Biophysical Perspective

In-Cell Solid-State NMR: An Emerging Technique for the Study of Biological Membranes

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ABSTRACT   Biological molecular processes are often studied in model systems, which simplifies their inherent complexity but may cause investigators to lose sight of the effects of the molecular environment. Information obtained in this way must therefore be validated by experiments in the cell. NMR has been used to study biological cells since the early days of its development. The first NMR structural studies of a protein inside a cell (by solution-state NMR) and of a membrane protein (by solid-state NMR) were published in 2001 and 2011, respectively. More recently, dynamic nuclear polarization, which has been used to enhance the signal in solid-state NMR, has also been applied to the study of frozen cells. Much progress has been made in the past 5 years, and in this review we take stock of this new technique, which is particularly appropriate for the study of biological membranes.

NMR is a noninvasive technique that can tackle individual molecules as well as whole animals. For NMR, three types of samples can qualify as in cell: living cells (in vivo NMR), intact cells, and intact cellular compartments such as organelles and cellular envelopes (in situ NMR). Biological cells were studied by NMR as early as 1955 (1), and 15 years later the focus shifted from looking at water molecules to examining lipids (2) and small metabolites (3). In the 2000s, larger soluble proteins within bacterial cells were studied by NMR (4), and in-cell NMR became a field in and of itself, with investigators identifying common obstacles and finding ways to circumvent them. In particular, a meaningful in-cell experiment requires a stable environment to avoid the degradation (e.g., molecular oxidation, hydrolysis, or proteolysis by proteases) that can occur in such samples in a matter of hours or days. Several approaches have been suggested to prolong sample survival (5,6), but the state of the sample should be regularly checked independently by biochemical methods, such as electrophoresis or activity assays. The first decade of in-cell solution-state NMR was reviewed in 2011 (7).

The weak signal/noise ratio provided by molecules in cells is due not only to their low abundance but also to environmental heterogeneity and the slow molecular tumbling of molecules that are either too large or are interacting with other molecules. It has been shown that the rotational correlation time (the time it takes for a molecule to rotate one radian) of proteins inside a cell is longer because of increased viscosity, but in most cases it is increased by only a factor of 2 (8), which should not be a major obstacle for solution-state NMR. Nevertheless, the molecular rotational correlation time can become prohibitively long for specific cellular or membrane interactions, in which case solid-state NMR and magic-angle spinning (MAS) may be the best option (9). In addition, MAS also averages out sample heterogeneity and magnetic susceptibility anisotropies (10), thereby improving the spectral resolution.

Solid-state NMR has been applied to a variety of biomolecules that are naturally solid, ranging from human biopsies (10) to amyloid fibrils (11), mussel byssus (12), whole nematodes (13), and whole living flies (14), but it has also proved useful for studying membrane-bound molecules, which become solid because of the boundary imposed by the membrane. Such molecules include lipids, membrane proteins, and components of the cell wall and extracellular matrix that can also be studied separately or reconstituted into model systems (15). In 2011, solid-state NMR joined solution-state NMR in the quest to determine the structure of proteins within cells (16). The concomitant development of dynamic nuclear polarization (DNP) for increased sensitivity (17) has made solid-state NMR an important player in this field, and over the past 5 years investigators have made much progress in optimizing sample preparation protocols and NMR and DNP conditions, and gathering important preliminary data.

In this review, we assess the state of the art of in-cell solid-state NMR and discuss the achievements that have been made in studying cell-envelope molecules such as lipids, membrane proteins, and lipopolysaccharides, as well as cell wall and biofilm extracellular matrix components, within their natural environment.

Sample preparation

As indicated above, in-cell NMR suffers from a major limitation: the short period of time before the sample irreversibly degrades. With some rare exceptions, in-cell solid-state NMR is often performed at low temperature to preserve the integrity of the membrane, and in certain cases, cellular samples are even frozen or lyophilized, which makes it impossible to probe dynamic phenomena. Although it is debatable whether results obtained in this way qualify as in cell, the important factor is that the studied molecules are never taken out of their native environment. Sample spinning has also been criticized as being too violent for cells because of its centrifugal and dehydrating effects, but spinning rates and times can be adjusted to preserve cellular integrity and even cell survival (14,18–20).

In addition, in-cell NMR faces the same obstacles as spectroscopy in general: sensitivity and resolution. Although in-cell solid-state NMR has benefited from recent developments such as MAS for high-resolution solid-state NMR and DNP for signal enhancement, the application of in-cell NMR to proteins also relies on one's ability to overexpress these biomolecules to ensure that they are 1) concentrated enough to be rapidly visible by NMR (on the order of 100 μM) and 2) more abundant than other molecules that may obscure them. Protein overexpression in bacteria has been improved throughout the years and is now very common, although it remains a challenge in the case of eukaryotic protein expression (21).

One can also achieve increased sensitivity to reduce the experimental time and improve the cell survival rate by using isotopic labeling. It is now quite simple to achieve uniform isotopic labeling, using mostly ammonium chloride for nitrogen labeling and glucose, glycerol, or sodium carbonate for carbon labeling. Carbon background labeling is often detrimental, and specific labeling for carbon may be preferable. For proteins, this can be done by using other labeled precursors (e.g., amino acids or ketoacids to label only the methyl groups), which reduces the background but presents other problems, such as metabolic scrambling. Investigators in this area of research are actively searching for alternatives, and even more specific labeling can be achieved by using auxotrophic bacterial strains that force bacteria to use the incorporated labeled molecule for a specific metabolic pathway (22). Although labeling is often unnecessary for lipids, researchers have used deuterated fatty acids to label lipids and observe them inside bacterial cell membranes (23).

Lipids and lipopolysaccharides

Pioneering work in solid-state NMR, before the advent of MAS, relied on low-resolution NMR spectra for protons and carbons, and wide-line NMR for phosphorous and deuterium (24). Lipids constitute approximately half of the membrane weight, and phospholipids are abundant in most membrane cells (except for some plants), making ^{31}P, an abundant and sensitive isotope, a nucleus of choice for in-cell NMR. In addition, rather than simply assigning signals, wide-line ^{31}P NMR provides structural and dynamic information about the lipid headgroups, such as phase changes, polymorphism, and changes in the order parameters. Therefore, ^{31}P NMR has been used to study a variety of cells ranging from rabbit muscle cells (25) to bacteria (26,27). Surprisingly, although MAS also improves the resolution in ^{31}P NMR and theoretically allows the separation of lipids according to their headgroups, ^{31}P MAS NMR has not been applied very often to cell membrane analysis (28,29).

As opposed to ^{31}P, ^{2}H is a very rare isotope that must be incorporated into the sample to be visible by NMR. Its natural background, on the other hand, is almost null. Throughout the 1970s, wide-line ^{2}H NMR was applied to various cell membrane lipids, starting with bacteria (30,31), and provided structural and dynamic information about lipid acyl chains by detecting changes in phases, order parameters, spectral moments, and membrane fluidity or curvature that complemented information obtained by ^{31}P NMR. This approach was still used in recent studies to probe whole sea urchin sperm cells (32) and the effect of antimicrobial agents on bacterial cell membranes (23,33). However, in 2015, in-cell ^{2}H MAS NMR was used to probe bacterial cell membrane lipids and was found to provide the same information as static NMR but within a shorter timeframe (20), making it compatible with cell survival.

The first attempt to observe whole red blood cells by ^{1}H NMR dates back 60 years ago (1), but 15 years later, the focus shifted toward cell membrane lipids that could be assigned by either proton (2) or carbon (34) NMR. ^{1}H and ^{13}C NMR were used to assign many cell lipids throughout the subsequent decades, and the resolution was greatly improved in the 1990s with the generalization of MAS, allowing solid-state NMR to become a diagnostic tool for the detection of diseases (10,35). Lipopolysaccharides (LPSs) were also scrutinized by ^{1}H MAS NMR directly on the surface of living bacterial cell membranes (36), which sometimes allowed their acetylation state to be determined in situ (19). More recently, we studied whole microalgae by dynamically filtered ^{13}C MAS NMR (37). Using polarization transfer schemes that are sensitive to molecular dynamics, such as refocused-insensitive nuclei enhanced by polarization transfer (RINEPT), which depends on scalar couplings, and cross-polarization (CP), which depends on dipolar couplings that can be averaged in the presence of motion, we were able to select mobile or rigid molecules, respectively. This strategy allowed us to identify the rigid cell-wall carbohydrates and membrane proteins, as well as the more mobile cell membrane lipids (see Fig. 1).
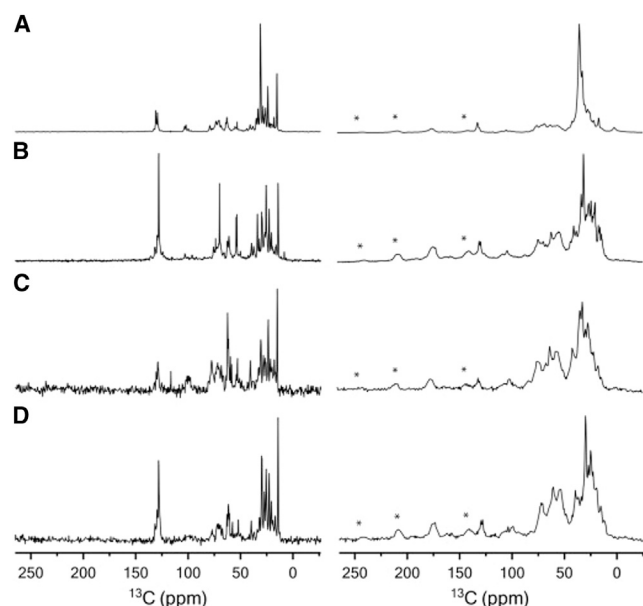
A

B

C

D

FIGURE 1 (*A–D*) Dynamically filtered ^{13}C NMR spectra of (*A*) *Nannochloropsis oculata*, (*B*) *Pavlova lutheri*, (*C*) wild-type, and (*D*) cell-wall-depleted *Chlamydomonas reinhardtii*. Dynamic and rigid molecules are detected by RINEPT (*left*) and CP (*right*), respectively. Spinning side bands are indicated by asterisks. Reprinted from (37) with permission from Elsevier.

Cell wall and extracellular matrix

In addition to membranes, many algae, fungi, and plant cells possess a cell wall composed of polysaccharides and (in bacteria) polysaccharides and peptidoglycan. As early as 1979 (38), the peptidoglycan of *Staphylococcus aureus* in intact cells was studied by ^{15}N and ^{13}C NMR, which can be used to assign resonances and measure relaxation times, and thus probe chain motion and packing. In the 1980s, the development of MAS, CP-MAS, and high-resolution solid-state NMR shifted the focus from lipids toward more rigid molecules such as polysaccharides and membrane proteins, and investigators sought to determine the atomic structures of these complex molecules. The advent of MAS and CP-MAS allowed a much more precise characterization of the molecular structure and metabolism of bacterial cell walls.

The groups of Jake Schaefer and Lynette Cegelski examined lyophilized isolated cell walls, and sometimes intact whole cells, by ^{15}N, ^{13}C, ^{19}F, and ^{31}P MAS NMR, measuring accurate distances within the peptidoglycan and between the peptidoglycan and antibiotic molecules (39–44). This strategy was recently extended to composition analyses of freeze-dried, isolated extracellular matrix from bacterial biofilms of *E. coli* (45) and *Vibrio cholerae* (46) by ^{15}N, ^{13}C, and ^{31}P MAS NMR.

Various isolated (but hydrated) cell walls have also been probed by ^{13}C MAS NMR and DNP. The group of Jean-Pierre Simorre compared the organization of peptidoglycan in various bacteria, including its metal-binding sites and constants, cross-linkage, flexibility, and dynamics (18,47). More recently, the same group (48) used ^{15}N, ^{13}C, and ^{31}P to determine the high-resolution structure of a complex made up of the *Bacillus subtilis* peptidoglycan and the L,D-transpeptidase protein (see Fig. 2). At the same time, the group of Mei Hong studied the organization of polysaccharides in the cell wall of the plant *Arabidopsis thaliana*, including interactions, dynamics, and hydration (49,50), with the help of solid-state ^{1}H and ^{13}C NMR techniques. With the additional benefit of DNP and an enhancement factor of 30 at 600 MHz, they were able to determine that the functional binding target of the protein expansin in the *A. thaliana* cell wall is a cellulose that is enriched in hemicellulose xyloglucan and has a different structure compared with bulk cellulose (51).

Membrane proteins

The first in-cell NMR study of a membrane protein is a special case, since *Halobacterium salinarium* is an archaeon in which the sole membrane protein, bacteriorhodopsin (bR), occupies nearly half of the purple membrane surface and is organized as a regular, homogeneous, and rigid 2D hexagonal lattice. In 1983, the group of Bob Griffin began an extensive study of bR by using MAS NMR to determine the bR retinal protonation state (52). The quest for intermediate structural states during the bR photocycle continues today, and ^{15}N and ^{13}C solid-state NMR, as well as DNP, have recently allowed investigators to disentangle several
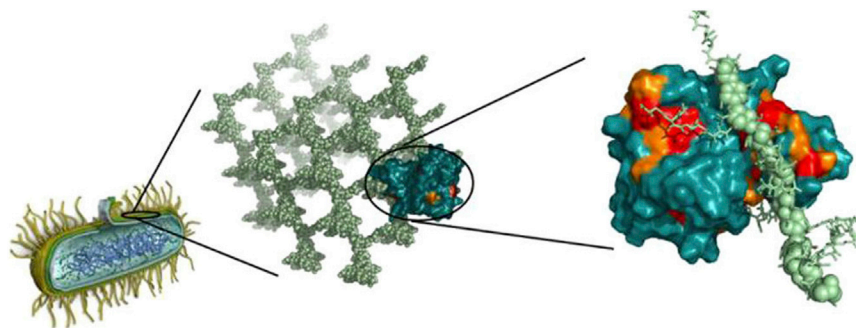
FIGURE 2 Cartoon showing the interactions between the highly cross-linked peptidoglycan of *B. subtilis* and the L,D-transpeptidase that performs this cross-linking. Reprinted with permission from Schanda et al. (48). Copyright 2014 American Chemical Society. To see this figure in color, go online.

coexisting states, including some that had never been observed previously, thanks to an enhancement factor of up to 90 at 380 MHz (17).

In 2011, Riqiang Fu and collaborators (16) published the first in-cell solid-state structural NMR study of a single membrane protein, the small transmembrane domain of the human lipoprotein receptor 11 (LR11) embedded in the purified *Escherichia coli* membrane where it was expressed. Several labeling schemes were tested (e.g., uniform labeling versus specific labeling of the alanines) and many parameters were optimized to reduce the intensity of lipid signals versus protein signals. Purified membrane signals were also compared with signals of the same protein reconstituted in detergent micelles.

The following year, in-cell ^{13}C and ^{15}N MAS solid-state NMR was applied to observe two small soluble proteins in *E. coli*, FKBP and Trx, which are invisible by solution-state NMR because interactions with other molecules slow their tumbling prohibitively (9). Using a similar approach, the group of Marc Baldus tackled the structure of the bacterial membrane protein lipid A deacylase of *Pseudomonas aeruginosa* (PagL) embedded in the *E. coli* outer membrane where it was expressed (53). Protein expression and labeling schemes were improved, and whole cells were compared with purified membranes and reconstituted proteoliposomes (see Fig. 3), allowing the assignment of PagL signals. Other signals were identified from LPS and peptidoglycan, as well as from another membrane protein, the endogenous Braun lipoprotein. Sample stability was also assessed, and it was found that whole cells were only stable for 36 h, whereas purified membranes were stable for about a week at low temperature. The same sample was subjected to DNP at low temperature, with an enhancement factor of 30 at 400 MHz, allowing faster data acquisition (and hence preservation of membrane integrity) as well as the detection of previously unseen low-abundance molecules such as nucleic acids (54).

High-field, solid-state DNP spectrometers have only been commercially available since 2009, and the first applications to cellular samples were mostly proofs of concept showing that despite the current resolution limitations at low temperature and the concomitant freezing of dynamics, the benefit of signal/noise ratio enhancement (on the order of 10–100, with an average of 30) allowed signals to be observed from very-low-concentration molecules. For example, in 2011, the group of Hartmut Oschkinat was able to detect by in-cell DNP the protein neurotoxin II bound to its naturally low-concentration receptor, the acetylcholine receptor in the synaptic membrane of the Pacific electric ray (55). In 2012, the same group was able to compare the structure of the membrane-associated protein mistic, determined by DNP directly in isolated native *E. coli* membranes, with a structure previously determined in detergent micelles (56). In a similar approach, the group of Ayyalusamy Ramamoorthy demonstrated the feasibility of using DNP on the membrane-anchored cytochrome b5, which contains a large soluble domain, in native *E. coli* cells, and compared the results with solid-state NMR data obtained in other membrane mimetics (57).

So far, in-cell solid-state MAS NMR and DNP have mostly been used to validate structures that were previously determined in model systems. For example, various structures of the M2 proton channel of the influenza A virus had been suggested from experiments performed in liquid crystalline lipid bilayers, detergent micelles, and detergent-based crystals (58). Solid-state NMR performed in native *E. coli* membranes could validate the tetrameric structure that had been proposed based on the results of solid-state NMR performed in synthetic bilayers (59). The diacylglycerol kinase structure had also been determined in detergent micelles, and Shi et al. (60) compared it with data obtained from the same protein in *E. coli* membranes, using modified amino acids incorporating ^{19}F labels. In the same line of thought, the structure of *Anabaena* sensory rhodopsin, which was previously determined in lipid bilayers, was validated against a structure determined in *E. coli* inner membranes. It was found that *Anabaena* sensory rhodopsin organized into trimers in both environments,
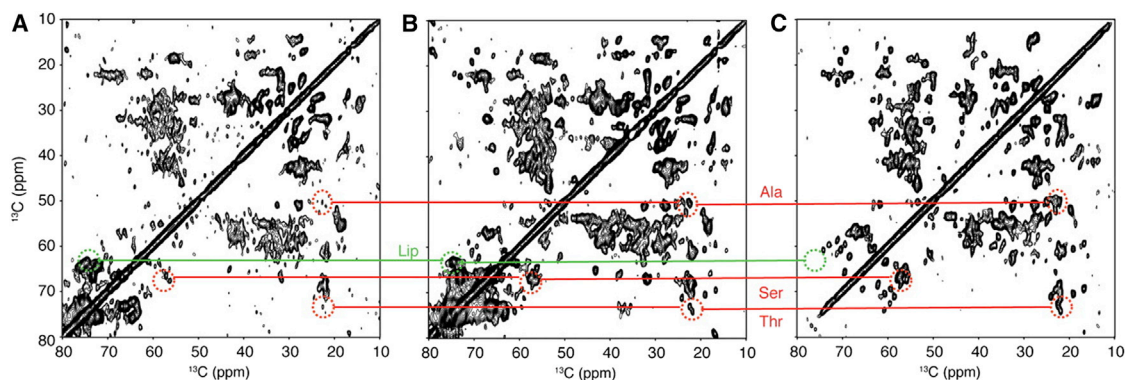

FIGURE 3 (*A–C*) ^{13}C-^{13}C NMR spectra of whole cells (*A*), cell envelopes (*B*), and (U-^{13}C,^{15}N)-labeled PagL-containing proteoliposomes (*C*). Characteristic crosspeaks of Ala, Ser, and Thr residues of PagL, and endogenous *E. coli* lipids (Lip) are indicated. Reprinted with permission from (53). To see this figure in color, go online.

but formed different crystal lattices (61). Baldus et al. (62) recently used in-cell solid-state NMR and DNP data to validate a proposed model structure, without relying on any previously obtained NMR data. Applied to the megadalton bacterial type IV secretion system core complex (T4SScc), their approach showed that T4SScc was well folded in its *E. coli* membranes.

CONCLUSIONS

Investigators in structural biology are making a significant step forward by tackling structures of molecules in their native environment, and solid-state NMR can contribute to this quest to determine the structures of cell-envelope-associated molecules. Although NMR has been used to study lipids in cellular membranes since 1967, progress in NMR techniques is now allowing researchers to gather much more precise information compatible with medical studies. Technical improvements in solid-state NMR have enabled us to focus on relatively mobile cellular molecules, such as lipids, as well as on much more rigid molecules, such as membrane proteins and cell wall and extracellular matrix components that were previously not accessible by any other structure-determination technique.

It is in the field of membrane protein structure determination that in-cell solid-state NMR has made the most significant leap in the past 5 years. Although most of the membrane proteins studied by in-cell solid-state NMR and DNP have already been studied in model systems, thanks to advances in this field, investigators have been able to optimize sample preparation, protein overexpression, isotopic labeling, NMR acquisition parameters, and general study protocols. In addition, they have provided much appreciated in-cell validations of model structures, and are now starting to tackle in-cell membrane protein complexes that have never been studied by NMR before.

Fast methods that benefit from MAS and DNP, among other technical developments, allow the acquisition of full sets of data within a timeframe that is compatible with cell survival. Therefore, the next step, which seems to be in reach, will be to develop in vivo solid-state NMR for the structural study of membrane-bound biomolecules.

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