In-Cell NMR Spectroscopy

Sina Reckel, Frank Löhr, and Volker Dötsch*[a]

Introduction

NMR spectroscopy combines two features that make it unique among all biophysical methods for the investigation of biological macromolecules. The first feature is its ability to provide information about molecules under physiological or at least "near-physiological" conditions. This ability has led to the development of entire new fields such as in vivo NMR spectroscopy, which focuses on the observation of metabolites and metal ions in systems ranging from suspensions of bacteria and other cells to entire perfused organs, and magnetic resonance imaging, which can provide information about entire organisms.[1–5] The second distinctive feature is the sensitivity of the chemical shift of an NMR-active nucleus to changes in its chemical environment. This ability has made NMR spectroscopy an excellent tool for studying the interaction of biological macromolecules with binding partners, ranging from other macromolecules to small ligands and medically important drugs.[6–13] Recently, we and others have started to combine these two advantages of NMR spectroscopy to obtain information about the conformation and dynamics of biological macromolecules inside living cells.[14–20]

Applications

"In-cell NMR" experiments do not aim at determining structures directly in the cellular environment, but use the sensitivity of the chemical shift towards changes in the environment to obtain information about the state of a macromolecule in its natural surrounding. Changes in this environment, caused by post-translational modifications, conformational changes, or binding events, result in changes in the resonance frequencies of the affected nuclei and can thus be detected in "in-cell NMR" experiments (Figure 1). If differences between the in-cell spectra and the in vitro spectra can be detected, the cause for these differences can be investigated by simulating the in vivo conditions in vitro, for example, by adding the suspected interaction partners to an in vitro sample. Using this strategy, Dedmon et al. showed that the bacterial protein FlgM, which is completely unfolded in vitro, is partially folded in the *E. coli* cytoplasm.[19] By adding high concentrations of either other proteins (bovine serum albumin) or small molecules (sugar) to an in vitro sample of FlgM, they could reproduce the spectral characteristics of this protein in the bacterial cytoplasm. Their conclusion was that the high concentration of other (macro-) molecules inside the cell, known as molecular crowding, is responsible for the observed partial folding.

Other investigations have focused on the interaction of proteins with metal ions. Hubbard and co-workers have used in-cell NMR spectroscopy to investigate the ion-binding status of

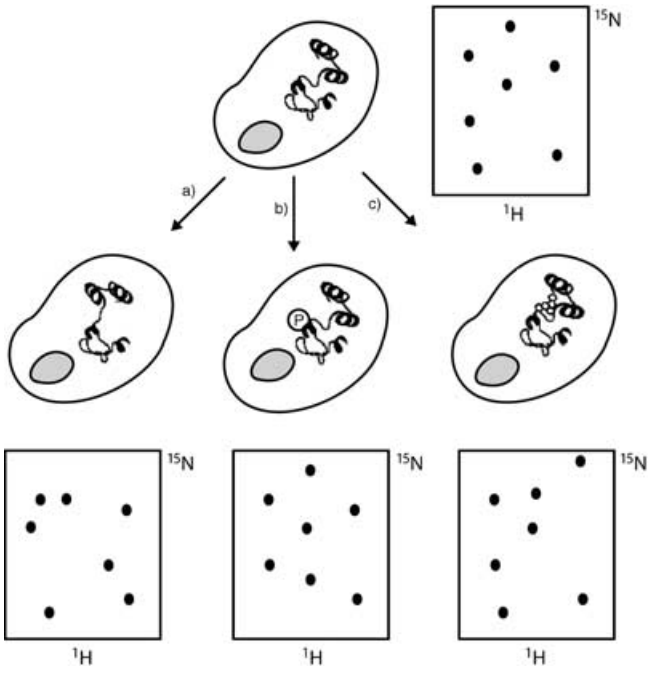

Figure 1. Potential applications for in-cell NMR experiments. Changes in the chemical environment of a protein's nuclei caused, for example, by a) conformational changes, b) post-translational modifications (here phosphorylation), or c) binding events can be detected by differences in chemical shifts in in-cell NMR experiments. Schematic HSQC spectra indicating the sensitivity of the chemical shift to the changes described above are shown below each cell.

the bacterial two-component signal transduction protein CheY in the bacterial cytoplasm.[18] By comparing an in-cell [^{15}N,^{1}H]-HSQC spectrum with in vitro spectra of the protein complexed with different ions, they could show that CheY preferentially binds Mg^{2+} ions in the *E. coli* cytoplasm. Small additional changes in the chemical shifts might indicate further interactions with other components; however, they cannot be conclusively interpreted so far. Our own investigations of calmodulin in living *E. coli* had indicated that calmodulin mainly exists in the apo-form; this shows that the intracellular Ca^{2+} concentration in bacteria is not high enough to make the calcium-bound form the major in vivo conformation.[16] Furthermore,

[a] *S. Reckel, Dr. F. Löhr, Prof. Dr. V. Dötsch*
Institute for Biophysical Chemistry and
Center for Biomolecular Magnetic Resonance
Johann Wolfgang Goethe University
Marie Curie Straße 9, 60439 Frankfurt (Germany)
Fax: (+49) 69-798-29632
E-mail: vdoetsch@em.uni-frankfurt.de

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additional peaks indicated that more than one conformation is present under these conditions in the bacterial cytoplasm.

Chemical-shift differences observed in HSQC spectra can also be used to detect and to characterize the interaction of proteins with drug molecules, and this method is widely used in the pharmaceutical industry as a screening tool. Using standard in vitro NMR experiments to screen for potential protein–drug interactions, however, has the disadvantage that an interaction that is observed might not occur in the same way in vivo. Such behavior could, for example, be caused by the inability of a drug molecule to cross the cellular membrane, its fast metabolization, its binding to other cellular components with higher affinity than to its intended target, or differences in the target-protein conformation between its in vitro and in vivo states. In principle, these disadvantages of in vitro screens can—at least partially—be overcome by using in vivo assays, for example, in-cell NMR experiments for screening. An example of the application of in-cell NMR experiments as a screening technique is reported by Hubbard et al. They could show that the drug BRL-16492PA, which binds to the bacterial two-component signal transduction protein CheY in vitro, also binds to the same protein inside living *E. coli* bacteria.[18] They based their conclusion on observing virtually identical chemical shift changes in the [^{15}N,^{1}H]-HSQC spectrum of CheY upon adding the drug either to a purified in vitro sample or to a slurry of *E. coli* overexpressing the protein. Other applications have focused on using saturation transfer difference methods (STD) for detecting interaction of receptors expressed on the surface of cells with external drugs.[21,22]

In protein–drug interaction screens, most often [^{15}N,^{1}H]-HSQC experiments are used based on the high chemical-shift dispersion of the amide protons and nitrogens. Alternatively, methyl-group-based NMR experiments can be employed due to their high sensitivity (see below) and their involvement in drug binding. Interestingly, a recent investigation has found that within a set of 191 crystal structures of protein–ligand complexes, 92% of the ligands had a heavy atom within 6 Å of a methyl group carbon while only 82% had a heavy atom within the same distance of a backbone nitrogen.[6] We have investigated the interaction of calmodulin with the known drug phenoxybenzamine hydrochloride, which is assumed to bind to a hydrophobic pocket that is lined with methionines.[17] We added the drug to an *E. coli* culture expressing calmodulin half an hour prior to sample preparation. No differences in chemical shifts between an in vitro sample and the in-cell sample could be detected; this indicates that no stable complex between the drug and the protein is created. However, some of the peaks in the in-cell spectrum showed increased line broadening; this suggests that a weak interaction with the drug exists. This result is in agreement with reports that phenoxybenzamine interacts with calmodulin only in its calcium-bound form and our own results that had demonstrated that calmodulin in the bacterial cytoplasm exists mainly in the calcium-free apo-form.[16] In order to investigate how much of the drug had been taken up by the bacteria, we harvested the cells by centrifugation. No drug resonances could be detected in a ^{1}H-1D spectrum of the supernatant. In contrast, the resuspended bacteria pellet showed strong signals of the drug, which remained associated with the cell debris after cell lysis. This result indicates that phenoxybenzamine is mainly associated with the bacterial membrane and that the high local concentration of phenoxybenzamine near the bacterial membrane is most likely responsible for the observed weak interaction. This example demonstrates the advantages of in-cell NMR experiments, which are able to detect both the resonances of the protein and of the drug and to characterize their interaction in a cellular system.

A further application of in-cell NMR spectroscopy is the investigation of the tautomerization and protonation state of histidines in the cellular environment.[23] Since histidines are frequently found in the active site of enzymes, their tautomerization and protonation states often determine an enzyme's activity. NMR spectroscopy has been used extensively to investigate the different states of histidines in vitro.[24,25] Recently, we have extended these investigations to proteins inside living *E. coli* cells based on determining the values of the C–N coupling constants of the $C^{\varepsilon 1}$ and $C^{\delta 2}$ carbon spins in histidines.[23] The exact values of these coupling constants depend on the form of the histidine side chain and can, therefore, be used to determine its tautomerization and protonation state. Alternatively, if the pK_a value of the histidine side chain is known, measurement of these coupling constants can be used to measure the intracellular pH value. Based on our measurements we have determined the pH in the bacterial cytoplasm under the conditions of our NMR experiments to be 7.1 ± 0.1.

Technical Aspects

The investigation and characterization of macromolecules in living cells by NMR spectroscopy has to overcome three main difficulties. First, the NMR signals of the molecule of interest must be distinguished from the NMR resonances of all other cellular components. Second, the macromolecule must be able to tumble freely, and third the cells have to survive the conditions inside the NMR tube at least for the time period of the experiment without significant changes of their metabolic state. These three difficulties will be discussed below in more detail.

1) Labeling

Distinguishing the NMR resonances of a macromolecule from all other resonances of the cell can be achieved by overexpressing the macromolecule and labeling with the NMR-active isotopes ^{15}N and ^{13}C. ^{19}F labeling has also been employed in some cases.[26,27] The advantage of ^{19}F labeling is the virtually zero background due to the low abundance of fluorine compounds in cells. For example labeling of phosphoglycerate kinase in yeast with 5-fluorotryptophan was used to investigate the binding of nucleotides to this protein in vivo.[27] However, the use of fluorine requires the chemical modification of amino acids by replacing a hydrogen with a fluorine; this changes the chemical properties of the amino acid and can therefore lead to a different behavior of the protein. In addi-

tion, fluorine-labeled amino acids are toxic for certain cell types.[27]

Observation of the macromolecule without chemical modification requires labeling either with ^{15}N or with ^{13}C. The specific labeling scheme will depend on the kind of macromolecule that is to be investigated as well as on the type of cells that are used. In some cases, for example *Xenopus* oocytes, it is possible to directly microinject a labeled protein into the cell.[28,29] Injection has the advantage that the only background signals are the signals produced by the natural abundance of ^{15}N and ^{13}C. Of these, only the natural abundance of ^{13}C (1.1%) plays a significant role. In most cases, however, the macromolecule of interest will be expressed directly inside the cells which—depending on the labeling scheme—can cause severe problems with background signals due to the labeling of other cellular components. The following discussion will focus on labeling strategies in *E. coli* since it is the most often used cell type so far. However, most considerations will also apply to other cell types such as yeast.

The experience with ^{15}N-labeling in *E. coli* so far has shown that only a minimum background level is produced that does not interfere with most protein resonances. The only requirement for the observation of a protein's backbone resonances in "in-cell NMR" experiments is that this protein is expressed above a certain threshold.[16] This threshold is approximately 1–2% of the entire soluble protein content of a cell or roughly 200 to 300 μM intracellular concentration. This minimal background level could be further suppressed to virtually zero by using an amino acid type-selective labeling scheme. However, not all amino acids can be used for selective labeling schemes in a standard *E. coli* strain. Good candidates for labeling in BL21 cells are lysine, arginine, and histidine, which are at the end of a biosynthetic pathway and do not serve as precursors for other amino acids.[30] For other amino acid types, cross labeling has to be suppressed either by the use of more complex media that do not only contain the labeled but also unlabeled amino acids or by the use of specialized auxotrophic strains.[30–32] Fortunately, at least one auxotrophic *E. coli* strain exists for each amino acid that can be used for selective labeling. In addition, many yeast auxotrophic strains have been created, albeit not for NMR labeling purposes. One disadvantage, at least of the auxotrophic bacterial strains, is that they often show a reduced expression level that decreases the quality of the in-cell NMR spectra.

To investigate side chains in general, carbon-based labeling schemes have to be employed. ^{13}C-based in-cell NMR experiments provide several advantages over ^{15}N-based experiments. First, the sensitivity of detecting methylene and methyl groups is higher based on the larger number of protons directly attached to the heteronucleus as compared to the single amide proton. Second, in contrast to amide protons, carbon-bound protons do not chemically exchange with protons of the bulk water. Fast exchange of the amide protons significantly reduces the signal intensity and can even result in complete signal loss. Probably the biggest advantage, however, is the fact that methyl groups have the most slowly relaxing spins based on their fast internal rotation. This slow relaxation further increases the sensitivity of methyl-group detection.[6,33,34] Since methyl groups also show the highest proton-to-heteronucleus ratio, they are the most attractive side chain probes for in-cell NMR experiments. These advantages predict that the sensitivity of methyl-group-detected in-cell NMR experiments should be at least three times higher than the sensitivity of amide-proton-detected experiments. These predictions could be confirmed by expressing calmodulin in *E. coli* and labeling it simultaneously with methyl group ^{13}C-labeled methionine and ^{15}N-labeled lysine (Figure 2).[17]

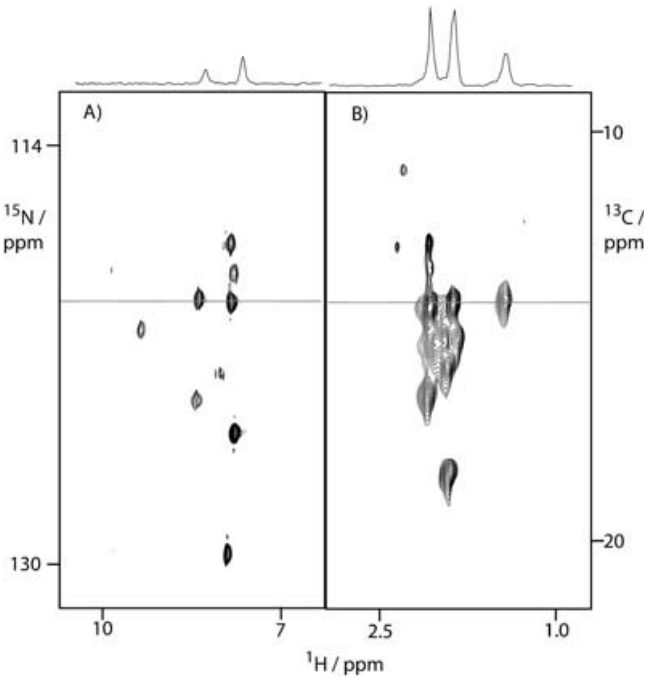

Figure 2. Comparison of an A) [^{15}N,^{1}H]-HSQC spectrum and a B) [^{13}C,^{1}H]-HSQC spectrum of calmodulin, selectively labeled with both ^{15}N-lysine and ^{13}C-methyl methionine in the bacterial cytoplasm. For a better comparison of the relative sensitivity, 1D cross sections are shown on top of each spectrum. Both spectra were measured simultaneously in an interleaved way with four scans per t_1 increment on a Bruker Avance spectrometer, operating at a proton frequency of 500 MHz and equipped with a cryogenic probe.

However, carbon-based labeling schemes also suffer from disadvantages. While the level of background signals in ^{15}N-based in-cell NMR spectra is minimal, full carbon labeling with ^{13}C-labeled glucose produces such a high background level that, with the exception of a few high-field-shifted calmodulin methyl groups, no protein resonances could be unambiguously identified.[17] The greater abundance of carbon than nitrogen in small molecules and the fast chemical exchange of many of the amide protons of small molecules with water protons are the most likely reasons for the high ^{13}C-background level. More selective labeling schemes, for example based on using ^{13}C-labeled pyruvate,[35] improve the situation but still produce a high level of background signals.[17] The lowest level of background signals can be achieved by amino acid-type-selective labeling. As mentioned above, labeling of methyl groups is—

with regard to the sensitivity of the experiments—the most attractive labeling option. However, similar to the ^{15}N-based amino acid type selective labeling, not all amino acids can be used equally well. While methyl-group-labeled methionine produces virtually zero background, the high background level caused by methyl-group-labeled alanine has to be suppressed by using special media compositions.[17,32]

^{13}C-based labeling schemes also offer the possibility of observing other biologically important macromolecules besides proteins. Lippens' research group has used ^{13}C-labeling to observe cyclic osmoregulated periplasmic glucan in *Ralstonia solanacearum*.[20] Similarly to experiments with proteins, they observed a high background level when using fully ^{13}C-labeled glucose, but could reduce it by using glucose that was selectively ^{13}C-labeled at the C1 position.

2) The rotational correlation time of proteins inside cells

A major limitation for the application of high-resolution liquid-state NMR spectroscopy of biological macromolecules is the requirement that these molecules have to tumble in solution with a sufficiently short correlation time. Long rotational correlation times lead to fast relaxation and, therefore, broad peaks. Since the rotational correlation time is proportional to the surrounding viscosity, the intracellular viscosity is an important parameter for the observation of macromolecules inside living cells. Diffusion measurements have indeed shown that the translational diffusion of a macromolecule inside cells can be severely restricted relative to an in vitro system with the purified molecule. However, investigation of the rotational correlation time by several different techniques, including NMR relaxation measurements,[36–38] EPR measurements,[39] and fluorescence experiments,[40–43] have shown that the intracellular rotational correlation time is only twice as long as the rotational correlation time of the same molecule in pure water. This increase by a factor of two of the rotational correlation time also increases the apparent molecular weight of the protein by a factor of two. Fortunately, the introduction of TROSY[44] and similar techniques[45,46] a couple of years ago has extended the applicability of NMR spectroscopy to large macromolecules with a molecular weight of 100 kDa and more. These technical advances, combined with the relatively low viscosity of the cellular cytoplasm, predict that the cytoplasmic viscosity is not a major limitation for the observation of proteins inside living cells. However, viscosity differs among the individual cellular organelles. While the viscosity in endosomes,[47] for example, is only slightly greater than the viscosity of the cytoplasm, the viscosity of the nucleus seems significantly increased.[48,49] In addition, the viscosity of the organelles and the cytoplasm can also change during different states of the cell, for example, different phases of the cell cycle.

The intracellular observation of proteins can, however, become impossible by binding of the proteins to other cellular components; this significantly increases the rotational correlation time of the protein. In particular, binding to large components, such as chaperones and nucleic acids, leads to the disappearance of a protein's resonances due to extensive line broadening. For proteins that are not observable by backbone-amide-based NMR experiments, we had good experience with the observation of ^{13}C-labeled methyl groups.[17] The fast internal rotation of these methyl groups makes proteins such as FKBP or thioredoxin, which are usually interacting with other components in the cell, observable.

If even larger protein complexes or intracellular aggregates (prion proteins, Alzheimer peptides) are to be investigated by in-cell NMR experiments, solid-state NMR techniques have to be employed. Preliminary experiments that we have conducted on proteins deposited in inclusion bodies in *E. coli* suggest that in-cell solid-state NMR experiments are technically feasible.

3) Cellular survival during NMR experiments

The third critical parameter that strongly influences the applicability of in-cell NMR experiments is the survival rate of the cells in the NMR tube. In particular, the high cellular density can cause problems through oxygen starvation and limiting the amount of available nutrients. If the sensitivity of the selected system (mainly the overexpression level) is high enough, the NMR spectra can be measured relatively quickly (less than an hour). During longer experiments or series of experiments such as relaxation studies, however, significant changes in the cellular status can occur. These changes can range from shifts of the intracellular pH in *E. coli* cells to cell death observed, for example, with insect-cell samples. On the other hand, in classical in vivo NMR experiments, cell cultures have been kept alive for long times.[50–52] This can be achieved by using modified NMR sample tubes that allow for a continuous exchange of the media. One problem that has to be solved for experiments with continuous flow of media is how to keep the cells in the NMR tube. While solutions with semipermeable fibers and microcarriers have been used, the easiest method for keeping unattached cells in an NMR tube is to encapsulate them, for example, in low-melting agarose.[50,53] Previous in vivo NMR experiments have shown that bacteria can be kept alive in these gels for long periods of time. In order to investigate if these encapsulating techniques would provide the necessary magnetic homogeneity for two-dimensional NMR experiments on proteins in bacteria, we have overexpressed and ^{15}N-labeled NmerA in *E. coli*, and mixed a concentrated bacterial slurry with an equal amount of low-melting agarose in an NMR tube. The resulting spectrum is shown in Figure 3B, and, for comparison, a regular in-cell NMR spectrum of NmerA measured with a resuspended bacterial sample is shown in Figure 3A. Comparison of both spectra shows that the quality is not decreased by the encapsulation process. Encapsulation will not only allow for perfusion of the bacterial sample, it will also solve the problem of cell sedimentation to the bottom of the NMR tube over time. Both problems are more severe with larger and more sensitive eukaryotic cells. However, the good experience of many metabolic studies with gel-entrapment methods combined with the fact that the spectral quality is not compromised also makes encapsulated cellular samples an interesting alternative for longer and more

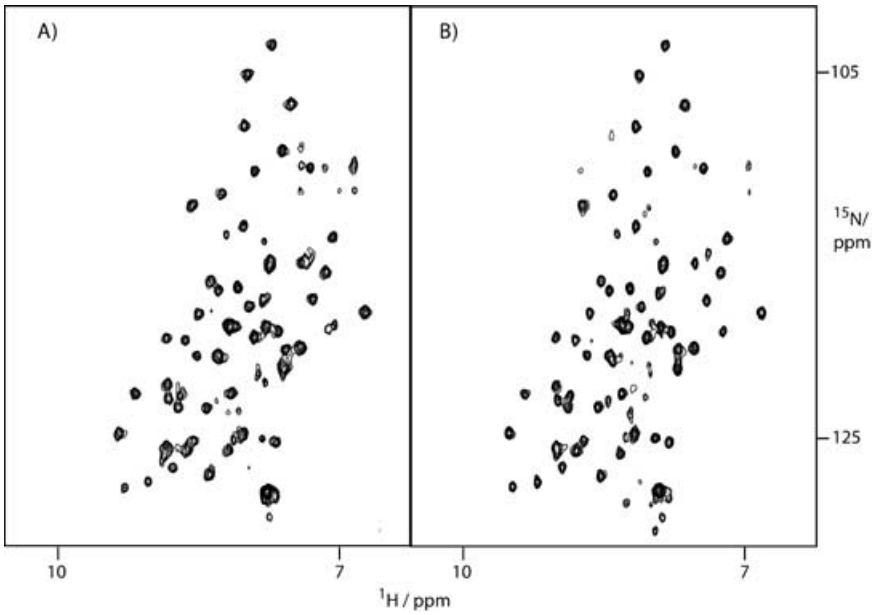

Figure 3. Comparison of the [^{15}N,^{1}H]-HSQC spectra of NmerA in bacteria A) resuspended in a phosphate buffer or B) encapsulated in low melting agarose. Both samples were measured at 37 °C with identical experimental parameters.

complicated in-cell NMR experiments with more sensitive cell types.

Limitations, Challenges, and Future Directions

The biggest disadvantage of NMR spectroscopy is its inherent low sensitivity. In particular, for the investigation of the behavior of biological macromolecules in their natural environment, it is important to keep the concentration of the macromolecule of interest as close to their natural level as possible. Currently, observation of proteins by "in-cell" NMR experiments requires their overexpression, and the detection limit is approximately 200 μM for amide-proton-based experiments and 70 μM for methyl-group-detected ones. For most proteins, these overexpression levels are at least one-to-two orders of magnitude higher than their natural intracellular concentration. This situation, of course, limits the possible applications of in-cell NMR spectroscopy. In particular binding studies with additional cellular components are limited to unspecific binding events, since the concentration of any natural binding partner would also be one-to-two orders of magnitude lower. A possible application of in-cell NMR, however, is for intracellular drug screens. These experiments do not depend on the formation of a stoichiometric complex with an intracellular component and can provide interesting information about the potential of a particular drug to cross the cellular membrane and interact with the protein inside living cells. Other possible applications include the investigation of the metal-binding state of a particular protein, its unspecific interaction with other cellular components (for example molecular crowding, chaperone binding), or its dynamics and intracellular stability. In addition, investigations of post-translational modifications are possible if an enzymatic relationship between the overexpressed protein and the modifying protein exists. In these cases, a low concentration of an intracellular enzyme can modify a considerably higher amount of the protein of interest; this makes this process observable by in-cell NMR spectroscopy.

The final goal, of course is the observation of proteins at or near their physiological concentration. In order to achieve this goal, however, further significant improvements of the sensitivity of NMR spectrometers have to be achieved. Fortunately, the introduction of oxygen probes has dramatically increased the sensitivity of NMR instruments over the last years, with further improvements expected. For in-cell NMR experiments, detecting proteins at their natural concentration, will, however, also create new challenges. In earlier investigations, we have shown that the most important parameter for the selective detection of a protein by amide-proton-based NMR experiments in living cells is the overexpression level. A protein's resonances will become detectable as soon as its expression level reaches a certain threshold.[16] Since the concentration of all other cellular components does not reach this threshold, these spectra show a low background level. ^{13}C-based in-cell NMR experiments had already demonstrated that the background level for spectra that detect carbon-bound protons is considerably higher, and more selective labeling techniques have to be used in order to observe the resonances of a particular protein inside living cells unambiguously.[17] An improved sensitivity of NMR spectrometers that reduces the threshold level and enables the observation of a protein's resonances at its physiological concentration will make the distinction between background signals and signals of the protein of interest more difficult, even for amide-proton-based experiments. Several possible solutions to this problem exist. One potential solution is the suppression of the expression of all intracellular proteins. This can, for example, be achieved with the drug rifampicin, which we have already used during some of our experiments.[15,16] This drug inhibits bacterial RNA polymerase but allows the expression of a plasmid under the control of the promoter of the bacteriophage T7.[54,55] Another very elegant approach that has very recently been presented is the degradation of all mRNA that contains ACA sequences by a mRNA interferase.[56] Changing all ACA triplets in the DNA sequence of the protein of interest by utilizing other codons enables the selective translation of the mRNA of a particular protein while all other mRNAs are degraded.

Another possibility is to use site-specific in vivo labeling schemes, as developed by the research group of Schultz.[57]

They have expanded the genetic code of bacteria and eukaryotic cells;[58] this enables the selective incorporation of a labeled amino acid at a specific site without any background signals from the bacterial (macro-)molecules. The disadvantage of this elegant method is, however, that it requires the use of non-natural amino acids, which have the potential to change the behavior of the protein.

The biggest challenge for in-cell NMR spectroscopy, however, is its extension to eukaryotic cells. Some preliminary experiments with yeast, insect cells, and, in particular, with *Xenopus* oocytes[28,29] exist that have shown that experiments with eukaryotic cells are, in principle, technically feasible. However, for sensitive eukaryotic cells such as insect cells or even mammalian cells, further improvements of the quality of in-cell NMR experiments and a concomitant reduction in the required overexpression level have to be achieved. For these cell types, improvements can be expected from the use of modified NMR tubes that allow for a continuous exchange of oxygenated and nutrient-rich media. Such devices will allow researchers to extend the measurement time of the experiments and further decrease the detection limit.

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