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Bioanalytical Methods

In-Cell NMR and EPR Spectroscopy of Biomacromolecules

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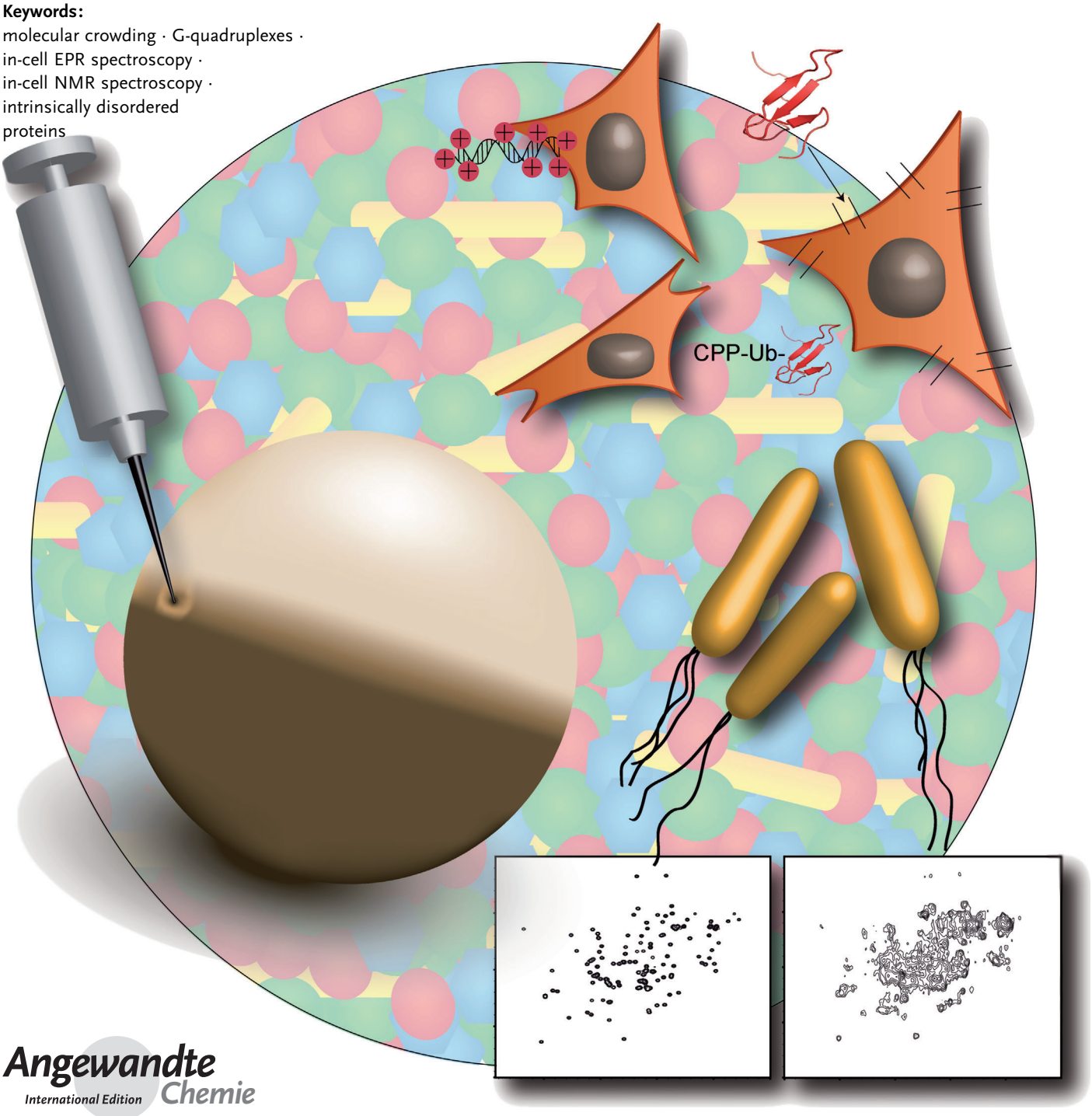

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The dream of cell biologists is to be able to watch biological macromolecules perform their duties in the intracellular environment of live cells. Ideally, the observation of both the location and the conformation of these macromolecules with biophysical techniques is desired. The development of many fluorescence techniques, including super-resolution fluorescence microscopy, has significantly enhanced our ability to spot proteins and other molecules in the crowded cellular environment. However, the observation of their structure and conformational changes while they attend their business is still very challenging. In principle, NMR and EPR spectroscopy can be used to investigate the conformation and dynamics of biological macromolecules in living cells. The development of in-cell magnetic resonance techniques has demonstrated the feasibility of this approach. Herein we review the different techniques with a focus on liquid-state in-cell NMR spectroscopy, provide an overview of applications, and discuss the challenges that lie ahead.

From the Contents

1. Introduction

The cellular interior is packed with macromolecules that differ in their shape, location, activity, and ability to interact. A detailed understanding of the biological function of macromolecules, such as proteins, DNA, RNA, or their complexes, often requires structural knowledge. NMR spectroscopy and X-ray crystallography are commonly used to solve the structures of these macromolecules with atomic resolution; however, these techniques are employed under in vitro conditions that support optimal spectral resolution and crystal growth, respectively. The focus on these technical aspects neglects effects of cellular factors on biomolecular conformation and dynamics, such as viscosity, molecular crowding, the activity of water, and interaction with other macromolecules, as well as the influence of the concentration of ions and other small molecules.[1–5]

The noninvasiveness of NMR spectroscopy and the development of in vivo NMR spectroscopy led to the establishment of the field of in-cell NMR spectroscopy in 2001 with investigations of the structural behavior of isotopically labeled proteins and osmoregulated periplasmic glucans inside living bacterial cells.[6–8] Since then, the method has been extended to several other cellular systems, including *Xenopus laevis* oocytes and mammalian cells, to study the conformation of biological macromolecules in their natural environment.[9–13]

The ability to detect changes in the conformation of a macromolecule between the in vitro situation and the interior of living cells is based on the sensitivity of the NMR chemical shift towards changes in the environment. Changes in the conformation, but also the binding of small or large molecules and posttranslational modifications, lead to such differences and can be detected by a shift of the resonance position in one- or two-dimensional NMR spectra. A significant challenge for in-cell NMR spectroscopy is the inherent insensitivity of NMR spectroscopy and the consequential need of high cellular amounts (50–250 μM) of the macromolecules under study. These concentrations are typically several orders of magnitude higher than the natural concentration of the macromolecule of interest (0.5–1 μM). Therefore, in-cell NMR spectroscopy is most often used to monitor enzymatic or "nonspecific" interactions, rather than for the investigation of specific interactions between a macromolecule and another cellular component. Enzymatic interactions include all sorts of posttranslational modifications, as well as enzymatic degradation. Since for this type of interaction a much lower cellular concentration of the enzymes is sufficient for the modification of a significantly higher concentration of the observed macromolecules, these effects can be studied by in-cell NMR spectroscopy. Nonspecific interactions are, for example, the effect of molecular crowding on the conformation of a macromolecule. In particular, intrinsically disordered proteins (IDPs) are believed to be driven to a more compact state by molecular crowding.[14,15] Since the total intracellular protein concentration can reach 400 $g\,L^{-1}$, a protein in living cells experiences a significantly more crowded environment than in a dilute solution as is typically used for in vitro experiments.[1]

In-cell NMR experiments with several small proteins that have been widely used in different in vitro NMR spectroscopic investigations have also revealed that many of these proteins interact nonspecifically with cellular components by weak attractive interactions.[16–20] These interactions often lead to a loss of the NMR signal, thus making it difficult to observe such macromolecules. These experimental difficulties can be overcome, and the intracellular behavior of such macromolecules can be monitored, for example, by selective

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labeling of methyl groups, which have a much higher sensitivity.[21] Furthermore, protocols for the observation of macromolecules by in-cell solid-state NMR spectroscopy and in-cell EPR spectroscopy have been developed.[22–24] Both in-cell solid-state NMR and in-cell EPR experiments are usually carried out at very low temperatures, and signal acquisition is not affected by the molecular size of the macromolecule or its complexes. The particular advantage of in-cell EPR spectroscopy is that distances within a macromolecule can be measured in a PELDOR experiment (pulsed electron double resonance; or double electron–electron resonance, DEER), and thus global conformational changes can be monitored.[25–27]

In this Review, we provide a description of general technical aspects of in-cell NMR and EPR spectroscopy (Section 2). We review all cellular systems used to date for in-cell NMR and EPR spectroscopy with respect to their applicability and discuss experimental design, control experiments, and limitations of the methods (Section 3). Finally, we discuss important contributions to the field and the potential impact of in-cell NMR and EPR spectroscopic methods in the near future (Section 4).

2. Technical Aspects of In-Cell NMR and PELDOR EPR Spectroscopy

2.1. Technical Principles

The observation of a particular macromolecule in the crowded intracellular environment requires that this macromolecule is labeled, in the case of in-cell NMR spectroscopy with NMR-active isotopes and in case of in-cell EPR spectroscopy with paramagnetic spin labels. For in-cell NMR spectroscopy, labeling with the isotopes ^{13}C (1.1% natural abundance) and ^{15}N (0.36% natural abundance) has proven to be the method of choice to overcome the cellular background signal of all hydrogen atoms and enable the extraction of information on the macromolecule of interest. By using experiments such as 2D [^{15}N,^{1}H] HSQC or 2D [^{13}C,^{1}H] HSQC, which select only hydrogen atoms directly bound either to ^{15}N or ^{13}C nuclei and suppress all other proton signals, NMR signals of the labeled macromolecule can be selectively detected. In particular, 2D [^{15}N,^{1}H]-HSQC, which measures the chemical shifts of the amide hydrogen and nitrogen atoms, provides information about the conformational state of the protein backbone.

Biological macromolecules can be labeled endogenously by overexpression (in the cell system used for in-cell NMR spectroscopy) or exogenously by chemical synthesis or cellular expression, purification of the labeled target, and

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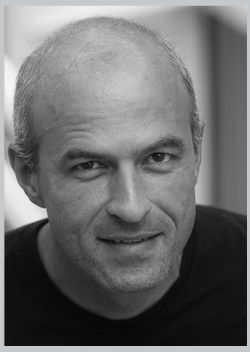
Robert Hänsel-Hertsch was born in 1982 in Berlin. He obtained his diploma in biochemistry in 2009 from Goethe University in Frankfurt, where he completed his PhD in 2013 under the supervision of Volker Dötsch on the use of NMR spectroscopy to investigate the structural behavior of telomeric DNA in living cells. He is currently pursuing EMBO-funded postdoctoral studies with Shankar Balasubramanian at Cancer Research UK in Cambridge, where he has been investigating G-quadruplex DNA and its role in cancer development by next-generation sequencing techniques.



Born in 1967 in Frankfurt, Volker Dötsch obtained his diploma in chemistry in 1990 from the University of Göttingen with Thomas Jovin and his PhD in 1994 from the ETH Zurich with Kurt Wüthrich. He was an EMBO postdoctoral fellow with Gerhard Wagner at Harvard Medical School, Boston (1994–1998) and began his independent career at UCSF in San Francisco in 1998 with a focus on the development of in-cell NMR spectroscopy. In 2003, he moved to Goethe University in Frankfurt, where he uses this and other techniques to investigate the behavior of biological macromolecules.

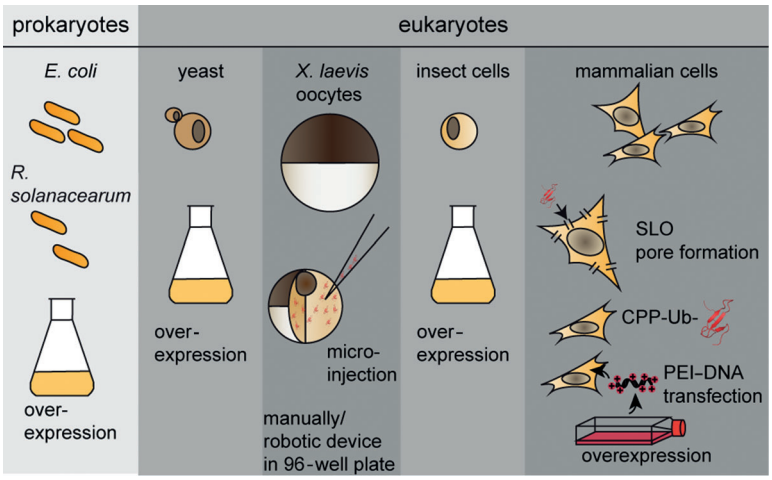


Figure 1. Summary of established methods for the preparation of samples for in-cell NMR or EPR experiments in prokaryotes and eukaryotes. In-cell NMR spectroscopy in bacteria, yeast, and insect cells is generally based on overexpression of the target protein. In contrast, for in-cell NMR experiments with *Xenopus laevis* oocytes, samples are prepared by the microinjection of macromolecules. The expansion of in-cell NMR spectroscopy to mammalian cells resulted in a variety of procedures, including pore formation with streptolysin O (SLO), the use of cell-penetrating peptides (CPPs), and simple overexpression preceded by polyethylenimine (PEI)-assisted transfection of a DNA plasmid encoding the protein of interest.

subsequent microinjection or transfection into the cell system for in-cell NMR spectroscopy (Figure 1). In the case that the labeled macromolecule is injected/transfected into the cell system for in-cell NMR spectroscopy, the cellular background signal will contain only signals due to the ^{13}C and ^{15}N natural abundance. If, however, labeling occurs within the cellular system also used for the NMR spectroscopic experiments, not only the macromolecule of interest, but all cellular components will be labeled. Originally, a complex procedure for the production of an in-cell sample was suggested that included growing the sample on unlabeled medium prior to transferring the bacteria to a medium containing the isotopic labels and inducing protein expression. The drug rifampicin, which inhibits the bacterial RNA polymerase but not the T7 RNA polymerase,[7] was also added to enable selective expression of the protein of interest. In the case of ^{15}N labeling, it turns out that almost no cellular background signals are visible even if the sample is grown from the beginning in isotopically labeled medium and expression is induced without the addition of rifampicin (Figure 2).

The only requirement for the observation of a [^{15}N,^{1}H] HSQC spectrum of the protein of interest is that it tumbles freely in solution (see below) and that its intracellular concentration reaches a certain level (estimated to be approximately 5% of total soluble protein in *Escherichia coli*). This surprising absence of a significant cellular background is due to the relatively low number of nitrogen-bound hydrogen atoms in biological molecules and the fast exchange of most of these hydrogen atoms with the bulk water, thus leading to the disappearance of their signals.[7] In contrast, ^{13}C-bound hydrogen atoms are far more abundant and do not exchange with water, thus resulting in a strong cellular background for samples generated by endogenous ^{13}C labeling (Figure 3A).[21] To overcome this problem, selective labeling techniques can be used (Figure 3B). Alternatively, experiments with coherence-transfer steps from ^{15}N nuclei to ^{13}C nuclei in combination with direct ^{13}C detection have been shown to produce high-quality in-cell spectra.[28]

For solution-state in-cell NMR spectroscopy, a second requirement must be met: The molecule of interest has to tumble sufficiently fast in the intracellular environment. Several investigations have shown that the rotational diffusion is only reduced by a factor of 2–3 in most cell types relative to measurements in water resulting in an increase of the apparent molecular weight of the biomolecule by the same factor.[7,29,30] However, interaction with other large cellular components, such as other macromolecules, complexes, or membranes, slows down the tumbling rate, thus resulting in broad or even undetectable resonance lines.[16,17] Intrinsically disordered proteins (IDPs) show often weaker interaction with their surroundings and can compensate the concomitant reduction in their tumbling rate with their inherently high internal flexibility. Therefore, in-cell NMR spectroscopic investigations have generated many high-resolution backbone chemical-shift profiles of IDPs or partly disordered proteins.[14,31–33]

In contrast, the backbone resonances of many globularly folded proteins are not detectable, thus suggesting that their tumbling rates are significantly slower in vivo, most likely as a result of nonspecific interactions with other cellular components.[16–18,20,34] One way to visualize these proteins in the cellular environment is to focus on methyl groups, which can be detected more readily because of their fast internal rotation.[21] The ^{13}C labeling of specific methyl groups has the additional effect that resonance overlap and problems with

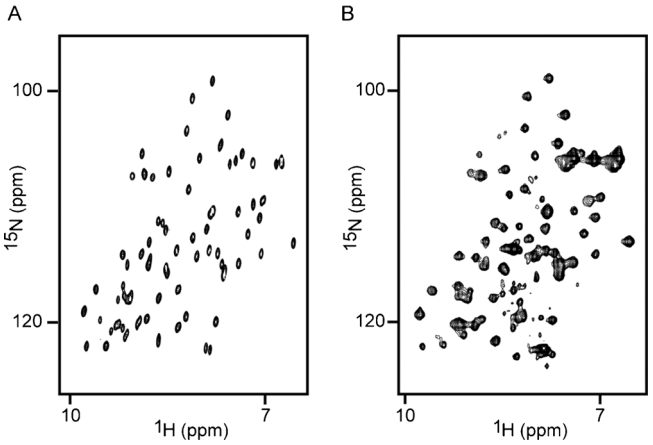


Figure 2. Comparison of A) an in vitro [^{15}N,^{1}H] HSQC spectrum of the purified N-terminal domain of the bacterial Tn*501* mercuric ion reductase MerA (NmerA) with B) the corresponding in-cell NMR spectrum in living *E. coli* cells. The peak patterns in the two spectra are very similar; however, the line width in the in-cell spectrum is significantly broader.

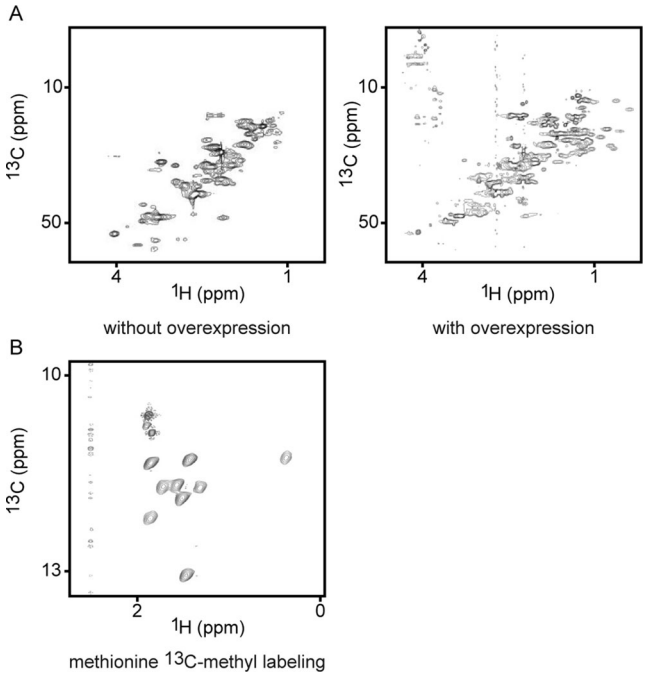


Figure 3. The level of background signals is significantly higher in in-cell [^{13}C,^{1}H] HSQC spectra than in in-cell [^{15}N,^{1}H] HSQC spectra. A) Spectra recorded with *E. coli* samples grown on ^{13}C-labeled glucose-containing media without (left) and with induction of the expression of calmodulin (right). Only the high-field shifted methyl signals can be assigned unambiguously as signals of calmodulin. The level of background signals can be largely suppressed by selective labeling. B) In-cell [^{13}C,^{1}H] HSQC spectrum of calmodulin labeled selectively with $^{13}CH_3$-labeled methionine.

background signals can be overcome (Figure 3). Particularly useful are the methyl groups of methionine and the δ methyl group in isoleucine, which relax very slowly.[35] In addition to site-specific ^{13}C labeling, ^{19}F labeling, for example, of tryptophan, phenylalanine, or tyrosine analogues, enables the characterization of sticky and disordered proteins by in-cell 1D ^{19}F NMR spectroscopic analysis. Besides its beneficial NMR sensitivity—the gyromagnetic ratio is similar to that of protons, and 1D experiments are more sensitive than multi-dimensional experiments—the advantage of ^{19}F over ^{13}C is the absence of any background signal in cells.[30,36] If even methyl groups with their fast internal rotation cannot be detected in liquid-state in-cell NMR spectroscopic experiments, in-cell solid-state NMR techniques recently established for the study of soluble and membrane proteins can be used.[22,37,38]

Besides weak nonspecific interactions with other cellular components, the loss of signals in in-cell NMR experiments can also be caused by the formation of large specific complexes, degradation, or conformational averaging. Specific-complex formation is in most cases unlikely, as the low sensitivity of NMR spectroscopy requires a high cellular concentration of the macromolecule of interest, which is typically several orders of magnitude higher than the cellular concentration of a specific binding partner. The degradation of macromolecules has mainly been observed in in-cell NMR experiments with nucleic acids.[39,40] Finally, signal loss can also occur as a result of conformational exchange processes. This phenomenon was observed in a study of an IDP in bacterial cells.[15] Whereas the in vitro spectrum of the protein FlgM showed the typical narrow chemical-shift dispersion and sharp resonance lines of an unfolded protein, the in-cell spectrum was characterized by broad lines and many missing signals. This loss of cross-peaks in the [^{15}N,^{1}H] HSQC spectrum was suggested to arise from conformational exchange within a molten globular state, as induced by the crowded intracellular environment.

Once conditions have been found that enable the acquisition of [^{15}N,^{1}H] or [^{13}C,^{1}H] HSQC spectra in cells, the peak positions in these spectra can be compared to those measured with the isolated macromolecule in dilute solution in in vitro experiments. Chemical-shift differences between the in vitro and in-cell spectra indicate differences in conformation, binding events, or posttranslational modifications. If the assignment of the resonances of the macromolecule is known, the interaction site (in the case of binding events or posttranslational modifications) can be identified and—if the structure has also been determined—can be mapped onto the structure. If significant overlap of the resonances prevents accurate interpretation of the spectra, site-specific ^{15}N labeling of single amino acid types can been used to reduce the complexity of the spectra.[7]

2.2. Dynamics

Many studies have recognized the importance of the dynamics of proteins for their specific function (see Refs. [41–44] for reviews). In most studies, the unique properties of high-resolution NMR spectroscopy were used to resolve dynamics on the picosecond-to-second timescale with atomic resolution.[45] However, the measurement of protein dynamics inside cells or cellular extracts turned out to be a major challenge, and only a few reported cases exist. The difficulties are mainly due to the limited lifetime of cells or the limited amount of time that cells can be kept in a defined metabolic state in the NMR tube. This time is often too short for the long measurement times required for relaxation experiments. Furthermore, the crowded cellular environment affects protein motion by the promotion of weak attractive interactions, which lead to slower tumbling of the target protein. The resulting slower longitudinal and faster transverse nuclear-spin relaxation promote signal broadening and lower sensitivity. Consequently, few proteins have yielded good in-cell NMR spectra and are thus suitable for the study of their dynamics under cellular conditions. Of particular interest are investigations of conformational exchange processes, which can be studied by relaxation dispersion (RD) NMR spectroscopic experiments. These experiments do not only provide kinetic information about the exchange process between ground and excited states, they can also be used to obtain structural information about these excited states.[46–48] Latham and Kay recently demonstrated the feasibility of this method with *E. coli* lysates.[35]

If the exchange kinetics between the different conformations is even slower, exchange spectroscopy (EXSY), which is

based on exchange of magnetization along the z axis, can be used.[49] The upper time limit depends on the longitudinal relaxation rate, so that longer T_1 values are of advantage, which makes this technique very suitable for in-cell NMR spectroscopic applications. However, spectral crowding and low sensitivity can make the interpretation of the spectra difficult. Site-specific labeling can be used to overcome these limitations. Takaoka et al. used ligand-directed tosyl (LDT) chemistry to fluorinate intrinsic human carboanhydrase I (hCAI) in red blood cells for EXSY studies.[50] In this way, the binding kinetics and thermodynamics of an enzyme and its inhibitor could be measured for the first time by in-cell NMR spectroscopy.

Finally, protein stability and conformational exchange can also be investigated by measuring proton/deuteron-exchange rates of amide protons. To assess the folding stability of proteins in the intracellular environment, Inomata et al. determined the exchange rate of the amide hydrogen atoms of ubiquitin in human cells.[11] Uniformly ^{15}N-labeled ubiquitin was unfolded by the use of guanidinium hydrochloride in D_2O at pH 7.3 and rapidly refolded and dialyzed in a dilute buffer solution containing H_2O. This method ensures that highly protected amides remain deuterated.[51] These deuterated amides exchange upon protein unfolding and therefore can serve as markers of protein stability. Partially deuterated ubiquitin was introduced into HeLa cells. To quantify the state of exchange by NMR spectroscopy, cells were lysed at pH 5 at different time points. [^{15}N,^{1}H] HSQC spectra were recorded for the different cell-lysate samples, and the intensity buildup of cross-peaks was plotted against the time the protein was present inside the cells. Fitted intensity-buildup curves from the lysate samples were compared with the buildup curves acquired in dilute solution to evaluate differences in exchange rates.[11]

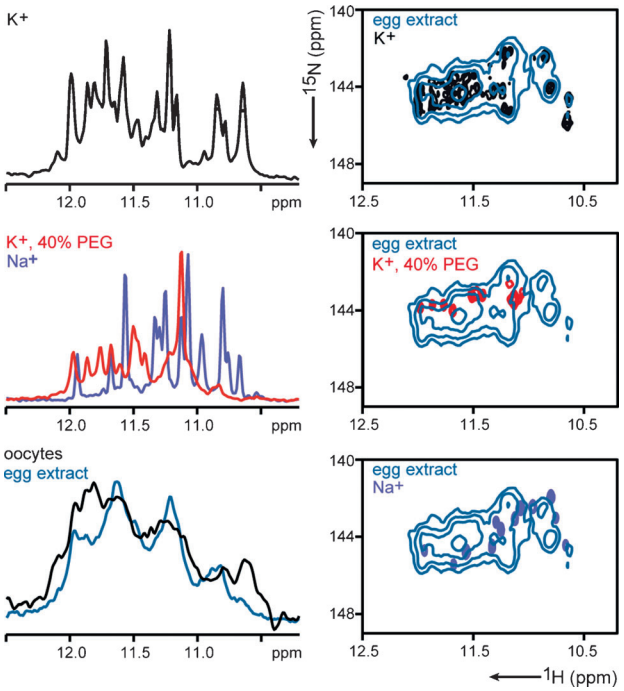


Figure 4. G-quadruplex conformations adopted by d(AG$_3$(TTAGGG)$_3$) in cells are exclusively observed in potassium-based dilute solution. Left: Overlay of ^{15}N-edited 1D imino proton spectra of ^{15}N/^{13}C-labeled d(AG$_3$(TTAGGG)$_3$) folded in dilute potassium-based solution (K^+), in dilute sodium-based solution (Na^+), in potassium-based solution supplemented with 40% PEG 200 (K^+, 40% PEG), in *Xenopus laevis* egg extract (egg extract), and with 1D spectra acquired inside *Xenopus laevis* oocytes (in-cell oocytes). Right: Comparison of 2D [^{15}N,^{1}H]-sfHMQC spectra of the imino region of ^{15}N/^{13}C-labeled d(AG$_3$-(TTAGGG)$_3$) folded in *Xenopus laevis* egg extract (egg extract) with the spectra obtained in dilute potassium-based solution (K^+), in potassium-based solution supplemented with 40% PEG 200 (K^+, 40% PEG), and in dilute sodium-based solution (Na^+).

2.3. Nucleic Acids

At present, in contrast to in-cell NMR spectroscopy of proteins, in-cell NMR spectroscopy of nucleic acids relies on the mechanical injection of nucleic acids into *Xenopus laevis* oocytes (Figure 4).[39] Whereas the detection of proteins in the intracellular environment requires isotopic labeling, imino hydrogen atoms of nucleic acids can be detected by simple 1D ^{1}H acquisition without the need for cost-intensive isotopic labeling, owing to the lack of endogenous background signals in the spectral region between 11 and 15 ppm. Imino proton signals from intracellular DNA and RNA are not detected, since most naturally occurring nucleic acids are too large and their concentration too low, whereas the imino proton signals of free nucleotides are lost as a result of fast exchange with the bulk water. Therefore, injected small and medium-sized DNA and RNA molecules can be investigated by in-cell NMR spectroscopy on the basis of imino hydrogen atoms that are protected from exchange in secondary-structure elements. By the use of imino proton resonances, which are unique reporters of base-pairing pattern, local and global folding-topology changes can be characterized by simple 1D ^{1}H NMR spectroscopic analysis.[52] Despite the poor resolution, the comparison of nucleic acid spectra obtained by in-cell and in vitro NMR spectroscopy enables the detection of structural changes.[39,40,52]

In-cell NMR spectroscopic investigations also showed that nucleic acids, such as DNA and RNA hairpins, are susceptible to degradation by endogenous nucleases, which limits the time available for observation of the intact molecules.[39] However, the degradation of DNA and RNA can be effectively diminished by chemical modifications, such as the replacement of the phosphate group in the backbone by a thiophosphate moiety or by methylation of the *O*2′-hydroxy group.[39] Besides the stabilization of DNA/RNA against degradation, the thiophosphate modification has the advantage that nucleic acids can be detected and studied by in-cell 1D ^{31}P NMR spectroscopy owing to the distinct chemical shift (ca. −54 ppm) of the thiophosphate group.[39]

More detailed structural characterization of nucleic acids with high resolution by in-cell 2D NMR spectroscopy focused on the imino, aromatic, or sugar regions requires ^{15}N or ^{13}C isotopic labeling. Owing to the accumulation of degradation products, the sugar and aromatic regions can be more challenging to interpret, since the observed signals might

arise from the intact or degraded target. The possibility to chemically synthesize nucleic acids enables the selective isotopic labeling of a specific nucleotide position, which simplifies the complexity of the 1D and 2D NMR spectra considerably. For example, site-specific ^{15}N labeling of a single base in an oligonucleotide sequence enables the assessment of whether the target adopts a single conformation or whether multiple folding topologies coexist.[53] Besides the study of imino proton signals, aromatic and sugar proton–carbon spin systems can be used as probes with [^{1}H,^{13}C] constant-time TROSY HSQC. Cross-peaks recorded in these regions appear with different signs, depending on the number of directly coupled ^{13}C neighbors.[54]

2.4. In-Cell EPR Spectroscopy

Whereas NMR spectroscopy makes use of the magnetic properties of the nuclear spin, EPR spectroscopy is based on the magnetic moment of unpaired electrons. One of the main advantages of EPR spectroscopy over NMR spectroscopy is its higher sensitivity. Of particular interest is a technique called PELDOR (or DEER), which measures the dipolar coupling between unpaired electrons that are separated by distances of 1.5–10 nm.[25,27,55] The primary components of proteins and nucleic acids are devoid of paramagnetic electrons; therefore, the target of interest must be labeled with a stable paramagnetic probe. Site-directed spin labeling (SDSL) is routinely used to attach small nitroxides, such as 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (3-maleimido-PROXYL, 5-MSL) or 2,2,5,5-tetramethyl-1-oxyl-3-methyl methanethiosulfonate (MTSL), to cysteine residues in proteins.[56,57] Nucleic acids, on the other hand, are modified at the base, sugar, or phosphate-backbone moiety during or after chemical synthesis.[58] Recently, it was shown that non-natural amino acids containing a paramagnetic center can be incorporated into proteins by the method of amber-codon suppression, thus enabling the intracellular expression of spin-labeled proteins.[59,60]

In contrast to liquid-state in-cell NMR spectroscopy, which is carried out at physiological temperatures, the fast relaxation of nitroxide spin labels at room temperature requires PELDOR experiments to be conducted at cryogenic temperatures (50 K), whereby the cells are shock-frozen in liquid nitrogen prior to the experiment. The use of new spin labels with better relaxation behavior might allow in-cell PELDOR EPR measurements at physiological temperatures in the future.[61] Although EPR spectroscopy is more sensitive than NMR spectroscopy, an extraordinarily high concentration of the target (50–250 μM) is needed for both methods to obtain the necessary sensitivity (Table 1).[23,62–64]

3. Cellular Systems for In-Cell NMR and EPR Spectroscopy and Their Applications

3.1. Escherichia coli

Since the first in-cell NMR experiments in 2001,[6] the bacterial *E. coli* cell has been the most explored system owing to the advantages of working with bacteria, for example, their rapid growth rates, robustness, straightforward manipulation, and high expression levels. Furthermore, in-cell NMR spectroscopic experiments with bacteria can be conducted at the relatively high temperature of 37 °C, which helps to offset the effect of the intracellular viscosity on the tumbling rate of the macromolecules.

The *E. coli* in-cell system requires intracellular expression of the macromolecule of interest in the presence of a suitable isotopically labeled medium.[65] To eliminate signal contribu-

Table 1: Summary of in-cell techniques.

	In-cell NMR spectroscopy	Solid-state in-cell NMR spectroscopy	In-cell EPR spectroscopy
labeling	^{15}N, ^{13}C, ^{19}F	^{15}N, ^{13}C	paramagnetic spin labeling, higher sensitivity
temperature	physiological	cryogenic or physiological	cryogenic
background	high for ^{1}H, ^{13}C	high for ^{1}H, ^{13}C, ^{15}N	low
dependence on tumbling	relies heavily on tumbling rate, background signals are not detectable for slowly tumbling molecules	slowly tumbling molecules or measurement in the frozen state	measurement in the frozen state
strategy	^{15}N uniform labeling: backbone analysis alternatively: site-specific detection of ^{15}N-, ^{13}C-, or ^{19}F-labeled residues	requires simultaneous labeling with ^{15}N and ^{13}C to filter for ^{15}N–^{13}C coupled pairs by cross-polarization	requires the attachment of spin labels
readout	chemical shift, spin relaxation, distances	chemical shift	long-range distances (1.5–10 nm)
information	conformations[12,15,66,67,78,98,99] dynamics[36,76] interactions[11,16–18,34,35,70,73,74,87,111] posttranslational modifications[10,31,86,87,112–115]	conformations[22,37,38] interactions[22,37,38]	distances[23,24,63,64,101] conformations[23,24,63,64,101] interactions dynamics

tions of leaked metabolites and target molecules, cells are subjected to extensive washing steps prior to the acquisition of the in-cell NMR spectrum. The bacterial cells are prepared as a thick slurry and transferred to an NMR tube. To maintain cell viability, short-term experiments (<3 h) are recommended, since nutrient and oxygen depletion might lead to cell death and the leakage of molecules into the supernatant.

The investigation of several small proteins in the bacterial cytoplasm has shown that many of them are not detectable by in-cell NMR spectroscopy owing to their interaction with other cellular components. The Pielak and Gierasch research groups studied this phenomenon in more detail and observed that the rotational but not translational movement of the target was significantly reduced.[16,18] They concluded that globular proteins with a net charge close to neutral form nonspecific weak attractive interactions with endogenous protein crowders inside the bacterial cytosol. Interestingly, the conventional macromolecular crowding effect, which is anticipated to promote the adoption of the most compact structural state of a macromolecule in the presence of cellular crowders that exclude volume, has recently been challenged by a bacterial in-cell NMR spectroscopic study.[66] Whereas a variant of the protein ProtL predominantly adopted its compact state in a dilute solution supplemented with sodium chloride (0.8 M), weak nonspecific attractive interactions of the target with the crowded cellular interior resulted in the observation of a partially disordered/folded conformation with a larger hydrodynamic radius. Furthermore, a DNA-binding protein, the MetJ repressor, was shown to nonspecifically bind genomic DNA next to its consensus sequence, thus suggesting that the permanent formation of nonspecific complexes might play a general role in protein biology.[19]

Up to now there has been a single reported case in which an intrinsically disordered protein, FlgM, was observed to gain α-helical structure in its C-terminal domain inside bacterial cells, while being fully disordered in dilute solution.[15] This study highlighted the potential of in-cell NMR spectroscopy to identify unique structural features not present in an in vitro situation. Interestingly, the exact opposite observation was recently made in a bacterial in-cell NMR spectroscopic investigation of the IDP α-synuclein. Whereas α-synuclein was observed in its monomeric fully disordered state in cells,[67] the recombinant expressed protein obtained by nondenaturing extraction from *E. coli* and endogenous α-synuclein obtained from red blood cells, different cultured neuronal cell lines, and human tissue was reported to occur predominantly as a native tetramer with well-defined α-helical segments.[68,69] It can be argued that a 56 kD well-structured tetramer is unlikely to be detected by means of in-cell NMR spectroscopy. However the predominant formation of a structured tetramer in cells would also result in notable peak broadening, which was not observed in these in-cell spectra. The detection of an exclusively unfolded conformation in bacteria suggests that tetramer formation might be an artifact of the in vitro conditions used in these studies.

In addition to the investigation of the physiologically relevant conformation, interactions such as protein–protein[70–72] and protein–small-molecule interactions[11,73] can be studied by in-cell NMR spectroscopy in bacteria. STINT-NMR is a technique in which the interaction partner of interest is expressed in a first step, followed by the overexpression of the target of interest.[71,72] The technique was also applied to screen the potency of drugs for the inhibition of protein–protein complex formation.[70] STINT-NMR was used to characterize the ubiquitin binding surface with respect to its interaction with a set of known protein interaction partners.[74] Astonishingly, whereas Shekhtman and co-workers obtained well-resolved backbone resonance profiles of free ubiquitin and its complexes with the expressed interaction partners,[74] other research groups failed to observe the backbone signals of ubiquitin by in-cell NMR spectroscopy in bacteria or other cells, thus suggesting that ubiquitin existed in larger complexes in cells.[11,75]

Liquid-state NMR spectroscopy is especially powerful in the characterization of macromolecular dynamics, which can be analyzed in the picosecond-to-second range. These techniques, however, require highly concentrated samples and time-consuming experiments. Therefore, only a single study has been reported, in which the side-chain dynamics of calmodulin was probed in bacterial lysate and compared to the observed dynamics in dilute solution.[76] Whereas the lysate had no effect on the picosecond–nanosecond (ps–ns) methyl-side-chain dynamics of Ca^{2+}-free CaM (apoCaM), striking differences were observed for intermediate dynamics in the microsecond–millisecond (μs–ms) time range. Attenuated dispersion in the presence of a metal chelator supported the idea that these differences resulted from exchange between apoCaM and a metal-bound intermediate of CaM in lysate. The same observation was made for the active, Ca^{2+}-loaded form of CaM. Whereas side chains of Ca^{2+}-bound CaM lacked dispersion in a buffer, significant dispersion was measured in bacterial lysate. This result is mostly due to low-affinity interactions with components of the lysate. Whether a lysate imitates reliably the dynamics of a protein in living cells remains, however, to be shown.

Finally, in-cell NMR spectroscopy was used to solve the first structure of a protein in living *E. coli* cells by a combination of fast 2D and 3D acquisition methods combined with nonlinear sampling,[77] which notably reduced the experimental time required for the usually time-consuming NMR experiments.[78] Very high intracellular protein concentrations (4 mM) had to be reached for the completion of this structure determination. Although an intracellular concentration of 0.8 mM was enough for the assignment of 70% of the protein backbone resonances, the general applicability of this approach is questionable, since the majority of proteins can not be expressed to these extremely high concentrations or do not maintain their in vitro tumbling behavior in cells. In general, NMR experiments that require longer experimental times also suffer from sedimentation of the cells. This effect can in principle be prevented by embedding the bacteria in agarose with a low melting temperature.[79–82]

3.2. Xenopus laevis

Xenopus laevis oocytes are a well-established cell system in the fields of developmental biology and electrophysiology. They were the first eukaryotic cells used in in-cell NMR experiments.[9] The main advantage of *X. laevis* oocytes is their size (ca. 1 mm), which allows microinjection of the protein or nucleic acid of interest. A typical NMR sample consists of roughly 200 oocytes that are either manually or automatically (by a robotic device) injected with 20–50 nL each.[65] Advantages of this approach are the much better control of the cellular target concentration, the ability to isotopically label specific sites of the macromolecule by chemical synthesis, and the ability to introduce modifications, such as paramagnetic spin labels, which are difficult to introduce in the overexpression mode. Furthermore, long-term experiments can be carried out with oocytes by using inert cosolutes, such as Ficoll, in the supernatant, which reduce the mechanical stress on the oocytes when packed in the NMR tube. To check the toxicity of the target, cell viability can be tested by the addition of progesterone. Progesterone triggers synchronized maturation to the egg state from the arrested prophase at the G2/M boundary of the first meiotic division to the metaphase of meiosis II. Another advantage is that cellular extracts from *X. laevis* oocytes or eggs can be readily obtained in a virtually undiluted form. These extracts show most of the biological functions of the intact cells.[83] They are frequently used as alternative cell-free systems for ex vivo analyses of cellular processes, since they enable even better control of the concentration and improved spectral quality owing to their higher homogeneity as compared to oocytes.[84,85] One disadvantage of the *X. laevis* oocyte system relative to bacteria, however, is that experiments have to be carried out at 18 °C (in contrast to 37 °C with bacteria) to keep the cells alive.

X. laevis oocytes have been used to study posttranslational modifications (PTMs) by in-cell NMR spectroscopy.[31,86,87] To investigate the phosphorylation of a specific target sequence, Selenko et al. introduced this sequence into a loop of the model protein GB1, which is suitable for the measurement of well-resolved in-cell NMR spectra.[86] The backbone resonances of the modified GB1 construct containing the introduced regulatory region of the SV40 large T antigen were analyzed in oocytes and in egg extracts. The kinase CK2, which is active in *X. laevis* oocytes and eggs, is known to phosphorylate two consecutive serine residues in this regulatory region. On the basis of the acquired real-time in-cell NMR spectroscopic data, Selenko et al. showed that CK2 phosphorylates a unique serine residue at a specific position in a first step, before the preceding serine residue is phosphorylated in the next phosphorylation event. Although this approach elucidated the phosphorylation mechanism of CK2, it is not clear if this phosphorylation of the regulatory region in the artificial GB1 scaffold imitates its natural processing by CK2.

Bodart et al. used a different approach. They investigated the backbone signals of the IDP tau in *X. laevis* oocytes under various in vitro conditions that promote the phosphorylation of different residues; however, they failed to identify the kinase that was responsible for the majority of the shifted backbone cross-peaks observed under in-cell conditions.[87] A further investigation targeted the so-called "unique" domain of the kinase c-Src, which is disordered. By real-time in-cell NMR spectroscopic analysis in oocytes and egg extract in combination with mass spectrometry and western blotting, Amata et al. showed that S69 and S75 were phosphorylated exclusively by cyclin-dependent kinases (CDKs), whereas the S17 site was targeted by both CDKs and protein kinase A in *X. laevis* oocytes.[31]

Recently, we demonstrated that the conformation of nucleic acids can be investigated in *X. laevis* oocytes by in-cell NMR spectroscopy.[39,40,88] These investigations of a DNA and a RNA hairpin showed that degradation by endogenous nucleases can be a severe problem. However, nucleic acids can be stabilized by chemical modifications, and alternative non-B-DNA structures, such as DNA G-quadruplexes, proved to be resistant to degradation. We used in-cell NMR spectroscopy to characterize human telomeric G-rich repeat sequences, which are known to form a diverse set of G-quadruplex structures in vitro.[89–92] Extensive in vitro studies have shown that the conformational state of these G-quadruplexes strongly depends on the environment, such as the nature of monovalent cations or a possible cosolute, as well as the base composition flanking the 5′ and 3′ ends of the quadruplex.[92] So far, six unique conformations have been found by NMR spectroscopy and X-ray crystallography, thus demonstrating the polymorphic nature of telomeric DNA.[89–92]

Molecular crowding simulated by a potassium-based solution supplemented with cosolutes, such as PEG 200 or ethanol, was shown to promote the formation of a parallel propeller-shaped G-quadruplex conformation.[90] Since molecular crowding is a permanent state in cells, it was suggested that the parallel G-quadruplex fold is most likely the physiologically relevant conformation of naked telomeric DNA in vivo. Interestingly, whereas in dilute solution exclusively antiparallel G-quadruplex topologies have been observed by solution NMR spectroscopy, an X-ray crystallographic study also revealed the formation of the parallel propeller fold.[93] To explain this result, it was suggested that the environment of a crystal better reflects the crowded cellular conditions than a dilute potassium-based solution.[90] However, our in-cell NMR spectroscopic analysis of various telomeric sequences in *X. laevis* oocytes and egg extract showed that the parallel G-quadruplex topology was not formed under these conditions.[40,88] Instead, the conformations that are adopted in dilute potassium-based solution were also found in cells.[53] Furthermore, we found that extended telomeric sequences capable of forming multiple G-quadruplexes on a single strand adopt the structure of G-quadruplex units in a beads-on-a-string architecture.[53] The analysis of site-specifically ^{15}N-modified guanine residues in these extended constructs showed that each G-quadruplex unit coexists in two distinct conformations, the 2-tetrad antiparallel basket and the hybrid-2 structure, both in dilute potassium-based solution and in *X. laevis* egg extract.[53]

3.3. Mammalian Cells

Another milestone in the field of in-cell NMR spectroscopy was the observation of the backbone resonances of uniformly ^{15}N labeled globular and intrinsically disordered proteins inside living mammalian cells. Since the typical expression level in mammalian cells is significantly lower than the level possible in bacterial cells, and because injection is not an option owing to the small size of these cells as compared to *X. laevis* oocytes, new methods had to be developed to enable the required high intracellular concentration to be attained. The research groups of Shirakawa and Shimada developed different procedures for the introduction of isotopically labeled proteins into adherent and suspension cells, respectively.[10,11]

In the procedure reported by Shirakawa and co-workers, the target of interest is C-terminally fused to a construct containing the Tat cell-penetrating peptide (CPP) and ubiquitin. Whereas the CPP localizes the target at the outer cellular membrane to promote efficient target transduction, the ubiquitin is recognized inside the cytosol by endogenous ubiquitin-specific C-terminal proteases (DUBs), which cleave ubiquitin from the construct to set free the target protein.[75] Furthermore, the CPP tag can be directly fused to the target of interest through an end-to-end cysteine disulfide bond, which is unstable under intracellular reductive conditions. Only the backbone amide signals originating from the processed target are visible in the recorded spectra, since exclusively the tag-free target tumbles sufficiently fast. In contrast, the noncleaved labeled protein interacts with membrane components and is therefore not detectable.

This CPP in-cell NMR spectroscopic procedure was employed to record spectra of two model proteins, a ubiquitin mutant that had lost its ability to form nonspecific weak attractive interactions with other proteins, and the *cis–trans* prolyl isomerase FKBP12.[11] The authors observed chemical-shift differences for FKBP12 before and after the addition of the ligand FK506, thus suggesting that the characterization of protein–ligand interactions by in-cell NMR spectroscopy in mammalian cells is feasible. Furthermore, wild-type ubiquitin and the triple alanine mutant were fully deuterated and transduced into HeLa cells, and amide H–D exchange rates were recorded both inside cells and in cellular lysate. These experiments revealed a notably faster exchange inside the cells than under the in vitro conditions. Importantly, wild-type ubiquitin exchanged significantly faster than the mutant, which is known to be incapable of forming nonspecific weak attractive interactions with endogenous crowders. These results suggest that the ability of ubiquitin to form nonspecific weak attractive interactions with other proteins makes the protein less stable inside cells. This phenomenon is in accordance with findings of the Pielak research group, who demonstrated that the dominant interactions in the cellular interior determine the stability of the protein structures: Proteins that participate in nonspecific attractive interactions form less stable/compact structural ensembles, whereas proteins that participate in nonspecific repulsive interactions tend to be more stable and compact.[4]

One disadvantage of this transfection method is that different CPPs, including the Tat-derived CPP, can have moderate toxic effects on mammalian cells. Cellular stress might lead to notable changes in the metabolic state of cells, which can in turn influence the results of in-cell NMR experiments. To monitor the state of the cells, the authors probed cell viability after each NMR experiment by trypane blue staining, which showed that more than 90% of cells were alive. This test, however, is incapable of detecting changes in the intracellular milieu. Recently, it was shown that the cytosolic pH value of CPP-treated mammalian cells was significantly lower than that of nontreated cells (6.2 ± 0.2).[94] The authors speculated that the observed acidification might be a result of the lack of oxygen in the in-cell sample in the NMR tube during sample acquisition. In principle, the intracellular pH value can also be measured by in-cell NMR spectroscopy. One possibility is to measure the coupling constants within the histidine ring, which are sensitive to the protonation and tautomerization state. On the basis of histidine residues in the bacterial protein NmerA, the pH value in the cytosol of living bacteria was thus measured to be 7.1.[95] Monitoring of the pH value will be important in future in-cell NMR spectroscopic experiments to avoid the misinterpretation of data.

An alternative to the CPP-based transfection system was developed by Shimada and co-workers as a tag-free procedure to enrich the labeled target of interest in HEK 293F suspension cells.[10] Prior to target transduction, cells are treated with the inducible pore-forming toxin streptolysin O (SLO), which accumulates at the cellular membrane upon treatment at lower temperature (4 °C). After the addition of the labeled target to the supernatant (at a concentration of approximately 1 mM), pore formation and thus target transduction is induced by incubation for 30 min at elevated temperature (37 °C). Pores formed by streptolysin O were shown to be permeable to macromolecules with a size of up to 150 kDa.[96] Once pores have been formed, the high concentration of the target outside the cells drives its passive diffusion into the cells. To seal the pores and proceed with in-cell NMR spectroscopic analysis, cells are treated with calcium, which triggers the internalization of the pores.

Similar to the trypane blue staining assay, flow cytometry can be used to measure cell viability and the incorporation of propidium iodide, a marker for late apoptosis.[10] The advantage of using flow cytometry is that besides quantification of the viability of the cells before and after in-cell NMR spectroscopic analysis, the transduction efficiency can be analyzed simultaneously, which is particularly useful during the optimization phase to attain the highest transduction efficiency and the lowest cell lethality.[10] The authors also measured negligible leakage of their ^{15}N-labeled IDP into the supernatant after recording the in-cell NMR spectra. Although the transduction efficiency per cell (50 μM) was shown to be high enough for the measurement of 2D NMR spectra for an ^{15}N-labeled IDP, only 50% of the cells in the NMR tube contained the target. Therefore, by considering the amount of cells and the volume of the buffer surrounding the cells, the actual detectable concentration of the protein in the NMR-active volume was estimated to be 4 μM, thus

calling into question the feasibility of this method for studying targets that tumble more slowly under in-cell conditions. However, the same research group recently reported an improved SLO-based transduction procedure that allowed them to characterize the structure and interaction surface of even slowly tumbling proteins by methyl-group labeling and detection by TROSY and sf-HMQC.[82]

To enable sophisticated and time-consuming experiments to be carried out, such as transfer cross-saturation (TCS),[97] Shimada and co-workers introduced a bioreactor system that constantly supplies the cells with fresh medium during in-cell NMR data acquisition.[82] They also observed that their SLO-treated cells under in-cell NMR conditions without a constant supply of fresh medium underwent changes in their ATP levels and severe target leakage. The bioreactor preserved in-cell homeostasis for up to 5–6 h and removed leaked molecules, which would otherwise would have resulted in false positive signals. With the bioreactor system, the protein–protein interaction surface of a 9 kDa microtubule-binding domain was successfully mapped in cells by the TCS method.[82]

Originally, the intracellular expression level of mammalian cells was thought to be too low for the concentrations required for in-cell NMR spectroscopic experiments to be reached. However, it was recently demonstrated that plasmid transfection and transient overexpression can yield ^{15}N-labeled IDPs and globular proteins in concentrations of up to (360 ± 30) μM in adherent HEK 293T cells.[98] Target-overexpression levels depended on the amount of transfected DNA. To reach a high transfection efficiency, plasmids were complexed with polyethylenimine (PEI) prior to transfection. This overexpression approach was used to study the complete posttranslational maturation process of human superoxide dismutase 1 (SOD1) by in-cell NMR spectroscopy.[99] Simple in-cell 1D ^{1}H NMR and in-cell 2D [^{15}N,^{1}H] sfHMQC NMR experiments were employed to monitor zinc binding, homodimer formation, and copper uptake. Strikingly, the coexpression of SOD1 with the copper chaperone for SOD1 (CCS) induced oxidation of the SOD1 intrasubunit disulfide bond, thus suggesting that the concept of STINT-NMR for mapping protein–protein interactions can also be applied to in-cell NMR spectroscopy with mammalian cells.

3.4. Yeast and Insect Cells

In addition to the *X. laevis* oocytes and different mammalian cells, yeast and insect cells were recently tested for their applicability for in-cell NMR experiments in eukaryotic cells.[12,13] Shekhtman and co-workers studied the fate of overexpressed ubiquitin in the yeast *Pichia pastoris*.[13] Overexpression for 48 h with an additional starvation period of 6 h was necessary for the observation of a well-resolved [^{15}N,^{1}H] HSQC spectrum of ubiquitin. However, a significant amount of background signals remained in these spectra and hindered the detection of resonances in the region of 7–8.5 ppm as a result of peak overlap. Interestingly, when *P. pastoris* was fed with different nutrients, all well-resolved resonances of ubiquitin disappeared in the corresponding in-cell NMR spectra, thus suggesting that ubiquitin was degraded or engaged in nonspecific binding with endogenous components of *P. pastoris*. Immunofluorescence experiments revealed that under these conditions, ubiquitin was immobilized in protein-storage bodies. This protein storage was not a ubiquitin-specific phenomenon, but was rather nutrition-related, since β-galactosidase ended up in the same storage compartments. In contrast to *P. pastoris*, Sf9 insect cells required only 24–25 h for baculovirus-induced overexpression, and the corresponding 2D in-cell NMR spectra had a significantly reduced background in the 7–8.5 ppm region with respect to that observed with the yeast cells.[12] These spectra could be further improved by subtraction of the background signals by the use of spectra acquired with non-overexpressing cells. The quality of the in-cell NMR spectra allowed the authors to perform 3D triple-resonance experiments and thus to assign 80% of the protein backbone. However, since most of the proteins studied by in-cell NMR spectroscopy tend to form nonspecific attractive interactions inside cells, the general applicability of NMR experiments beyond 1D and 2D correlation is uncertain.

3.5. In-Cell EPR Spectroscopy of Proteins and Nucleic Acids

EPR spectroscopy cannot provide the same amount of detailed information about the conformational and dynamic state of a macromolecule as NMR spectroscopy. However, the advantages of in-cell EPR spectroscopy are its greater sensitivity, the lack of cellular background signals, and the possibility of measuring distances by the PELDOR EPR method independently of the tumbling rate, and therefore the molecular weight, of the macromolecule.[23,62]

Detection of a macromolecule in the cellular environment requires the labeling of this macromolecule with a spin label (most often MTSL attached to a cysteine residue in the case of proteins), and the measurement of distances is based on the introduction of two spin labels at different sites of the molecule. With the *X. laevis* oocyte microinjection system, the PELDOR EPR method was used to measure distances (1.5–8 nm) between spin labels that had been attached to ubiquitin and nucleic acids.[23,24] Longer distances of up to 10 nm were measured by using deuterated soluble proteins.[100] Instead of 200 oocytes, as required for in-cell NMR experiments, only 30–50 injected oocytes are needed to occupy the active volume of the EPR resonator. Injection and loading can be performed within 10–15 min. Distance measurements in PELDOR experiments are most successful when the target is as immobile as possible, since the flexibility of the spin labels and the conformational flexibility of the macromolecule make the obtained distance information less precise. Thus, for the detection of long-range dipolar coupling between the spin labels, the samples are typically shock-frozen prior to measurement at 50 K.

Igarashi et al. observed that the nitroxide spin label attached to the target under study was quickly reduced once present in the cellular environment, with an estimated half-life of 1 h.[24] They further demonstrated in their proof-of-concept study that spin-labeled ubiquitin (with nitroxide-

based 3-maleimido-PROXYL) can be analyzed by means of in-cell PELDOR EPR spectroscopy. Injection and immediate sample preparation resulted in distance profiles that were virtually identical to the in vitro profiles. However, measurements made after incubation for 1 h at a physiological temperature of 18 °C yielded PELDOR time traces with low signal-to-noise ratios (modulation depths). This reduction in modulation depth is expected, since from the moment the spin labels are inside the oocytes, they are recognized by reducing agents, such as small molecules or enzymes, which actively reduce the nitroxide and thus diminish the EPR signal.

By using in-cell EPR spectroscopy, we investigated several spin-labeled DNA and RNA molecules and were able to record in-cell PELDOR time traces.[23] Whereas the in-cell distances measured for all three investigated nucleic acid molecules were reminiscent of the distances observed under in vitro conditions, thus suggesting that all of them adopted their known in vitro folding topologies in the cellular environment, PELDOR measurements of a DNA duplex resulted in the detection of an additional larger distance that could not be observed in in vitro experiments. This larger identified distance also showed a significantly broader distribution. The observation that the modulation depth of the in-cell time traces was notably deeper than expected also suggested that the dipolar interaction of more than two coupled spins occurred.[23] Taking these results together, we concluded that the monomeric B-DNA duplex and multimeric duplexes coexisted in cells.

In-cell EPR spectroscopic investigations of DNA duplexes were also reported by Drescher and co-workers.[62,63,101] Each strand of a 7-mer DNA duplex was modified at the 5′ end with a six-membered-ring nitroxide and analyzed in *X. laevis* oocytes. In contrast to the measured in-cell lifetimes in our study (50–60 min), the lifetime of the spin-labeled DNA investigated by Azarkh et al. was estimated to be 1.1 min. The difference in the lifetimes could be attributed to the different nature of the spin labels used in these studies.[63] Indeed, we and Azarkh et al. showed that five-membered-ring nitroxides are much more stable to reduction in cells than six-membered-ring nitroxides.[23,101] Investigation of the heptameric oligonucleotide sequence revealed that in contrast to the in vitro situation, in which a defined duplex is observed, the broad distance distribution measured in cells indicated a melted DNA state.[63]

4. Future Perspectives of In-Cell NMR Spectroscopy

No other spectroscopic method is capable of providing the same amount of detailed information on the conformational state and the dynamic behavior of a biological macromolecule as NMR spectroscopy. This ability combined with the low energy of the radiofrequency radiation used for excitation and the noninvasiveness of the technique makes NMR spectroscopy the perfect tool for studying macromolecules in their natural environment. Detailed protocols for the use of many different cell types, including bacteria, mammalian cells, and other eukaryotic cells, have been developed and established.[12,13,102–105]

The advantage of the low energy needed for excitation is at the same time, however, also the biggest disadvantage of NMR spectroscopy. The low excitation energy correlates with a low energy gap between the ground and the excited state and therefore only a small population excess in the ground state, thus leading to the inherently low sensitivity of the method. To overcome this low sensitivity, NMR samples usually require concentrations in the upper micromolar range or even higher. The biggest challenge for in-cell NMR spectroscopy in the future will be to perform experiments at intracellular concentrations that are close to the endogenous levels: in most cases, in the upper nanomolar to lower micromolar range. The next generation of ultra-high-field magnets and cryogenic probes will certainly help to diminish this problem, but other technical developments will be necessary to fully reach the endogenous level. Dynamic nuclear polarization (DNP) has been shown to be able to increase the sensitivity of solid-state NMR experiments dramatically,[106] and first applications of DNP to the study of membrane proteins in membrane preparations have been reported.[107] Deuteration and the focus on methyl groups can also reduce the need for high concentrations in liquid-state in-cell NMR spectroscopic applications. Only if the goal of getting close to relevant intracellular concentrations can be met will in-cell NMR spectroscopy be able to provide information about specific interactions of the observed macromolecules. For all in-cell NMR experiments in which higher intracellular concentrations are used, the biological relevance of the results has to be considered.

Besides the cellular concentration, intracellular localization is also very important for obtaining physiologically relevant information. Proteins or nucleic acids that are not in the correct cellular compartment will function differently. For small oligonucleotide fragments it was shown that after injection into the cytoplasm of mammalian cells they accumulate in the nucleus.[108–110] For most proteins it is assumed that they are localized in the cytoplasm,[98] but in the future more detailed investigations of their intracellular localization will be necessary to assure that physiologically meaningful experiments are performed.

A further challenge is that the formation of specific complexes but also nonspecific interactions with other cellular components increase the rotational correlation time,[16–18] thus resulting in significant line broadening or even complete disappearance of the signals. A focus on methyl-group detection can alleviate this problem to a certain extent, but if the rotational correlation time becomes too large, even methyl-group detection will fail. In this case, solid-state in-cell NMR spectroscopy could be the method of choice. To increase the sensitivity of solid-state experiments that rely on magnetization transfer by dipolar coupling, these experiments are best performed at low temperatures with frozen cells. Most cells will not survive such a procedure, and the goal for solid-state in-cell NMR spectroscopy will most likely be to prepare a certain cellular state and preserve it by flash freezing. When the sample is kept in a frozen state, cellular processes such as degradation are slowed down or

even prevented, and thus longer measurement times are possible. Most solid-state NMR experiments are based on the detection of ^{13}C or ^{15}N nuclei. The significantly lower sensitivity of these detection schemes as compared to the ^{1}H detection usually used in liquid-state NMR experiments makes longer measurement times necessary. Investigations of membrane proteins or other molecules that tumble very slowly are also possible at temperatures higher than 0 °C, depending on the efficiency of the magnetization transfer through dipolar coupling and the stability of the cellular system at these temperatures.

Similarly, in-cell PELDOR EPR experiments are (with a few exceptions) also performed with frozen cellular samples. The biggest challenge for EPR spectroscopy is the fast reduction of the currently available spin labels in the cellular environment, which limits the measurement time. Sterically more protected spin labels are currently being developed and will have a major impact on the application of EPR spectroscopy in cellular systems.

Finally, monitoring of the exact state of the cells in the NMR tubes will be important to obtain biologically meaningful results. To keep the cells in a stable metabolic state, the further development and optimization of bioreactors that maintain constant levels of oxygen, CO_2, and nutrients will also be essential. With such bioreactors and stable conditions, longer experiments, such as relaxation series or experiments with low concentrations, will also become possible.

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