficiencies (affinity vs. number of atoms) can be further enhanced into lead-like molecules through the addition of chemical functionalities, and/or the linking of ligands that target disparate sites. However, fragments are inherently low-affinity (micromolar affinity) molecules that do not easily crystalize and are difficult to identify in functional assays. In contrast, NMR is not affinity limited and can directly determine binding constants during compound screening to rank them for optimization and lead selection. Binding kinetics directly determine accurate quantification of drug affinity by NMR (for detailed texts on NMR kinetics, see [23–25]). The particular exchange regime of a binding reaction can be readily identified by observing each peak throughout a titration; it is important to note that each atom can exhibit its own rate of exchange for a particular binding reaction.

First spearheaded by Fesik and colleagues, structure-activity relationship (SAR) by NMR [26] has matured into a

moderate-throughput screening technology. Titrations can be performed in two different formats: (1) ligand-based or (2) target-based. Ligand-based screening relies on excess concentrations of molecular fragments and monitors chemical shift changes through one-dimensional spectra collected in minutes. A positive binding interaction alters the chemical shift and/or the line shape of a given resonance. Details of various ligand-based experiments are reviewed here [27], for example, pulse sequences that monitor changes in the ligands' diffusion coefficient (diffusion-ordered spectroscopy, DOSY), or only allow visualization of atoms within a particular distance of the target protein (such as saturation transfer difference spectroscopy, STD). The high resolution of small molecules allows batch screening of 5–10 molecules to improve throughput (for details, we recommend this excellent review [27]). Ligand-based screening is further advantageous as the target protein does not need to be isotopically labeled and usually only studied in small concentrations. Conversely, target-based screening follows changes to on the protein side. Once the chemical shift assignments of the target protein are known, isotopically enriched protein can be titrated with a putative ligand or binding partner and the resulting chemical shift changes in monitored by two-dimensional correlation spectroscopy (Fig. 2c) to determine binding constants and stoichiometry [28]. Highlighting perturbed residues on the surface of a known protein structure, called *chemical shift mapping*, can be used to identify the binding pocket (Fig. 2d). Two-dimensional ^1H - ^{15}N heteronuclear single-quantum coherence (HSQC) spectroscopy is the most common method for mapping backbone chemical shifts because every residue type (except proline) contains a highly sensitive probe to changes in local magnetic environment. HMQC [29] and TROSY [30] are alternative pulse schemes that supply equivalent spectral information and provide advantages in certain situations such as large molecular weight proteins. As most protein-ligand interactions are side-chain mediated, two-dimensional ^1H - ^{13}C HSQCs can provide additional information, but side-chain signals typically suffer from overlap in large target proteins. Thus, methyl-selective ^1H , ^{13}C -labeling of Ile, Leu, Ala, and Val residues can significantly improve resolution without compromising sensitivity [31].

Intrinsically disordered proteins

Since the initial discovery of functional proteins without a stable architecture, it is been estimated that $>30\%$ of the human proteome constitute partially, or completely, unstructured polypeptides [32]. Intrinsically disordered proteins (IDPs) exist in an ensemble of high-energy states that may kinetically sample globular structures and often only in the presence of specific binding partners. They evolved to maintain a level of solubility required for optimal function and have significant biological roles in signaling and regulation—in part due to

their promiscuous binding activity. A large proportion of the peptides and proteins responsible for aberrant misfolding diseases are intrinsically disordered in their free soluble forms—amyloid- β in Alzheimer's [33], α -synuclein in Parkinson's [34], and amylin in type II diabetes [35]. Whereas X-ray crystallography may obtain structures when IDPs adopt well-ordered bound states, NMR spectroscopy can report on both the bound-state and the apo, high-energy ensemble. Besides NMR, small-angle X-ray scattering (SAXS) is now increasingly used to characterize IDPs. Their high flexibility results in spectroscopic properties that are characteristic of small molecules, allowing for backbone chemical shift assignment even for high molecular weight proteins. Once assigned, one can monitor changes upon interaction with a particular ligand using chemical shift mapping as described in the SBDD section. Although NMR has monopolized their structural characterization, IDPs do present unique technical challenges that are beginning to be addressed by low-gamma nuclei (^{13}C and ^{15}N) detection experiments. In brief, using low-gamma nuclei instead of traditional ^1H -detected experiments potentially results in larger signal dispersion, sensitivity, and resolution. Interested readers are directed towards an excellent review by Takeuchi and colleagues [36].

Integral membrane proteins and nanodisc technology

Proteins embedded into plasma membranes are critical to the molecular mechanisms of life, responding to extracellular stimuli and communicating changes in the external environment to intracellular machinery. Some estimates suggest that integral membrane proteins (IMPs) comprise one third of the genome and nearly half of the current pharmacology targets [37, 38]. Despite being ubiquitous in healthy and pathological systems, structural studies of IMPs have been limited. Until recently, X-ray crystallography was unable to handle the fluid-like environment of lipid membranes. In the past decade, lipidic cubic phase-based studies have revolutionized crystallography of membrane proteins with more than 200 structures, nearly half of which were determined in the last 5 years [39]. In contrast, NMR spectroscopy has steadily contributed to IMP structural information—being limited primarily by isotope labeling, molecular weight, and detergent stability. However, the size limitations have decreased significantly following the invention of TROSY methods and improved isotopic labeling schemes. At this point, a major limitation is the loss of structure and function that accompanies purification in detergent and removal of the native lipid environment. If a suitable detergent environment can be found, an additional challenge arises in characterizing protein-protein interactions which are frequently compromised by detergents. A promising strategy is to incorporate IMPs into discrete phospholipid-bilayer mimetics called nanodiscs.

Nanodiscs are an elegant solution created by Sligar and colleagues [40]. Modeled after high-density lipoprotein (HDL) particles, they comprise of a discoidal lipid bilayer stabilized by a polypeptide belt, termed a membrane scaffold protein (MSP). In 2013, Hagn et al. modified the MSP primary sequence to reduce the disc diameter and subsequently rotational correlation times—critical to making nanodiscs accessible to the NMR community [41]. The nanodiscs range from 4 to 10 nm (approximately equivalent to 52–124 kDa) and dominate the IMP's relaxation properties assuming there are minimal extramembrane portions. In 2017, an empty nanodisc structure was determined by NMR spectroscopy [42]. As suggested by previous bioinformatics and biochemical experiments, MSP dimerizes and adopts a series of alpha-helical repeats that wrap around the lipids in an anti-parallel alignment. One suggested limitation of nanodisc technology is inherent heterogeneity of the disc diameter and difficulty in controlling the stoichiometry of embedded IMPs. Recently, Wagner and colleagues have modified the MSP construct to be covalently circularized using sortase technology to control 95% of discs to within a 1 nm size distribution [43]. MSP-based nanodiscs rely on detergent molecules to solubilize protein: lipid mixtures prior to disc formation. An alternative styrene maleic acid polymer is capable of directly incorporating IMPs without detergent solubilization and may gain traction in the future once disc diameters can be faithfully formed below 15 nm [44, 45].

Technical considerations in brief

Sensitivity The magnitude of a NMR signal is dependent on the population difference between high and low-energy states. The population difference is directly proportional to the static magnetic field strength—which is the primary reason for building higher-field spectrometers. For a given field strength, signals scale linearly with sample concentration and signal-to-noise (S/N) ratio scales as a $\sqrt{2}$. It is important to recognize that signal strength reflects the total mole fraction of NMR-active spins rather than the molar concentration, which means the spectra of a 200 μl sample at 100 μM is effectively equivalent to a 400 μl sample at 50 μM (in the absence aggregation, oligomerization, etc.). Sample volumes are typically around 300–500 μl . However, smaller tubes are available that reduces sample volume to 175–200 μl . Modern spectrometer hardware has reduced the minimum protein concentration to $\leq 15 \mu\text{M}$ (in 500 μl) for collecting a two-dimensional spectrum within a couple of hours, although higher concentrations will directly result in shorter experimental times. Samples are tolerant to a wide range of solution conditions and temperatures—limited mainly by high salt concentrations (special tubes should be used at $>200 \text{ mM}$ salt). Temperatures are only limited by the solvent's freezing point and the spectrometer's

hardware boundary; modern cold probes operate at temperatures between $-40\text{ }^{\circ}\text{C}$ and $80\text{ }^{\circ}\text{C}$.

Molecular weight The details of spin physics are beyond the scope of this review; however, it is important for any scientists to have an idea of the limitations of each technique. For an accessible, yet thorough text, we recommend understanding NMR spectroscopy by James Keeler [46]. The maximum theoretical signal is proportional to the number of NMR-active spins within a sample. This signal is then modulated by exponential decay functions that reflect *relaxation*, or the return of a spin system to thermodynamic equilibrium. The relaxation source divides signal decay into two types: T1 (aka longitudinal or spin-lattice relaxation) and T2 (aka transverse or spin-spin relaxation). T2 relaxation is particularly important in biomolecular NMR spectroscopy—with the decay rate increasing as a function of molecular weight. Many researchers are quick to note that T2 relaxation limits the application of NMR to low molecular weight systems. However, as we have detailed in this review—although *de novo* structure determination becomes challenging at higher molecular weights—the sensitivity of each nuclei to changes in protein structure and dynamics can provide invaluable information without requiring complete structure determination.

Isotope labeling The major limitation of NMR spectroscopy is the requirement of NMR-active atoms, specifically spin-1/2 nuclei. Luckily for biological systems, hydrogen's most common isotope is spin-1/2 (^1H ; 99.9% natural abundance); however, samples must be enriched with ^{15}N and ^{13}C -atoms as their natural abundance is $\leq 1\%$. The details of isotope enrichment depend on the specific recombinant protein expression system. In brief, *Escherichia coli* cultures can be cheaply grown with simple isotopically labeled carbon and nitrogen sources (typically, glucose and ammonium chloride, respectively) whereas eukaryotic systems require supplementation with costlier labeled amino acids. Depending on yields, isotopic labeling can be prohibitively expensive. We recommend these articles for *E. coli* [47], yeast [47], insect cell [48], or mammalian expression systems [48]. As molecular weights increase (typically ≥ 30 kDa), non-exchangable hydrogens must be partially or fully deuterated (^2H) to improve T2 relaxation properties (as reviewed in [47]).

Conclusion

NMR has always been chemists' default technique for robust small-molecule structure determination, but its role in the biomolecular arena has been slightly more contested. X-ray crystallography remains the workhorse of structural biology—providing 90% of the Protein Data Bank's (PDB) entries. Although crystallization has contributed priceless amounts

of architectural information, it inherently stabilizes the lowest-energy conformation at the expense of eliminating protein conformational dynamics. It requires a variety of schemes to trap the reaction substrates or coax all molecules within the crystal to adopt the same substrate conformation. Even then, the crystallization procedure itself has the potential to modify the structure, or introduce artifacts in the form of oligomerization, non-specific protein-ligand interactions, or occlusion of binding interfaces. In contrast, solution-state NMR spectroscopy can be performed at physiological temperatures and buffer conditions. Experiments exist to quantitatively probe motions over all timescales from picoseconds to days, and most of these experiments provide atomic resolution information about conformations without the need to determine the complete structure. One glaring absence in this review is the description of NMR experiments probing conformational motions. Even cursory explanation of NMR-based dynamics requires in-depth discussion of spin physics for numerous experiments (Fig. 1). Interested readers are directed to the texts *Spin Dynamics* [25], *Structural Biology: Practical NMR Applications* [49], and *Protein NMR Spectroscopy* [50]. Protein structure determination methods have improved considerably over the past two decades as a result of improved hardware, software, and isotopic labeling strategies. Hardware advancements such as cryogenic probes increase the sensitivity allowing for low concentration studies. Data processing advancements such as non-uniform sampling (NUS) allow faster data collection or the allocation of a given time to collect more data for higher resolution or signal: noise ratio. Novel pulse sequences, especially transverse relaxation-optimized spectroscopy (TROSY) continue to push the molecular weight limits to nearly 1-megadalton complexes [51, 52]. NMR spectroscopy will continue to be a valuable tool for not only exploring fundamental biological principles, but also understanding the molecular details of a variety of diseases and provide critical aid for designing robust medical therapies.

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