Spectroscopic Methods

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Solid-State NMR Spectroscopy on Complex Biomolecules

*Marie Renault, Abhishek Cukkemane, and Marc Baldus**

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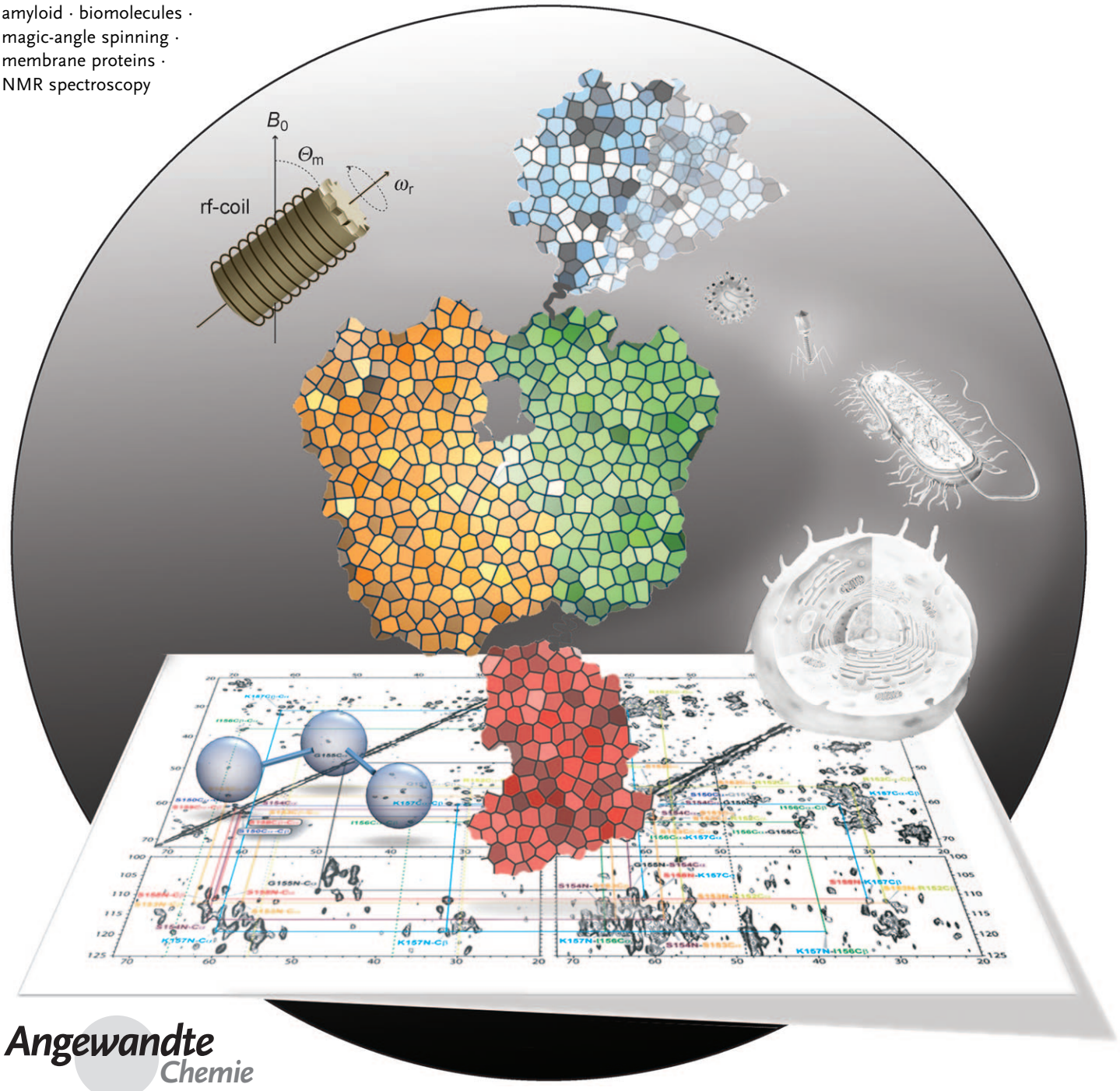

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Biomolecular applications of NMR spectroscopy are often merely associated with soluble molecules or magnetic resonance imaging. However, since the late 1970s, solid-state NMR (ssNMR) spectroscopy has demonstrated its ability to provide atomic-level insight into complex biomolecular systems ranging from lipid bilayers to complex biomaterials. In the last decade, progress in the areas of NMR spectroscopy, biophysics, and molecular biology have significantly expanded the repertoire of ssNMR spectroscopy for biomolecular studies. This Review discusses current approaches and methodological challenges, and highlights recent progress in using ssNMR spectroscopy at the interface of structural and cellular biology.

From the Contents

1. Introduction

The ability of X-ray crystallography and NMR spectroscopy to determine conformations of individual molecules has greatly influenced our current view of chemical and biological processes as well as how to combat diseases related to their failure.[1] These studies usually involve a reductionist approach, in which molecular entities are separated from their natural environment. Today, we know that most physiological processes involve the interaction of molecules within functional modules on different spatial and temporal scales.

In a biological context, increasingly accurate maps based on genetic, physical, and functional interaction data have revealed a higher level of molecular organization in which different molecular players and their spatiotemporal interactions are critical to biological functioning.[2] For example, the cellular response to outside stimuli, such as light or nutrients, or the process of protein aggregation in the context of Alzheimer's or Parkinson's disease take place in a more complex and dense cellular environment than previously envisioned. "Holistic" structure-determination methods that can be applied in a complex molecular environment are of prime importance to understand these fundamental processes at atomic resolution and restore them in a pharmacological context.

The potential of solid-state NMR (ssNMR) spectroscopy as a method capable of delivering such information was revealed more than 30 years ago. Pioneering applications involved heterogeneous biomolecules such as collagen,[3] bone,[4] nucleoprotein complexes,[5,6] and protein gels.[7] In addition, lipid bilayers[8] and membrane proteins[9] (MPs) were early on investigated by solid-state NMR spectroscopy. The following years saw further progress as ssNMR and biological solution-state NMR spectroscopy underwent revolutionary developments through the introduction of multidimensional correlation methods,[10] combined with general concepts to determine entire 3D structures,[11] and the widespread use of isotope labeling.

The availability of dedicated high-field instruments in the late 1990s resulted in such concepts recently been developed for ssNMR spectroscopy of biological systems. In parallel, progress in the area of microscopy[12] and advancements in diverse areas, such as theoretical chemistry, molecular modeling, and biophysics, in general offer the potential to greatly expand the utility of ssNMR spectroscopy for the investigation of complex molecular systems. Thanks to these developments, ssNMR spectroscopy today offers the potential to bridge the gap between classical structural biology methods (such as X-ray or electron crystallography) and imaging methods that can be applied in a cellular context such as X-ray and electron tomography or light microscopy (Figure 1).

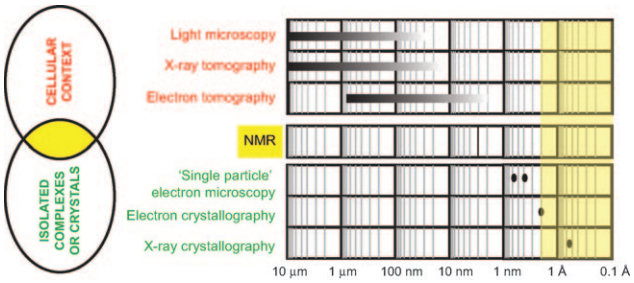

Figure 1. Solid-state NMR spectroscopy can bridge the gap (as indicated by the yellow strip) between conventional structural biology methods that provide high-resolution structures of isolated complexes or crystals (green) and cellular imaging methods (red). The bars and dots reflect typical and maximal structural resolution, respectively, of the techniques. Adapted from Ref. [12].

In the last few years, the ssNMR spectroscopic technique involving magic angle spinning (MAS)[13] has made particularly strong progress in probing the molecular structure of complex biological systems. The versatility of ssNMR spectroscopy in biological and material sciences means that an adequate discussion about all research studies is outside the scope of this Review. Instead, we consider conceptual aspects of ssNMR spectroscopy on complex biomolecules. In addition, we discuss challenges related to such studies and we give an update on recent applications in areas ranging from amyloid proteins and other protein aggregates to membrane-embedded proteins and entire bacterial cells.

[*] Dr. M. Renault, Dr. A. Cukkemane, Prof. Dr. M. Baldus
Bijvoet Center for Biomolecular Research
Utrecht University
Padualaan 8, 3584 CH Utrecht (The Netherlands)
Fax: (+31) 30-253-7623
E-mail: m.baldus@uu.nl

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2. Methodological Challenges

2.1. Preparative Aspects

Many of the preparative methods to deal with spectral crowding in the context of biological solution-state NMR spectroscopy are also of value for use in ssNMR spectroscopy. In particular, the advancements in biochemistry and molecular biology in combination with isotope labeling techniques, and in a more general sense the ability to design biomolecular sample preparations for ssNMR studies, has played a critical role.

Probably one of the first approaches to deal with larger molecules in ssNMR spectroscopy was the use of dedicated isotope-labeling techniques. The production of recombinant proteins expressed either in *E. coli* or by in vitro transcription/translation in cell-free expression systems[14] offer the possibility to incorporate isotope labels within the desired amino acid type, either uniformly or at specific atomic sites, thus allowing the implementation of structurally diagnostic labeling strategies.

A much larger degree of labeling is obtained if the growth medium contains glucose or its derivatives (glycerol, acetate, pyruvate, succinate) as the sole carbon sources and ammonium salts (chloride, nitrate, sulfate) as the sole nitrogen source. The versatility of the carbon sources stems from their primordial role of fueling the cell. Apart from serving as an energy source, glucose supplies an array of intermediates for most of the biochemical pathways. The fate of glucose in all cell types is met invariably in the linear glycolysis pathway. In this pathway, the six-carbon glucose is eventually metabolized into two molecules of three carbons—3-phosphoglycerates—which are converted into pyruvate. The conversion of pyruvate into acetate serves as the intermediary link between glycolysis and the citric acid cycle (TCA cycle). The two pathways together account for all the cellular energy and biomolecule requirements. As we discuss in the following, all the intermediates of these two pathways can provide a biochemical entry point for dedicated ^{13}C labeling, but cost-related factors have to be considered in practical applications of the carbon sources mentioned above.

Uniform ^{13}C,^{15}N labeling, also referred to as global labeling, is attractive since this strategy potentially generates the maximum amount of spectroscopic information from a single sample. However, with increasing size, the analysis may suffer from spectral overlap. Furthermore, larger biomolecules can be characterized by a high repetitiveness of hydrophobic amino acids (as regularly seen in membrane proteins) and a dominant influence of a single type of secondary structure (such as membrane proteins or amyloids). Such effects lead to additional ambiguities in the interpretation of the spectra, thus calling for more refined labeling approaches for protein production, such as specific and selective isotope labeling.

By contrast, bacteria fed with labeled or unlabeled versions of the amino acids just prior to cell induction will produce proteins with the corresponding amino acid labeling pattern as a result of direct incorporation during protein synthesis.[15,16] Selective amino acid labeling can thus not only reduce spectral overlap, but may also reveal specific protein topological regions or domains of special interest in the NMR spectra. Such "forward" labeling has, for example, been used extensively to study membrane proteins and other large biomolecules (see, for example, Ref. [17] for early demonstrations).

In a next stage, position-specific labeling can be achieved by substitution of uniformly labeled glucose by [1,3-^{13}C]-glycerol) or [2-^{13}C]-glycerol in the minimal volume of growth medium. This leads to a characteristic distribution of ^{13}C and ^{12}C isotopes within each amino acid.[18] Such labeling patterns can provide more highly resolved spectra because of the removal of most one-bond ^{13}C-^{13}C scalar couplings, and might facilitate sequential resonance assignment arising from the appearance of characteristic cross-peak patterns for specific types of amino acids.[19] Another example includes characteristic ^{13}C-labeling patterns within methylated and aromatic residues by using [1-^{13}C]-glucose[20] as the sole carbon source. Alternatively, it is also possible to generate protein with specifically ^{13}C-enriched backbone nuclei ($^{13}C'$-$^{13}C\alpha$ pairs) or to incorporate an isolated ^{13}C spin label at methyl sites by using [1,2-^{13}C]-pyruvate[21] and [3-^{13}C]-pyruvate,[22] respectively. Such isotope-labeling strategies can play a critical role in establishing structural constraints by using CC, CHHC, or related correlation methods with larger biomolecules (for reviews, see, for example, Refs. [23,24]).

Another attractive strategy—though not yet demonstrated for ssNMR spectroscopy—is segmental isotope labeling, which involves a protein trans-splicing mechanism.[25] Alternatively, the two polypeptides can be chemically ligated by synthesizing an N-terminal peptide with a thioester end and a C-terminal peptide with a cysteine residue at its amino terminus.[26]

Marie Renault studied Biochemistry at the University of Toulouse, and completed her PhD at IPBS Toulouse/Bruker Biospin France in 2008. Since then, she has been carrying out postdoctoral research at Utrecht University in the group of Marc Baldus on the structural and functional characterization of integral membrane proteins under native conditions.

Abhishek Cukkemane studied Microbiology at the University of Pune, India, and completed his PhD studies at the Research Centre Juelich and the University of Cologne, Germany, in 2007. This was followed by postdoctoral research in Juelich. He joined Utrecht University in 2009. His research concerns the application of NMR spectroscopy techniques to comprehend biochemical and biophysical aspects, with an emphasis on membrane protein–ligand interactions.

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Additional mixed-labeling strategies may be required to identify intermolecular interactions. For example, such approaches use samples in which one protein is ^{15}N labeled while the second, which binds the protein, is produced separately in a ^{13}C-enriched medium.[27] The protein interface is then analyzed by ssNMR experiments that encode magnetization transfer between ^{15}N and ^{13}C. Segmental labeling can also be an option as the molecular size increases. In this approach only a fraction of the protein is studied and data are compared to larger constructs. Such "divide-and-conquer" strategies were, for example, employed with reassembled proteins[28]and multidomain membrane proteins.[29] Along the same lines, spectral crowding can be reduced by studying biomolecules after protein segments have been removed by enzymatic cleavage.[30] A graphical representation of the labeling approaches discussed here is given in Figure 2.

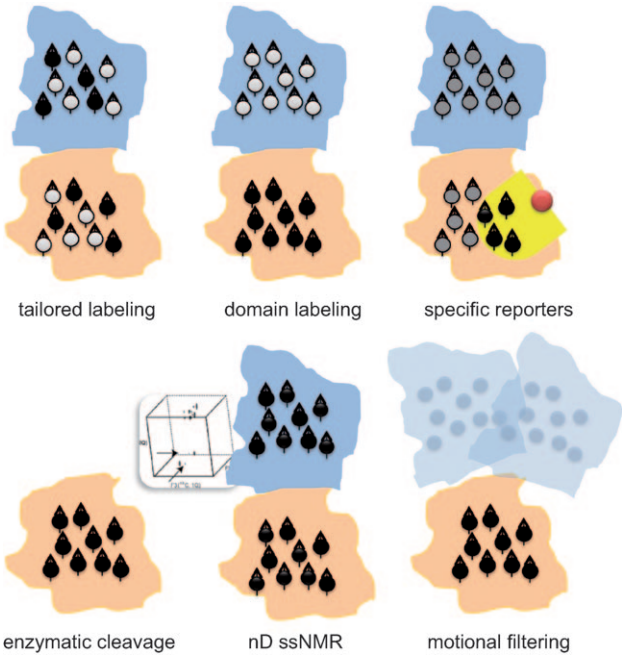

Figure 2. Preparative routes and NMR approaches to reduce spectral complexity in ssNMR studies of large biomolecules.

In addition to the tailored incorporation of ^{13}C,^{15}N-labeling patterns, sample deuteration has been widely used in macromolecular studies. The use of deuterated samples in biomolecules was pioneered by Crespi, Katz, and co-workers in the 1960s.[31] Since then, deuteration has been recognized as a powerful approach to probe macromolecular structure by solution[32] and solid-state[33] NMR spectroscopy. Today, exquisite labeling schemes involving the combination of methyl labeling and deuteration are widely in use in solution NMR spectroscopy.[34] However, protein deuteration often dramatically reduces protein expression levels, influences NMR resonance frequencies and cross-polarization transfer efficiencies, and compromises the possibility to probe structurally relevant ^{1}H-^{1}H distance constraints. As a result, the application of ssNMR spectroscopy to complex biomolecules has thus far been limited. Recent results obtained on a membrane-embedded ion channel in our research group (as yet unpublished) suggest that future applications may involve a combination of fractional deuteration (in which protonated precursors are used during bacterial expression), ultrahigh-speed MAS, and the use of dedicated multiple-pulse schemes. Under such conditions, ssNMR studies may benefit from enhanced ^{1}H resolution, and may readily deliver structural information if proton-detected, multidimensional ssNMR spectroscopy is used.

Marc Baldus studied Physics at the TU Darmstadt and at the University of Florida. He completed his PhD at ETH Zurich and the University of Nijmegen in 1996. After postdoctoral research at MIT and a lecturer position at Leiden University, he worked as group leader at the MPI for Biophysical Chemistry. In 2008, he joined Utrecht University as full professor for structural biology. His research focuses on establishing structure–function relationships in complex biomolecular systems using NMR spectroscopy.

After expression and isotope labeling, there are in principle alternative routes to prepare large biomolecules for ssNMR spectroscopic studies. In the by far most utilized approach, the biomolecules of interest are purified before the ssNMR study. This procedure is particularly challenging in the case of membrane proteins because of their characteristic hydrophobic nature. Protocols involving protein lyophilization or precipitation have been employed for ssNMR studies. In addition, the use of nanodiscs for ssNMR studies of membrane-associated systems has been demonstrated.[35]

However, the preferred method is reconstitution into lipid bilayers. The incorporation of detergent-solubilized membrane proteins in such lipid bilayers occurs spontaneously in the presence of liposomes when the concentration of detergents is reduced. Parameters such as lipid composition, nature of the salts, pH value, and temperature or the presence of an endogenous ligand can be varied to maximize a functional reconstitution at a high protein to lipid ratio. Alternative procedures relying on cell-free expression systems[36] have emerged as efficient and versatile tools for the production of isotope-labeled samples, and were also recently utilized in the context of ssNMR spectroscopy.[37] Membrane proteins can be synthesized directly in the hydrophobic environment such as detergent micelles or lipid vesicles, which greatly facilitates procedures for the preparation of samples of membrane proteins. Purification and reconstitution procedures may finally become obsolete when cell extracts or whole-cell preparations are used. In this case, however, measures must be taken to ensure dominant isotope labeling of the protein of interest. Sample quality can be further improved by reducing or suppressing cellular protein synthesis during the induction phase. By using T7 vector systems, the *E. coli* RNA polymerase can be selectively inhibited by addition of rifampicin to the growth medium.[38] Alternatively, the overexpression of the mRNA interferase MazF, which cleaves ssRNA at ACA nucleotide sequences, can lead to a substantial reduction of the overall host-protein content. As a prerequisite, both the

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gene of interest and the expression vector have to be engineered to be devoid of an ACA codon. This technique, referred to as the single protein production (SPP) method,[39] was recently applied successfully to integral membrane proteins. This approach is particularly attractive since the strong suppression of host-protein synthesis might favor both the expression efficiency of membrane proteins as well as the targeting and correct insertion in the host membrane,[40] thus opening a new route for the structural study of integral membrane proteins by ssNMR spectroscopy. Furthermore, other heterologous expression systems such as *Kluyveromyces lactis*,[41] *Pichia pastoris*,[42] other cell lines, such as Sf9 insect cells,[43] and mammalian cell lines, such as CHO[44] and HEK[45] cells, may become more accessible to NMR studies in the future.

2.2. Sensitivity and Resolution

The intensity of an NMR signal is proportional to the population difference between the spin states, as defined by Boltzmann statistics. Since the spin states are separated by slight energy differences (in the radio-frequency range), the differences in the population between the spin states are small. This makes solid-state NMR spectroscopy a relatively insensitive technique compared to other spectroscopic methods such as EPR or FTIR spectroscopy. The population difference increases with magnetic field strength (B_0) and, thus, one of the benefits of high magnetic fields is improved sensitivity. In practice, the signal-to-noise ratio of modern NMR spectrometers scales approximately with $B_0^{1.5}$, thereby leading to a gain in sensitivity of about 2 when comparing a 18.7 T (800 MHz) spectrometer to a 11.7 T (500 MHz) instrument. At the same time, spectral resolution improves, since line-broadening mechanisms involving dipolar or scalar couplings only appear in higher-order perturbation terms. These aspects and the availability of dedicated high-field ssNMR instruments not only greatly facilitated work on microcrystalline proteins in the more recent years, but also enhance the prospects of studying other heterogeneous systems. For example, Figure 3 shows the beneficial effect of working at higher magnetic fields in the study of the membrane-embedded KcsA-Kv1.3 ion channel. Here, spectral resolution enhancement was critical to obtain resonance assignments.[30]

According to the Boltzmann Equation, the population difference between spin states also increases on conducting experiments at low temperatures (often referred to as cryo-NMR spectroscopy). For example, performing experiments at 128 K can increase the sensitivity by almost a factor of 3 compared to spectra acquired at 0 °C (Figure 3, bottom). Note that significant gains in sensitivity can be obtained at the expense of spectral resolution in protein amyloids as well as in the proteoliposomal preparations. It will be important in future applications to control the line broadening, that is, spectral resolution at low temperatures (see for example, Ref. [46]).

A classical means to reduce spectral complexity involves the use of multidimensional NMR spectroscopy.[10] In the last few years, a series of multidimensional correlation experiments have been developed in the field of ssNMR spectroscopy which can be readily applied to proteins or other biomolecular systems (Figure 2). These experiments, for example, correlate N–C moieties or C–C–C spin networks in three[47] or even four[48,49] spectral dimensions. In parallel, alternative sampling schemes that reduce the experimental time or modify the spectral dispersion without sacrificing spectral information have been explored.[50] Spectral resolution can also be increased by the use of decoupling schemes that remove scalar or residual dipolar couplings. Such schemes are used increasingly to study complex biomolecules (see, for example, Ref. [51]). Another powerful means to deduce structural information in larger biomolecules relates to ssNMR filtering approaches. For example, the addition of molecular reporters that have specific NMR properties, such as reduced relaxation or enhanced abundance, including bulk H_2O, paramagnetic quenchers, or NMR-active nuclei of the lipid bilayer can be used to probe molecular surfaces, interfaces, or local moieties close to the reporter spin of interest (Figure 2). One of the early demonstrations in ssNMR spectroscopy involved the use of magnetization transfer between selectively excited water or lipid spins[52] and protein spins, which was subsequently expanded to multidimensional ssNMR spectroscopic methods.[53–55] Such approaches have been of considerable use in the context of membrane-associated peptides and proteins (see, for example, Ref. [56]) and also offer novel means to examine protein assemblies, including amyloid proteins, by using water[57] or paramagnetic relaxation agents.[58,59]

Even without the addition of dedicated reporter molecules, the differential motional properties often observed in complex biomolecules can help to simplify the analysis by ssNMR spectroscopy. Indeed, molecular motion is a param-

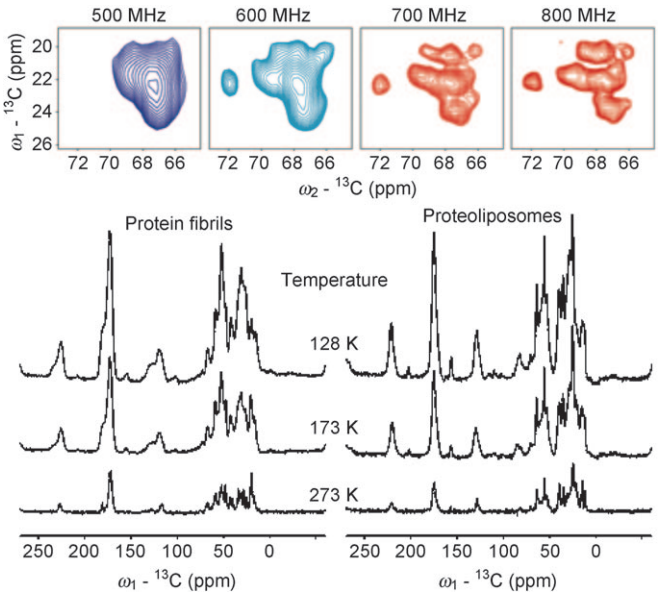

Figure 3. Top: Enhancement of spectral resolution in the ssNMR spectroscopic analysis of the chimeric KcsA-Kv1.3 channel in lipid bilayers. Bottom: Signal enhancement in biological ssNMR spectroscopy by using low temperatures (unpublished results).

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eter that has been investigated for a long time in larger biomolecules by ssNMR spectroscopy.[60] Recently, such studies examined the conformational dynamics of an intact virus[61] or were used to study molecular dynamics in functionally crucial segments of membrane protein.[62,63] A complete discussion of recently developed methods and their particular application to microcrystalline proteins (see, for example, Refs. [64,65]) would be outside the scope of this Review. Briefly, ssNMR pulse schemes that encode changes in spectroscopic parameters, in particular relaxation times including T_1, T_2, or $T_{1,rho}$, are often used. Alternatively, polarization transfer steps that exclusively invoke scalar or dipolar transfer (see, for example, Ref. [66]) and, in the latter case, probe the motional-induced scaling of a known (usually one-bond dipolar) ^{1}H-^{15}N, ^{1}H-^{13}C, or ^{13}C-^{13}C spin pair interaction are incorporated. In general, intermolecular interactions play a prominent role in the solid state.[67] Structural studies on microcrystalline proteins or amyloid fibrils have employed dedicated labeling patterns that separate polarization transfer dynamics arising from intra- or intermolecular transfer[67] and the quenching thereof.[58] Indeed, mixing molecular species with different labeling patterns furthermore offers a route to probe intermolecular contacts by ssNMR spectroscopy.[27,68] Similar to solution-state NMR spectroscopy, an additional reduction in spectral complexity may be obtained by using $^{1}H/^{2}H$ exchange experiments.

An additional increase in spectroscopic sensitivity is necessary to further enhance the applicability of ssNMR spectroscopy to complex biomolecules. A particularly attractive idea is the use of fast repetition methods, in which the necessity of strong radio-frequency fields is lifted by using ultrafast MAS and/or molecular dilution. Favorable relaxation properties are established by adding paramagnetic sites (see, for example, Ref. [59,69]). The theoretical influence of such probes, their effects upon ssNMR spectroscopic data, and their structural interpretation are a particularly interesting field of research. Importantly, the use of such methods has already been demonstrated with amyloid proteins.[59] Even in the absence of paramagnetic relaxation agents, the differential relaxation of biomolecular systems under high-resolution MAS conditions[70] may provide a route to optimize the use of multidimensional ssNMR spectroscopy.[71]

Much larger signal enhancements are possible by using dedicated methods such as Photo-CIDNP (chemically induced dynamic nuclear polarization[72]) and the use of parahydrogen[73] or polarized noble gases including xenon.[74] In principle, such methods are also applicable to the ssNMR spectroscopic analysis of complex biomolecules (see, for example, Ref. [75]). A particularly elegant and potentially widely applicable approach relates to dynamic nuclear polarization (DNP). This technique involves the creation of a large, nonthermal spin polarization that is transferred to NMR-detectable nuclei of interest.[76] Efficient polarization transfer is possible if microwave irradiation under low-temperature conditions is used, which leads to signal enhancements larger than a factor 100 in ssNMR spectroscopy.[77] Efforts to polarize liquids have employed flow methods.[78] In addition, rapid dissolution methods have been developed that permit signal enhancements in the solution state and for in vivo imaging applications.[79]

Recently, dedicated ssNMR DNP spectrometers have become commercially available and DNP enhancements of 20–100 have routinely been obtained on such instruments. A recent result obtained in our research group (unpublished results) confirms that DNP can also be applied to cellular preparations. Clearly, the widespread availability of such instruments will spark the development of additional routes to the preparation of samples. Furthermore, the development of custom-made polarization agents will not only enhance the overall signal intensity,[80] but the polarization source may also be placed close to the molecular area of interest by using endogenous radicals or by chemical modification.

2.3. Integrated Approaches

In the last few years it has been clearly demonstrated that entire 3D molecular structures can be obtained from ssNMR data alone (see, for example, Ref. [24] for a recent review) and that ssNMR spectroscopy can be used to probe biomolecular motions over different time scales (see, for example, Ref. [64]). At the same time, classic structural biology methods (Figure 1) continue to provide exciting insights into the atomic details of the isolated/solubilized biomolecular complexes, and molecular modeling approaches have made remarkable progress in predicting entire molecular structures.[81] These developments along with the increasing utility of biophysical techniques such as electron microscopy (EM), Förster resonance energy transfer (FRET), and FTIR spectroscopy strongly suggest that future biomolecular applications of ssNMR spectroscopy will profit from applying hybrid concepts to solve challenging problems in complex biomolecules.

The ability to predict the NMR frequencies from first principles[82,83] or the use of hybrid strategies (see, for example, Ref. [84]) has already changed the ways in which (isotropic and anisotropic) chemical-shift information is today used in solution-state and solid-sate NMR spectroscopy. In general, chemical shifts depend on several factors, such as hydrogen bonds, electric field effects, ring currents, and backbone and side-chain torsion angles. A variety of NMR concepts have been developed to assess protein structures and, in favorite cases, to guide the process of determining the 3D structure by both solution-state and solid-state NMR spectroscopy.[29,85] On the other hand, chemical shifts provide a rich source for probing intermolecular interactions in molecular crystals[83] and in certain biomolecules, such as photosynthetic membrane proteins, with a high density of chromophores.[86] Under such conditions, the combination of molecular modeling studies and explicit chemical-shift calculations may be required to establish reliable structure–function relationships (see, for example, Ref. [87]).

Moreover, the combination of solution-state and solid-state NMR data can provide a rapid means to structurally resolve multidomain biomolecules, including protein assemblies, and multidomain membrane proteins[63,88–90] or can offer

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a route to sample different motional regimes. In many cases, X-ray crystallography and SAXS data can provide structural information that can often be directly compared to ssNMR results (see for example, Ref. [91]). In addition, the entire arsenal of state-of-the-art biophysical methods including FTIR[90] and Raman spectroscopy,[92] fluorescence methods,[93,94] and mass spectrometry[95] can provide a valuable reference for ssNMR studies on complex biomolecules.

Finally, there is an increasing number of examples were ssNMR studies are combined with computational methods including molecular docking,[96–98] in silico modeling,[29] or molecular dynamics studies.[99] Such strategies suggest that many future applications of ssNMR spectroscopy on complex biomolecular systems will involve an iterative approach (Figure 4). In these, structural models obtained from NMR spectroscopy or other resources are evaluated using ssNMR data, and procedures described in Sections 2.1 and 2.2 are used to refine the resulting analysis of biomolecular structure, motion, and, ultimately, functionality in a complex environment.

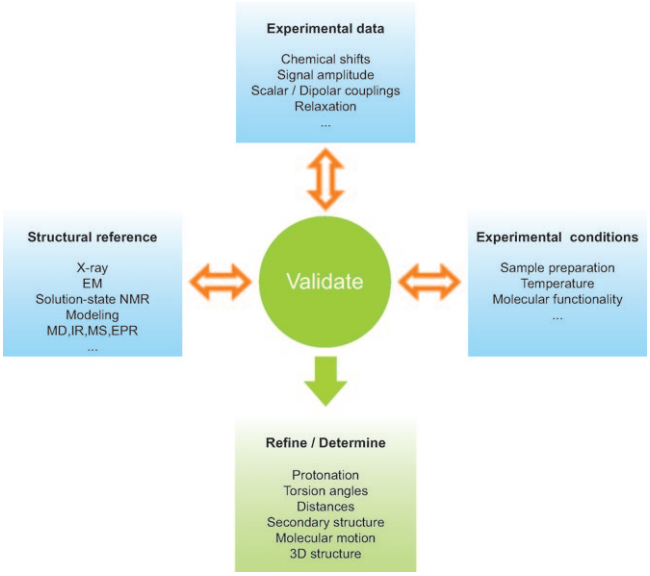

Figure 4. Towards integrated approaches in the ssNMR spectroscopic study of large biomolecules.

3. Applications

3.1. Amyloids and Other Aggregates

ssNMR spectroscopy has become the premier structural method to study amyloid fibrils.[100,101] Three-dimensional structures have not only been obtained for several amyloid proteins of medium size but high-resolution studies were also possible for significantly larger proteins, such as those related to α-synuclein[102] and even the full-length HET-s prion.[103] These studies employed pulse sequences that are sensitive to different motional regimes. In addition to the structural characterization of amyloid fibrils, ssNMR spectroscopy also becomes increasingly successful in the study of aggregation intermediates. Recent reports[104] have studied variants of the β-amyloid peptide and α-synuclein, and can provide crucial insights into the folding landscapes of amyloid and other aggregating proteins. Similar to other biophysical methods, these experiments pave the way towards in situ studies of protein folding and aggregation by solid-state NMR spectroscopy. A particularly interesting step in this direction was reported by Tycko and co-workers, who investigated β-amyloid fibrils that were grown on seeds from fibrils derived from brain of Alzheimer's patients.[105]

While these and other ssNMR studies focussed on disease-related aspects of amyloid proteins, there is also a growing interest in the functional role of "amyloid-type" folds. Such states are naturally seen in the case of prion proteins[101] and also relate to protein components of the extracellular matrix.[106] In addition, ssNMR spectroscopy also recently established a structural link to proteins of the nuclear pore complex.[93] In particular, the 62 kDa nuclear porin Nsp1 that forms functional hydrogels under in vitro conditions is stabilized by protein sequences and structural motifs that are amyloid-like. Again, the versatile nature of ssNMR spectroscopy to probe both structural and dynamic aspects allowed the examination of protein assembly and organization on the atomic level. In addition, the development of a dedicated ssNMR probe enabled the assembly process to be tracked during data acquisition.[93] Other recent ssNMR studies on complex biomolecules relate to HIV-1 capsid protein aggregates,[107] αB-crystallin,[88] microtubules,[89,108] and proteins that form part of the type-three secretion machines[90].

Of course, molecular organization is not restricted to protein networks, but potentially also involves novel, possibly biologically inspired, materials. In these cases, the combined application of methods that can probe different length scales (such as X-ray crystallography/electron microscopy and ssNMR spectroscopy; Figure 1) already contributes to a structure-based analysis of functional (bio)materials (see for example, Ref. [109]).

3.2. Membrane Proteins

Membrane proteins have been the subject of ssNMR spectroscopic investigations for more than three decades and pioneering studies on bacteriorhodpsin (bR or rhodopsin)[9,16,110] and gramicidin[15] established its utility for studying molecules which are otherwise untenable by other structural tools. In addition to the early studies,[6] ssNMR investigations have also been conducted on membrane-embedded coat proteins of filamentous bacteriophages (see Ref. [111] for a recent review) and have also played an important role in the field of antimicrobial peptides[112,113] and membrane fusion.[114]

In the last few years it has also become clear that ssNMR spectroscopy is capable of delivering comprehensive information about larger membrane proteins. For example, after light activation, retinal proteins shuttle through various states associated with conformational changes, both in the protein and in the chromophore. In the case of rhodopsin, it has been possible to dissect the topology[115] and dynamics[116] of the retinal ligand as well as the activation profile of the receptor

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itself.[45] Different functional states of bR have also been characterized by ssNMR spectroscopy by using DNP-enhanced ssNMR and irradiation with laser light.[117] In the case of sensory rhodopsin II, ssNMR spectroscopy was used to examine receptor topology, complex formation, and activation in a natural membrane environment.[55,63] In addition, novel families have been studied by ssNMR spectroscopy.[118] These studies demonstrate that, even in the ground state, a protein may exist in several conformations and it may be possible to identify the different species.

ssNMR spectroscopy has also played a prominent role in determining the structural biology of the M2 channel, both in terms of the channel structure and function[119] as well as ligand binding[120]. Indeed, ssNMR spectroscopy has for a long time been used to study ligand binding in functional membrane protein. Such ligands, including toxins from various spiders and scorpions, represent an important source of natural drugs. It is essential to understand ligand–protein interactions at atomic resolution for a rational improvement of the biological and therapeutic use of such drugs. In fact, 3D structural information was obtained on a toxin bound to a potassium channel.[97] Different functional and ligand-binding states for natural and synthetic blockers have been probed using ssNMR spectroscopy.[54,96,121] Further studies on interactions between ligands and membrane proteins involved other ion channels[122] and G-protein-coupled receptors (GPCRs).[123]

Many other membrane proteins have also been investigated using ssNMR spectroscopy, including membrane-embedded enzymes,[49,98,124] histidine kinases,[29] ABC transporters,[125] and bacterial outer-membrane proteins.[126] Again, an appropriate discussion on this research area is beyond the scope of this Review, and the reader is referred to recent reviews on this subject.[113,127]

3.3. Cell Walls, Biomaterials, and More

An ultimate goal in biomolecular spectroscopy and microscopy is to bridge the gap between structural and cell biology. Solid-state NMR spectroscopy is a method that is capable of providing atomic information under such in situ conditions and has already been utilized in this direction for more than two decades.[128] In the following, we focus on recent applications in this area of research (Figure 5).

Firstly, ssNMR spectroscopy offers the possibility to infer biological structure and cellular organization, for example, of rigid cell walls of bacteria, fungi, or plants. Indeed, high-quality solid-state NMR spectra could be obtained on intact *E. coli* peptidoglycan sacculi. Through-bond 2D ssNMR correlation experiments revealed atom-resolved information about the structure and the dynamics of the bacterial peptidoglycan and its interactions with its protein partners.[129] Antibiotics that target peptidoglycans have been of great scientific interest since the discovery of penicillin. ssNMR spectroscopy, in particular, has been very effective in deducing the mode of action of these drugs in intact cells and cell wall preparations of pathogenic bacteria, for example, by probing intermolecular dipolar couplings under MAS conditions.[130] Moreover, the tertiary structure of the peptidoglycan has been investigated by using ssNMR ^{13}C spin-diffusion experiments[131] by transferring magnetization from the labeled pentaglycl bridges to neighboring natural abundance ^{13}C in the main chain. Similar strategies have also been employed to study virulence factors from labeled bacterial lipo-polysaccharides[132] and cell-wall precursors in fungal pathogens.[133] The details of the tertiary structure of biopolymers, notably the plant cell wall, are much sought of in the food industry. Soluble[134] and insoluble[135] extracts of plant biopolymers relevant for food storage and cell-wall synthesis indicate highly dynamic polymorphic structures with a variety

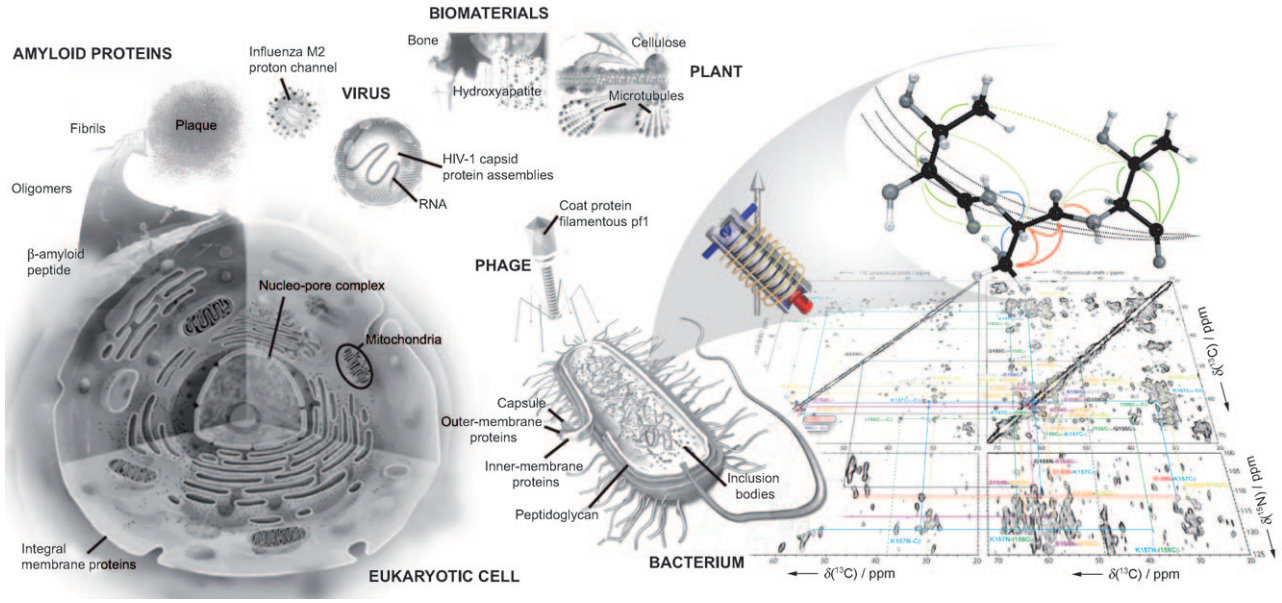

Figure 5. ssNMR spectroscopy is a versatile tool to study complex biomolecules in vitro and, as demonstrated in several of the indicated cases, under in situ conditions.

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of molecules holding the system together. One recent study in which the structure of cellulose, which is the structural facet of the plant cell wall, was examined by 2D ssNMR dipolar recoupling experiments[136] revealed a considerable degree of polymorphism.

ssNMR spectroscopy has also contributed significantly to the atom-resolved description of the structure of the cell walls of diatoms.[94] The siliceous cell walls of diatoms are held together by unique peptides in the form of silaffins[137] and polyamines.[138] Understanding the architecture of the ornate cell wall of diatoms is central to nanotechnology for the development of nanoelectronics. ssNMR spectroscopy also provided structural insight into other biomaterials, such as apatite minerals. For example, ^{1}H-^{31}P HETCOR and $^{15}N(^{31}P)$-REDOR experiments were used successfully to determine the hydroxy ion content of biological apatite crystals of bone and to probe molecular interactions between salivary protein and the surface of hydroxyapatite crystals found in the dentine cementum (see, for example, Ref. [139] for recent work).

Another area of research relates to cell organelles. Sivertsen et al. recorded 2D ^{15}N-^{13}C correlation experiments on gas vesicles from cyanobacteria[140] which revealed that the major protein component was organized into an asymmetric dimer. In addition, ^{31}P ssNMR spectra were obtained on mitochondria from potato tuber[141] to study lipid composition under various stress conditions. ssNMR spectroscopy provided the first atomic description of the molecular organization of inclusion bodies[142] and its spectroscopic principles contributed to the metabolic profiling of complex biomolecules.[143]

4. Conclusions and Outlook

For more than three decades ssNMR spectroscopy has offered atomic insight into the structural organization of complex biomolecules. With recent advancements in NMR spectroscopy, biophysics, and related research areas, ssNMR spectroscopy has the potential to study whole cells or other complex systems at the highest (that is, atomic) structural resolution and in an increasingly comprehensive manner. A central aspect of whole-cell functioning is the concerted actions of interacting proteins or other biomolecules that assemble into molecular complexes and networks. For example, there is increasing evidence that cellular biogenesis involves molecular players at the interface of several molecular compartments. So far, the structural organization of such macromolecular entities has been elucidated by incorporating structural models from single-particle electron microscopy, cellular electron tomograms, or by utilizing high-resolution information obtained from X-ray crystallography on isolated crystals (see for example, Ref. [144]). Since ssNMR spectroscopy can deliver atomic-resolution information in complex molecular systems, such models could in the future be replaced directly by structural or dynamic data obtained in situ.

ssNMR spectroscopy is already the leading technique to study amyloid aggregation and fibril formation; atomic models from monomeric proteins may be inadequate or simply not available. Even if reference information and high-resolution structural information is accessible from isolated molecular subunits, molecular assembly and function may require structural reorganization, as seen in recent ssNMR studies on the type III secretion system.[90] Likewise, ssNMR spectroscopy offers the potential to follow structural rearrangements in different functional states in the intact molecule.

For such studies, it is also clear that increasing the molecular complexity will require further advancements in both spectroscopic sensitivity and resolution. Importantly, efficient signal-enhancement methods that are crucial to provide structural information in a cellular environment have already been applied successfully to amyloid systems and membrane proteins. The combination of isotope and paramagnetic labeling, the introduction of non-natural amino acids, and the tailored use of polarization agents will provide new possibilities to study biomolecules of increasing complexity. At the same time, further advancements in ssNMR methods and instruments are likely to push the current boundaries of biomolecular ssNMR spectroscopy, thereby bridging the gap between traditional structural biology and cell biology.

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