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In-Cell NMR Spectroscopy†

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ABSTRACT: The recent development of "in-cell NMR" techniques by two independent groups has demonstrated that NMR spectroscopy can be used to characterize the conformation and dynamics of biological macromolecules inside living cells. In this article, we describe different methods and discuss current and future applications as well as critical parameters of this new technique. We show experimental results, compare them with traditional in vitro experiments, and demonstrate that differences between the in vitro and the in vivo state of a macromolecule exist and can be detected and characterized.

Nuclear magnetic resonance (NMR)[1] phenomena have provided information about molecules under physiological or "near-physiological" conditions since the first spectra of biological molecules were measured (*1*, *2*). Over the last 2 decades, NMR not only has yielded atomic resolution structures of macromolecules in solution but also has enabled researchers and clinicians to study living systems ranging from cell suspensions to humans, giving rise to whole new fields including in vivo NMR and magnetic resonance imaging. While MRI deals with macroscopic objects and phenomena such as organ morphology and fluid dynamics within vascular tissues, in vivo NMR spectroscopy provides information about the behavior of molecules inside living organisms and cells. So far the focus of in vivo NMR spectroscopy has been on small molecules. To distinguish a specific small molecule from all other molecules inside a cell or an entire organism, these techniques rely either on introducing molecules that have been labeled with NMR-active isotopes of low natural abundance, or on the fact that the molecule of interest is highly abundant and its NMR signals can be distinguished simply based on their intensity (*3*−*8*). These powerful methods have allowed chemists and biologists to obtain a wealth of information about the behavior of small molecules in living systems and to map entire metabolic pathways.

Investigations of biological macromolecules in living organisms have, so far, been rare and mainly confined to one-dimensional NMR experiments of proteins labeled site-selectively (*9*). Recently, two research groups using different NMR techniques and different biological systems have demonstrated that biological macromolecules can be studied inside living cells by NMR spectroscopy (*10*−*13*). In this article, we provide an overview of current and future applications of this technique and discuss the different

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[1] Abbreviations: NMR, nuclear magnetic resonance; MRI, magnetic resonance imaging; HSQC, heteronuclear single-quantum coherence; *E. coli*, *Escherichia coli*; NmerA, N-terminal 7 kDa domain of MerA; NFAT, nuclear factor of activated T-cells; IPTG, isopropyl β-D-thiogalactopyranoside; LB medium, Luria−Bertani medium; TROSY, transverse relaxation optimized spectroscopy; *R. solanacearum*, *Ralstonia solanacearum*; Cys, cysteine; Ala, alanine; HRMAS, high-resolution magic angle spinning.

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experimental approaches. To distinguish these macromolecular NMR investigations from the traditional, small molecule in vivo NMR experiments, we will refer to these new approaches as "in-cell NMR spectroscopy".

What Can We Learn from In-Cell NMR Spectroscopy? While NMR experiments can be carried out in buffers mimicking physiological conditions, a recent survey of structures determined by NMR spectroscopy revealed that they rarely are (*14*). Instead buffers are chosen to optimize solubility and minimize the number of protons in the buffer molecules that can obscure the proton resonances of the investigated macromolecule. Consequently, the chosen buffer conditions can significantly differ from the natural environment of the macromolecule. In-cell NMR spectroscopy goes beyond trying to create buffers that resemble the natural environment of a protein: it investigates the protein directly inside this natural environment. Does this mean that in the future we will determine structures of biological macromolecules directly inside living cells? The answer is most likely no. While in-cell NMR spectroscopy would allow us to eliminate the often time-consuming purification procedures, traditional in vitro methods offer several key advantages such as sharper line width and more stable conditions over the course of a several day multidimensional experiment. In addition, structure determination of the same protein in different buffers by both NMR spectroscopy and X-ray crystallography has shown that the structure of single protein domains is remarkably insensitive to the surrounding conditions (*15*−*17*).

The power of in-cell NMR spectroscopy lies not in determining structures de novo, but in observing changes in the structures of biological macromolecules and in their interaction with other cellular components. The exact position of a resonance line of an atomic nucleus (the chemical shift) is a sensitive function of its magnetic environment. Changes in this environment, caused by posttranslational modifications, conformational changes, or binding events, result in changes in the positions of the resonance line (Figure 1). As an example, Figure 2A shows an in-cell [^{15}N,^{1}H]-HSQC spectrum of calmodulin selectively labeled with ^{15}N on all lysine residues measured in living *E. coli* cells. Figure 2B shows the in vitro spectrum of the same labeled protein after lysing the cells. While some resonance positions are identical, several peaks have very different chemical shifts in the two conditions. In addition, the in-cell spectrum contains more than the expected eight peaks given the eight lysines in calmodulin, suggesting that different conformations of calmodulin exist in *E. coli* (*11*). Most likely the conformational differences between the in vivo and in vitro states of the protein are due to differences in the occupation of the four calcium binding sites of the protein. While the in vitro sample represents the fully calcium-bound state, not all binding sites are occupied in the in-cell sample, consistent with the tight regulation of the calcium concentration inside living cells (*18*) and the known differences in the calcium affinity of the N- and C-terminal domains of calmodulin (*19*). The spectra in Figure 2 also show that selective labeling of only certain amino acid types is possible in in-cell NMR experiments, thereby reducing the complexity of NMR spectra of larger proteins.

While demonstrating the principle that conformational differences between the in vitro and in vivo states of a protein exist and can be detected by in-cell NMR spectroscopy, bacteria do not have endogenous calmodulin, so this example is of limited biological relevance. In contrast, Figure 3 presents a more subtle, yet more biological relevant example. Figure 3A shows part of an in-cell [^{15}N,^{1}H]-HSQC spectrum of the N-terminal domain of the bacterial mercury-detoxification protein MerA (*20*). This N-terminal 7 kDa domain (NmerA) binds metal ions with the help of two cysteines located in a metal binding loop for subsequent transfer to the C-terminal catalytic unit (*20*−*22*). The arrow in Figure 3A indicates the in-cell resonance position of one of the two cysteines in the metal binding loop, cysteine 11. Figure 3B shows the same part of an HSQC measured with a purified in vitro sample of the same protein, and the arrow indicates again the position of the cysteine 11 peak. While the chemical shifts of the surrounding residues do not change significantly,

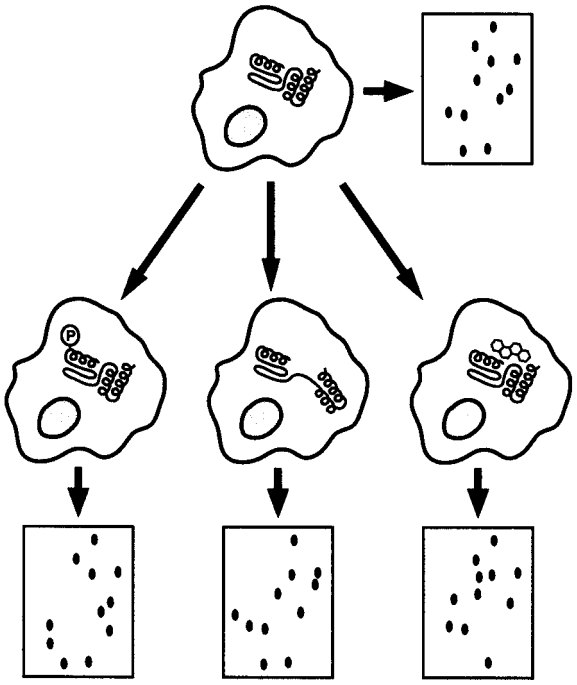

FIGURE 1: Examples of potential applications of in-cell NMR spectroscopy. Posttranslational modifications (left), conformational changes (middle), and binding events (right) can be monitored relative to the unaffected protein (top) through changes in the resonance positions of peaks in NMR spectra. Schematic NMR spectra are shown next to each cell.

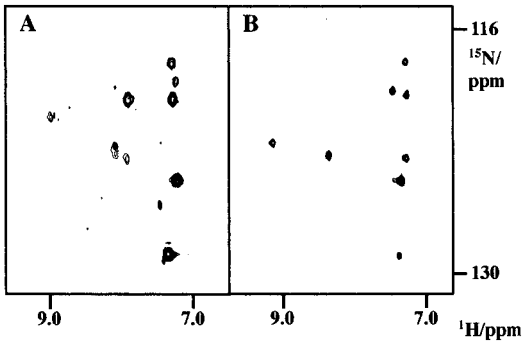

FIGURE 2: Comparison of an in-cell NMR [^{15}N,^{1}H]-HSQC spectrum of calmodulin (A) with a corresponding in vitro spectrum (B). In both cases, only lysine resonances are ^{15}N-labeled. The strong peak with chemical shifts of 129 ppm in the ^{15}N dimension and 7.6 ppm in the proton dimension represents a metabolic product of lysine. All other peaks are calmodulin resonances.

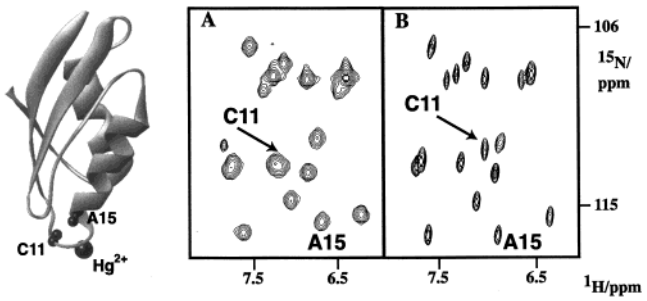

FIGURE 3: Section of an (A) in-cell and (B) in vitro [^{15}N,^{1}H]-HSQC spectrum of NmerA. Significant differences in the chemical shifts between both spectra are observed for cysteine 11 and alanine 15, which are both located in the metal-binding loop. A model of the protein based on the structure of MerP (*65*), indicating the positions of Cys 11 and Ala 15, is shown to the left of the spectra.

the resonance position of cysteine 11 moves by 0.19 ppm in the proton dimension and by 0.53 ppm in the ^{15}N-dimension. In the same spectrum, smaller differences can also be detected for alanine 15, which is part of the metal binding loop as well. Although these change are small, they indicate differences in the magnetic environment of residues in the metal binding loop between the in vitro and the in vivo states of the protein. This interpretation is further supported by preliminary relaxation experiments on purified and in-cell samples that show differences in the dynamic behavior of this metal binding loop. MerA is a bacterial protein from the TN501 strain, which is highly homologous to the endogenous BL21 *E. coli* protein. In response to mercurials, expression of MerA is elevated and can reach levels of up to 6% of the entire soluble bacterial protein (*22*). While the overexpression level of NmerA in our in-cell NMR experiments is roughly 3 times higher, the naturally high expression level observed in wild-type bacteria enables our experiments to mimic the normal environment of the protein closely. Therefore, we consider these differences in the chemical shifts and dynamic behavior of the protein biologically relevant. Experiments to further characterize these differences and to determine their cause are currently being carried out.

Both examples show that binding of metal ions can change the conformation or the dynamics of these proteins. Conformational changes can also be triggered by other mechanisms. For instance, posttranslational modifications, and, in particular, phosphorylation-induced conformational changes, are at the heart of activation processes in many signaling cascades in eukaryotic cells in some bacterial signaling events. In vitro investigations of conformational changes caused by phosphorylation are often hindered by ignorance of the exact kinase(s) involved. Thus, to study conformational switches and concurrent behavioral changes by traditional structural approaches, one must first know the sites of phosphorylation, the kinase(s) responsible, and the physiological effects induced. The protein must be purified and treated with the appropriate purified kinases in the hopes of reconstituting a biologically relevant conformational state. The difficulties entailed are reflected by the dearth of structural investigations of phosphorylation-induced changes, despite their obvious biological importance. Investigations of conformational changes by in-cell NMR experiments do not suffer from these same problems because, in the protein's natural environment, all factors that control its activity, including kinases, are present even if their identity is not exactly known. One need know only which signaling pathway has to be activated in order to phosphorylate the protein of interest. One example for which in-cell NMR spectroscopy could provide important information is the regulatory domain of the nuclear factor of activated T-cells (NFAT) (*23*−*26*). In resting T-cells, this protein resides inside the cytoplasm in a highly phosphorylated form. Activation of NFAT requires its dephosphorylation. One model says the phosphorylation masks the basic amino acids in the nuclear localization signals (*23*). This mechanism predicts that the phosphorylated form contains structured parts whereas the unphosphorylated form is unfolded (*26*). The direct observation of NFAT in the cytoplasm of suitable host cells by in-cell NMR spectroscopy could prove or disprove this model.

An additional subject for in-cell NMR studies are multidomain proteins. While the in vitro determined structure of a single domain (without posttranslational modification) will in most cases not significantly differ from its structure inside the cell, the relative orientation of individual domains of a multidomain protein can show strong differences, especially when one domain regulates the activity of another through an intramolecular mechanism. A striking example in which such an intramolecular regulatory mechanism may play an important role is the tumor suppressor protein p53 (*27*−*29*). This protein, which consists of an N-terminal transactivation domain, a central DNA-binding domain, an oligomerization domain, and a short C-terminal regulatory domain, exists inside cells in an inactive, latent form. Different models for the activation of p53 are proposed, one of which is based on an intramolecular interaction between the C-terminal regulatory domain and, most likely, the DNA-binding domain. While an array of results supports this conformational switch model, a very recent in vitro NMR investigation contradicts it (*30*, *31*). In-cell NMR experiments of p53 in its natural environment could help resolve this controversy.

Furthermore, given that domains within a protein can tumble independently of one another, it may be possible to observe changes induced in the inter- or extracellular domains of cell surface receptors. In other words, while the tumbling rate of any transmembrane domain will surely be too slow for study by liquid-state NMR, domains tethered to these transmembrane regions play an important role in signaling and may be measurable due to their discrete mobility.

Potential applications of in-cell NMR spectroscopy are not limited to intramolecular binding events. Binding of any molecule will change the chemical and magnetic environment of particular residues and can, therefore, be detected by changes in chemical shifts. While binding to macromolecules will increase the mass of the molecule under investigation and can cause certain problems (see below), in-cell NMR experiments could become a useful tool for drug discovery projects. Due to the sensitivity of the chemical shift to binding small organic molecules, conventional in vitro NMR experiments are already a standard tool for screening potential drug candidates for binding to macromolecular targets in the pharmaceutical industry (*32*, *33*). In-cell NMR combines the screening power of conventional NMR experiments with the added capability to select from a pool of potential drugs only those molecules not only that bind to a certain macromolecule but also that can penetrate the cellular membrane and reach its target inside a cell. Therefore, in-

cell NMR spectroscopy could be used to combine two screening steps in one and make searching for biologically active drugs more efficient.

The new field of in-cell NMR spectroscopy is just starting to emerge. Some of the applications described above are already reality while for others further techniques have to be developed. To provide the reader with an overview of the current state of the technique, the technical limitations, and the potential future solutions, the following sections focus on the technical aspects of in-cell NMR and summarize the results that have so far been reported by two different research groups.

Technical Aspects of In-Cell NMR Spectroscopy. Observation of a specific protein species inside living cells by NMR spectroscopy has to overcome two main problems: First, a method must be used to distinguish the NMR signals of the protein species of interest from the NMR resonances of all other proteins, nucleic acids, and other cellular components. Second, the protein must tumble freely inside the cell, and its rotational correlation time must be within a range that allows the observation of narrow resonance lines.

I. Selective Observation of a Protein Inside Living Cells. Identification of the NMR resonances of a specific macromolecule species among the resonance lines of all cellular components relies on labeling of the macromolecule with NMR-active isotopes of low natural abundance. Both nonradioactive ^{15}N and ^{13}C isotopes can be used for this labeling purpose since their percentage of all nitrogen and carbon isotopes is 0.2% and 1.1%, respectively. In addition, ^{19}F can be employed for labeling purposes (*9*). Although ^{19}F is the only naturally occurring fluorine isotope, its low abundance in living organisms creates a virtually zero background. However, labeling with fluorine requires chemical modification of the building blocks of biological macromolecules by replacing a hydrogen atom with a fluorine atom, thus creating unnatural systems with potentially different behavior.

In contrast to classical in vivo NMR experiments, biological macromolecules cannot, in most cases, be labeled externally and then injected into the cell of interest (for exceptions, see below). Instead, the protein has to be produced and isotopically labeled simultaneously inside the cell. Techniques for overexpressing proteins and labeling them with NMR-active isotopes in bacteria have been well established for many years, and labeling protocols and special media are also available for other organisms, including yeast and insect cells. Originally we thought using the same cells for protein overexpression and subsequent NMR experiments would lead to a huge background signal caused by labeling not only the protein of interest with the NMR-active isotope, but also virtually every cellular component. To avoid these potential pitfalls, we designed experimental schemes to reduce the amount of background labeling. Rifampicin is a drug that inhibits the bacterial RNA polymerase but does not block the RNA polymerase of the bacteriophage T7 (*34*−*36*). Since our proteins of interest are under the control of a T7 promoter, we could use rifampicin to suppress production of all endogenous bacterial proteins. Adding the drug to the media some time after IPTG induction, thereby allowing the cell to produce sufficient T7 RNA polymerase, encourages near-exclusive production of the exogenous protein. To avoid labeling of cellular components during the growth phase prior to induction, we first grew the bacteria in unlabeled LB medium to the desired optical density, harvested them by centrifugation, and resuspended them in medium containing the NMR-active isotopes. NMR spectra measured by placing these bacteria in a normal 5 mm NMR tube and into an NMR instrument showed no sign of background signals from endogenous bacterial macromolecules, but contained a number of strong resonance lines with proton chemical shifts in the 8.0−8.5 ppm range (*10, 11*). The fact that these resonance lines had a much narrower line width than the peaks of the overexpressed protein suggested that they were caused by incorporation of ^{15}N into small molecules, e.g., amino acids. The same resonances were present even when no protein overexpression was induced. Surprisingly, however, in control experiments neither the inclusion of rifampicin nor the switching of the media had a substantial influence on the amount of background signals (*11*). Protein overexpression without rifampicin and using the same labeled medium for the bacterial growth and protein overexpression phases resulted in virtually the same spectra, consisting of sharp resonance lines presumably from small molecules. Although surprising, this result is consistent with other studies that have revealed that only resonances of the overexpressed protein are visible in crude cell lysates despite the presence of all bacterial macromolecules (*37*−*39*). Moreover, the absence of detectable signals from endogenous bacterial macromolecules in the in-cell NMR experiments is not a consequence of the overexpression. NMR samples of bacteria which had not been induced showed the same absence of signals from bacterial macromolecules.

These results suggest that instead of background signals, the overexpression level of the protein is the most important parameter for the spectral quality of in-cell NMR experiments. We did obtain a noticeable improvement of the signal intensity in the spectra when we harvested the bacteria after growing them to the desired optical density and resuspended them in fresh medium followed by induction with IPTG (*11*). The sensitivity of the experiments could be further increased 2−3-fold if the medium used for overexpression was labeled full medium (e.g., algae extract) instead of labeled minimal medium. Based on our results, we have adopted a protocol that calls for growing the bacteria first in unlabeled LB medium and after harvesting them by centrifugation resuspending them in fresh labeled medium (Figure 4). We use unlabeled LB medium during the growth phase not to suppress potential background signals, but for cost-effectiveness, to keep the initial growth period short, and to improve the protein yield.

A similar labeling protocol was used by the laboratory of Guy Lippens in their in-cell NMR investigations of the cyclic osmoregulated periplasmic glucan of *Ralstonia solanacearum* (*13*). For production of the sample, they grew the T11 strain of *Ralstonia solanacearum* first in unlabeled LB medium and then harvested them and resuspended them in labeled medium. While the results published so far indicate that the exact labeling protocol is not a crucial parameter for in-cell NMR with highly expressing bacteria, different cell types might require more sophisticated procedures.

II. The Rotational Correlation Time of Proteins Inside Cells. Another crucial parameter that influences the success of in-cell NMR experiments is the rotational correlation time or tumbling rate of the macromolecule in the cellular environment. The tumbling rate of a molecule is a key

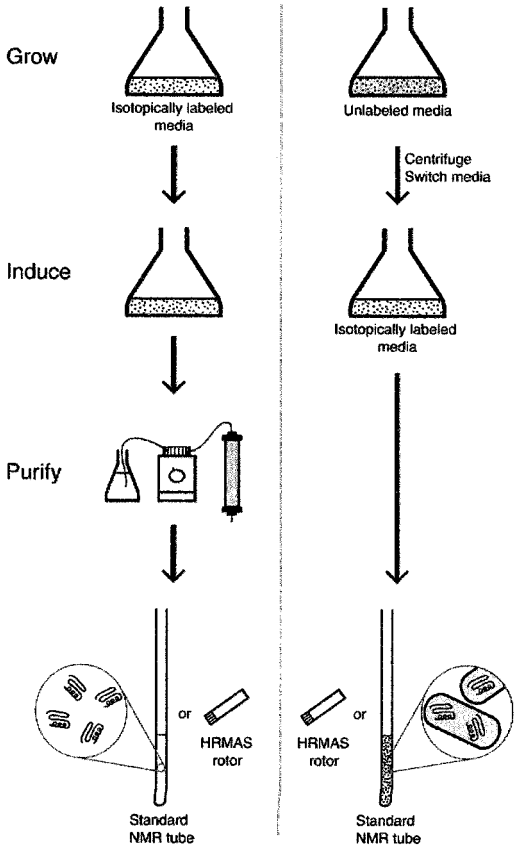

FIGURE 4: Schematic representation of the sample preparation procedure (left) for conventional in vitro NMR spectroscopy with purified protein and (right) for in-cell NMR experiments. For conventional in vitro NMR spectroscopy, cells are grown and induced in isotopically labeled media. The protein is then purified and placed in an NMR tube or HRMAS rotor. For in-cell NMR, the cells are first grown in unlabeled media and then transferred to isotopically labeled media prior to induction. Intact cells harboring the overexpressed protein are then prepared as a slurry for NMR studies.

parameter that strongly influences the sensitivity of NMR experiments since slower tumbling of a molecule causes broadening of the resonance lines which can lead to their complete disappearance. Since the rotational tumbling rate is a function of the viscosity of the medium the macromolecule is dissolved in, the cellular viscosity becomes a crucial parameter for in-cell NMR experiments. So far several techniques have been used to study the intracellular viscosity of different cell types. In yeast cells, an intracellular viscosity twice that of water was observed through measurements of ^{19}F relaxation times of selectively tryptophan-fluorinated enzymes (*9*). In fibroblasts, viscosities as low as 1.2−1.4 times that of water determined by fluorescence polarization experiments (*40*−*44*) were reported, and other measurements in higher cells using ^{13}C relaxation (*45*), ^{1}H line width (*46*), and electron spin resonance (*47*) also showed an upper limit of the intracellular viscosity for most cell types of twice that of water. Due to the linear relationship between the viscosity, the rotational correlation time, and the molecular mass of a protein, this 2-fold increase in the viscosity leads to a 2-fold increase in the apparent molecular mass of a macromolecule. The recent introduction of TROSY (*48*) and similar techniques (*49*) into the field of high-resolution NMR spectroscopy has significantly increased the molecular weight range of proteins that are amenable to NMR spectroscopy. In particular, the [^{15}N,^{1}H]-HSQC experiment, which is arguably the most important NMR technique for in-cell NMR studies, can in its TROSY version be applied to macromolecules beyond the 100 kDa mark. The combination of TROSY and the relatively low viscosity of the cellular medium suggest that for virtually all proteins investigated so far by traditional NMR methods the intracellular viscosity will not be a limiting factor for in-cell NMR experiments.

The situation changes, however, if interaction between the macromolecule of interest and cellular components occurs. In particular, binding to large cellular components such as membranes or DNA increases the rotational correlation time, causing line broadening and potential loss of signals. As an example, we were unable to observe in-cell NMR signals from an unfolded domain that was highly overexpressed in bacteria, but was, however, also bound by cellular chaperones.

III. NMR Techniques. Two different classes of NMR methods have so far been used in in-cell NMR experiments: traditional high-resolution liquid state (*10, 11*) and high-resolution magic angle spinning techniques (*13*). Magic angle spinning is a technique that was originally developed for solid-state NMR applications. In these experiments, the sample is contained in a small rotor and tilted inside the NMR magnet by an angle of 54.7° relative to the *z*-axis of the magnet (the magic angle) and spun fast (kilohertz range) around the long axis of the rotor. Recently, this technique has been adopted for work in high-resolution liquid applications where it has been successfully used for solid-phase organic chemistry, peptide synthesis, and in vivo NMR experiments with tissue samples (*50*−*54*). In these heterogeneous systems, magic angle spinning eliminates the effects of magnetic susceptibility gradients that are generated at the interfaces of regions with different magnetic properties such as water and cells. By spinning a sample of living *R. solanacearum* at rates of 6 kHz, the research group of Guy Lippens was able to obtain high-resolution [^{13}C,^{1}H]-HSQC spectra of a periplasmic glucan (*13*).

In our laboratory, we have used a different strategy. To prepare a sample for in-cell NMR experiments, we produce a 20−30% bacterial slurry by gentle centrifugation and transfer it into a standard 5 mm NMR tube (*11*). Although the rotational correlation times of the cellular proteins do not depend on the macroscopic viscosity of the entire sample and a denser packing of cells should in principle be feasible, the quality of the NMR spectra of tightly packed cells decreases. We attribute this result to nonuniform cell distributions in the more densely packed samples that increase the inhomogeneity of the entire sample and contribute to line broadening. Similar results have been observed by the laboratory of Guy Lippens (*13*). However, higher cell densities in liquid-state in-cell NMR experiments might be feasible if more uniform and homogeneous samples could be prepared. Experiments with different media and sample preparation protocols are currently being tested.

IV. Future Directions. The in-cell NMR experiments that have been published so far differ in many experimental aspects. While the Lippens laboratory has characterized the behavior of glucans in the periplasm of the bacterial strain *Ralstonia solanacearum* with magic angle spinning experiments, our laboratory has focused on the proteins NmerA and calmodulin in the cytoplasm of common *E. coli* BL21

Table 1: Comparison of the Published In-Cell NMR Investigations, Using either Magic Angle Spinning or Liquid-State NMR Techniques

	high-resolution magic angle spinning NMR	high-resolution liquid-state NMR
host organism	*R. solanacearum*	*E. coli* BL21
biological macromolecule	cyclic osmoregulated periplasmic glucan	proteins: NmerA, calmodulin
cellular compartment	periplasm	cytoplasm
heteronucleus	^{13}C	^{15}N
cell density	~80%	20−30%

cells by standard liquid NMR methods (Table 1). Clearly, different bacterial strains can be used as host cells for in-cell NMR experiments, and it also shows that the behavior of biological macromolecules can be studied both in the cytoplasm as well as in the periplasm. Since bacteria are easy to handle, grow fast, and can express exogenous proteins and other biological macromolecules to high levels, they will play an important role in future in-cell NMR applications. The technical advantage of bacterial host systems could further increase if thermophiles would be used as host systems. Their higher temperature tolerance would allow an increase in the temperature during the actual NMR measurements which would result in faster rotational tumbling rates of proteins and concomitant sharper resonance lines.

While bacterial strains are, from a technical standpoint, the most attractive host system, from a biological standpoint, a eukaryotic model system would be more exciting. Several different eukaryotic cell types have already been used to overexpress exogenous proteins for structural studies. Although we are just starting to obtain experience with these nonbacterial cells, the key factors that will determine the suitability of a certain cell type as a host for in-cell NMR experiments can be deduced from our experience with the bacterial systems. With the current sensitivity of NMR instrumentation, the most critical parameter will be the overexpression level of a certain protein. High overexpression levels of up to 30% of total protein have been obtained in yeast, in particular in *Pichia pastoris* (*55*, *56*). In addition, the insect cell/baculovirus system can produce high overexpression rates of up to 25−50% of total cellular protein (*57*). Fortunately, for both cell types, media that can be used to label overexpressed proteins with NMR-active isotopes are commercially available. One potential advantage of a yeast system is that they grow naturally in much higher densities than bacteria. The 20−30% slurry that we currently use in our in-cell NMR experiments will, therefore, resemble more closely a natural environment for yeast than for bacteria.

Another potentially interesting biological host system is *Xenopus* oocytes. The huge dimensions of these cells make them attractive for injecting purified proteins that were expressed and labeled with the desired NMR-active isotopes in another recombinant system. Our preliminary experiments with this model system have revealed that the biggest problem is the inhomogeneity of the sample, causing the resonance lines to become much broader than in the bacterial samples.

The final goal of in-cell NMR experiments is to observe the behavior of biological macromolecules inside their natural environment. The need to overexpress a macromolecule sometimes far beyond its natural concentration, however, creates an artificial situation. In addition, the tight packing of the cells in the NMR tube or the magic angle spinning rotor leads to oxygen starvation which influences the metabolism of the cells. These conditions can affect the behavior of the macromolecule inside the cells. Potential problems that could be caused by these conditions have to be carefully considered for each individual experiment. In the case of NmerA, for example, its naturally occurring high expression level means that our in-cell NMR experiments deviate only slightly from its true environment.

Technical advances, some of which already exist, can improve both the external environment as well as the intracellular one and make them resemble more closely the natural surrounding of a given macromolecule. The external parameters such as oxygen and nutrient levels can, for example, be controlled if, instead of regular 5 mm NMR tubes, flow cells and bioreactors are used (*4*, *58*, *59*). These types of NMR tubes were designed for classical in vivo NMR studies in which different cell types including higher eukaryotic cells are kept alive and constantly supplied with fresh medium for extended periods of time.

The sensitivity of NMR instruments has dramatically increased over the last 10 years, and this process has even been accelerated since the introduction of the cryoprobes 3 years ago. These cryoprobes achieve a more than 3 times higher sensitivity than regular room-temperature probes (*60*−*63*). Consequently, the concentration of the investigated macromolecule can be reduced by a factor of 3, or, if the same sensitivity as with a standard room-temperature probe should be achieved, the measurement time can be reduced by a factor of 9 (*60*). From our experiments, we have estimated that the detection limit of the protein NmerA on our 500 MHz NMR instrument, equipped with a cryoprobe, is just a few percent of the total soluble protein and that 5% will produce in-cell NMR spectra of good quality (*11*). This year's introduction of the 900 MHz instrument will, if cryoprobes are used, increase the sensitivity by another factor of 2−3 relative to our 500 MHz machine. Future improvements of the technique will undoubtly lead to even more sensitivity increases. At a detection limit of 50 μM for in vitro samples, the concentration of a macromolecule inside a cell in a 30% slurry of an in-cell NMR sample will be approximately 150 μM. Assuming that 18% of the cell consists of protein (*64*), this number corresponds to only roughly 1% of the entire cellular protein, for a 10 kDa domain. Based on the published overexpression levels of up to 30% of total cellular protein in yeast and 25−50% in insect cells, these numbers predict that the method will be tractable for cells from higher organisms, making in-cell NMR spectroscopy a practical new tool for structural cell biology.

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