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# Solid-State NMR-Based Approaches for Supramolecular Structure Elucidation

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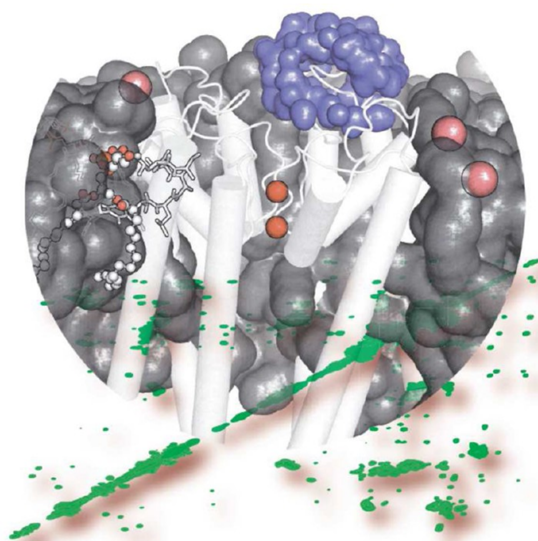
## CONSPECTUS

Supramolecular chemistry provides structural and conformational information about complexes formed from multiple molecules. While the molecule is held together by strong intramolecular contacts like covalent bonds, supramolecular structures can be further stabilized by weaker or transient intermolecular interactions. These interactions can confer a great diversity and sensitivity to exogenous factors like temperature, pressure, or ionic strength to multimolecular arrangements.



Solid-state nuclear magnetic resonance (ssNMR) can provide atomic-scale structural and dynamical information in highly disordered or heterogeneous biological systems, even in complex environments such as cellular membranes or whole cells. In these systems, the molecule of interest no longer exists as a separate unit, but it entangles with its surroundings in a dynamic interplay. Researchers have long accounted for the complexity of these intermolecular arrangements through a rather phenomenological description. But now the focus is shifting toward a detailed understanding of supramolecular structure at atomic resolution, constantly expanding our understanding of the stunning influence of the environment.

In this Account, we discuss how ssNMR can help to dissect the remarkable interplay between intra- and intermolecular interactions. We describe biochemical and spectroscopic strategies that tailor ssNMR spectroscopic methods to the challenge of supramolecular structure investigation. In particular, we consider protein−protein interactions or the protein−membrane topology, and we review recent applications of these techniques. Furthermore, we summarize methods for integrating ssNMR information with other experimental techniques or computational methods, and we offer perspectives on how this overall information allows us to target increasingly large and intricate supramolecular structures of biomolecules. Advancements in ssNMR methodology and instrumentation, including the incorporation of signal enhancement methods such as dynamic nuclear polarization will further increase the potential of ssNMR spectroscopy, and together with additional developments in the field of NMR-hybrid strategies, ssNMR may become an ideal tool to study the heterogeneous, dynamic, and often transient nature of molecular interactions in complex biological systems.

## 1. Introduction

Solid-state nuclear magnetic resonance (ssNMR) has recently made remarkable progress in providing detailed insight into the structural and dynamic organization of complex biomolecules. To name a few, applications range from biomaterials,[1] protein assemblies,[2−8] and amyloids[9−12] to membrane proteins,[13−16] as well as cellular preparations.[17] Not surprisingly, ssNMR methodology developed for such studies shares many analogies to what have become standard protocols to determine molecular structure by NMR methods in solution. For example, the three-dimensional structure determination of proteins today involves resonance assignments as well as the collection of a sufficient number of structural constraints irrespective of whether the molecule is tumbling in solution and exhibits reduced or no local overall mobility.

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However there is an important difference that distinguishes ssNMR structural investigations from NMR studies in solution. It relates to the fact that with decreasing diffusional motion the influence of molecular components surrounding the molecules of interest become relevant. In preparations such as fibrils or protein hydrogels, these interactions are mediated by intermolecular contacts among identical monomer units and may be further stabilized by small molecules including water or ions. Moving to more physiological conditions such as cellular membranes, organelles, or whole cells, the complexity among intermolecular interactions further increases.

Solid-state NMR has developed into a spectroscopic method that can provide structural information even in such complex environments. The availability of high-field and high-sensitivity ssNMR instrumentation and an expanding methodological repertoire have increased the potential of ssNMR to infer structural information in a complex molecular environment. Together with other biophysical methods such as electron microscopy (EM) and developments in computational structural biology, powerful approaches are currently being developed to place ssNMR data into a macromolecular context. In such studies, ssNMR approaches that are tailored to obtain information about the supramolecular arrangement of biomolecules are critical.

Here, we will describe ssNMR approaches to study protein−protein and protein−ligand interactions with a special emphasis on membrane systems. Subsequently, we exemplify how computational structural biology can be used to aid ssNMR studies. Finally, we discuss recent applications that underline the growing utility of ssNMR in studies ranging from self-assembled biomaterials to whole cells to obtain information about the supramolecular arrangement in a biological context.

## 2. Sample Preparation and ssNMR Spectroscopic Setup

Experimental approaches and applications discussed here rely on the use of magic angle spinning (MAS, refs 18 and 19) and the combined application of heteronuclear decoupling and polarization transfer schemes. The combination of one-dimensional experiments and selectively or sparsely labeled biomolecules can be sufficient to probe intermolecular structural constraints (e.g., see refs 20 and 21). In the following, we will however largely concentrate on applications that employ two- or higher-dimensional correlation experiments. With increasing molecular size and complexity, variations in the degree of isotope labeling and the combination of differently labeled molecules (see ref 22 and references therein for a detailed discussion of labeling strategies) has become an essential aspect in the study of protein−protein interactions and membrane protein organization. In the latter case, ssNMR also profits from using isotope-labeled lipids and by varying the bilayer composition from single synthetic lipid types to native membranes. In the case of cellular preparations, different preparative routes exist to produce isotope-labeled proteins in a cellular setting (e.g., see refs 17 and 23).

## 3. Protein−Protein and Protein−Ligand Interactions

Comparing chemical shifts before and after complex formation is a convenient means to probe protein binding interfaces. For example, this strategy has been employed to map the binding interface in the protein/peptide complex Bcl-xL/Bak.[24] In membranes, binding of kaliotoxin to the chimeric KcsA−Kv1.3 channel,[13] as well as the formation of the sensory rhodopsin/transducer complex (SRII/HtrII),[25] were investigated. Of course, these approaches are analogously applicable to small molecular ligands. For example, amantadine (ref 15)-binding sites of the influenza M2 proton channel or the binding mode of porphyrin[14,26] to the chimeric KcsA−Kv1.3 channel were studied by ssNMR.

While chemical shift perturbation studies provide useful insights into residues affected by complex formation, specific ssNMR pulse schemes in combination with isotope labeling strategies have been developed to obtain distance restraints across molecular interfaces (Figure 1a). The direct detection of intermolecular contacts at the protein−protein interface in uniformly labeled samples is usually prohibited by spectral crowding and dipolar truncation. Hence, differential labeling strategies (involving species X and Y in Figure 1) are usually utilized to probe protein interfaces. A frequently applied strategy refers to the use of equimolar mixtures of $^{13}$C- and $^{15}$N-labeled proteins in combination with a $^{15}$N−$^{13}$C transfer schemes.[27] In general, polarization transfer across the molecular interface, which may be facilitated by the application of low temperatures to freeze out dynamics, can be brought about either via the relatively small dipolar $^{15}$N−$^{13}$C couplings by REDOR- or TEDOR-based transfer schemes[28,29] (Figure 1b) or by involving protons in the context of NHHC[27,30] and PAIN[31] experiments (Figure 1c,d). Also, combinations of such schemes have been employed to study protein interfaces of uniformly ($^{13}$C,$^{15}$N) and $^{15}$N-labeled proteins.[6]

As indicated in Figure 1, in principle any combination of spin 1/2 species X and Y can be studied by schemes shown

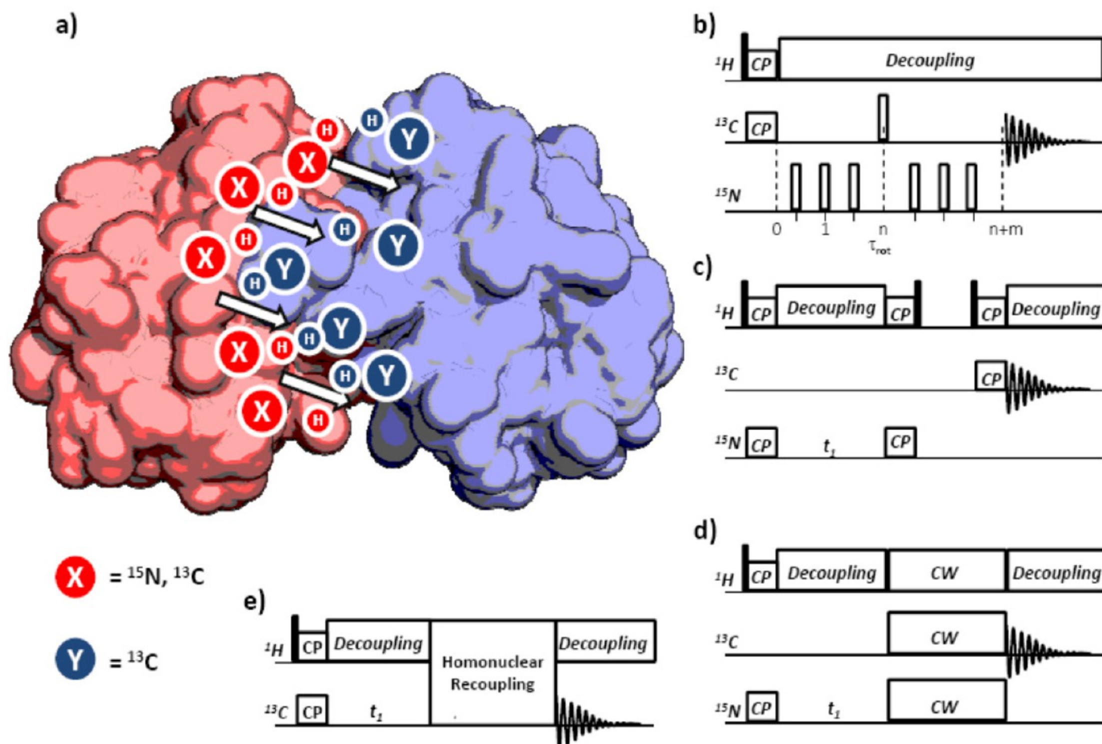


**FIGURE 1.** (a) Identification of protein–protein binding interfaces by using mixtures labeled with species X and Y and dedicated ssNMR schemes. For X = $^{15}$N to Y = $^{13}$C, polarization transfer can be brought about by heteronuclear mixing sequences such as (b) REDOR,[56] (c) NHHC,[30] or (d) PAIN.[31] Homonuclear recoupling sequences can be used for X–Y transfer (such as X labeled on the basis of [1-$^{13}$C]-glucose and Y labeled using [2-$^{13}$C]-glucose). Filled and unfilled rectangles represent 90° pulses and 180° pulses, respectively, if not indicated otherwise.

in Figure 1. For example, in complexes involving nucleic acids, $^{31}$P nuclei are a convenient source to establish heteronuclear transfer and can yield unambiguous intermolecular information.[32] Using protein mixtures labeled on the basis of [1-$^{13}$C]- and [2-$^{13}$C]-glucose has emerged as another strategy to probe intermolecular contacts of larger proteins. As indicated in Figure 1e, homonuclear recoupling sequences can be employed and spectroscopic sensitivity and dispersion of the $^{13}$C dimension usually compare favorably to $^{15}$N spectroscopy. Applications have been reported for the determination of the αB-crystallin core domain homodimer[3] and helped to define supramolecular interfaces of the type III secretion needle.[5]

Next to polarization transfer across the interface, site-specific labeling with paramagnetic tags can be used to define protein–protein interfaces. The presence of unpaired electrons accelerates longitudinal and transversal relaxation in proximity of the tag and induces electron–nucleus distance-related chemical shift perturbations, which can be used to deduce long-range structural restraints.[33] Such strategies have been used to study microcrystalline proteins,[34] and they have recently been exploited to reveal the oligomeric state of membrane protein ASR.[35]

Finally, molecular complexation is also accompanied by changes in protein dynamics and plasticity. Tailored ssNMR methods can then be applied that are sensitive to fast (nanosecond), medium (microsecond to millisecond) or slow motion. Already in the case of microcrystalline proteins, a variety of motional degrees of freedom can be probed that may at least in part be related to the molecular environment.[25,36]

## 4. Protein–Membrane Topology

The ssNMR tool box offers a wide variety of experiments to investigate structural, topological and dynamical aspects of intermolecular interactions of membrane proteins, for example with lipids, ions or water (ref.[37–39]). In addition, $^{2}$H and $^{31}$P ssNMR allow probing phase, lateral diffusion, composition, homogeneity and other biophysical characteristics of the membrane itself[40] (Figure 2a). Similar to proteinaceous complexes, investigating the state of the membrane in the presence and absence of a protein yields information about plasticity and dynamics of membrane–protein interaction. The orientation of membrane peptides, in particular, and proteins can be established using oriented samples and exploiting the anisotropic character of ssNMR interactions

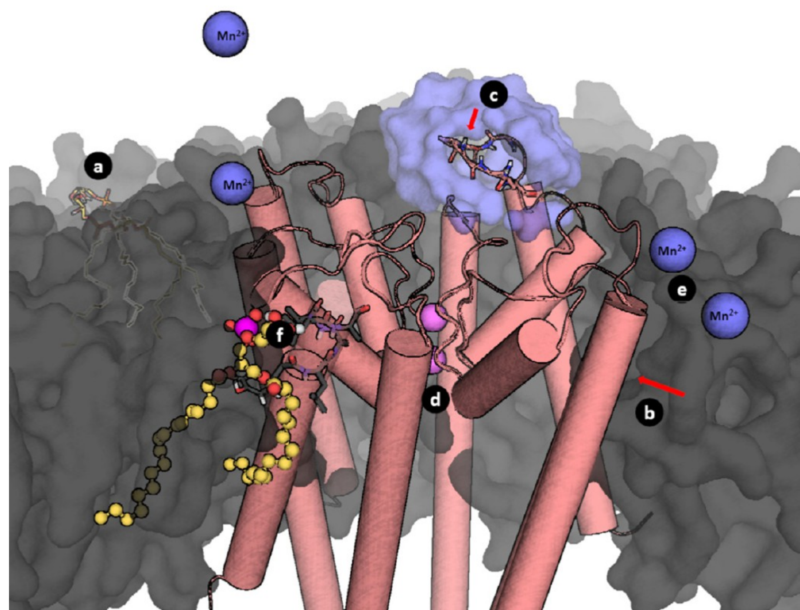


**FIGURE 2.** Pictorial representation of ssNMR methods to study supramolecular membrane protein topology. Studies may target membrane lipids as a function of the molecular environment (a). Furthermore, (b) lipid- and (c) water-accessible protein residues may be investigated by $T_2$-edited experiments or (c) H/D exchange. Alternatively, ssNMR can detect interactions between protein and diamagnetic ions (d). Finally, PRE experiments (e) or $^{31}P/^{2}H-^{13}C$ polarization transfer[39] (f) help define the supramolecular arrangement in membranes.

(see ref 41 for a review). Moreover, the rotational diffusion of membrane proteins at elevated temperatures has been used in MAS ssNMR.[16]

Many MAS ssNMR experiments allow probing protein membrane topology semiquantitatively by selectively i dentifying surface-accessible residues. This selection can be made by appropriately tailored pulse sequences that measure magnetization transfer from the environment (i.e., lipids, water, ions (Figure 2b–d), or other proteins) to the protein. For instance, $T_2$-edited $^{1}H(^{1}H)^{13}C/^{15}N$ experiments[37−39] exploit the wide range of molecular dynamics present in a membrane environment to initially select magnetization of mobile water or lipid residues. Polarization is then transferred to rigid protein protons in a $^{1}H-^{1}H$ mixing step and subsequently relayed to heteronuclei in a short dipolar-based polarization transfer step. On a macroscopic level, this allows distinguishing integral and surface-bound membrane peptides and proteins, and it can also be employed to determine peptide or protein membrane insertion depth semiquantitatively.[37−39] Membrane–protein topology can also be deduced from site-specific proton/deuterium exchange experiments (Figure 2c), in which bulk-water-accessible exchangeable protons can selectively be monitored.[42] Furthermore, paramagnetic relaxation enhancement (PRE) using ions such as $Mn^{2+}$, $Gd^{3+}$, and $Dy^{3+}$, which locate to the surface of lipid bilayers and induce distance-dependent line broadening, can be employed to study membrane protein insertion depth. In this approach, assuming that lipid and protein exhibit comparable mobility, protein insertion depth can be semiquantitatively estimated by comparison of the PRE effects on the protein to that on the lipids, whose segmental depths of insertion are usually known (Figure 2d).[43] By asymmetric application of paramagnetic ions on only one leaflet of the bilayer, PRE has further been used to determine the membrane topology of specific protein residues.[44] Finally, topological details can be deduced from direct lipid–protein polarization transfer via the $^{31}P$ of the lipid headgroups[39] (Figure 2e).

## 5. Supporting Supramolecular Structure Investigations by Computational Methods

Computational methods can support the investigation of protein supramolecular structure by ssNMR from the sample preparation stage to the supramolecular structure calculation. Often, a supramolecular structure analysis by ssNMR deals with increased spectral complexity, for example, resulting from the presence of several molecular components. To address these challenges, significant progress has been made to support and automate the ssNMR data analysis process. One example is the software environment and web portal FANDAS, which allows for the rapid prediction of NMR data sets in multiple spectral dimensions.[45] The resulting data sets permit a direct comparison to experimental

spectra, which can be used, for example, to validate assignments, distinguish different molecular components, and tailor ssNMR experiments.

Once chemical shifts have been assigned, the classical route to NMR structure calculation is to collect distance restraints, a usually time-consuming procedure. Alternatively, procedures such as CS-ROSETTA (ref 46) have emerged, which allow for the *de novo* structure generation from chemical shifts only. The inclusion of chemical shifts in the ROSETTA framework[47] was shown to significantly improve on the selection of molecular fragments. Examples for ssNMR applications of the ROSETTA[47] and CS-ROSETTA[46] algorithm are the DcuS-PAS$_C$ domain of a membrane-embedded sensor kinase,[48] the type III secretion needle,[5] and, more recently, the G-protein coupled receptor CXCR1.[16]

Given that structures of the interaction partners are available, structural insights into molecular complexes are desirable. Here, computational techniques like molecular docking and molecular dynamics (MD) simulations can be of tremendous help. Docking programs such as HADDOCK[49] or the ROSETTA[47] based "dock-and fold"[50] allow for some protein flexibility during the docking and can incorporate experimental data like ssNMR chemical shifts or chemical shift perturbations.[49] Molecular flexibility is an important consideration, since in NMR monomer structure calculations, structures are usually ranked according to lowest energy, which does not necessarily represent the lowest-energy state of a monomer in the complex.

Unlike docking programs, MD simulations can assist both structural and topological refinement of protein structure and dynamics. On a structural level, MD simulations using ssNMR restraints can add a crucial refinement stage,[51] and chemical shifts combined with MD is a powerful means to obtain insights into conformational space and diversity of proteins and peptides.[52,53] Moreover, we have recently developed an approach where MD simulations guide the search to specific protein sites, for example, sites critical for protein structure or involved in lipid–protein interactions.[54] These "hot spots" are subsequently investigated by tailored ssNMR experiments including spectrally selective ssNMR pulse schemes.[55,56] We used this approach, applied as a supramolecular refinement stage, to refine the tertiary structure of a peptide in a membrane setting (Figure 3).

Generally, ssNMR-derived protein/peptide–membrane topologies assume a rigid, planar bilayer. However, protein/peptide–membrane interactions can considerably modulate bilayer structure and dynamics, which in turn strongly influences ssNMR-derived structural parameters such as surface accessibility. We have developed a strategy for MAS ssNMR to reconcile the dynamic nature of membrane–peptide/protein interactions by back-calculating experimental ssNMR observables including the protein–water interface (Figure 3) over long MD trajectories. Once ssNMR and computational data match, the supramolecular structure (which is rather a trajectory) of the peptide/protein is determined (Figure 3). This method was recently used to dissect the supramolecular structure of the Shaker B peptide, representative of voltage-gated potassium channel N-type inactivating domains, in a dynamic membrane setting.[54]

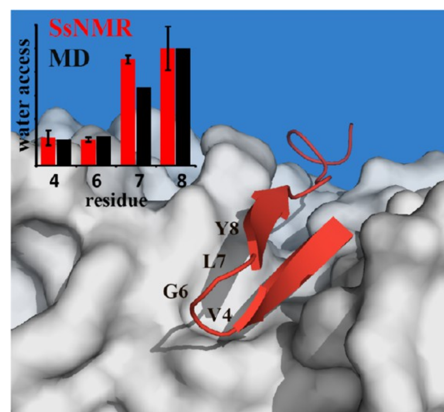


**FIGURE 3.** Supramolecular structure of membrane-associated Shaker B peptide by back-calculation of MAS ssNMR measured water access over MD trajectories. See ref 54 for further information.

Atomistic MD simulations are currently limited to system sizes and simulation times of about 10 nm and a few hundreds of nanoseconds, respectively. These requirements can impose very strong dependencies on the starting conditions, so that atomistic MD simulations allow only qualitative insights in many aspects of the protein–membrane interplay. In principle, mesoscopic coarse-grained (CG) MD simulations[57] could overcome these limitations, and the combination with ssNMR data could emerge as a powerful approach to explore membrane protein supramolecular structure. Indeed, we have recently integrated CGMD simulations, MAS ssNMR, and electrophysiological measurements to probe nonannular lipid-binding to potassium channels.[58]

## 6. Applications

In small molecules, intermolecular interactions may influence crystallinity and can result in polymorphic assemblies that are difficult to characterize by diffraction methods alone. Correspondingly, recent structural studies in the field of material science have profited from what is now termed NMR crystallography[59,60] in which ssNMR is combined with

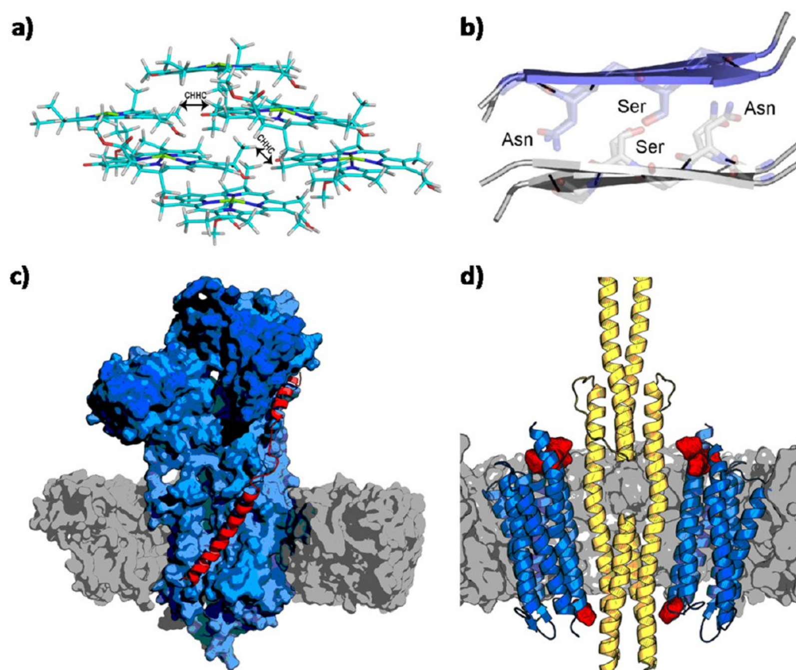


**FIGURE 4.** (a) The alternating *syn*−*anti* stack arrangement of bacteriochlorophyll, found in chlorosomes,[1] as revealed by ssNMR. Figure kindly provided by H. de Groot. Intermolecular interstack ssNMR contacts are indicated by arrows. (b) A structural model of interchain $\beta$-sheets between NQTS-rich spacers of nucleoporin Nsp1p of *S. cerevisiae*. The model was created using molecular docking (HADDOCK),[49] consistent with NHHC[30] interchain contacts. (c) Docking model of phospholamban (AFA-PLN) and SERCA1a (PDB ID 2AGV, blue surface) on the basis of ssNMR structural information and biochemical data. See ref 63 for further information. (d) Structure model of membrane-embedded sensory rhodopsin II/transducer Htr II complex.[25] The transducer is shown in yellow. Sensory rhodopsin II residues probed by ssNMR upon complexation are highlighted in red.

X-ray diffraction techniques, first-principles calculations, or both to obtain structural information that would prove difficult to determine using any one of these techniques alone. A similar strategy was applied to obtain the stacking model of self-assembled bacteriochlorophyll in chlorosomes[1] (Figure 4a). By combining ssNMR distance restraints measured by CHHC[30] experiments and cryo-EM, a 3D molecular view in which chlorosomes self-assemble into coaxial cylinders to form tubular-shaped elements could be established.[1] A similar approach using molecular docking in combination with intermolecular ssNMR distance restraints was used to generate the 3D atomic model of the type III secretion needle, notably by simultaneously integrating volumetric data derived from EM.[5] Alternatively, SAXS data combined with ssNMR restraints were used to model the molecular organization of the $\alpha$B oligomer.[3]

Molecular heterogeneity may also be a result of strong molecular dynamics in the supramolecular structure of interest. Such conditions apply, for example, to hydrogels, which form three-dimensional molecular networks that exhibit a semisolid morphology. Again, intermolecular interactions are critical for assembly and structural stability. SsNMR has been successfully used to probe such interactions in protein-based hydrogels related to the nuclear pore complex using techniques described in section 3. In the case of nuclear proteins (NUPs) related to *Xenopus*, ssNMR results are consistent with a prominent role of glycolysation for network formation.[61] On the other hand, intermolecular $\beta$-sheets are formed that stabilize the protein mesh (Figure 4b) in the case of *Saccharomyces cerevisiae*.[2] Similar interactions also form the essential building blocks of fibrils formed by a variety of amyloid proteins. Using methodology described above, ssNMR has been critically involved in probing the higher-order architecture of fully fibrillized amyloid proteins (e.g., see refs 10 and 28) or amyloid oligomers.[9,11]

Probing molecular motion is a convenient means to monitor molecular interactions. In the case of membrane-embedded protein complexes such effects can be readily studied by comparing ssNMR data obtained for the individual membrane-embedded units to data obtained on the complex provided that resonance assignments are available or dedicated labeling schemes are employed. Previously it was shown that such studies could be used to compare structural views of phospholamban in a membrane environment[62] as well as in the complex with the sarco(endo)plasmic

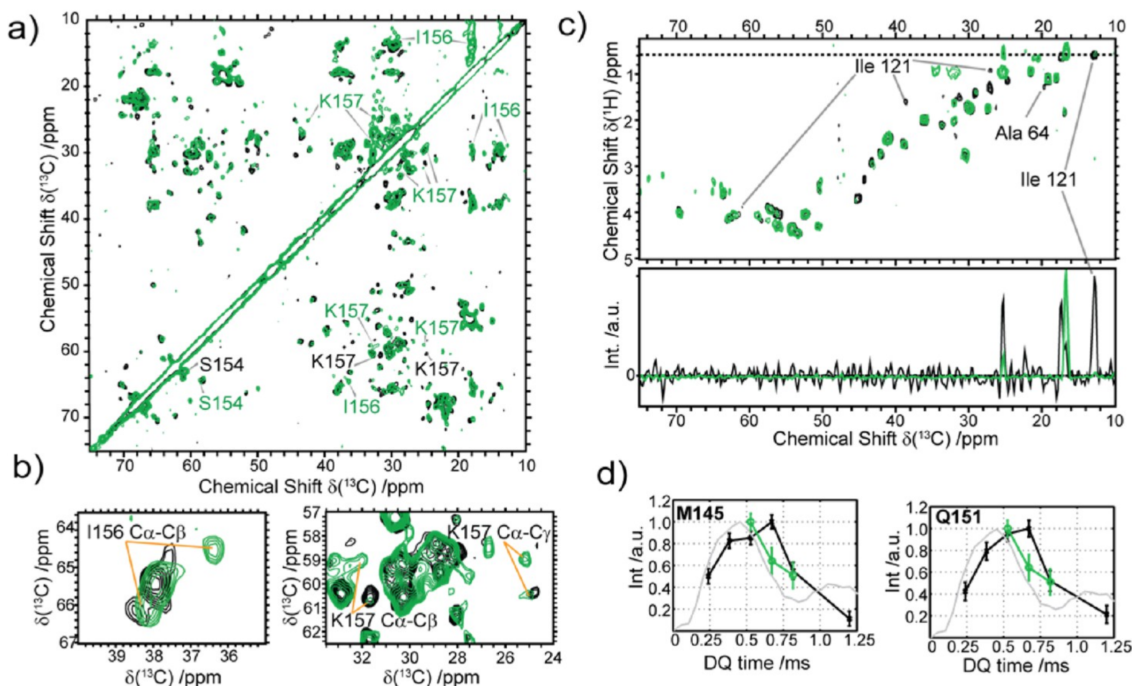


**FIGURE 5.** (a) ($^{13}C$,$^{13}C$) ssNMR correlation spectrum of SRII before (black) and after (green) complex formation with specific chemical-shift changes given in panel b. (c) Through-bond correlation spectrum before (black) and after (green) complex formation. A one-dimensional slice along the indicated f1 position is shown below the 2D spectrum. (d) Variation of (Cα,Cβ) double-quantum mixing efficiency in SRII residues Met145 and Gln151 (EF loop region) of free (black) and complexed (green) SRII (see also ref 25).

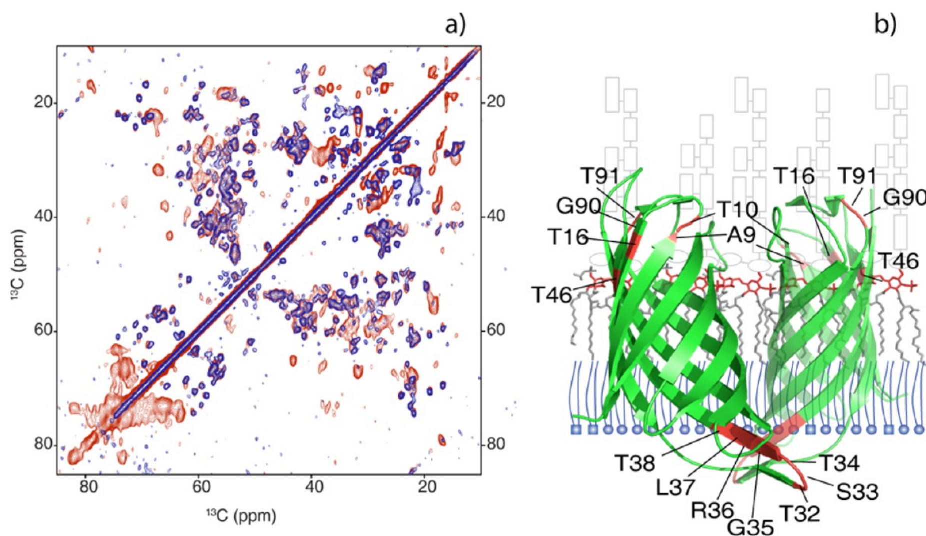


**FIGURE 6.** (a) Overlay of two-dimensional ($^{13}C$,$^{13}C$) spin diffusion spectra obtained on isotope-labeled PagL after isolation of cellular envelopes from IPTG-induced whole cells (red) and proteoliposomes (blue). (b). PagL protein segments indicated in red on the crystal structure (PDB code 2ERV) exhibit chemical shift changes when data shown in panel a are compared. Figure adapted from ref 17.

reticulum calcium ATPase SERCA1a (Figure 4c). The resulting structural model of the complex was obtained using a combination of ssNMR data and biophysical and biochemical data using flexible protein–protein docking simulations.[63] In the case of the sensory rhodopsin/transducer complex (SRII/HtrII), complexation led to a reduction of fast protein dynamics as monitored by through-bond spectroscopy[62] and changes in medium time scale motion (probed by 2Q spectroscopy[36]) upon complex formation[25] (Figure 4d). As visible from Figure 5, the formation of the SRII/HtrII complex yields high-resolution ssNMR spectra in the context of through-space (Figure 5a,b) or through-bond (Figure 5c) experiments,

which facilitates the analysis of motional parameters such as those deduced from the 2Q dipolar excitation profile (Figure 5d).

A higher level of molecular complexity is reached when other essential building units such as nucleic acids, sugars, or lipids are involved in macromolecular structure formation. For example, when using synthetic lipids, the insertion of proteins into lipid suprastructures such as micelles, bicelles, or liposome is influenced by molecular interactions among protein, lipids, and water. Such interactions are even more heterogeneous in the case of cellular preparations, starting with natural lipid bilayers that usually represent lipid mixtures, extending to cellular envelopes that may span several molecular compartments to, ultimately, whole cells. In the latter case, not only do molecular interactions dictate structure and function in cellular membrane layers, but the sheer molecular density leads to molecular crowding in virtually all cellular compartments.[64] Ultimately, the effect of entire cellular compartments can be investigated as was recently shown for Gram-negative bacteria.[17,65] When cellular ssNMR spectra obtained on the outer membrane protein PagL (Figure 6a, red) are compared to data obtained in proteolilopsomes (Figure 6a, blue), alterations in ssNMR signal intensity were detected for PagL residues that are located in protein segments potentially exposed to other major membrane-associated cellular components, that is, lipopolysaccharides (LPS) and the peptidoglycan (PG) layer (Figure 6b).

## 7. Conclusions

Structural biology has recently made remarkable progress to determine folds of soluble proteins and to design de novo fold following general principles.[66] Following early work by Anfinsen, a thermodynamic view has emerged in which molecular structure is closely related to a folding landscape. Structural findings in amyloid fibrils, oligomers, or protein-based hydrogels suggest that in the presence of intermolecular interactions the thermodynamic details are more appropriately described by a supramolecular folding landscape.[67] Similar principles have emerged in the context of membrane proteins where three-dimensional folds are determined not only by the amino-acid sequence but also by the molecular environment and the functional state of the molecule of interest.[68] In this contribution, we have discussed methodological approaches and some recent applications that underline the growing potential of ssNMR to study such heterogeneous biomolecular systems.

Advancements in ssNMR methodology and instrumentation, including the incorporation of signal enhancement methods such as DNP[69] for applications to complex molecular systems,[65,70] will further increase the potential of ssNMR spectroscopy. Together with additional developments in the field of NMR-hybrid strategies, ssNMR may become an ideal tool to study the heterogeneous, dynamic, and often transient nature of molecular interactions in a complex environment.

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ABBREVIATIONS

SEM, scanning electron microscopy; FANDAS, fast analysis of multidimensional NMR data sets; MAS, magic angle spinning; MD, molecular dynamics; ssNMR, solid-state nuclear magnetic resonance; SAXS, small-angle X-ray scattering; PAIN, proton-assisted insensitive nuclei cross polarization; REDOR, rotational echo double resonance; TEDOR, transferred echo double resonance; PRE, paramagnetic relaxation enhancement;

BIOGRAPHICAL INFORMATION

**Markus Weingarth** studied biochemistry at Greifswald University. He did his doctoral studies at ENS (Paris) and received his Ph.D with G. Bodenhausen and P. Tekely. He works currently as postdoc with M. Baldus at Utrecht University, focusing on integrating ssNMR and computational methods to study membrane protein supramolecular structure.

**Marc Baldus** obtained his doctoral degree in 1996 with R. R. Ernst and B. H. Meier at ETH Zürich. After a postdoctoral stay at the MIT/Harvard Center of Magnetic Resonance with R. G. Griffin and a lecturer position at Leiden University, he worked as tenured group leader at the Max-Planck-Institute for Biophysical Chemistry at Goettingen/Germany. In 2008, he joined Utrecht University as full professor for structural biology where he currently heads the NMR Section. His research focuses on establishing structure–function relationships in complex biomolecular systems using NMR spectroscopy.

FOOTNOTES

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The authors declare no competing financial interest.

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