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Current Opinion in Structural Biology

Solid-state NMR of membrane proteins *in situ*

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Membrane proteins have evolved to function as part of specialized biological membranes, and their structures and activities are highly susceptible to their local environment. Detergents and lipid mimetics replicate certain aspects of biological membranes, and have been used to produce an exceptional body of structural data, but do not fully capture the complex, asymmetric properties of the native environment and can alter structure and function. Here, we review recent advances in nuclear magnetic resonance (NMR) that enable the examination of membrane protein structure and activity *in situ*, within native membranes. The development of optimized protein expression strategies, isotopic labeling schemes, powerful instrumentation and specialized pulse sequences offer new opportunities for exploring the new frontier of *in situ* structural biology. By outlining the framework for *in situ* NMR of membrane proteins from conceptualization to experiments we hope to inspire new research in this growing and important area.

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Current Opinion in Structural Biology 2025, **94:**103129

This review comes from a themed issue on VSI: Biophysical Methods (2025)

Edited by **Hoi Sung Chung** and **Francesca M. Marassi**

For a complete overview see the Issue and the Editorial

Available online xxx

https://doi.org/10.1016/j.sbi.2025.103129

Introduction

The direct analysis of proteins in their native environment has long been a goal of structural biology and a major driver of technology development in electron microscopy (EM), X-ray diffraction, electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR). While structural biology has advanced significantly by applying these techniques to purified proteins in crystalline, water-soluble, or lipid-reconstituted states, the importance of environmental effects on protein structure has been appreciated for some time. It is well established that a protein's "native conformation is determined by the totality of inter-atomic interactions and hence by the amino acid sequence, in a given environment", and that "a protein molecule makes only stable, structural sense when it exists under conditions similar to those for which it was selected — the so-called physiological state" [1]. This knowledge underpins the development of approaches that enable structural biology studies *in situ*.

NMR is exceptionally well suited for *in situ* structural studies. Since its origin, NMR has continued to grow as a technology with broad applications in physics, chemistry, biology and medicine, its versatility stemming from its ability to provide atomic-level information for molecules in heterogeneous and complex environments, including living systems. Because NMR signals are highly susceptible to the local environment of their atomic sites, they are capable of reporting on structure, dynamics and even very weak intermolecular interactions, with little or no perturbation of the natural structure. Taking advantage of these many capabilities, the growing fields of *in situ* and in cell NMR [2–5] offer new opportunities for examining protein structure, function and drug interactions in native biological contexts, including cells, intra- and extra-cellular aggregates, native membranes and sub-cellular compartments, tissues and other biomedical specimens.

In situ structural studies are particularly important for membrane proteins, which have coevolved with their lipidic membrane components for specialized inside-outside membrane functionalities [6]. While micelles, nanodiscs and liposomes replicate some aspects of the membrane environment and are widely used for structural studies, these mimetics fail to fully capture the complex anisotropic and asymmetric properties of native biological membranes and can destabilize proteins or obscure conformational states important for function [7].

One of the earliest demonstration of *in situ* NMR dates to 1977, when Lee, Inouye, and Lauterbur examined the *E. coli* lipoprotein Lpp directly in the bacterial cell envelope [8]. Exploiting the fact that Lpp is the predominant

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Given the role as Guest Editor, Francesca M. Marassi had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full responsibility for the editorial process of this article was delegated to Hoi Sung Chung.

outer membrane (OM) protein produced under His and Trp starvation because it lacks these residues entirely, they achieved selective incorporation of ^{13}C- or ^{19}F-labeled Tyr at its sole tyrosine position. The resulting spectra revealed distinct chemical shifts that reflect both the conformation and local environment of the protein. This landmark study established the feasibility of examining membrane proteins in their native setting and presciently anticipated that the method could “be extended to a number of other membrane protein systems, as well as to intact microorganisms and tissues, especially if the more sensitive NMR spectrometers now available, which accept larger samples and employ higher magnetic fields, were to be used” [8].

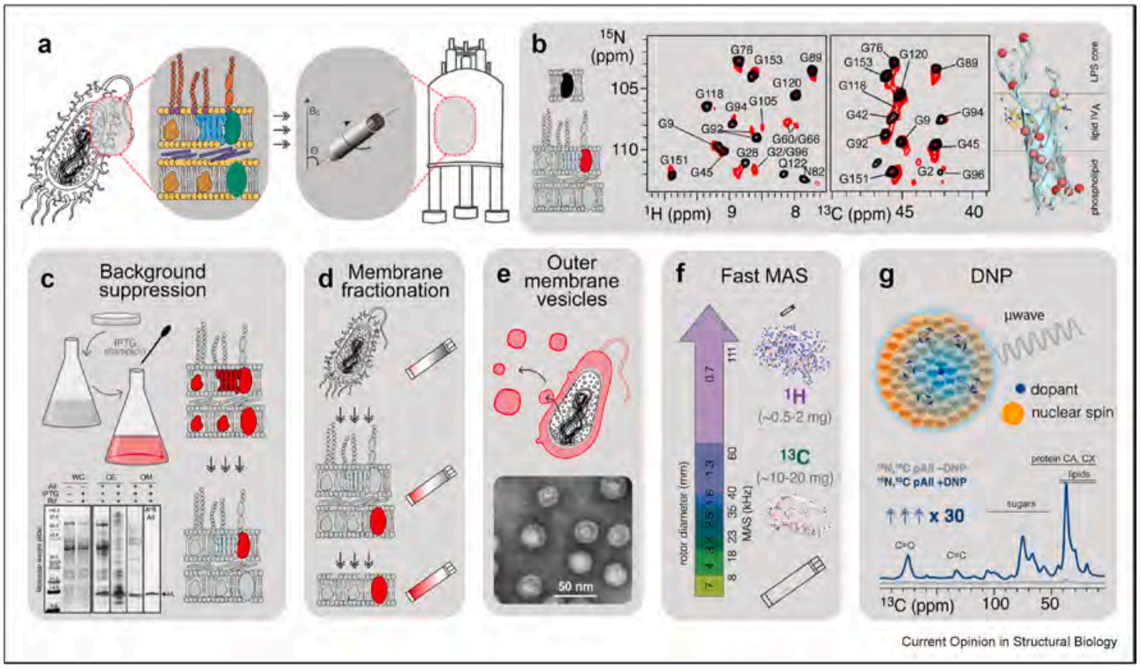
While the promise of this vision was clear, early applications of *in situ* solid-state NMR were limited by low sensitivity due to the scarcity of labeled target proteins, and by high spectral overlap from isotopically enriched endogenous background. Today, these obstacles can be effectively overcome through a combination of optimized expression strategies, isotopic labeling schemes, and powerful instrumental and methodological advances, including high magnetic fields, proton detection, and dynamic nuclear polarization (DNP). As the subsequent sections illustrate, this early prediction has indeed come to pass with remarkable success.

A key enabling technology for these studies is magic-angle spinning (MAS), which allows high-resolution spectra to be obtained from immobilized proteins in their native membrane environments. When combined with targeted isotopic labeling, MAS techniques make it possible to investigate structure and dynamics without requiring purification or reconstitution. Figure 1 provides an overview of the framework for *in situ* solid-state NMR of membrane proteins, from the conceptual basis to the methodological tools that now support routine structural investigations in native contexts.

Optimizing selectivity, resolution and sensitivity

Protein expression and isotope labeling. A major strategy to enhance selectivity and sensitivity is optimization of sample preparation. For studies in native bacterial cell envelope (Figure 1a and b), non-specific labeling of endogenous proteins can be minimized using dual-media protocols, in which cells are first grown in unlabeled LB media and subsequently switched to isotope-labeled M9 media just before induction. Coupled with rifampicin treatment, which blocks endogenous transcription while maintaining T7-promoter-driven expression, this approach enables targeted ^{15}N and ^{13}C

Figure 1



Concepts and tools for *in situ* solid-state NMR of membrane proteins. **(a)** Core concept: membrane protein structure and dynamics are probed directly in their native environments using magic angle spinning (MAS) NMR. **(b)** Example ^{13}C−^{15}N correlation and ^{1}H−^{15}N correlation spectra (900 MHz, 30 °C, 57 kHz MAS) of *Y. pestis* Ail in native cell envelopes (black) vs. purified Ail reconstituted in liposomes (red). Amide chemical shift differences (0−0.15 ppm) are mapped on a structural model of Ail in the native outer membrane (OM). **(c−g)** Methodological tools for enhancing selectivity, resolution, and sensitivity: **(c)** Background suppression by tuning expression and selective isotope labeling; **(d)** membrane fractionation for sample enrichment; **(e)** isolation of native OM vesicles (OMVs); **(f)** fast MAS using small-diameter rotors to enhance spectral resolution and sensitivity through detection of ^{1}H signals; **(g)** dynamic nuclear polarization (DNP) boosts signal sensitivity through microwave-driven polarization transfer. Adapted from Refs. [9−11].

labeling with excellent suppression of background signals [12].

For bacterial OM protein studies, background NMR signal can be further reduced by using mutant *E. coli* strains lacking the major OM proteins OmpA, OmpC, and OmpF [13,14]. In studies of the OM proteins Ail and PagC, optimized IPTG and rifampicin pulses minimized transcription of endogenous RNA yielding high-quality spectra with minimal background (Figure 1c), even in wild-type strains [9–11]. *E. coli* cell envelope preparations of Ail yielded high-quality $^{15}N/^{13}C$ solid-state NMR spectra, with ^{13}C linewidths in of 0.6–0.8 ppm and ^{15}N linewidths of 1.3–1.8 ppm, comparable to those from purified proteins reconstituted in liposomes (Figure 1b).

Background labeling from lipids, lipopolysaccharide (LPS), and other cell envelope components can be further reduced by using individually labeled $^{13}C/^{15}N$ amino acids instead of ^{13}C-glucose and ^{15}N salts [13]. Treatment with cerulenin, an inhibitor of fatty acid biosynthesis, inhibits ^{13}C labeling of *E. coli* lipids and simplifies $^{13}C/^{13}C$ correlation spectra of proteins [15]. Moreover, spectroscopic approaches based on ^{15}N filtering can be used to silence lipid signals [16], and NMR data acquisition above the gel-to-liquid phase transition of the lipids has also been shown to suppress lipid signal intensity [17]. Nonetheless, these background signals can also serve as reporters of specific interactions between the target protein and its native membrane environment.

Membrane fractionation. Separation of inner membrane (IM) and OM components by density gradient centrifugation both reduces background signal and significantly increases the fraction of labeled protein in the sample, thereby enhancing sensitivity (Figure 1d). This was recently accomplished for the mechanosensitive channel of large conductance from *Methanosarcina acetivorans* (MaMscL), as well as for the sugar transporters *Vibrio* sp. SemiSWEET [18]. In these studies, a combination of dual-media labeling, BL21(DE3) expression, and membrane fractionation, yielded effective suppression of nearly all background protein signals, while also enabling near-complete backbone assignments directly in the native IM of *E. coli*.

Native Vesicle Platforms. For OM proteins, natively secreted OM vesicles (OMVs) offer an attractive platform for NMR (Figure 1e). OMVs preserve the asymmetric architecture of the OM and maintain native protein-LPS and protein-lipid interactions. Originally exploited for solution NMR studies after genetic deletions and vesicle extrusion [19], native *E. coli* OMVs were used recently, to study the *Salmonella* protein PagC by solid-state NMR, without reconstitution or detergent treatment, yielding high-resolution $^{15}N/^{13}C$ and $^{13}C/^{13}C$ spectra with linewidths comparable to those from purified reconstituted samples [11]. This platform enables structural studies without purification artifacts, while preserving functional membrane asymmetry. This is especially important in cases where membrane composition critically affects protein structure and function, as shown for the β-barrel protein AlkL [20]. These approaches have also been extended to eukaryotic systems. In a landmark study [21], solid-state NMR was used to probe full-length epidermal growth factor receptor (EGFR) in extracellular vesicles (EVs) derived from human A431 cells. These EVs also preserve native lipid–protein interactions and enabled observation of ligand-induced conformational changes in EGFR in its native membrane context.

^1H detection. *In situ* NMR studies of membrane proteins rely on solid-state MAS techniques to overcome the correlation time limitations posed by molecular immobilization on the μsec time scale or longer. Recent developments in MAS probes capable of spinning above 60 kHz (Fig. 1F) have dramatically improved resolution and sensitivity for ^1H detection [22]. This approach now enables site-specific assignments of membrane proteins in cellular environments [23,24]. 2D ^1H–^{15}N spectra, which report selectively on nitrogen-containing groups, help suppress background from lipid and carbohydrate. With MAS rates exceeding 100 kHz, fully protonated membrane protein samples can now be studied routinely, while partial deuteration is still beneficial at spinning rates around 60 kHz [25]. In the case of Ail, where perdeuteration and back-exchange are not feasible *in situ* due to membrane insertion, partial deuteration involving D_2O growth followed by induction of gene expression in H_2O enabled the acquisition of excellent 2D $^1H/^{15}N$ CP-HSQC spectra (Figure 1b) with resolved linewidths of ∼0.15 ppm for ^1H and ∼1.5 ppm for ^{15}N, with *22* peaks assigned through comparison with purified protein spectra [26].

Dynamic nuclear polarization (DNP). Finally, DNP provides a key avenue for overcoming the challenges associated with the inherently low sensitivity of NMR spectroscopy, and has been used to study a wide range of biomolecular systems including intact cells [27–29], native bacterial membranes [10] and eukaryotic EVs [21]. By transferring polarization from unpaired electrons to nearby nuclei, DNP can boost signal intensity by 30-fold or more (Figure 1g). DNP experiments are performed under MAS at cryogenic temperatures (90–100 K), conditions that are essential for efficient polarization transfer. Samples are typically cryoprotected in a solution of glycerol-d_8, D_2O, and H_2O containing a polarizing agent (commonly nitroxide-based biradicals) in a formulation referred to as “DNP juice” [30]. A key challenge is incorporating these polarizing agents into native membrane preparations without disrupting structural integrity while freezing the sample. Nevertheless, DNP has enabled groundbreaking studies, including EGFR activation in eukaryotic EVs [21]. Although freezing-induced line broadening is a tradeoff, DNP remains an invaluable tool for accessing weak or transient interactions in native-like conditions. For example, DNP was employed to study Ail interactions with LPS directly in the bacterial OM [10], revealing contacts that would otherwise remain undetectable by conventional NMR.

Recent examples and applications

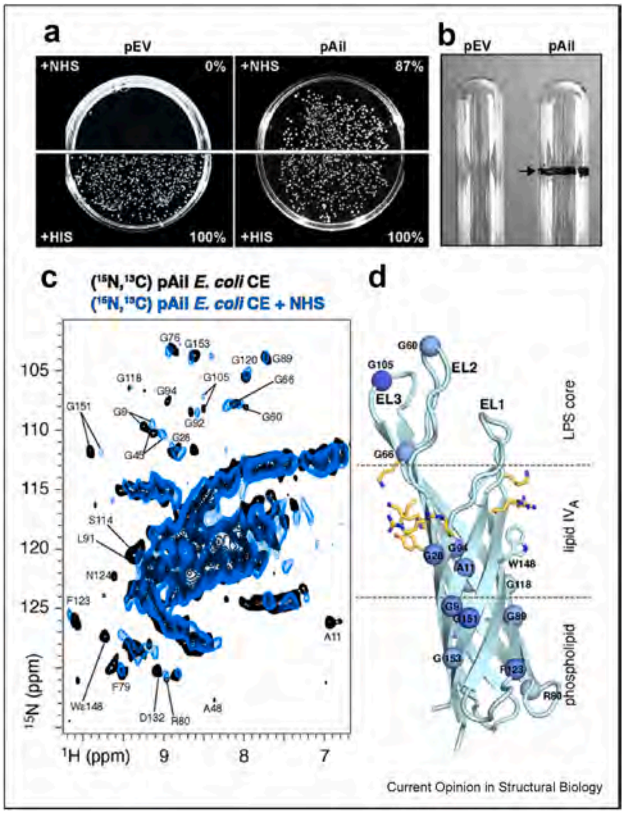
Thanks to the combined progress on sample preparation protocols, labeling strategies and improved instrumentation, *in situ* solid-state NMR has now enabled structural investigations of a broad range of membrane proteins in their native environments. Early studies provided insights into the bacterial OM proteins Lpp [8], PagL [13], YadA [17], the β-barrel assembly machinery (BAM) complex [31], and Ail [9,10], as well as the IM proteins ATP synthase subunit c [15], diacylglycerol kinase (DAGK) [32], YidC [24], aquaporin [33], MaMscL [18], bacteriorhodopsin [34,35], the sensory rhodopsins from *Anabaena* and *N. pharaonis* [36,37], and membrane-active antimicrobial peptides [38,39]. In addition, the *E. coli* IM has served as a useful model system for studying eukaryotic and viral membrane proteins such as the SorLA/LR11 transmembrane domain [40] and the influenza M2 ^{1}H channel [41], in a context that more closely mimics physiological conditions. Recently, this list has grown to include more examples that have significantly advanced both the technical capabilities and biological reach of *in situ* NMR.

Work on Ail, the virulence-associated OM protein of *Yersinia pestis*, revealed similarity between the solid-state NMR spectra from *E. coli* cell envelopes versus purified liposomes and nanodiscs, confirming preservation of the β-barrel fold *in situ* [9]. However, further analysis revealed that the native membrane induced clear conformational ordering, as reflected by enhanced resolution, line narrowing, and additional cross peaks from dynamic loop regions and membrane-facing sites. Spectral differences, especially in the extracellular loops and membrane—water interface, correlated to clusters of positively charged residues on the barrel surface that form LPS-recognition motifs (Figure 1b). These findings were extended in a more recent study using DNP [10], which enabled detection of weak interactions between LPS sugars and Ail sidechains (notably His, Lys, Arg, and Tyr). These contacts were absent from control spectra lacking Ail expression, underscoring the protein-specific nature of these interactions.

Functionally, the same Ail-containing cell envelope preparations retained hallmark virulence phenotypes of *Y. pestis*, including serum resistance, autoaggregation and pellicle formation, and binding to the human protein vitronectin (Figure 2a and b). Importantly, solid-state NMR spectra recorded after exposure to normal human serum revealed chemical shift perturbations in the Ail β-barrel interior and LPS-binding loops (Figure 2c and d), suggesting that the β-barrel indirectly senses host factor interactions. These results exemplify the power of *in situ* NMR to monitor both structure and function within the same biologically active sample.

Structure determination of the *E. coli* aquaporin Z (AqpZ) water channel in the *E. coli* IM represents a major milestone [33]. Using a combination of uniform and sparse isotopic labeling, complete chemical shift assignments could be obtained for nearly all backbone and sidechain atoms (Figure 3a–c), as well as over 1000 long- and medium-range ^{13}C—^{13}C distance restraints for structure

Figure 2



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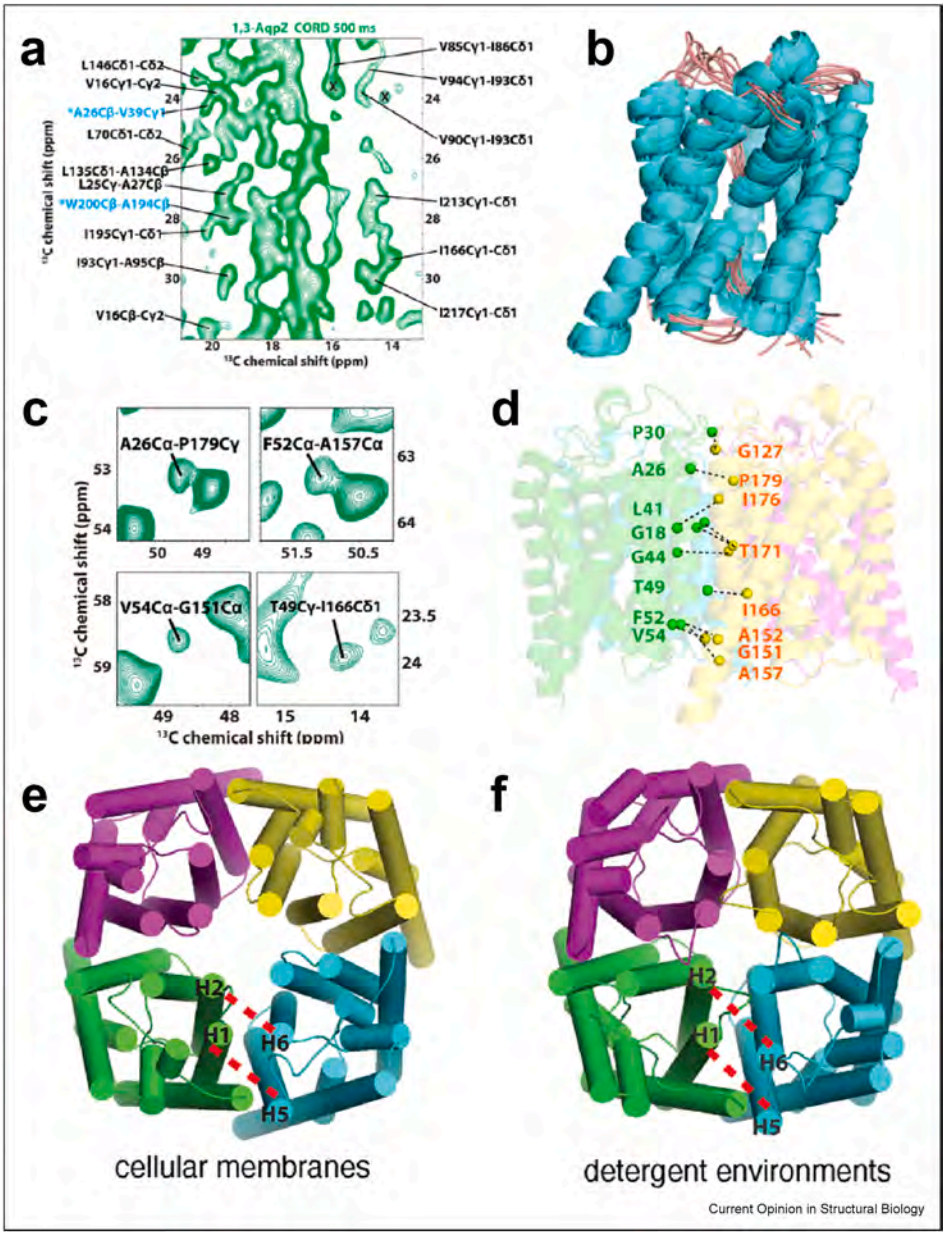
NMR analysis and native phenotypes of Ail-expressing *E. coli*. (a) Serum resistance assay of *E. coli* Lemo21(DE3) cells transformed with Ail (pAil) or empty vector (pEV) lacking the Ail gene. Cell survival (%) was assessed after incubation with normal human serum (NHS) or heat-inactivated serum (HIS). **(b)** Pellicle formation observed in pAil but not in pEV cells by crystal violet staining. **(c)** ^{1}H–^{15}N CP-HSQC spectra (900 MHz, 30 °C, 57 kHz MAS) of pAil cell envelopes before (black) and after (blue) NHS exposure. **(d)** Ail residues showing NHS-induced amide chemical shift perturbations (cyan to blue, 0–0.2 ppm) mapped on an MD-derived membrane structure. LPS-recognition sidechains are shown in yellow, and lipid IVA and LPS core regions are indicated. Adapted from Refs. [9,10].

Figure 3



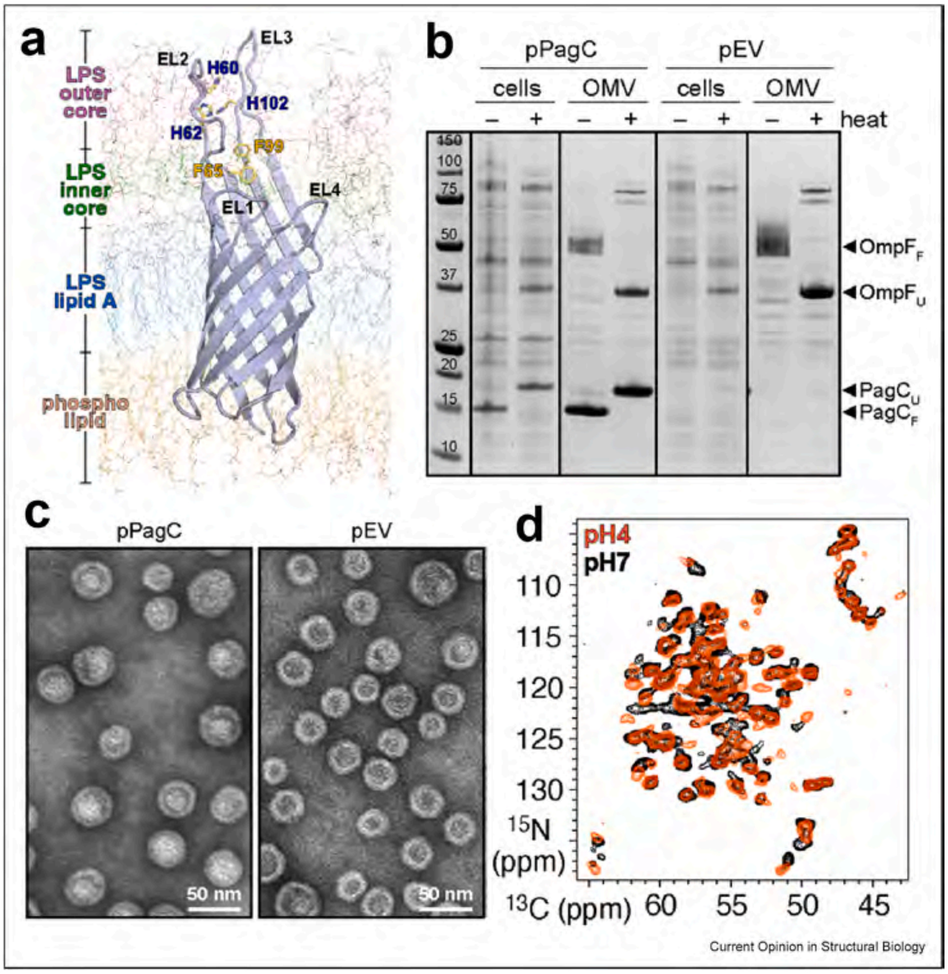
Solid-state NMR structure of AqpZ in native *E. coli* membranes. (a) 2D ^{13}C−^{13}C correlation spectrum (CORD, 500 ms) of 1,3-AqpZ in the *E. coli* IM, highlighting methyl-methyl and long-range contacts. **(b)** Ensemble of the 10 lowest-energy AqpZ monomer structures (cyan) derived from chemical shifts and distance restraints. **(c)** Examples of unambiguous inter-monomer contacts extracted from the CORD spectrum. **(d)** Key long-range contacts mapped onto the AqpZ structure, indicating interhelical connections critical for bundle packing. **(e–f)** Comparison of AqpZ tetramer packing in cellular membranes **(e)** and detergent micelles **(F)**, showing rearrangement of helices H5–H6 at the monomer-monomer interface. Adapted from Ref. [33].

determination in the native membrane (Figure 3b). The resulting structure, a first solved *in situ* by solid-state NMR, achieved a backbone RMSD of 1.7 Å, comparable to the structural precision obtained in synthetic bilayers. Mapping of long-range contacts onto the AqpZ fold highlighted key interhelical connections stabilizing the four-helix bundle architecture (Figure 3d). Importantly, a comparison of the AqpZ tetramer in the native IM and detergent micelles revealed substantial differences at the monomer–monomer interface, particularly involving helices H5 and H6 (Figure 3e and f). These rearrangements, likely influenced by lipid packing and bilayer thickness, also impacted the geometry of the selectivity filter, suggesting functionally relevant modulation of pore size under native conditions.

A recent application targeted the heptameric MaMscL channel inserted into the *E. coli* IM [18]. A combination of multiple preparation strategies yielded high-resolution spectra with minimal background, enabling residue-specific assignments for over 70 % of the sequence, secondary structure prediction and chemical shift comparisons to previously reported liposome and crystal structures. While the global fold was preserved, differences in hydration and lipid interactions emerged at specific residues, supporting the conclusion that local environmental factors impact protein conformation even for structurally stable channels.

Finally, the OM protein PagC from *Salmonella* was recently examined in biogenic OMVs produced by engineered *E. coli* [11]. PagC adopts a β-barrel fold with long extracellular loops that are sensitive to their local environment (Fig. 4). NMR experiments, performed at both neutral and acidic pH, revealed specific chemical shift perturbations in extracellular residues, and three His in the extracellular loops displayed pH-dependent changes consistent with a His-based pH sensor. These residues are essential for the protein's ability to drive OMV production, linking the structural response of PagC to a biological function at the cellular level. The data link conformational adaptation to OMV biogenesis and highlight the way solid-state NMR can be used to probe protein function in physiological samples, including those from human pathogens.

Figure 4



Solid-state NMR of the *Salmonella* OM protein PagC in native bacterial OMVs. (a) MD-derived model of His-neutral PagC embedded in a native OM (LPS and phospholipid layers shown). His and Phe sidechains are in yellow. **(b)** Coomassie stained SDS-PAGE of cells and OMVs from *E. coli* expressing PagC (pPagC) or empty vector (pEV), with and without heat treatment to reveal folded (F) and unfolded (U) protein bands. **(c)** Negative-stain EM of OMVs from pPagC and pEV cells at pH 7. **(d)** ^{15}N–^{13}C TEDOR NCA spectra of OMVs at pH 7 (black) and pH 4 (red), acquired at 700 MHz and 4 °C. Adapted from Ref. [11].

Conclusions and outlook

The ability to probe the molecular structures, dynamics and functions of membrane proteins within their native cell membranes is critical for gaining mechanistic insights that impact human health. Collectively, the examples described here illustrate the growing capabilities and resolution of *in situ* solid-state NMR for studying membrane proteins in their native lipid environments. They demonstrate the ability to capture structural, dynamic, and functional properties that remain inaccessible to conventional reconstitution approaches.

In the near future, advances in instrumentation are expected to further amplify these capabilities. Although MAS spinning rates of 100 kHz have not yet been widely implemented *in situ*, their combination with proton detection holds the potential to deliver unprecedented resolution even for densely protonated samples [22]. Moreover, even higher MAS rates (exceeding 160 kHz) have recently become available, and this technology seems ripe for application to membrane proteins. Recent work on AqpZ, for instance, demonstrates sub-70 Hz proton linewidths in native-like bilayers, enabling near-complete backbone observation in sub-milligram samples [42]. Parallel progress in magnetic field strength (now reaching up to 1.2 GHz/28.2 T) has yielded superlinear resolution gains for multiple membrane systems [43].

DNP continues to extend the frontier of *in situ* sensitivity. The integration of faster MAS (40–60 kHz) at cryogenic temperatures with high magnetic fields [44], improved biradicals [45], hyperpolarization at near-ambient temperature [46], and targeted labeling strategies such as selective ^{19}F labeling for background-free detection [47], will ease the detection of weak protein-lipid or protein-ligand interactions within cellular membranes.

Future directions may also include refined isotope-labeling schemes (e.g., reduced adjoining protonation [48], inverse fractional deuteration [23], SAIL [49]) and exploitation of engineered cell strains depleted of background proteins to increase selectivity.

In parallel, the implementation of relaxation-based NMR measurements in native membranes represents an exciting perspective. These experiments can provide residue-specific insights into molecular motions over biologically relevant timescales [50], but have not yet been widely applied *in situ*. Their future development and integration could offer a powerful new dimension to the study of protein dynamics in complex environments.

Taken together, these developments will expand the application of *in situ* NMR to a wider range of membrane protein systems, including more complex eukaryotic models. Solid-state NMR is thus poised to play a central role in integrated structural biology, bridging the gap between atomic-level insights and physiological relevance.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by grants from the National Institutes of Health (GM 118186; AG 081167;, OD 028716), the European Research Council (ERC-2015-CoG; GA 648974 “P-MEM NMR”), the European Union’s H2020 research and innovation programme (GA 101008500 “PANACEA”), the Agence Nationale de la Recherche (ANR-23-CE11-0024-02 “CTRbyNMR”), and the CNRS (Infranalytics FR2054).

Data availability

The authors do not have permission to share data.

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* * of outstanding interest

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