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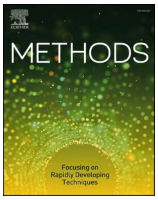

NMR techniques for investigating antimicrobial peptides in model membranes and bacterial cells

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ABSTRACT

AMPs are short, mainly cationic membrane-active peptides found in all living organism. They perform diverse roles including signaling and acting as a line of defense against bacterial infections. AMPs have been extensively investigated as templates to facilitate the development of novel antimicrobial therapeutics. Understanding the interplay between these membrane-active peptides and the lipid membranes is considered to be a significant step in elucidating the specific mechanism of action of AMPs against prokaryotic and eukaryotic cells to aid the development of new therapeutics. In this review, we have provided a brief overview of various NMR techniques commonly used for studying AMP structure and AMP-membrane interactions in model membranes and whole cells.

1. Antimicrobial peptides and biological membranes: Key features of molecular interactions

Antimicrobial peptides (AMPs) are found in all living organisms [1,2]. They act as sentinels or immune response modulators that prevent bacterial infections or colonization. Their chemical and structural properties are extremely diverse, with particular scaffolds used for *in silico* design, and providing a large library of potential therapeutics [3,4]. The hallmark of membrane-active AMPs is their amphipathic nature that induces an affinity for lipid membrane interfaces [5–7]. This has led to extensive research on the interplay between these membrane-active peptides and the variety of lipid membranes found in Nature.[8,9] Indeed, elucidation of the key biophysical and biochemical parameters regulating the interaction between AMPs and biomembranes is considered to be a significant step for understanding the specific mechanism of action of AMPs against prokaryotic and eukaryotic cells.

AMPs are short (usually under 50 residues), mainly cationic peptides that can adopt several secondary structures depending on their chemical composition or on the local environment. For instance, many AMPs are linear peptides with random coil structures in solution but spontaneously form helical structures in the presence of lipid membranes [10,11]. The accumulation of AMPs at the membrane interface triggers their lytic activity, with peptide self-assembly as a key event for the formation of macromolecular structures between lipids and peptides (Fig. 1). [6] The driving forces for the specific affinity towards bacterial membranes are the electrostatic interactions. Indeed, biological membranes are extremely diverse, with significant differences found between eukaryotic (neutral membranes, mostly unsaturated lipids) and prokaryotic (negatively charged membranes, mostly branched and saturated lipids) cells and within cell types and organelles.[12–14] The chemical structure of phospholipids modulates their response to pH, temperature, hydration level and lateral pressure. Moreover, natural membranes are asymmetric, with the inner and outer leaflets are often presenting diverse lipid compositions. For instance, Gram-negative bacteria present a membrane with an outer layer mainly made of lipopolysaccharides (LPS) while the inner layer is made of phospholipids [15,16]. This leads to the diversity of lipid structures found in membranes, such as rigid or fluid domains and bilayer or hexagonal assemblies. Furthermore, the interactions between AMPs and lipids can form complex assemblies that deviate from the physiological bilayer structures. This is in part the molecular mechanism of the antimicrobial activity, thereby pore formation, cell aggregation or membrane thinning can induce cell death and prevent bacterial infections (Fig. 1). Thus, deciphering the key biophysical and biochemical factors regulating AMP activity is a critical step for their therapeutic development. Crafting systems to understand the complex interactions at a molecular level has continuously evolved with the advances in biophysical techniques. By using asymmetric lipid membranes [17,18], ghost cells [19], or live

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organisms [20–25], a wealth of structural and dynamic information has been obtained in the attempt to decipher the key interactions between AMPs and cells. This work will focus on the use of nuclear magnetic resonance (NMR) spectroscopy to extract structural and dynamic information on the complex interactions between AMPs and lipid membranes, from model membrane systems to live bacteria.

NMR relies on the angular momentum property of nuclear spin found within atoms. Thus, NMR is a non-destructive technique that can provide information at atomic resolution without the need for chemical modification, such as the addition of a fluorescent probe. [26] However, NMR is not very sensitive and often lacking in resolution for *in situ* investigations due to significant signal overlap since each biomolecule have many NMR-active reporters, such as ^{1}H or ^{13}C nuclei, that have similar chemical shifts.[21,22] For instance, ^{13}C carbonyl signals from phospholipids, amino acids or metabolites, all present in cellular systems, are all contributing to the NMR spectrum with modest chemical shift dispersion. Also, some isotopes have low natural abundance, such as ^{13}C, ^{15}N or ^{2}H, which can be counteracted by isotopic enrichment. Isotopes can also be introduced, such as ^{19}F, which does not significantly perturb biological functions but provides a background-free reporter for high-resolution studies.[25,27] Furthermore, the tumbling rate of the molecules modulates the linewidth of the NMR signal. A fast reorientation on the NMR timescale (ns) significantly averages out all orientation-dependent interactions with the magnetic field such as dipolar couplings or chemical shift anisotropy, thereby providing sharp NMR signals for high-resolution studies (i.e., for determination of AMP structure); while large systems, such as liposomes with diameter of *ca.* 1 µm, slowly re-orient leading to broad anisotropic signals that can be exploited using solid-state NMR techniques to monitor the structure and dynamics of lipid assemblies (*i.e.*, planar bilayers versus regions of high curvature).

Magic angle spinning (MAS) of the NMR sample within the magnetic field can mechanically average the orientation of the biomolecules, and reintroduce sharp isotropic signals for high resolution solid-state NMR experiments. To obtain solution-like NMR signals, the MAS frequency needs to be greater than that of the NMR interactions that cause line broadening, such as dipolar couplings between ^{13}C and ^{1}H spins which are in the order of tens of kHz. Typically, 4 mm diameter rotors (~80 µL volume) can spin up to 15 kHz, while recent advances in hardware allow rotors with a diameter as small as 0.5 mm to reaching spinning frequencies above 150 kHz.[28] Hence solid-state NMR enables the investigation of biological systems that are large, heterogenous, and amorphous. Thus, NMR is a powerful technique to study the complex macromolecular assemblies found within cell membranes, providing information on the spatial distribution of atoms and on their motions or dynamics.[29].

Historically, the complexity of NMR signals from native systems, coupled with the preservation of the cellular integrity, has been a

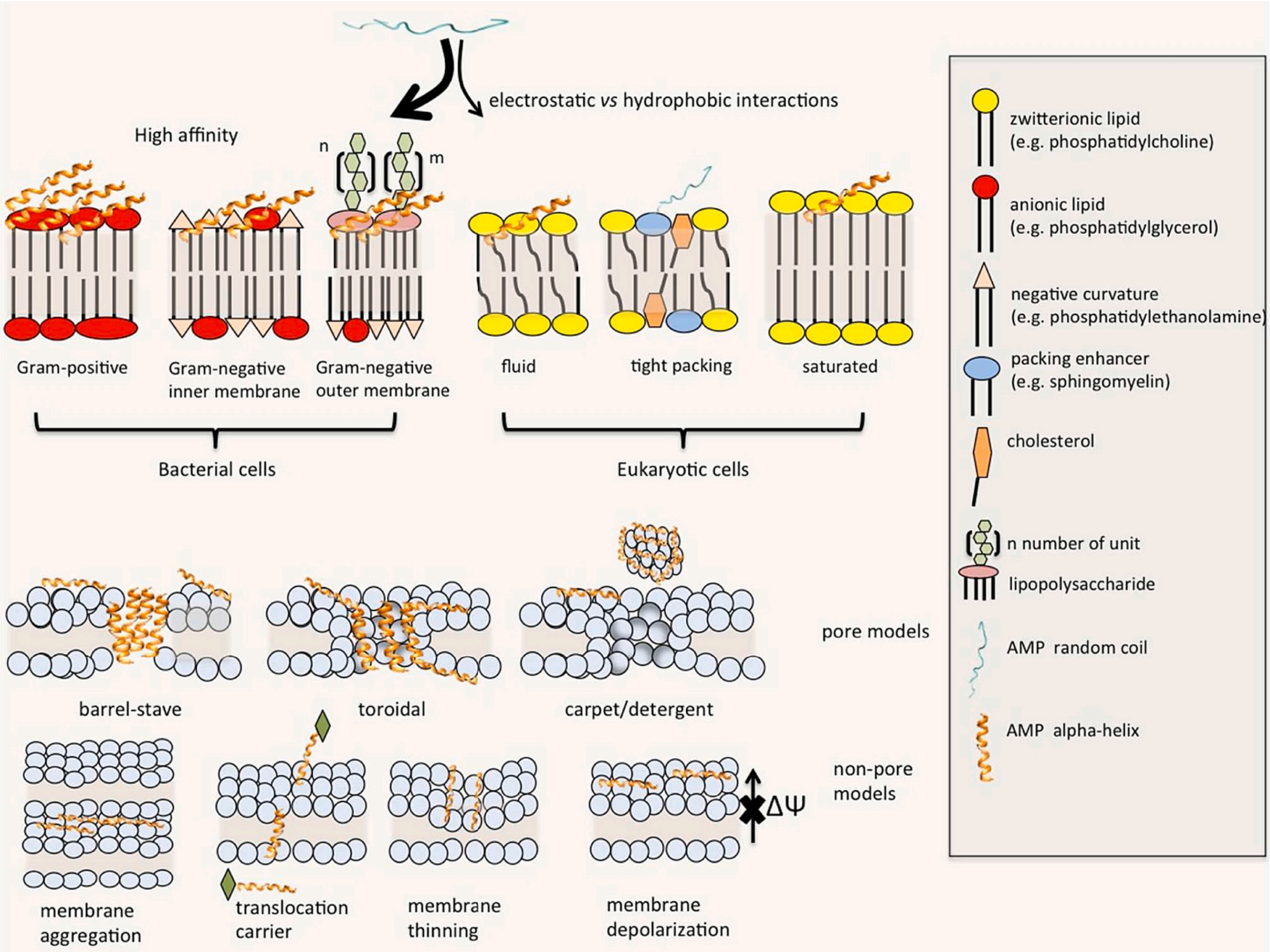

Fig. 1. Key features from biomembranes and their impact on the regulation of the interactions with AMPs, with various modes of action of membrane-active AMPs [6]. Electrostatic interactions are a key driving force for the affinity of AMPs towards bacterial membranes, in particular for anionic lipids such as phosphatidylglycerol, cardiolipin or lipopolysaccharide. Once accumulated at the membrane interface, AMPs can cause disruptions such as pore formation, cell aggregation, membrane thinning or loss of membrane potential (ΔΨ).

limiting factor for *in vivo* studies, although progress in methods and hardware (e.g. dynamic nuclear polarization NMR, discussed later) is rapidly developing. Model membrane systems have, therefore, been developed to simplify experimental parameters while attempting to maintain the key properties of native environments. The crafting of a model system is determined by factors such as investigating AMPs in models of prokaryotic or eukaryotic cell membranes, and the resolution required to determine their structure and/or dynamics in the AMP-membrane complex. For instance, solution-state NMR takes advantage of fast-tumbling systems (e.g. micelles, bicelles, small unilamellar vesicles, nanodiscs, etc.) for structure determination of AMPs using 2D NMR methods such as ^{1}H TOCSY and ^{1}H NOESY techniques.[30,31] By contrast, slow tumbling systems, e.g. multilamellar vesicles (MLVs), anisotropic bicelles, are suitable for solid-state NMR to investigate the architecture of phospholipid assemblies (^{31}P or ^{2}H static NMR) and their alteration upon interaction with AMPs, or to determine the peptide orientation within lipid bilayers *i.e.*, tilt angle in mechanically aligned bilayers or magnetic aligned anisotropic bicelles, using ^{15}N, ^{2}H or ^{19}F labelled peptides. [31–34].

For each model system, several biophysical and biochemical parameters can be evaluated by NMR. Since the electrostatic interactions between cationic amino acids and anionic lipids are a key feature of AMPs, membranes with variable amounts of negatively charged lipids or peptides with changed lysine, arginine or histidine and hydrophobic residues are commonly tested. Furthermore, pH, temperature and importantly lipid to peptide (L/P) ratios can have a large impact on AMP-lipid interactions.

Most commonly, eukaryotic membranes are mimicked using neutral phospholipids, such as zwitterionic palmitoyloleoylphosphatidylcholine (POPC) for liposomes or dodecylphosphocholine (DPC) for micelles. Prokaryotic membranes are mainly composed of saturated neutral and negatively charged lipids, such as zwitterionic dimyristoylphosphatidylethanolamine (DMPE) and anionic dimyristoylphosphatidylglycerol (DMPG) for liposomes or sodium dodecyl sulfate (SDS) for micelle systems.

Bicelles or bilayer-micelles are another frequently used model membrane system. Bicelles are composed of a combination of long-chain and short-chain phospholipids, and can also include bile salts, polymers or detergents. The ratio between the long-chain phospholipids and the detergent or short chain lipid component, generally referred to as the q ratio, has a significant impact on the shape of the bicelles. Disc-like bilayer species are known to exist for $q > 0.25$ and small fast-tumbling isotropic bicelles are observed at q </= 0.5. Bicelle structures are also dependent on the lipid concentration [35]. Numerous studies of peptide structure and membrane interactions using solution NMR have employed isotropic bicelles as the biomembrane mimics. On the other hand, bicelles with high q-value, which readily align in the external magnetic field to form a lyotropic liquid crystalline phase, have been frequently used for solid-state NMR studies. In addition, magnetically aligned bicelles have also been used to solubilize membrane proteins and measure residual dipolar couplings.[31].

Mechanically oriented bilayers are another aligned membrane model system where the inherent ability of hydrated phospholipids to align on the surface of thin glass plates, with bilayer normal perpendicular to the glass plane, has been exploited to align membrane-active peptides and proteins in the external magnetic field of the NMR spectrometer. Numerous studies have employed such mechanically aligned membrane bilayers to determine the structure and orientation of AMPs using solid-state NMR techniques.[36–39] In particular, extensive work on ^{15}N labelled peptides and proteins have demonstrated the power of solid-state NMR using separated local field type NMR experiments, such as PISEMA [40]. These experiments are performed to obtain the parallel or perpendicular orientations and tilt angles within oriented lipid membranes.

Although less common for NMR studies of AMPs, lipid nanodiscs [41], amphipols[42] and lipidic cubic phases[43] have also been investigated as fast tumbling systems with bilayer-like properties to provide high-resolution spectra. However, these model systems do not mimic the complexity of the native environment, thus, more complex or physiological environments have been investigated using solid-state NMR. For instance, whole cells [22,44–46] or ghost membranes of red blood cells [19] have provided *in situ* systems for the study of membrane active peptides or membrane proteins. In this article, we have attempted to provide a brief overview of the application of NMR techniques used to study AMP-biomembrane interactions in model membrane systems followed by the application of NMR to whole bacterial cells to provide insights into the mechanism of action of AMPs.

2. NMR studies of AMPs in model membrane systems

2.1. Probing the configuration and orientation of AMPs in model membranes

AMPs are thought to form aggregates that disrupt the microbial membranes via a variety of mechanisms, with three main widely accepted categories being formation of a barrel-stave pore, formation of a toroidal pore and general disruption of the microbial membrane via a carpet model involving detergent-like aggregation of AMPs on the surface of the membrane.[6,12] (Fig. 1) A range of techniques is available to study various parameters pertaining to the AMP-peptide interactions.[47–49] Specifically, determination of the configuration adopted by AMPs upon interacting with biomembranes and their orientation with respect to the model membranes can provide valuable insight into the mechanism of AMP-membrane interactions. In addition, the effect of different membrane lipid compositions, representative of different cell types, on AMP structure and orientation can be studied. In this section, we briefly discuss the application of solution- and solid-state NMR techniques, together with oriented samples as discussed in the previous section, to provide insights into the mechanism of action by studying the configuration and orientation of AMPs with respect to the model membranes.

Based on their structure, AMPs can be broadly classified into three main categories: linear amphipathic α-helical, amphiphilic β-sheets and turns, or extended structures, which may be concomitant with differing modes of actions. Since, AMPs are relatively short peptides and, therefore, are amenable to standard solution-state NMR methods, such as through bond ^{1}H–^{1}H homonuclear DQF-COSY and TOCSY, through space ^{1}H–^{1}H NOESY or heteronuclear correlation ^{13}C–^{1}H or ^{15}N-^{1}H HSQC, for elucidation of the structure of the peptides [50], many structural studies have employed these techniques to calculate distance-based constraints and elucidate 3D structures for a vast number of AMPs in numerous solvents. It is noteworthy that as AMPs are usually polymorphic, *i.e.*, capable of adopting different conformations in different environments, the choice of the solvent or model membrane system for these structural studies is of key significance. As AMPs usually exhibit random coil conformations in aqueous buffers, which bear little relevance to the mechanism of action, and the signals are hard to assign by standard NMR methods, many early structural studies were carried out in aqueous solutions containing fluorinated solvents such as trifluoroethanol or hexafluoroisopropanol that promote an α-helical conformation which can then be studied by standard NMR techniques.[51–53] However, the structure determined in the presence of these solvents may not be biologically relevant as the peptide may exhibit an entirely different structure in the presence of membranes.

As the interaction of AMPs with biomembranes is a key aspect of their mechanism of action, solution-state NMR structural studies of AMPs are nowadays carried out in the presence of fast tumbling model membrane systems such as micelles (dodecylphosphocholine, DPC, or sodium dodecylsulfate, SDS)[53,54] or isotropic bicelles. It is noteworthy that structural studies have also been performed with dioctanoylphosphatidylglycerol and LPS to mimic more accurately the inner and outer membrane of Gram-negative bacteria, respectively

[55,56]. LPS is a key lipid for understanding the first interactions with Gram-negative bacteria, and has often been pointed towards resistance mechanisms against cationic molecules since it can possess several anionic phosphate groups. Bhunia and Bhattacharjya have obtained evidence using saturation transfer difference NMR methodology that AMPs can be structured in the LPS environments by mapping residue-specific contacts of polymyxin B with LPS [57]. Micelles, however, are small spherical monolayers and their monolayer structure and curvature can impose an artificial curvature on the peptide, resulting in a structure different from the biologically relevant form. Bicelles more closely simulate the bilayer structure of biomembranes and are, therefore, the preferred choice for the application of solution-state NMR methods. Thus, bicelles have been widely used as model systems for structural studies of AMPs.

Further, the orientation or depth of the peptide in a micelle or bicelle can be determined using paramagnetic relaxation enhancement (PRE) techniques.[30] Titration of the peptide-model membrane mixture with paramagnetic agents, e.g. gadolinium complexes, results in different PREs and the resultant PREs or signal attenuations for different peptide atoms, which depend on their distance from the micellar or bicellar surface, are then used to determine the orientation and depth of the peptide within the model membrane system.[58] Using the NMR techniques discussed above and complementary techniques such as CD spectroscopy, the AMP Mac1 was shown to adopt an α-helical structure in zwitterionic as well as anionic micelles and to insert into the micelles with the N-terminus of the peptide closer to the surface of the micelles using titration with the PRE agent (Fig. 2).[10] A detailed procedure for structure elucidation of AMPs in micelles or isotropic bicelles and using PRE techniques to determine the orientation and depth of insertion of peptides can be found here[59].

Model membrane systems such as anisotropic and magnetically oriented bicelles provide a closer approximation of the bilayer structure of biomembranes and have, therefore, been frequently used to study peptide-biomembrane interactions by solid-state NMR. As discussed above, oriented samples facilitate molecular alignment and allow access to structural and orientational information, which is inherent to anisotropic spin interactions within peptides embedded in membrane bilayers. The samples used for such studies may either be mechanically aligned on glass plates[36–38,60–62] or magnetically aligned in solution. The elucidation of the structure and orientation of the peptide is carried out by taking advantage of the rigidity of peptide amide bonds which allows the determination of the orientation of the amide N-H—O = C bond relative to the molecular symmetry axis by following the chemical shift tensor of the peptide bond, e.g. helix axis or long axis of the β-hairpin β-sheet.[62–65] Peptide analogues with ^{15}N and ^{13}C labelled amide N-H and C = O groups are employed to reduce complexity of the ^{15}N and ^{13}C NMR spectra. Many studies have also used ^{19}F labelled amino acids for this purpose [25,27,34,60,66,67], albeit with an effort to reduce the reorientation of the sidechain for greater accuracy of the angular determination[60,68]. These techniques are,

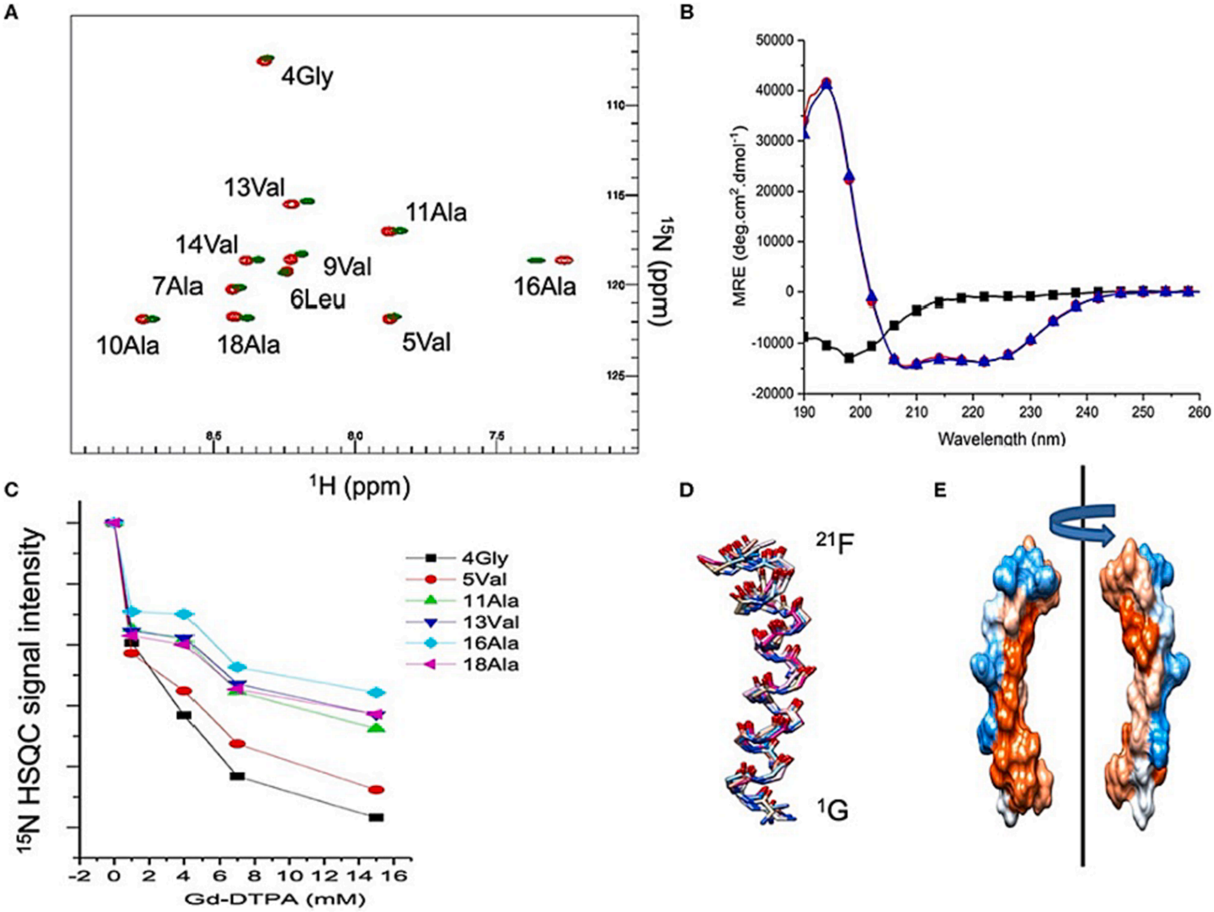

Fig. 2. A) ^{15}N HSQC spectra of 1 mM maculatin 1.1 (Mac1) in the presence of zwitterionic d$_{38}$-DPC micelles (green) or anionic d$_{38}$-DPC/lyso-myristoylphosphoglycerol (9:1) micelles (red) B) CD spectra of Mac1in aqueous buffer (black squares), in the presence of zwitterionic DPC micelles (blue triangles) or anionic SDS micelles (red circles) C) Resonance peak intensity of ^{15}N labeled Mac1 residues as a function of the Gd-DTPA concentration in the presence of d$_{38}$-DPC/LMPG (9:1) micelles D) 10 lowest energy structures of Mac1 calculated from the solution NMR experiments E) Electrostatic surface representation of Mac1 averaged structure with the hydrophobic (red), hydrophilic (blue) and neutral (white) residues.[10]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

however, laborious and subject to disadvantages such as peptide aggregation or disruption of the membrane bilayer due to lytic activity of the peptide. Many studies are, therefore, carried out in MLVs that are amenable to MAS solid-state NMR spectroscopy.

2.2. Probing the location of AMPs in lipid bilayers by investigating lipid perturbations of model membranes

As discussed above, the mode of action of most AMP peptides involves the disruption of microbial membranes. Therefore, numerous studies have been conducted to elucidate AMP-membrane interactions from the perspective of their impact on the lipid bilayer dynamics and order by using ^{31}P and ^{2}H solid-state NMR methods.

A picture of the interaction of AMPs with the membrane surface or headgroup region of a phospholipid bilayer can be constructed by monitoring the changes to ^{31}P isotropic chemical shift and ^{31}P chemical shift anisotropy in static NMR spectra, or by investigating ^{31}P relaxation rates under MAS, upon addition of AMPs to the model membrane system, usually MLVs or liposomes (Fig. 2). The static (i.e., non-spinning) ^{31}P NMR spectrum of unoriented phospholipid liposomes is a powder pattern where the chemical shift anisotropy (CSA) is observed. The fast axial rotation of lipids in the liquid crystalline lamellar phase gives rise to a symmetric CSA peak, with an overall width due to headgroup dynamics and re-orientation on a 10^{-4} s timescale, and a decrease in the signal width indicative of a change in the orientation of the headgroups.[70] The appearance of an isotropic signal upon addition of AMPs to the model membrane system indicates the formation of lipid domains with rapid reorientation, e.g. toroidal pore or micellar structures (Fig. 3). In the case of ^{31}P MAS NMR, changes observed in the spin–lattice T_1 relaxation rate are indicative of an increase or decrease in the intensity of motions of lipid headgroups on a ns timescale, e.g. changed rate of lipid rotation along the lipid long axis. Further, changes observed in the spin–spin ^{31}P T_2 relaxation rate measured by MAS NMR are representative of changed lipid dynamics on the μs timescale, e.g. lipid diffusion or membrane fluctuations. Since ^{31}P is naturally abundant in the phospholipids, it is easy to apply ^{31}P NMR techniques to study AMP-membrane interactions as reflected by numerous reports in literature.[69,71,72].

The effect of AMP peptides on the hydrophobic core or acyl chains of the lipid bilayer can be investigated via ^{2}H solid-state NMR by using lipids containing deuterium labelled acyl chains.[75,76] ^{2}H is a quadrupolar nucleus with spin 1 and by static solid-state NMR techniques gives rise to a complex line shape, which may be described as a sum of Pake powder patterns comprising a central pair of doublets from the terminal CD_3 and pairs of doublets from each CD_n position along the lipid acyl chain (Fig. 4). The spectrum is “de-Paked” to obtain the order parameter S_{CD} for each CD_n position to elucidate the order profile along the lipid acyl chain and observe changes following the addition of AMPs or by varying experimental conditions such as L/P ratio, pH and temperature (Fig. 3.) This information is then used to determine the nature of AMP-membrane interaction. For example, an uniform increase in the order along the acyl chains may suggest insertion of the AMP into the bilayer. The observation of a single set of quadrupolar splitting in the presence of an AMP is indicative of reasonably fast and homogeneous diffusion of the peptide in the bilayer without the formation of more rigid or ordered domains.

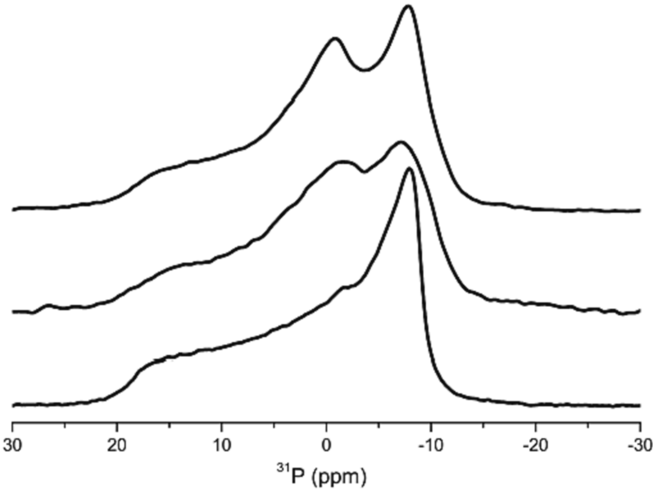

Fig. 3. Solid-state ^{31}P static NMR spectra of E. coli PG/CL MLV at 37 °C in the absence of Mac1 (bottom spectrum), at 50:1 (middle spectrum), and at 25:1 L/P (top spectrum).[69].

^{31}P and ^{2}H NMR techniques together with other complementary biophysical techniques have been employed in numerous studies to provide insights into AMP-biomembrane interactions.[54,73,77,78] For example, ^{31}P and ^{2}H NMR studies of the AMP aurein 1.2 with the model membranes indicate that aurein 1.2-membrane interaction is consistent with the carpet-model.[71,79] Similar studies performed on Mac1 and analogues with glycine or alanine residues replacing the native proline residue in position 15 (P15G and P15A) of the peptide demonstrate the significance of the proline residue in differentiating between eukaryotic and prokaryotic membranes.[72].

3. NMR studies of AMPs in live bacteria

The molecular mechanism of AMPs has been extensively studied using model systems that provide a controlled environment. This has enabled NMR studies with higher resolution and thus have given greater understanding of the molecular interactions between AMPs and specific lipid membranes. Conversely, the complex cellular environment is a challenge for high-resolution solid-state NMR studies. Yet, several elegant solution NMR studies have revealed how AMPs can adopt their amphipathic structures in the presence of live bacteria, such as *C. neoformans* [80], or key component such as LPS [81]. Among the difficulties, the cell background signals can mask the signal of interest as numerous molecules possess similar NMR resonances, albeit modulated by their tumbling rate. Furthermore, due to the intrinsic low sensitivity of the technique, greater amounts of material and longer experimental times are required to obtain structural information, eventually imposing additional experimental hurdles such as cellular degradation during acquisition. Hence, fast signal acquisition and/or better sample stability is critical for in-cell solid-NMR studies of AMPs. The use of DNP is emerging as an exciting technique for in-cell NMR studies of peptides and proteins, providing tremendous NMR signal enhancement, while cryogenic temperatures greatly preserve cell integrity.[20,22].

3.1. Using DNP NMR for in-cell studies

3.1.1. Advantages and limitations

DNP is a hyperpolarization technique that transfers the magnetization from electrons to nuclear spins under microwave irradiation, providing a signal enhancement by up to two orders of magnitude.[82,83] The high-power microwaves are usually imposed at low temperature, *ca.* 100 K, to preserve the electron source, usually a radical. The cryogenic conditions are beneficial but also detrimental. By using cryo-protectants, cell viability can be maintained for longer measurement. However, the filling factor is reduced as most of the sample volume is occupied by the cryoprotectant, with a strong background signal, especially for ^{13}C detected experiments. For *Escherichia coli* with glycerol or trehalose as cryoprotectants, over 75 % viability was obtained after more than 24 hr of acquisition under MAS and continuous microwave irradiation.[84] This compares well with similar studies using mammalian cells to study membrane proteins.[44,85] Better cryoprotection to improve filling factor and reduce background signal is under investigation in our laboratory. One potential avenue is the use of

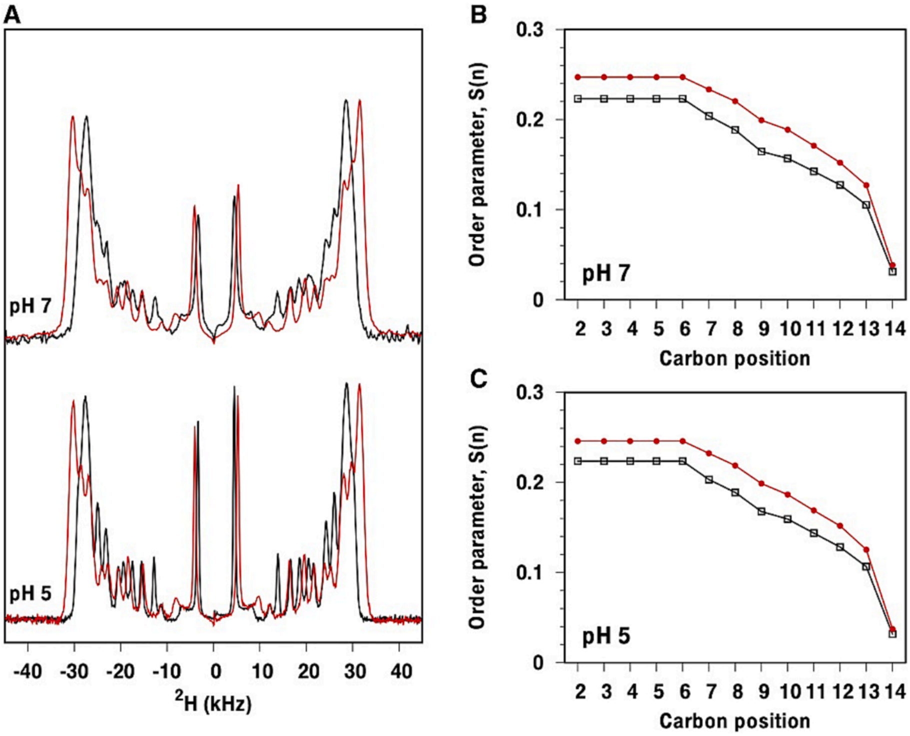

Fig. 4. ^{2}H solid-state NMR study of the effect of pH on the interaction of caerin 1.1 with deuterated phospholipid bilayers at 30 °C: A) De-Paked ^{2}H NMR spectra of D_{54}-DMPC MLVs (black lines) at pH 7 (top) and 5 (bottom) and in the presence of Cae-1 at a lipid to peptide molar ratio (L/P) of 30:1 (red lines), B) the lipid order profiles S(n) at pH 7, and C) at pH 5 calculated using $\Delta\nu_Q = (3e^2qQ/4h).S(n)$, where$e^2qQ/h = 167$ kHz and $\Delta\nu_Q$ is the quadrupolar splittings at the CD_n site. [73,74]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lyophilized samples with a lyoprotectant preserving the critical structure of the cellular environment. For AMPs which first encounter the outer bacterial membrane, questions around this primary target can be answered if the native membranes are preserved.

While cell preservation is essential, the signal enhancement is also critical for extracting structural information. For example, the Cegelski and Schaeffer groups have studied complex interactions between antibiotics and the cell wall of Gram-positive bacteria under lyophilized conditions and without DNP. The weak interactions required long experimental times, i.e., several days for a single REDOR experiment.[86] Using DNP, Sani *et al.* ran 16 REDOR experiments in a day, benefiting from a signal enhancement factor of 80.

Since the enhancement factor is modulated by the proximity of the radical source and magnetization diffusion via the ^{1}H pool, the intracellular components of the cell often display weaker enhancements than the extracellular environment. This can be an advantage if the AMPs are interacting solely with the external membranes but could also mask interactions with the intracellular components of the bacteria since the radical source may not penetrate the cell membrane. Targeted DNP using radicals that can be confined to a particular environment, such as the lipid membrane, can offer several advantages: a membrane-localized radical source is less prone to degradation by antioxidant or enzyme reactions and the signal enhancement is not homogenous, which reduces unwanted background signals.[84,87].

Finally, since the NMR experiments are performed at about 100 K using nitrogen boil-off gas, limited MAS spinning frequencies, currently below 25 kHz, are accessible. Thus, broad signals due to large anisotropic and dipolar interactions drastically reduce the resolution of the NMR spectra. Typical MAS linewidths of several ppm prevent higher resolution, which can be partially improved by incorporating specific isotopes in the molecules of interest or eventually acquiring complex multi-dimensional solid-state NMR experiments. Overall, the design of the DNP NMR experiment requires careful setup to obtain high enhancements and spectral resolution with limited background signals and acceptable cell survival rate.

3.1.2. Design of experiments

NMR studies of AMPs in native environments are challenging but rewarding as *in situ* observations can identify key interactions often not seen in simpler environments, such as intracellular secondary targets. Several considerations for sample preparations are necessary to ensure the full benefit of an in-cell environment.

Since the mode of action of AMPs is primarily to lyse the bacterial cell membranes, the composition and structure of the bacterial membrane are important factors to consider. For instance, the type of bacterial strain, the media used to grow the cells and the methods used for recovery of the cell pellets have impacts on the physiological AMP-bacteria interactions. To grow bacteria in a controlled way, growth media often contain an antibiotic and the cells have been engineered to contain a gene that confers resistance to the chosen antibiotic, commonly ampicillin, thereby only the wanted strain is grown without contamination. This is a limiting factor since clinical strains cannot be easily genetically modified to harbor resistance to the chosen antibiotic. Many studies, including ours, have taken advantage of the widely used BL21 (DE3) strain, easily transformed with a plasmid providing ampicillin or other antibiotic resistance. However, the question arises whether the BL21 (DE3) bacteria present the same lipid membrane composition and architecture as that of typical clinical strains. BL21 (DE3) is an *E. coli* B strain that does not possess a critical protease, such as the outer membrane OmpC, or does not express motility-related proteins that are necessary for flagella formation.[88] More importantly for the study of AMPs, B-strains display a truncated O antigen structure.[89] Furthermore, the synthesis of capsular colanic acid and biofilm formation is drastically reduced due to the absence of the Rcs phosphorelay system (rcsADB).[90] Thus, observations from in-cell NMR studies using *E. coli* B-strains must take into consideration the difference in the composition of the outer leaflet of the outer membrane (OM) and, if possible, should use other strains to compare the effect of different lipopolysaccharide (LPS) structure on AMP activity. While Gram-positive bacteria are not widely used for large scale production, limiting accessibility to engineered strains and protocols for controlled growth, some strains such as *Staphylococcus aureus* or *Bacillus subtilis* have also been labelled, and studied either as whole cells or specific isolated parts.[91].

Indeed, growing cells in controlled media provide the opportunity to use isotopically enriched nutrients to label the cellular components, either in a specific or nonspecific manner. This is commonly used for protein overexpression using minimal media containing ^{13}C carbon sources and/or ^{15}N salts and, more rarely, in D_2O for ^{2}H enrichments. Interestingly, the Marcotte's group has successfully ^{2}H enriched bacterial membranes using deuterated palmitic acid in the growth media [92].

Sani *et al.* took the advantage of enriched media to simply label the cellular components without overexpressing a target protein by using an ^{15}N salt. The ^{15}N spectra of *E. coli* BL21 (DE3) displayed all molecules containing nitrogen atoms, i.e., amino acids, nucleic acids, and phospholipids. Thus, the full spectrum can be used to assess the AMP perturbation on a global scale but at atomic resolution, placing in-cell NMR as a unique technique. DNP experiments that take advantage of ^{15}N and/or ^{31}P for extracting information on the AMP perturbations of cellular components also side-step the issue of background signal.[46].

Cegelski *et al.* produced a Gram-positive ^{13}C-^{15}N labelled strain to study antibiotic actions on the bacterial cell wall. [91,93]. As Gram-positive bacteria are surrounded by a thick cell wall essential for cell survival, it is a common target of antibiotics. Therefore, NMR studies of the cell wall structure and interactions with antibiotics are crucial for therapeutic development. For instance, in-cell NMR studies can provide important molecular details of antibiotic modes of action by profiling intact whole cells using particular isotopes, such as ^{13}C [94], ^{2}H [23]., or ^{15}N [46]. Similarly, Wang and co-workers have studied the extracellular matrices and cell walls of algae, bacteria, fungi, and plants by solid-state NMR. [95].

Not only can the cells be labelled, but AMPs can also be isotopically enriched. Partial or full labelling can enable the determination of the peptide structures, and eventually their location within bacteria, by using labelled pairs such as ^{13}C for the peptide and ^{31}P for the bacterial lipids. Solid phase peptide synthesis, or more recently expression systems, have been used to produce labelled AMPs that are C-amidated. [96] C-amidation is an important feature of AMPs that can modulate the interplay between the peptides and the lipid membranes.[97].

DNP NMR experiments performed at 100 K require the cell to be cryo-protected for maintaining viability. Fortunately, the so-called 'DNP juice', a mixture of 60 % glycerol and 30:10 D_2O/H_2O, necessary to homogenously distribute and preserve the radical source in a glassy matrix, is also a good solvent for bacterial cryoprotection. In fact, a glycerol stock is commonly used for storage of bacteria. Notably, in-cell DNP studies using mammalian cells report that, while the common hydrophilic radical AMUPol has no effect on membrane permeability, glycerol was shown to have dehydrating properties that induced apoptosis. Glycerol can be replaced by 10 % dimethylsulfoxide (DMSO) with similar preservation of cell integrity and DNP performance.[85] However, reducing the glycerol content to 15 % has also been shown to provide similar enhancement performance and cell survival rate as 10 % DMSO.[45].

To date, there is no extensive study of bacterial cryoprotection and DNP performance but optimizing the DNP medium is of importance for *in situ* DNP NMR studies of AMPs, especially since the large volume occupied by the cryoprotectant not only greatly reduces the sample filling factor but also increases the ^{13}C background signal. Furthermore, the cryoprotectants modulate the distribution of the radical source, which is critical for the DNP signal build-up and the NMR scan repetition rates.[98].

Improving the radical source is a fast-growing area of research, especially for DNP NMR studies at high magnetic fields where the DNP performance significantly drops compared to signal enhancements observed at moderate magnetic fields (*ca.* 9.4 T).[99] Sani *et al.* designed a radical tagged peptide TOAC-MacW based on an AMP (Mac1) sequence, which was found to locate within the bacterial cell membrane. This allowed high retention and radical protection [100] during peptide incubation and bacterial harvesting[84,87], with the DNP enhancement more localized at the membrane interface. At the moderate magnetic field of 9.4 T, the in-cell ^{13}C and ^{15}N signal NMR enhancements and ^{1}H spin–lattice T_1 relaxation times showed that TOAC-MacW and TOAC-TOAC-MacW performed better than the commonly used biradical AMUPol. Designing such radical carriers can deliver targeted NMR experiments, which have been applied to other cellular studies.[101] Overall, by use of labelling strategies, in-cell studies of AMPs can be achieved with high resolution and provide atomic details of cellular component and the perturbations induced by interaction with AMPs.

3.2. *Monitoring bacterial perturbations under antimicrobial stress*

NMR studies of bacteria were performed by the Davis group in the early 1980 s. Using membrane-deuterated bacteria, they provided the first ^{2}H NMR spectra of live bacteria, which paved the way for the methodology used to follow the effect of AMPs on bacterial membranes in near native conditions [102,103]. In 2012, Booth and co-workers investigated the effect of the AMP MSI-78 on lipid acyl chain order in intact cells of engineered *E. coli* that did not metabolize or synthesize fatty acids, thereby forcing the incorporation of deuterated palmitic acid (PA) provided in the growth media.[104,105] The Marcotte group refined the protocol to avoid employing mutants, providing a solid methodology for acquiring reproducible ^{2}H NMR spectra from bacteria incubated with antimicrobial agents.[92] They demonstrated that a lot more of the AMP MSI-78 per lipid was needed to see the same lipid disruption as seen in the corresponding model lipid membrane NMR studies.[106].

Similarly, the deuteration of the Gram-positive bacteria *Bacillus subtilis*, treated with the AMPs CAME and BP100, have also been studied using whole-cell NMR. [24,107]. The AMP-induced effects were similar to those observed in *E. coli*, suggesting similar lipid disruption mechanisms for the peptides and similar effects on both Gram (+) and Gram (−) bacteria. However, the AMPs caerin 1.1 and aurein 1.2 disrupted *B. subtilis* less than *E. coli* which suggested that some cell wall components present in *B. subtilis* - such as TA and PGN - could reduce the peptide local concentration at the lipid membrane and reduce their membrane activity [108].

Recently, in-cell DNP NMR studies of AMPs interacting with bacterial cells grown in ^{13}C, ^{15}N and ^{2}H reported new insights on the impact of the peptides on bacteria. The *E. coli* cells were grown in enriched minimal media to exhaustion, washed and freeze-dried. Interestingly, the cells preserved their diameter (estimated by dynamic light scattering as ∼ 3 μm). The addition of the pore-forming AMP induced greater signal enhancements of the intracellular content and decreased T_1 values of ^{13}C and ^{15}N intracellular signals, which indicated that the integrity of the bacterial membranes was preserved.[84].

Regarding the impact of the AMP on structural features of *E. coli*, in-cell NMR studies using REDOR experiments (Fig. 5) showed that the peptide Mac1 disrupted the inter-molecular hydrogen bonds between the charged ^{15}N ammonium group of a phosphatidylethanolamine lipid headgroup and neighbouring lipid ^{31}P phosphate groups.[46] Two main ^{15}N-^{31}P distance populations were observed, at *ca.* 3 Å and 4.5 Å corresponding to intra and inter-molecular pairs. In the presence of Mac1, the inter-molecular population was significantly reduced, which was reproduced using a model lipid membrane made from ^{15}N labelled *E. coli* lipid extracts. The peptide insertion into the bacterial membrane induced a disruption of these lipid-lipid bridges, which perturbed the bilayer packing, a typical AMP mode of action.

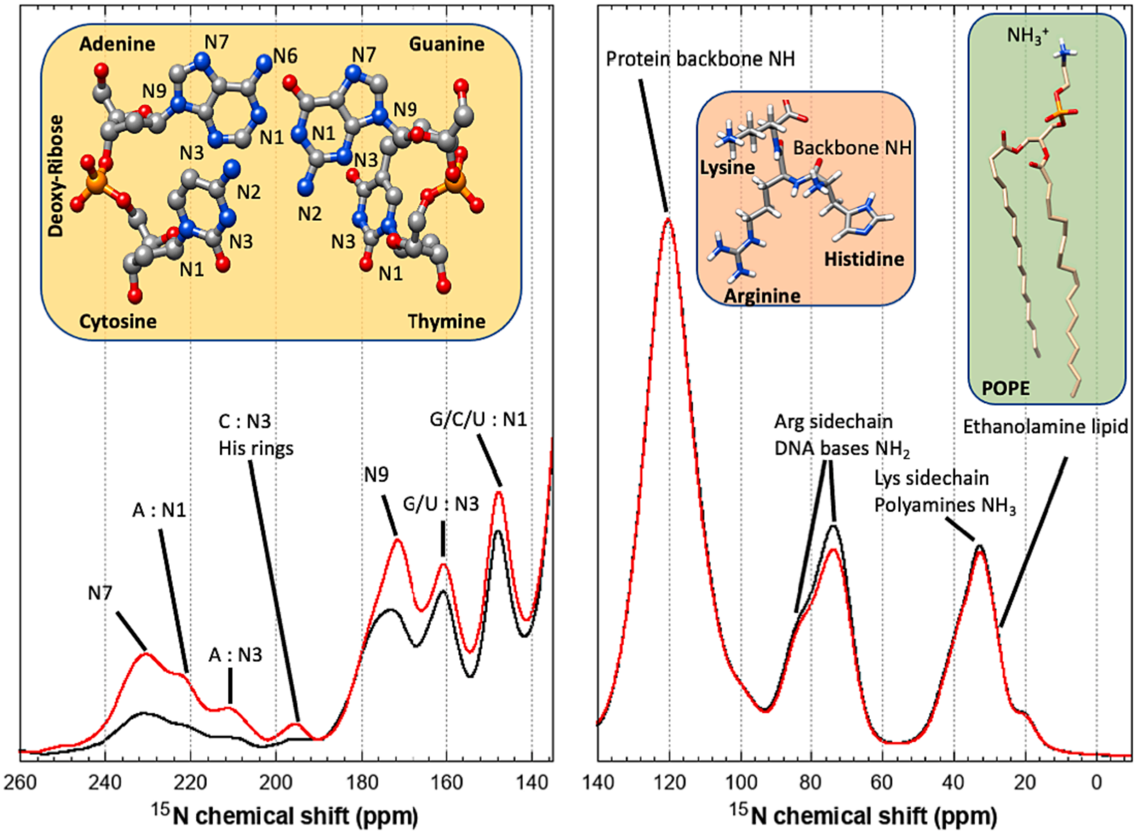

Fig. 5. ^{15}N spectra of E. coli with (red line) and without (black line) the AMP Mac1. Some cellular component structures are displayed with their ^{15}N chemical shift assignments. [46]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

However, the peptide was also shown to induce nucleic acid condensation by shortening the distance between nitrogen atoms within nucleotides and the ^{31}P atoms within the phosphate group linking the nucleic acid chains. A similar response was previously reported using electron microscopy, which showed bacterial DNA condensation upon incubation with Mac1.[109] Remarkably, these observations were obtained at peptide concentration well below the minimum inhibitory concentration (MIC). This was unexpected since most models depict a lysis effect when AMPs reach a threshold concentration. However, these in-cell NMR studies demonstrate that AMPs can form pores and impact on the DNA packing without incurring bacterial cell death. It is likely that repair mechanisms and damage tolerance modulate the MIC, and the ability of AMPs to reach intracellular secondary targets, which may explain the difference in the AMP structure–activity relationship. Interestingly, using dye uptake assays with fluorescence monitoring, Sani *et al.* had demonstrated previously that pore formation can occur below the MIC.[110]This was also reported with MSI-78 peptides using ^{2}H labelled *E. coli*, which supported AMP binding to non-lipid targets and/or non-lipid components of the cell envelope protecting the lipid bilayer from AMP-induced disruption.[104,105].

Whole cell NMR studies of ^{13}C-^{15}N labelled Gram-positive *S. aureus* were also performed to investigate the action of the antibiotics fosfomycin (a cell-wall biosynthesis inhibitor) and chloramphenicol (a protein synthesis inhibitor). The NMR experiments showed that incubation with fosfomycin resulted in a decreased peptidoglycan signal but those incubated with chloramphenicol had a higher percentage of peptidoglycan. In-cell NMR is thus a powerful technique to monitor the action of antimicrobials by profiling the whole cell cellular components using ^{13}C and ^{15}N labelling. The modes of action of many different antibiotics have been examined, including lipoglycopeptides such as vancomycin and its derivatives.[111,112] By site-specifically labelling ^{13}C-alanine and ^{15}N-glycine or ^{13}C-glycine and ^{15}N-lysine, and incubating with specifically ^{19}F-labeled antibiotics such as vancomycin and derivatives, REDOR NMR experiments were able to probe cell wall structure and drug binding in bacteria. These antibiotics had heavy impact such as thinning effects or reduced crosslinking in the cell walls. This structural knowledge was used to design a derivative that exposed its hydrophobic sidechain to stabilize the binding to the cell wall in a vancomycin-resistant strain of *S. aureus*.[113].

DNP NMR was also employed to detect interactions between antibiotics and lipid II *in situ*.[114] Finally, a growing amount of in-cell DNP NMR studies are being used to investigate extracellular matrices (ECMs) and demonstrate the importance of the cell walls and biofilms in supporting cell integrity, function, and regulation of intercellular communication, which in turn provide insights for the development of antimicrobial treatments.[95] DNP NMR is well suited to provide key understanding of the conformational structure and supramolecular assembly of carbohydrates and other biomolecules in extracellular matrices found in many organisms spanning across plants, bacteria, fungi, and algae [95].

4. Conclusions and perspectives

NMR has provided a wealth of structural and dynamic information on the mode of actions of AMPs. NMR studies of the complex interplay between AMPs and cells have allowed mapping of the change in peptide or lipid membrane structures or identifying key affinities for cellular targets, such as LPS or nucleic acids. With advances in instrumentation and NMR techniques, in-cell NMR studies of AMPs will certainly increase in frequency, which will further advance the knowledge needed to combat bacterial infections. To date, a limited number of AMPs have been investigated by in-cell NMR, usually with only a single type of bacterium. Thus, a large sphere of research remains open to understand the complex mode of action and the differences in activity observed across bacterial strains.

Finally, there is clearly a lack of AMP structural studies within whole bacteria, and advances in peptide labelling will help fill the gaps in determination of AMP structure–function relationships *in situ*. Indeed, since the action of AMPs onto bacterial membranes often necessitate peptide self-assembly, it is imperative that methods for routine structure determination of the multitude of AMPs that have been described can be performed in-cell, and eventually in infection models where bacteria and host tissue are present. New labelling schemes for the cellular component of bacteria could provide a route for supporting high resolution NMR studies of AMPs. For instance, by introducing ^{19}F labelling of residues, specific contacts could be determined with greater resolution *in situ*, and thereby key cellular interactions resolved to develop better therapeutics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

review, all data has been published

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