Minireviews
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When Small becomes Too Big: Expanding the Use of In-Cell Solid-State NMR Spectroscopy

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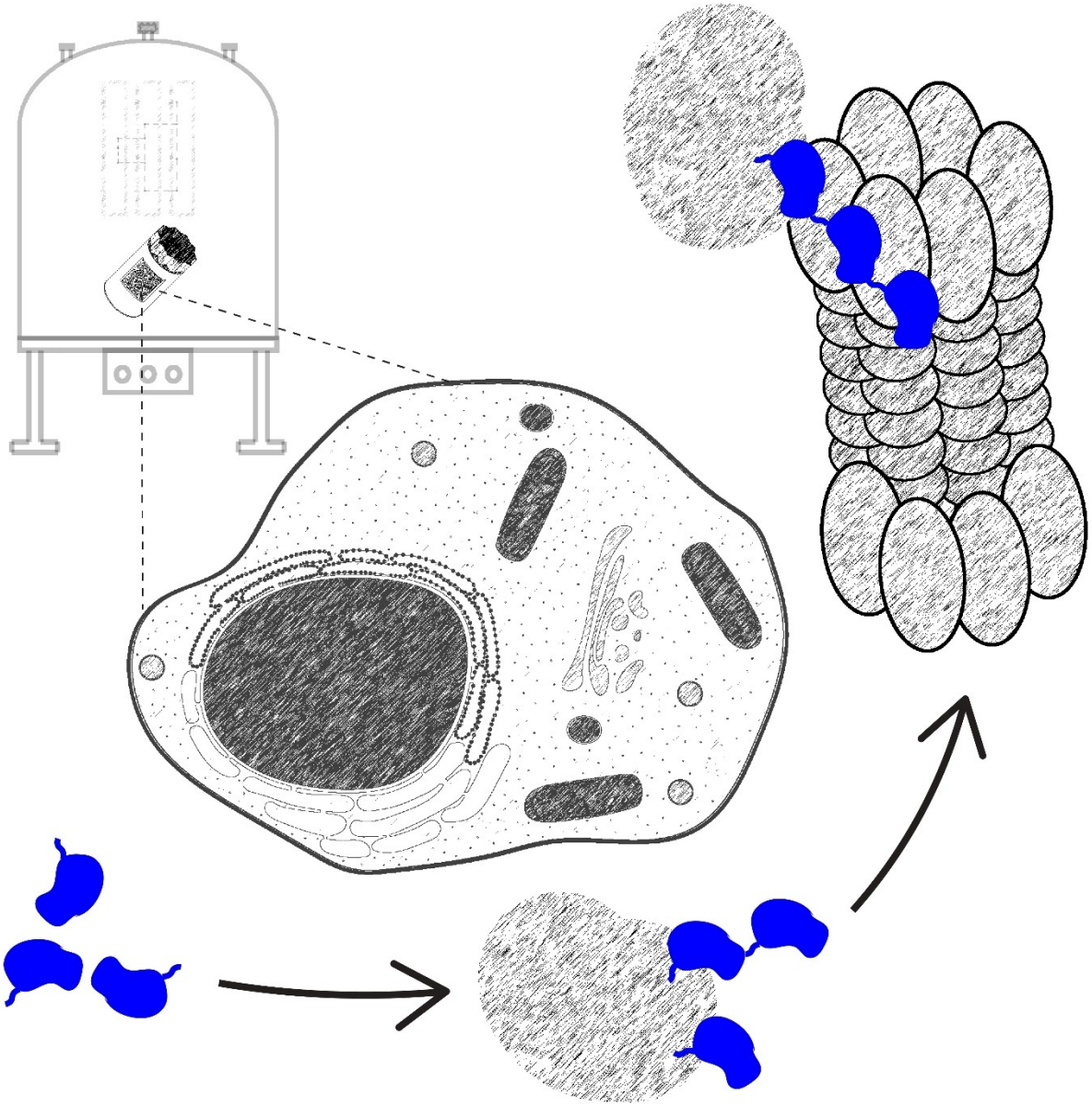

Solution-state NMR spectroscopy has become a powerful tool to study soluble proteins in cells, provided that they tumble sufficiently fast. In addition, cryo-electron tomography (cryo-ET) has recently displayed a tremendous potential to probe structures of large proteins and assemblies in their native cellular environments. However, challenges remain to obtain atomic-level information in native cell settings for proteins that are small, disordered, or are strongly engaged in intermolecular interactions. In this Minireview, we discuss recent progress in using sensitivity enhanced solid-state NMR spectroscopy methods in the context of cellular structural biology.

Introduction

For almost a century, structurally biology has vitally contributed to our current view of life and disease at the molecular level.[1] In the recent years, structural biologists began to realize the need to validate their in-vitro findings by correlative studies on the biomolecule of interest in native environments. Such efforts are motivated by the notion that the structure solved in-vitro, for example in controlled buffer conditions, may not always reflect the true state of the protein in its native environment. The native environment thus remained a significant "blind spot", triggering an incentive to bring together the fields of cell and structural biology.

The potential for conducting "cellular structural biology" has emerged in the last two decades with the advent of in-cell solution-state NMR spectroscopy. This approach enables to non-invasively study small, soluble and well-tumbling proteins in the cellular environment.[2–5] The tumbling requirement excludes three biologically relevant categories of proteins, i.e., insoluble proteins (including membrane proteins), large protein assemblies and small proteins that promiscuously and/or covalently engage in (multiple) intermolecular interactions. In-cell Cryo-Electron Tomography (Cryo-ET) has in the recent years enabled the studies of large protein assemblies.[6–8] In parallel, solid-state NMR spectroscopy has been successfully implemented to study membrane proteins in native lipids,[9–13] also in combination with cryo-ET methods.[14]

This leaves us with proteins which are either too small for detection in cryo-ET and/or too immobile for solution-state NMR. As a result, such biomolecules have for a long time remained invisible for structural methods (Summarized in Figure 1). Next to the field of in-cell EPR[4,15,16] (Electron Paramagnetic Resonance), solid-state NMR in combination with sensitivity enhancement techniques such as ^{1}H detection (see, e.g., refs. [17–20]) and dynamic nuclear polarization (DNP)[21–26] have in recent years shown potential to address such systems. For example, solid-state NMR has recently been applied to whole human cells[27,28] and currently offers prospects for studying many other promiscuous proteins. In this review, we highlight the evolution of NMR in cellular structural biology leading up to the recently developed application of solid-state NMR to study soluble proteins in mammalian cells. We discuss various aspects involved in sample preparation as well as current trends and future directions.

The advent of NMR – adding cellular context to proteins

Until the recent "resolution-revolution" in cryo-EM, the structural characterization of very large proteins and complexes remained challenging for structural biology. Thus, the number of structures deposited in the PDB every year through using any method other than cryo-EM has reached a plateau (Figure 2). A possible explanation for this trend may be the existence of a finite number of unique structures that are of biological relevance. In fact, as per the statistics collected by the CATH database,[29] no new unique topologies have been deposited in the PDB since the year 2012. However, the important question of the biological relevance of many of these structures especially in their native cellular contexts still remains.

With the advent of biomolecular NMR spectroscopy, solving structures without crystallization was already seen as an

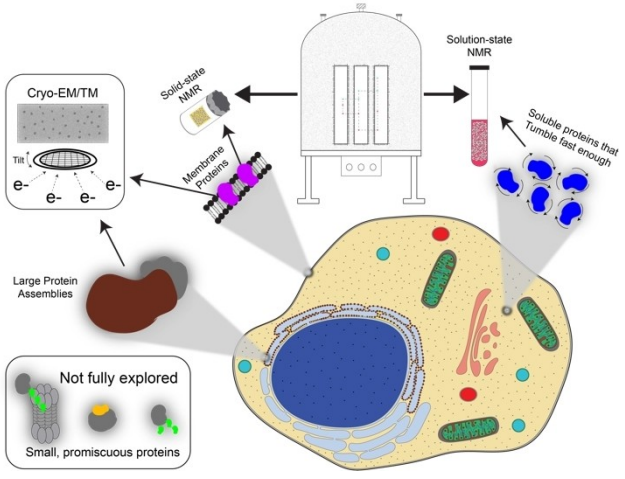

Figure 1. Cartoon depiction of proteins that are amenable to NMR (shown in blue and magenta) or cryo-ET studies (shown in brown and magenta). Small proteins that form larger assemblies or interact with larger proteins (bottom left box, shown in green and orange) were not detectable until recently. One such example discussed in this review, Ubiquitin, is depicted in bright green, covalently linked to substrates and bound to the proteasome (leftmost cartoon in the box). Elements of the figure were reproduced from ref. [28] with permission.

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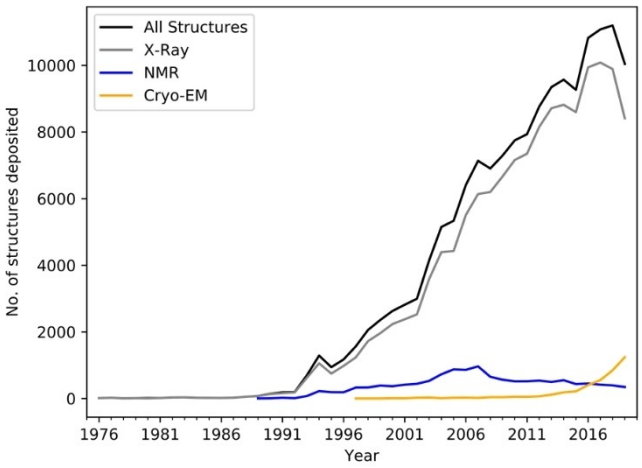

Figure 2. Overview of the number of structures obtained using different structural biology methods that have been deposited every year in the protein data bank (PDB) until late 2019.

innovative leap towards probing a more functional state of macromolecules. The logical, albeit non-trivial, next phase in biomolecular NMR then was to enable acquisition of NMR data or even the ability of solving molecular structures within the cellular environment, denoted as "in-cell NMR".[2] The ability to solve complete structures of most biologically relevant proteins in cells is indeed a difficult endeavor to pursue. Nevertheless, the ability of NMR to determine entire 3D structures of proteins in the cellular environment has already been demonstrated.[30,31] As pointed out already in 2001 by Serber et al., NMR may also be a powerful tool to map out changes on biomolecules due to the influence of the cellular milieu (see ref. [32]). Indeed, for a long time, NMR has been used to seek structural or chemical information of molecules in their native states as it will be highlighted in the next two sections where we review applications of NMR in-situ or in cellular environments throughout the last decades.

Early demonstrations of in-situ NMR spectroscopy

Prior to the early 1990s, structure determination was never the only goal of most NMR spectroscopists in the context of biological applications. As highlighted in the historic overview in Figure 3, experiments were instead aimed at uncovering the (bio)chemical underpinnings of biological processes in-situ by exploiting the non-invasive nature of NMR. This holds especially true for magnetic resonance imaging (MRI), which employs magnetic resonance for visualizing complex systems such as vertebrates and plants non-invasively. In a similar spirit, early in-situ NMR (not excluding in-cell) was focused on acquiring precise chemical information at the atomic scale that complemented imaging at millimeter resolution. The first in-situ experiments, including those targeting intact cells, in fact predate the invention of multidimensional NMR. For example, the lack of mobility of $Na+$ ions was confirmed as early as 1967 in eukaryotic tissue samples, which beforehand had been scientifically disputed.[33]

The following decade (Figure 3) saw an increased application of NMR metabolomic studies, which were elegantly designed to trace metabolic processes over time. Given the high proton (^{1}H) density in biological samples, and the absence of multidimensional experiments at this time, ^{31}P 1D-NMR was used for detection of phosphorus nuclei which were much less abundant in cells as compared to ^{1}H. The ^{31}P nucleus is an obvious choice as it is isotopically 100% abundant and many phosphorus containing biomolecules display unique ^{31}P chemical shifts. ^{31}P NMR was hence used to study a range of complex samples such as bacterial cells,[34] yeast cells,[35] tumor cells,[36,37] cultured eukaryotic cells,[37] rat organs[34] and to track metabolic

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Gert Folkers obtained his doctoral degree from the Hubrecht Institute in 1998, working on retinoic acid receptors. Following this, he worked as a postdoc at the NMR spectroscopy research group in Utrecht University. During this time, he worked on biochemical characterization and structure determination of several proteins. In 2006, he was appointed as an associate professor in the life science faculty at Utrecht University. Since then he has worked on diverse areas of research including studying DNA-damage repair mechanisms and developing novel expression methods for in-situ NMR.

Marc Baldus completed his PhD at ETH Zurich in 1996. After postdoctoral research at MIT and a lecturer position at Leiden University, he worked as tenured group leader at the Max Planck Institute 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 in combination with other spectroscopic, biophysical and cell biology methods.

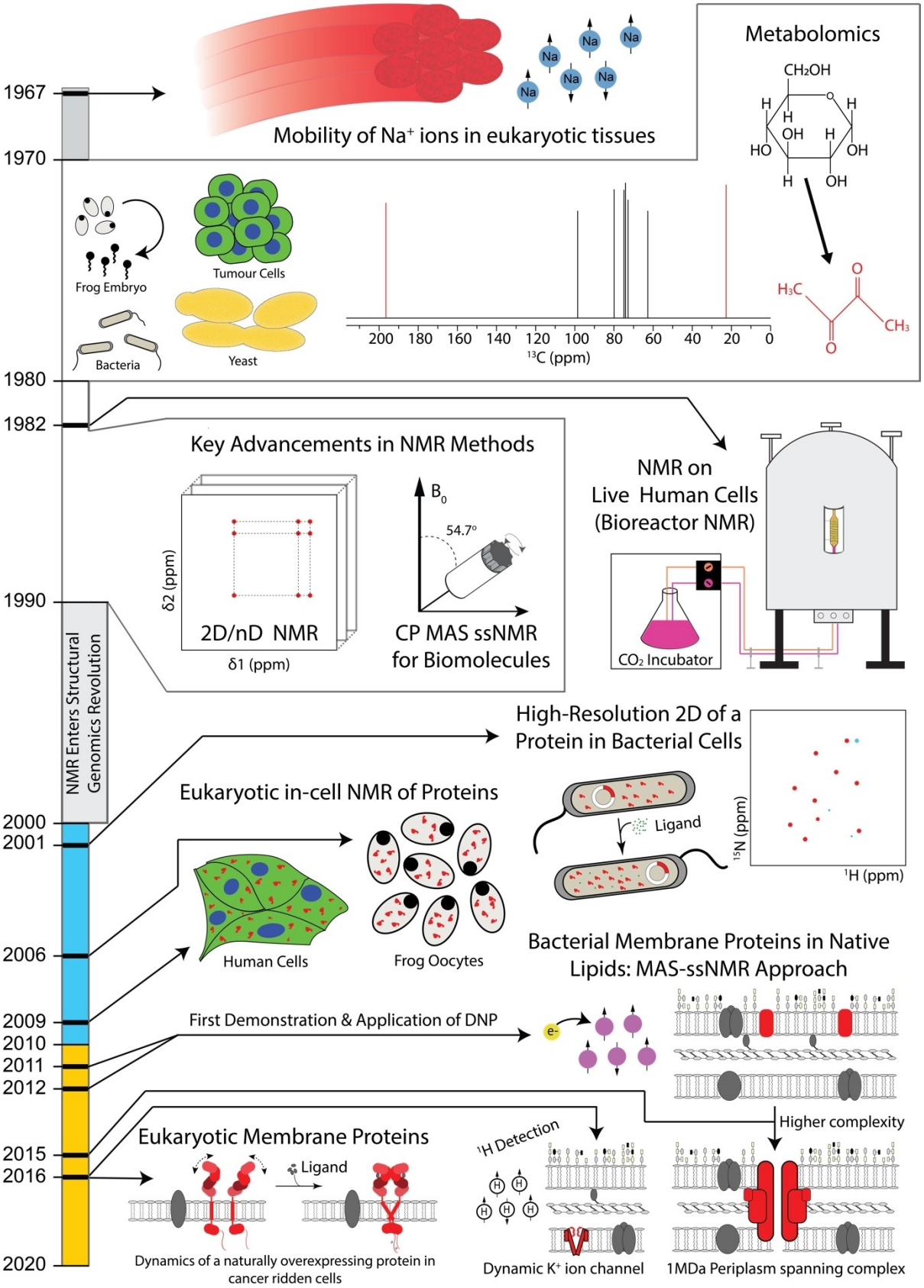

Figure 3. Evolution of NMR spectroscopy for cellular/in-cell and in-situ applications starting from the late 1960s.

changes during the development of *Xenopus laevis* embryos.[38] Next to ^{31}P, ^{13}C NMR spectroscopy emerged as another powerful spectroscopic tool including the use of isotope-enriched glucose and glycerol to track metabolic processes inside cells.[34] Similar approaches have evolved over time and continue to be in use until today. For example, a recent study was reported where fully automated methods were used to track metabolism in real time in viable patient samples over long periods of time.[39]

In the late 1970s through the 1980s, a series of three landmark innovations were made facilitating in-situ NMR studies of complex systems: 1) The development of a continuous flow reactor which allowed for tracking metabolism in live cells upon application of stress and stimuli.[40,41] 2) The development of multi-dimensional NMR experiments[42] which provided the basis for NMR-based structural biology that enables the determination of biomolecular structure and dynamics (see, e.g. refs. [43,44]). Much later, specific methodological advancements including fast multidimensional NMR experiments such as the SOFAST-HMQC[45,46] sequence became of particular use for high-resolution in-cell/in-situ NMR experiments. 3) Cross-Polarization[47] greatly improved spectroscopic resolution and sensitivity in solid-state NMR applications including metabolic studies on immobile parts of the cells such as membranes and/or the peptidoglycan layer.[48]

In-cell/cellular NMR

In-cell solution-state NMR for soluble proteins

The increasing ability of NMR spectroscopists during the 1990s to determine high-resolution structures of proteins played a significant role in structural genomics studies. These in-vitro structures currently serve as valuable references for cellular studies. An early report that linked NMR studies in-vitro and in a cellular environment was published in 1996, where an approach was designed to monitor the integrity of recombinantly overexpressed proteins prior to arduous purification on cell lysates.[49] In the early 2000s, the prospects of structural investigations on biomolecules in-cell entered a new phase with studies of proteins in whole cells. For example, tailored isotope labelling schemes that were developed to obtain complete assignment and hence the structure of the proteins in-vitro, were extended in innovative ways to in-cell structural biology.

In 2001, the first 2D in-cell NMR experiment in bacteria using solution NMR of a metal binding protein, NmerA and the changes following metal introduction into cells were reported.[3] Following this demonstration, the same research group detailed all parameters that must be taken into account to carry out in-cell solution-state NMR for a protein of interest via overexpression in bacterial cells.[50] As eukaryotic systems tend to be more complex, protein overexpression was not initially employed for such systems. Instead, the isotope labelled protein was delivered exogenously into unlabeled cells to ensure complete obliteration of background labelling. This approach was for the first time demonstrated on frog eggs (*Xenopus laevis* oocytes), as they were large enough to be subjected to microinjection.[32,51]

As most eukaryotic cells are usually much smaller than frog eggs, protein delivery through microinjection would not be feasible. Therefore, other means such as pore forming toxins[52] (e.g. Streptolysin-O), protein fusions containing cell penetrating peptides[53] (CPP) and electroporation were explored.[4,54] In their first demonstration of multi-dimensional NMR in HeLa cells, Inomata et al. showed that using a cell penetrating peptide to deliver exogenously produced isotope-enriched proteins had no deleterious effect on the cells.[53] This observation largely holds true also for pore forming toxins such as SLO when used in moderate amounts and if the pores are repaired in time through Ca^{2+} treatment.[52] Using pore-forming toxin offered the additional advantage that modifications to the delivered proteins were unnecessary. Electroporation mediated protein delivery which also does not require the protein to be altered came into prominence later.[4,54] The advantage of this technique is that one retains control over the amount of protein delivered into the cells thereby increasing experimental reproducibility. Complementary to electroporation, mammalian expression systems evolved over time allowing for direct protein overexpression through transient transfection. This approach has, for example, been demonstrated on the metalloprotein superoxide dismutase (SOD) whose maturation process and metal binding properties have been studied using in-cell NMR.[5] Note that the studies on protein folding or maturation are not possible when folded isotope labelled proteins are delivered into the cells.

The protein of interest needs to be relatively inert in cells for detection by solution-NMR studies.[55] Examples of proteins that adhere to such criteria are SOD (mentioned above) or the protein alpha-synuclein (α-syn) which can exist in a multitude of states, ranging from disordered monomers[4,56] to a membrane associated state[57] or forming an aggregated fibril.[58] Next to structural insights as well as experiments that examine transient interactions, such in-cell NMR studies have also provided novel and unexpected insights into largely unexplored chemical impact of the cellular environment including the occurrence of post-translational modifications (PTMs). These processes were first observed in the form of phosphorylation in *Xenopus laevis* oocytes.[59] Later on, N-terminal acetylation of exogenously delivered proteins was detected for the proteins thymosin β4[52] and α-syn.[4] N-terminal acetylation of exogenous proteins is particularly interesting as it has been reported to occur purely by co-translational means,[60] which has been disproved by two separate in-cell NMR studies.[4,52] Due to its ability to study PTMs, NMR hence offers great potential to uncover labile PTMs or other transient chemical processes in cells which are difficult to study in-vitro.

Cellular solid-state NMR of membrane proteins

As discussed above, in-cell solution-state NMR continues to provide a powerful tool for studying small/medium sized soluble proteins if they tumble sufficiently fast in cells. The

molecular size limitation and solubility requirements described above have triggered research into the potential use of biomolecular MAS (Magic Angle Spinning)-ssNMR for cellular studies. When using MAS-ssNMR for in-cell measurements, one must keep in mind that experimental conditions such as temperature or the significant G-force present during sample spinning may limit applications for certain cell types such as cultured mammalian cells in 2D. Such restrictions do not apply to membrane proteins, leading to the development of cellular or in-situ ssNMR. Since membranous components only represent a fraction of the total amount of the whole cell, isolating intact membranes proved to be beneficial for NMR sensitivity because other unwanted cell fractions do not occupy the NMR-active sample volume. Using intact membranes and membrane-containing organelles instead of cells not only improves sensitivity and signal specificity, but also allows one to use high MAS rates without risking cell lysis which may subsequently be followed by degradation via intracellular proteases. Obtaining insoluble cell fractions is rather straightforward and entails mechanical or osmotic cell lysis procedures followed by pelleting of the insoluble material by centrifuging at the appropriate G-force.[61] The two major advancements which have facilitated ssNMR studies on diverse groups of membrane proteins are discussed below.

1)_From small to large proteins: Early progress in membrane proteins was made in bacterial membranes in 2012. Renault et al. managed to successfully implement DNP in cellular-ssNMR studies of membrane proteins, which enabled the measurement of spectra of whole cells enriched with a particular membrane protein of interest.[10] Next to β-barrel proteins[9,62] inserted into the bacterial outer membrane (OMPs), additional studies were done on proteins embedded in the inner membrane of bacteria such as ion- and proton channels[13,63] or chaperones,[14,64] retinal proteins,[65] electron transport proteins,[66] globular proteins[67,68] and peptides.[17] In addition, periplasmic proteins associated with the peptidoglycan layer were studied.[67]

In 2015, it was furthermore demonstrated that such concepts can be applied to large proteins. Kaplan et al. studied a 1MDa periplasm spanning protein complex by combining sample preparation approaches from Renault et al. with amino-acid selective isotope labelling to reduce spectral crowding.[11] In 2016, the approach was extended to eukaryotic systems. This research involved studying the dynamic changes brought about by a ligand-mediated activation followed by autophosphorylation of a large eukaryotic membrane protein, the epidermal growth factor receptor (EGFR, 150 kDa). EGFR is found in high endogenous concentrations in the native membrane environment of the cancer-derived A431 cells.[12] Importantly and as recently shown, the isotope-labelling approach used to produce labeled EGFR is also applicable to other human proteins.[69]

2)_Complete suppression of background labeling: In 2015, Baker et al., demonstrated targeted labelling of membrane protein of interest, by timed expression using non-native RNA polymerases and the subsequent suppression of native *E. coli* RNA polymerases.[70] In the following year, this approach was applied to study the potassium channel protein KcsA in native membranes using high sensitivity & resolution ^{1}H detection schemes at very high MAS frequencies.[13] Targeted labelling is almost mandatory for ^{1}H detected methods as high sensitivity combined with high proton density in biological samples would lead to enhancement of sparsely labelled cellular background. Additionally, by fractional[71] or inverse fractional deuteration[13] and amino-acid selective labelling approaches,[20] it is possible to further focus on specific protein regions using ^{1}H detected ssNMR.

In conclusion, ssNMR methods to probe membrane proteins in native membranes and cells have made significant progress in terms of sample preparation and make use of currently available high sensitivity ssNMR approaches. On the other hand, very large membrane protein assemblies can be detected in the EM tomograms of whole cells[6] and cell envelopes[14] (Figure 1). Increasingly, EM-based studies are delivering in-cell structures of mostly very rigid parts of large protein machineries[6] at progressively higher resolution. In the spirit of cellular structural biology, combination with ^{1}H-detected motion filtered experiments, should make it possible to detect the flexible parts of such protein machineries irrespective of the size. For example, Baker et al. have demonstrated the combined application of ^{1}H-detected ssNMR and cryo-TM to study membrane proteins in their native environment.[14]

Expanding the scope for ssNMR to soluble proteins

As depicted in Figure 1 and discussed above, if one is interested in observing the behavior of most types of proteins in their native habitat, there are increasing possibilities available from either NMR or cryo-EM. This leaves us with one unexplored territory: small protein units or peptides that either assemble into larger complexes or aggregates, often in an inhomogeneous manner, or interact strongly with one or many binding partners. For instance, hub proteins that have been described in interactomes are involved in many key regulatory processes by interacting with other biomolecules. Reduction or even complete removal of molecular tumbling upon complex formation would hinder detection by in-cell solution-state NMR. If the proteins are small and/or disordered they often remain undetectable by EM/ET methods and hence have continued to be elusive to in-cell structural studies. An example of such a protein is Ubiquitin, which is inherently small (8.5 kDa) and thus easy to study in-vitro. But due to its ability to be conjugated to almost any protein and additionally to form complexes with other proteins in the cell (Figure 4), only an inert population of wild-type Ubiquitin is detectable in solution-NMR.[53]

A protein like ubiquitin would be an ideal candidate for solid-state NMR as the small size reduces spectral crowding, enabling the detection of both bound and free populations of Ub. However, attempting to study whole cells with MAS-ssNMR not only poses the problem of applying significant G-forces on eukaryotic and some prokaryotic cells, but one would also be confronted with low sensitivity due to smaller sample volumes containing NMR active nuclei. Both issues can be circumvented by employing low-temperature DNP enhanced ssNMR experi-

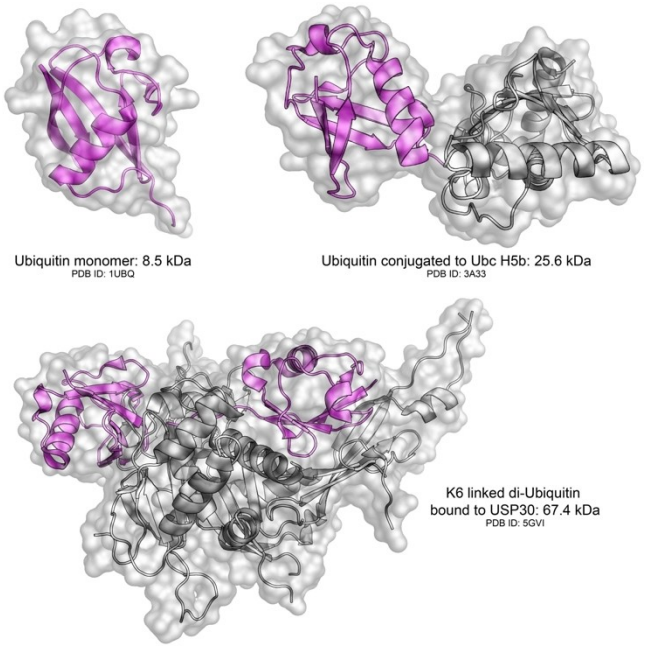

Figure 4. Ubiquitin (magenta, 8.5 kDa) can be associated with multiple proteins and complexes, leading to heterogeneous structural states including higher molecular weight complexes making it difficult to detect them by in-cell solution-state NMR.

ments, as demonstrated on whole cells recently.[27,28] Since DNP experiments in ssNMR are performed below 110 K, the effect of the G-force is negligible on the frozen sample subjected to MAS. Notably, the latter aspect was already exploited in 2012 when biomolecules inside bacterial cells were examined by low-temperature solid-state NMR experiments.[68]

DNP-enhanced ssNMR of whole mammalian cells

The advent of a new generation of water soluble DNP biradicals such as AMUPol and PyPol[22] have enabled a variety of biological applications for DNP-ssNMR (See, e.g. Refs. [12,21,24,25,27,28]). However, these radicals were primarily developed and tested in the context of in-vitro biological applications. The foremost hurdle to overcome for implementing DNP in whole cells is to deliver DNP agents into the cells while ensuring that they are not reduced due to the cellular components.[27,28] Previous theoretical studies in our group in which we calculated the distance range of polarization enhancements around currently used DNP biradicals[72] suggested that in the case of human cells (with a typical size of few tens of micrometers) the radicals must enter the cells to guarantee strong and uniform DNP enhancements within an average mammalian cell.

Albert et al. in 2018 observed that a cell penetrating peptide coupled to a DNP agent allows the DNP agent to spread throughout the cytoplasm and the nucleus in isotopically enriched HEK293 cells.[27] In the following year, our lab demonstrated that DNP radicals such as AMUPol rapidly diffuse into cells, providing the basis for molecular studies on exogenously produced and isotope labelled Ubiquitin in HeLa cells (See Figure 5) using protein concentrations that may approach endogenous levels.[28] The in-cell solution-state NMR spectra served as a vital control to monitor unwanted degradation of the isotope labelled protein (Figure 5A). As indicated in Figure 5A, besides sidechain amine signals, the only visible backbone correlations in our ^{1}H-^{15}N correlation spectrum emanated from the flexible C-terminal tail of unconjugated ubiquitin. On the other hand, in the 2D (see ref.[28]) and 3D DNP-ssNMR spectra (Figure 5B) we could readily detect residues located throughout the protein, including both the structured and unstructured domains (Figure 5C). To this end, the multidimensional DNP experiments including 2D ^{15}N-^{13}C and ^{13}C-^{13}C correlation experiments as well as the 3D double quantum filtered ^{13}C, ^{13}C correlation data set[73] together required about a week of measurement time using a concentration of isotope labelled ubiquitin that was comparable to endogenous levels.[28] Despite the high signal enhancements obtained due to the presence of the radicals throughout the cells (Figure 5D), we were limited by resolution even in higher dimension spectra (Figure 5B). The lack of resolution, which is inherent to frozen protein DNP preparations that exhibit dynamics or conformational disorder[74] can be reduced by using amino-acid specific isotope labelling to reduce spectral crowding. Indeed, obtaining DNP spectra at increased spectral resolution is an active field of research.

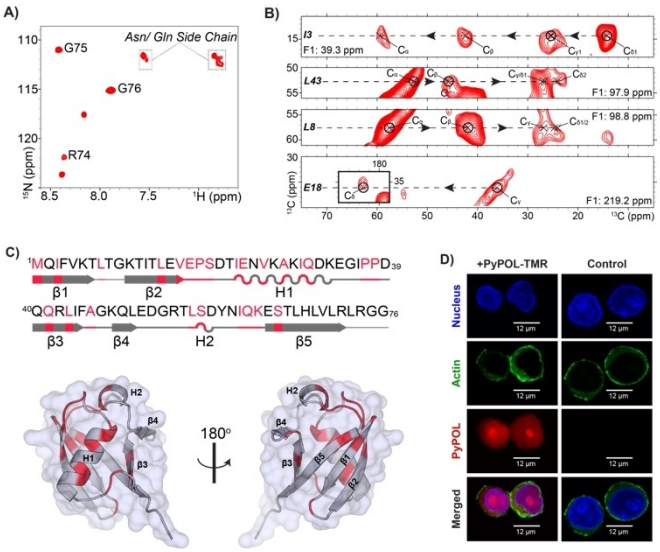

Figure 5. Summary of in-cell NMR studies on Ub reproduced from ref. [28] with permission. A) 2D ^{15}N-^{1}H solution-state spectrum shows the absence of any degraded proteins, and the three peaks identified in the backbone region emanate from the C-terminal tail of the unconjugated Ub. B) Example slices from a 3D double quantum filtered spin-diffusion spectrum recorded under DNP-ssNMR conditions. In combination with 2D experiments, we could probe significantly more residues than in-cell solution NMR (indicated in red on PDB structure 1UBQ in panel- C). D) Fluorescently tagged DNP agents (TMR-PyPol) could readily diffuse intothe cells.

Conclusion and Future Prospects

In this review, we have discussed progress in utilizing NMR to conduct in-cell studies. With recent advancements in DNP solid-state NMR, such studies can now be conducted for a variety of biomolecular systems. Yet, there is clearly room for further improvements for ssNMR-based cellular studies and its combination with other structural modalities.

For example, the short-lived nature of the radicals in cells at room temperature is of a great hindrance to improving DNP enhancements. For in-cell DNP-NMR applications, it would be ideal to prevent the reduction of the radicals by shielding them from reducing agents. Such radicals have been developed for use in in-cell electron paramagnetic resonance (EPR)[15,16,75] and they are in a preliminary stage of development for in-cell NMR applications.[27] Development of such sterically shielded/caged radicals could potentially allow us to incubate the cells in the DNP buffers for longer times resulting in a higher overall concentration of active radicals inside cells. Hence, advancements in radical development could further reduce intracellular protein concentrations than can be studied by DNP-ssNMR methods. In a similar vein, taggable radicals that would irreversibly conjugate to the proteins of interest harboring an inert unnatural amino acid have recently been developed.[76] In complex and heterogeneous biological environments, it has already been shown that tagging the DNP agents directly to the protein of interest is advantageous in obtaining molecule-specific DNP enhancements.[77,78]

In addition to inherent requirements related to in-cell NMR studies, DNP experiments using water-soluble biradicals such as AMUPol and PyPol could thus far not exploit the potential of higher magnetic field due to an unfavorable magnetic field dependence.[79] For example, moving from 400 to 800 MHz DNP conditions in cells leads to a reduction in the DNP enhancements in cells by a factor ~3.7 (see ref. [28]). Such a decrease is similar to values seen for in-vitro DNP applications on complex biomolecules[80] and currently still compromises the practical use of high-field DNP where spectral resolution is increased. Instead and as demonstrated in ref. [28], performing 3D experiments at a lower field where DNP performance is maximized is still often preferred. Current chemical synthesis efforts are underway to design DNP radicals such as biradicals that contain trityl-nitroxide moieties which show superior performance at high magnetic fields.[81]

Next to advancements within the field of NMR and its combination with DNP methods, there are further opportunities to conduct correlative studies and design hybrid approaches under in-cell conditions. For example, we have already demonstrated the potential to combine cryo-ET and NMR studies.[14] Previous work in the field of in-cell NMR as well as our recent work on ubiquitin inside human cells furthermore underline the growing potential to correlate NMR data with light microscopy results in line with the expanding field of Correlative Light and Electron Microscopy (CLEM).[82] Moreover, specific, long-range distance information may be obtained by including data obtained using EPR[4,15] and/or fluorescence resonance energy transfer[83] (FRET) methods that offer specific distance information in cellular studies. Taken together, a powerful toolbox is hence arising that provides a high-resolution structural and dynamical view of cellular life and disease from atomic distances to the micrometer range.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: cellular structural biology · dynamic nuclear polarization · in-cell NMR · magic angle spinning · solid-state NMR

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