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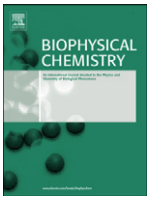

Review

The structural and topological analysis of membrane-associated polypeptides by oriented solid-state NMR spectroscopy: Established concepts and novel developments

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ABSTRACT

Solid-state NMR spectroscopy is a powerful technique for the investigation of membrane-associated peptides and proteins as well as their interactions with lipids, and a variety of conceptually different approaches have been developed for their study. The technique is unique in allowing for the high-resolution investigation of liquid disordered lipid bilayers representing well the characteristics of natural membranes. Whereas magic angle solid-state NMR spectroscopy follows approaches that are related to those developed for solution NMR spectroscopy the use of static uniaxially oriented samples results in angular constraints which also provide information for the detailed analysis of polypeptide structures. This review introduces this latter concept theoretically and provides a number of examples. Furthermore, ongoing developments combining solid-state NMR spectroscopy with information from solution NMR spectroscopy and molecular modelling as well as exploratory studies using dynamic nuclear polarization solid-state NMR will be presented.

Contents

1. Solid-state NMR approaches for membrane protein structural analysis

The structural investigation of membrane polypeptides remains a challenging task although the number of deposits to the PDB data base and the rate by which these proteins are investigated by classical approaches, including X-ray crystallography and multidimensional solution NMR spectroscopy, has considerably increased during the last decade [1,2]. Structural studies on membrane proteins are hampered by problems of solubility, difficulties associated with their over expression and purification, and their limited stability in the absence of lipid bilayers. In addition, among those systems that can be made available many are too large for a detailed solution NMR spectroscopic analysis and/or they are difficult to crystallize in their native

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Abbreviations: DHPC, 1, 2-dihexanoyl-*sn*-glycero-3-phosphocholine; DMPC, 1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine; DNP, dynamic nuclear polarization; DOPC, 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine; MAS, magic angle spinning; MD, molecular dynamics; MM, molecular modelling; PDB, protein data base; POPC, 1-palmitoyl-2oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; VASS, variable angle sample spinning.

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conformation. In particular, very few crystal structures of G protein-coupled receptors have so far been solved (e.g. [3]), despite the importance of these proteins in pharmaceutical and medical research. Therefore a number of other biophysical approaches have been developed that allow one to gain insight into the structure, dynamics and function of this important family of proteins.

Whereas multidimensional solution NMR spectroscopy is a well-established technique to determine the structure of globular proteins in isotropic environments, including membrane proteins in micelles [4–6], solid-state NMR spectroscopy is probably the only high-resolution technique available to date for the investigation of the structure, topology and dynamics of polypeptides that are associated with phospholipid bilayers in their liquid disordered state [7–13]. In addition solid-state NMR spectroscopy provides a complete view of the membrane structure as it also allows for the investigation of the lipid macroscopic phase properties, the detailed order parameter of the lipid fatty acyl chains [14–19] or lipid phase separation in the presence of polypeptides [20,21].

Two fundamentally different solid-state NMR approaches are used during the structural studies of biomolecules. On the one hand, magic angle spinning (MAS) averages the orientation-dependent NMR interactions by mechanically spinning the sample at frequencies of currently up to ≥ 70 kHz. As a consequence relatively narrow resonance line shapes are obtained of proteins in the solid state or when associated with extended membranes, under the condition that the sample exhibits a high degree of local order [22]. In such cases multidimensional high-resolution MAS solid-state NMR spectra provide a large number of angular and distance constraints and protein structure determination follows related strategies to the ones well established for biomacromolecules in solution [12,23–29]. Using this technique valuable information about the structure and dynamics of some globular proteins in nanocrystals such as Crh (10.4 kDa) and the alpha spectrin SH3 domain have been obtained [23,30,31]. Furthermore, membrane-inserted potassium channels [32–34], ABC transporters [11], outer membrane proteins [35], sensory rhodopsin II [36] and phospholamban [37] have been investigated, the spectra (partially) assigned and their structures analyzed using MAS techniques. As in the case of multidimensional solution NMR spectroscopy, the analysis of multispanning helical membrane proteins is hampered by the lack of chemical shift dispersion. Nevertheless, partial assignments of a seven-transmembrane helical protein has been achieved [38]. It has also been possible to follow the conformational changes of the voltage sensor within a mostly helical Shaker potassium channel during the binding of a proteinaceous ligand [39]. Other examples of MAS solid-state NMR investigations in the context of membrane proteins include the scorpion toxin KTX when associated with the KscA-Kv1.3 chimeric potassium channel [39], a neurotensin peptide bound to its GPCR [40,41], and of neurotoxin II associated with the nicotinic acetylcholine receptor [42]. Furthermore, using dipolar recoupling techniques distances can be obtained, at very high accuracy (typically ±0.1 Å) between pair-wise labelled sites (e.g. ^{13}C–^{15} or ^{13}C–^{13}C) [43–47], or within uniformly labelled proteins [48].

Whereas spinning around the magic angle (54.7°) efficiently averages the anisotropy of the NMR interactions a variation of the technique is to rotate the sample slightly off this direction. In this case averaging is incomplete and residual contributions are observed comparable to the residual dipolar couplings observed in solution NMR spectroscopy [49]. In such variable angle sample spinning (VASS) experiments, the magnitude of these residual anisotropies of membrane-associated peptides can be controlled in a flexible manner by using variations of the spinning angle [50–52].

A complementary solid-state NMR approach, which shall be discussed in more detail in this review, takes advantage of the orientation dependence of NMR interactions by investigating membrane samples that are uniaxially oriented relative to the magnetic field of the NMR spectrometer (Fig. 1D, E). In this manner angular restraints are established that can be recombined for a topological and structural analysis of membrane-associated polypeptides [7,8]. This approach works best when polypeptides are reconstituted into lipid bilayers that are oriented with their normal parallel to the magnetic field direction (Fig. 1E) and has been successful in the structural analysis of e.g. bilayer-associated gramicidin A, Vpu, alamethicin and phospholamban [7,9,53–55], antimicrobial peptides [56–61] as well as to larger and functionally more complex membrane proteins (e.g. [62–64]). Whereas for some polypeptides very accurate structures have been determined [7,9,65], this approach also provides detailed information about the tilt and rotational pitch angles of membrane-inserted helices and can be used to monitor even small changes (e.g. of 1°) in structure or tilt angles [53,54,66]. Thereby the investigation of oriented membrane samples by static solid-state NMR spectroscopy provides information not only about the structure but also about the topology of membrane-associated polypeptides.

2. Basic concepts of oriented solid-state NMR spectroscopy

The fundamental concept of membrane protein structure determination using oriented solid-state NMR spectroscopy is based on the inherent anisotropy of NMR interactions. Whereas in solution fast molecular tumbling ensures their isotropic averaging, the reorientational correlation times of molecules associated with extended phospholipid bilayers are slow when compared to the corresponding time scales of chemical shifts, dipolar couplings or quadrupolar interactions [8]. Therefore, the spectral properties depend on the alignment of molecules and bonds relative to the magnetic field direction and can be used to deduce angular information from solid-state NMR spectra. The interactions and angular correlations of a number of biologically interesting nuclei are well characterized for this purpose. For example the ^{15}N chemical shift of a peptide bond exhibits an anisotropy of about 170 ppm (reviewed in [67]) and this value exceeds by an order of magnitude the ^{15}N chemical shift dispersion typically observed for a protein in solution [8]. The angular dependence is described by $\sigma_{zz} = \sigma_{11}\ \sin^2\Theta\ \cos^2\Phi + \sigma_{22}\ \sin^2\Theta\ \sin^2\Phi = \sigma_{33}\ \cos^2\Theta$, where σ_{zz} is the chemical shift, σ_{11}, σ_{22} and σ_{33} are the main tensor elements, and the Euler angles Θ and Φ position the chemical shift tensor relative to the magnetic field direction of the NMR spectrometer (B_o). In case of the ^{15}N amide bond the size of the chemical shift main tensor elements are about 60, 80, and 225 ppm (typically ±5 ppm) and their alignments relative to the molecular coordinate system have been characterized [67].

Similar dependencies have been established for the dipolar splitting [8] $Dzz = \frac{\mu_o}{4\pi}\frac{\gamma_1\gamma_2\hbar}{r^3}(3\cos^2\ \Theta - 1)$, where r is the distance between the two interacting nuclei and Θ the angle between the dipolar interaction vector and B_o. The $\frac{\mu_o}{4\pi}\frac{\gamma_1\gamma_2\hbar}{r^3}$ term is called dipolar coupling constant where γ_1 and γ_2 are the magnetogyric ratios of the two interacting nuclear dipoles, and μ_o represents the free magnetic inductivity. Depending on the alignment of the dipolar interaction vector Θ the ^{1}H–^{15}N coupling within an amide bond typically varies between −20 kHz and 10 kHz [8].

In the case of the deuterium quadrupolar splitting the investigation of the methyl group of alanines has proven useful as it is directly attached to the peptide backbone and the Cα–Cβ bond exhibits a well-defined alignment relative to the helix long axis (59.4°, cf. reference [68]). Furthermore the three equivalent deuterons are in fast exchange at ambient temperatures [69] and the superposition of the intensities from three sites increases the sensitivity of the ^{2}H NMR measurement. The orientation dependence of this interaction is $\Delta\nu_Q = \frac{3}{2}\left(\frac{e^2qQ}{h}\right)\left(\frac{3\cos^2\Theta - 1}{2}\right)$, with Θ being the angle between the Cα–Cβ bond and B_o. At room temperature, when the three deuterons of the methyl group are equivalent, and depending on the molecular

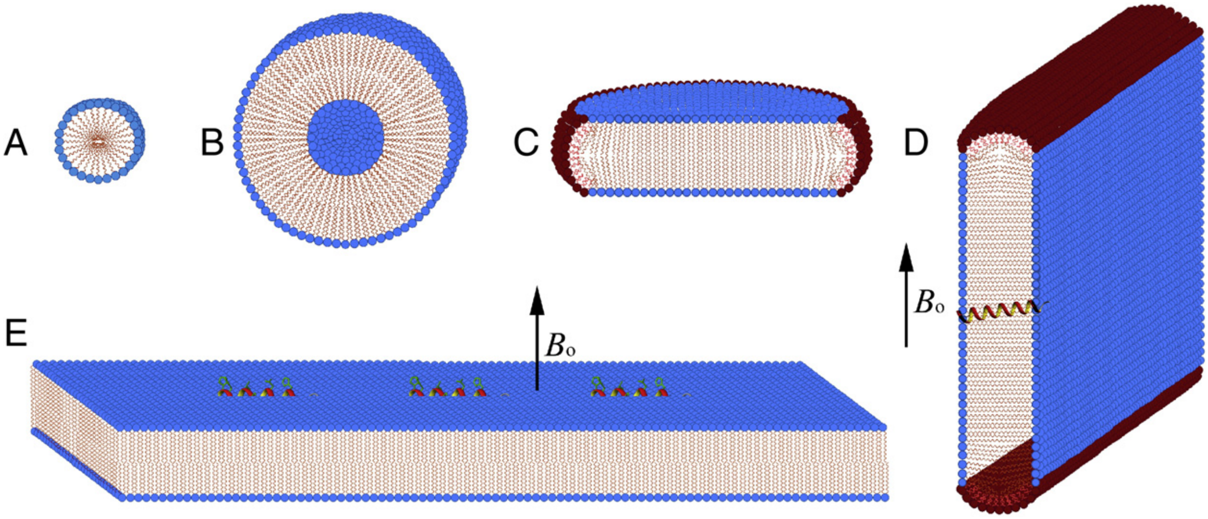

Fig. 1. Schematic representation of (A) a detergent micelle, (B) a small phospholipid vesicle, (C) bicelle, (D) the representation of a transmembrane helical peptide associated with a ribbon-like assembly formed by mixtures of short-chain/long-chain phospholipids under conditions of magnetic alignment and (E) a planar phospholipid bilayer as it occurs in mechanically oriented systems.

alignments quadrupolar splittings of up to 80 kHz are observed for the deuterated methyl group of alanine [69].

3. Investigations of the structure, dynamics and topology of membrane-associated polypeptides by oriented solid-state NMR spectroscopy

The orientational dependence of the solid-state NMR measurables has been used to restrict the possible spatial alignment of peptide bonds and of given molecular structures relative to the magnetic field direction (B_o). In cases where the bilayer normal is oriented parallel to B_o the topology of membrane-associated peptides and proteins is thus obtained (Fig. 1E). In such an arrangement the unique properties of the ^{15}N chemical shift tensor of the amide bond allow one to read the approximate helical tilt angle directly from the ^{15}N chemical shift [8]. Using this approach the approximate topology of the antimicrobial peptide derma distinctin K was recently determined [70]. When additional measurements are obtained from such a sample it is possible to further restrict the possible membrane alignment of bonds, domains or the peptide as a whole. In general the most probable orientation can be selected from a limited number of topologies by molecular modelling (MM) and energetic considerations [57,59,70–73]. For example, early on the secondary structure and membrane alignment of the antimicrobial peptide magainin 2 has been analyzed by labelling eight residues with ^{15}N [74]. Among the low energy secondary structures only a right-handed α-helix oriented parallel to the membrane surface agreed with the experimental ^{15}N chemical shift and ^{1}H–^{15}N dipolar couplings.

More recently it has been demonstrated that the ^{2}H quadrupolar splitting of deuterated methyl-alanines provides information on the alignment of the Cα–Cβ bond which ideally complements the ^{15}N chemical shift information from a backbone labelled site. Combining the two measurements not only restricts the possible tilt angles but allows one to also define the rotational pitch angle of helical domains quite accurately [59,71,75]. This approach has been used previously to analyze in considerable detail the membrane topologies of several helical peptides [59,66,71] and will be illustrated in Fig. 2 where the orientation of the helical domain of the cationic antimicrobial peptide phylloseptin-2 (PS-2) [76] is determined. This antimicrobial peptide was originally isolated from the skin secretion of the tree frog *Phyllomedusa hypochondrialis*.

In a first step the peptide is prepared by solid-phase peptide synthesis carrying a ^{15}N label at the backbone position 18 and a deuterated methyl group at the alanine-11 position, reconstituted into oriented bilayers and the ^{15}N chemical shift and ^{2}H solid-state NMR spectra recorded (Fig. 2A, C). Additional information can be obtained from a two-dimensional separated local field spectrum which reveals the ^{1}H–^{15}N dipolar coupling in addition to the ^{15}N chemical shift (Fig. 2B). For the following spectral simulations the peptide structure (PDB 2JPY) is rotated around two axes in a stepwise manner (180×180 steps) thereby screening all possible tilt and rotational pitch angles of the helix, and for each alignment the chemical shift, dipolar coupling and quadrupolar splitting are calculated. If these agree with the measurements, including experimental errors, the angular pair is indicated by the contour plot shown in Fig. 2D. Topologies that agree with the ^{15}N chemical shift are shown in black, those that fit the dipolar coupling in turquoise and those that fit the deuterium quadrupole splitting in red. A closer inspection of Fig. 2D reveals some interesting features. First, the ^{15}N chemical shift by itself restricts the tilt angle but not the rotational pitch. Second, the dipolar coupling largely confirms the tilt angles from the ^{15}N chemical shift measurements but the combination of both, represented by the overlap of the areas in black and turquoise, still leaves a continuum of tilt/pitch angular pairs. In contrast the combination of ^{15}N chemical shift and ^{2}H quadrupolar splitting results in only five possible combinations which are circled in Fig. 2D illustrating the highly complementary nature of the two measurements. Third, among the five possible solutions which agree with two restraints simultaneously, the in-plane alignment IV is the most favorable one as it is the only topology that does not show the insertion of polar residues in the membrane interior. Finally, when all three parameters are combined with each other topologies I and IV are the ones that agree with all three experimental restraints. Although the topology I is very unfavorable energetically, it is possible to resolve the remaining ambiguity experimentally by preparing an additional peptide labelled with $^{2}H_3$ at the alanine position 8. The additional experimental quadrupolar splitting of this site ($\Delta\nu = 50$ kHz) results in the angular restraints which are represented by the contours shown in magenta in Fig. 2D. Indeed the combination of ^{15}N chemical shift (position 18) and two ^{2}H quadrupolar splittings (positions 8 and 11) only agrees with topology IV.

By extending the concept of using orientational restraints for membrane protein structure determination furthest Tim Cross and coworkers early on prepared a series of pentadecameric gramicidin A sequences by solid-phase peptide synthesis and labelled specific sites with stable isotopes. After reconstitution into oriented membranes the structure of this peptide in its bilayer-associated state was

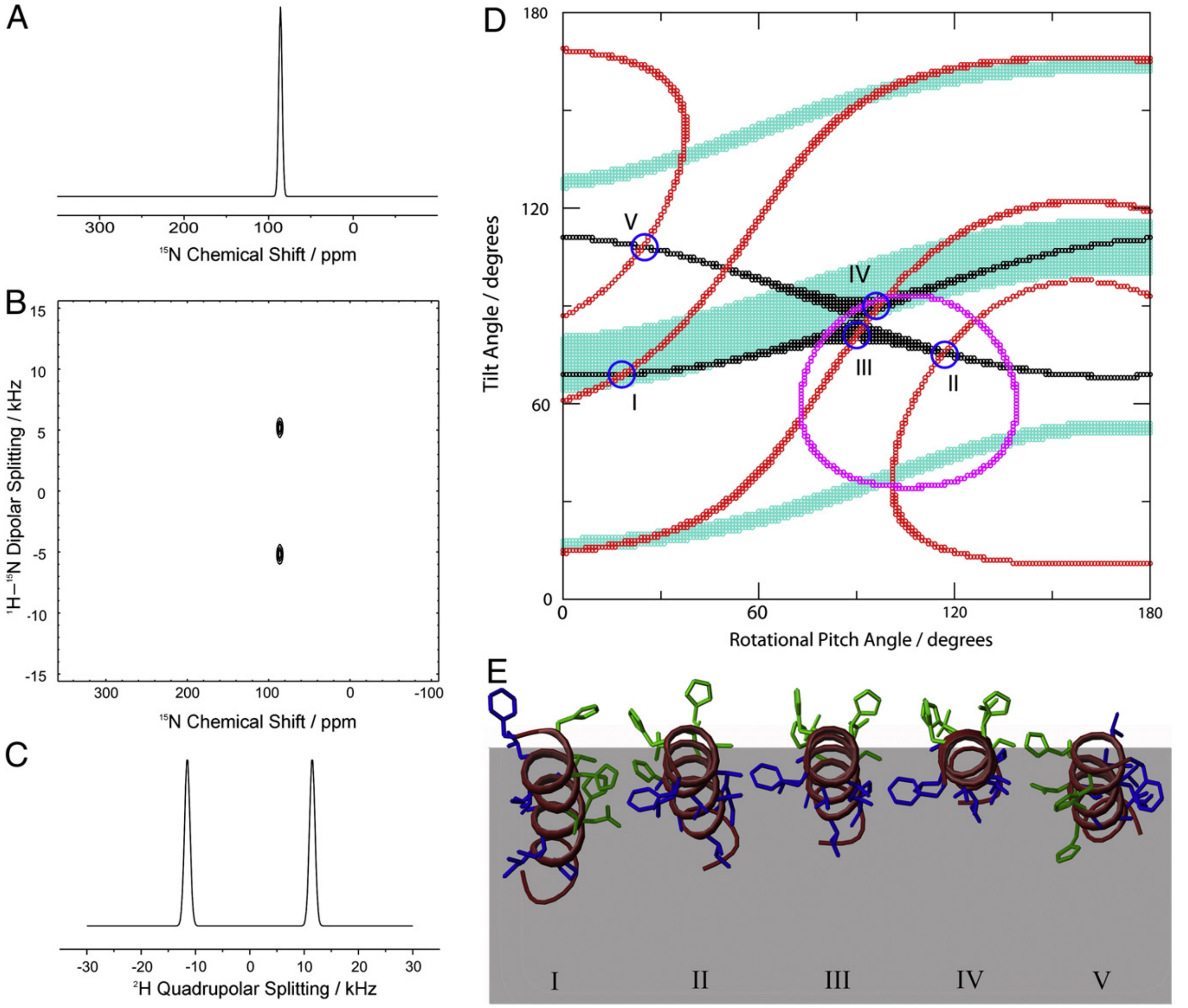

Fig. 2. (A) The proton-decoupled ^{15}N chemical shift-, (B) separated local field- and (C) deuterium solid-state NMR spectra are shown that would result from a perfect in-planar alignment (tilt angle/pitch angle 90°/96°) of the antimicrobial peptide PS-2 (PDB structure 2JPY) labelled with ^{15}N at the backbone position 18 and with 2,2,2-$^{2}H_3$-alanine-11. (D) The contour plot that results from experimental measurements of the ^{15}N chemical shit (86 ± 1) ppm are represented in black, the ^{2}H quadrupolar splitting of alanine-11 (23 ± 1) kHz in red, of alanine-8 (50 ± 1) kHz in magenta and the ^{15}N–^{1}H dipolar coupling (10.4 ± 1) kHz in turquoise. The angular pairs that agree with both the ^{2}H quadrupolar splitting of alanine-11 and the ^{15}N chemical shift are circled and the corresponding topologies relative to the membrane illustrated in panel (E). Among these, the perfect in-plane alignment shown in IV agrees with all these measurements.

determined at high resolution by taking into account 120 orientational restraints [7].

Whereas solid-phase peptide synthesis is ideally suited to introduce isotope labels at specific sites an alternative approach consists in preparing polypeptides biochemically and labelling them uniformly or selectively (one type of amino acid). As many membrane-active peptides and proteins are toxic to bacterial cells special precautions, such as their neutralization by fusion to other proteins or their production as inclusion bodies, have to be taken (e.g. [77], and references cited therein). However, with increasing length of the sequence the biochemical preparation becomes a more and more attractive alternative as the chemical preparation of long polypeptides is time consuming, expensive and associated with low yields.

With the availability of uniformly ^{15}N labelled sequences it is possible to measure many correlations between ^{15}N chemical shift and the ^{1}H–^{15}N dipolar coupling in a two-dimensional solid-state NMR experiment (Fig. 3) [78] albeit at present assignment of resonances from such spectra is far from routine [62,63]. Although the unique elements of the dipolar interaction and the chemical shift tensor (σ_{33}) deviate only by about 18 degrees [67], and therefore the ^{15}N chemical shift and the ^{1}H–^{15}N dipolar coupling exhibit a closely related angular correlation with respect to the magnetic field direction (cf. Fig. 2D), the measurements of many such cross peaks from a uniformly labelled sample can confirm and detail models for the secondary structure (Fig. 3) [56,79,80]. In the case of helical structures also the tilt angles relative to the membrane normal are directly calculated from 'PISA helical wheels' that are visible in these spectra (Fig. 3A–D) [79]. When combined with the selective labelling of one or a few amino acid types the cross correlation peaks can be assigned and the PISEMA spectra provide information about the tilt and the rotational pitch angles [53]. Furthermore, deviations from perfect helical structures such as kinks or gaps have been analyzed [53,81].

It should be noted that under conditions of fast rotational diffusion around the membrane normal the proton-decoupled ^{15}N and ^{13}C solid-state NMR spectra of non-oriented samples can also be analyzed to extract angular information about the membrane-associated peptides as the resulting partially averaged 'powder pattern line shapes' are strongly dependent on the axis around which motional averaging occurs

[8,82,83]. The effects of motional averaging have also been used to follow peptide oligomerization in the membrane [84]. This approach requires that the same sample used for structural analysis is tilted by 90° thus that the bilayer normal is aligned perpendicular to the magnetic field direction (e.g. Fig. 1D). For example, when a deuterated methyl group of alanine is investigated in this arrangement many different orientations of the Cα–Cβ vector coexist and contribute to the ^{2}H solid-state NMR spectrum. Whereas the spectra of oligomers are thus characterized by a distribution of resonances fast diffusion of monomers results in spectral averaging and a single quadrupolar splitting is again observed [84]. It is also possible to study such 90-degree tilted samples by proton-decoupled ^{15}N solid-state NMR spectroscopy. As the time scales needed to reduce the ^{15}N chemical shift anisotropy is considerably slower the transition from fast to slow averaging occurs when complexes of considerably bigger size form [84].

In order to better understand the effects of averaging on anisotropic parameters such as ^{15}N chemical shift and ^{1}H–^{15}N dipolar couplings solid-state NMR spectra have been simulated for helical and β-sheet proteins when motions occur [84–86]. Furthermore, an extensive analysis of the dynamic aspects of a single residue of alamethicin has recently been obtained using three orientation-dependent parameters [87]. Finally, the side chain dynamics of membrane-associated proteins can be evaluated using dedicated pulse schemes and/or line shape analysis [62,88,89].

4. Existing limitations and ongoing developments

Whereas lipid bilayers provide the ideal 'native' environment for the structural investigation of membrane polypeptides the preparation of samples and the concomitant solid-state NMR approaches often remain technically challenging and time consuming. Due to the inherently low sensitivity of NMR spectroscopies large quantities of pure and difficult to prepare proteins need to be over-expressed, labelled with stable isotopes and purified. Furthermore, additional complications arise from the requirement to reconstitute these membrane proteins into oriented lipid bilayers and to keep them functional during many days of preparation and NMR signal averaging.

As the resonances are broadened by dipolar and other anisotropic interactions most solid-state NMR measurements have to rely on the acquisition of the less sensitive hetero-nuclei such as ^{13}C, ^{15}N and ^{2}H rather than ^{1}H, which among all stable nuclei exhibits the highest γ, thereby the highest sensitivity but concomitantly also the strongest dipolar couplings. Furthermore, when compared to solution NMR spectroscopy the signal intensity of solid-state NMR spectra is distributed over a broader width, which further reduces the signal-to-noise-ratio. These problems are most pronounced for static oriented (and non-oriented) membrane samples where the line width and spectral window is typically again an order of magnitude increased when compared to MAS solid-state NMR spectroscopy. At the same time, as detailed above, the line width and shape of these resonances contain valuable information on the conformational and topological heterogeneity of the membrane-associated polypeptide, which are often functionally important and inherent properties of these sequences [90–92].

Solid-state NMR spectroscopy has already in the past provided valuable structural information and major advancement of the technique can be expected when it is possible to overcome the present lack of sensitivity where recording a two-dimensional spectrum can take many days even for samples consisting of several milligrams of labelled polypeptide [54,92]. Whereas high magnetic fields have already ameliorated the situation [93,94] novel developments such as dynamic nuclear polarization (DNP) promise to boost the sensitivity of solid-state NMR spectroscopy by about two orders of magnitude [48]. Notably DNP/solid-state NMR has already been performed on oriented bilayer samples [95] as well as on membrane proteins [48]. With such developments the application of three- and four-dimensional NMR experiments should become possible [96] and much extend the ease of structural investigations on oriented membranes as well as and the conformational details that can be obtained.

5. Micelles, bicelles, and bilayers

In the mean time solid-state NMR spectroscopy has been combined with other approaches such as solution NMR spectroscopy and

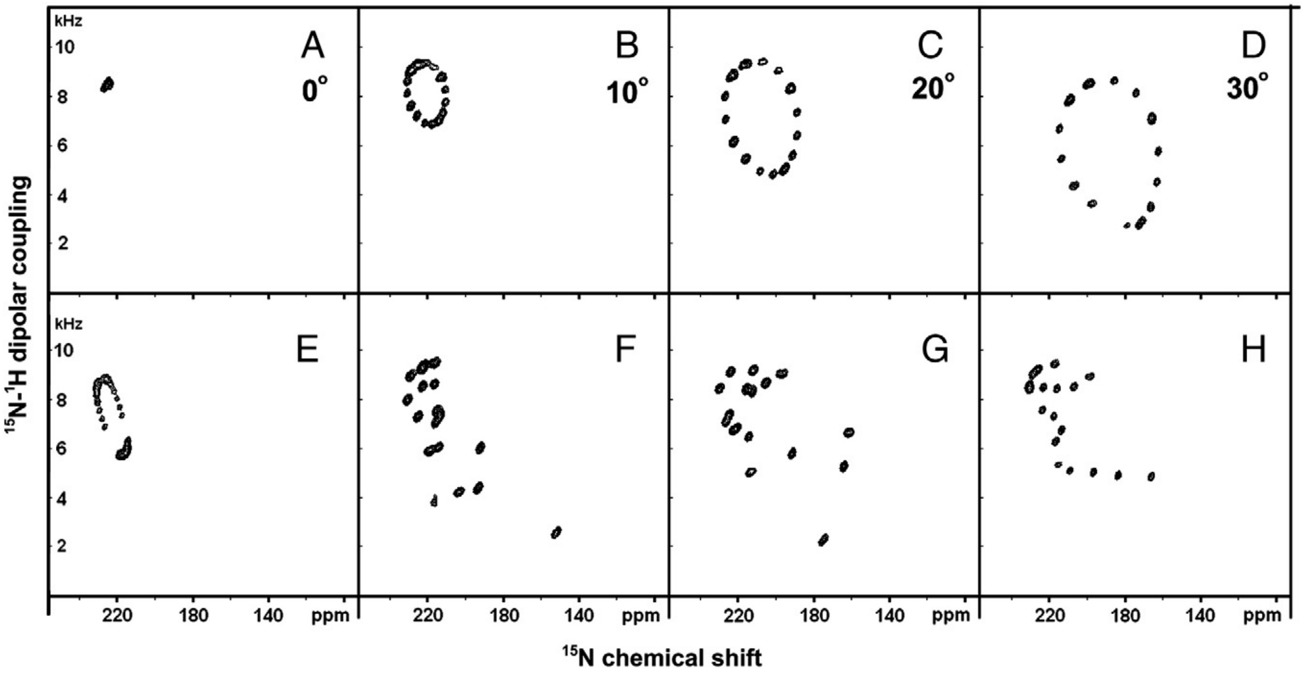

Fig. 3. The figure illustrates the changes of PISEMA spectra upon alterations in tilt angle and/or structure. Panels A–D are from α-helical structures with the indicated tilt angles (0–30°). Panel E. is the spectrum resulting from a 3_{10} helix oriented at 10° and F–H are simulations of the spectra obtained from the three alamethicin conformers that are found in the crystal structure (1AMT). The N-terminal helix of alamethicin was oriented at 10° in F–H.

computational techniques. In some cases it has been possible to investigate the structure of polypeptides associated with lipid bilayers by transfer NOE measurements [97] provided that the off-rate into the isotropic environment is fast when compared to the relaxation time T_1 [98]. Alternatively valuable information on the structure of bilayer-associated melittin was obtained by investigating the proton-deuterium exchange in a position-dependent manner using solution NMR spectroscopy [99]. However such techniques need to be tested and implemented for each polypeptide-membrane system individually and they are not always applicable.

In order to apply the well-established solution NMR spectroscopic techniques to the investigation of biomolecules the reorientational correlation times have to be short in order for the anisotropy of NMR interactions to average and for the observation of sharp well-resolved lines. In the simplest approach hydrophobic or amphipathic membrane polypeptides are dissolved in organic solvents, which mimic to a limited extent the hydrophobic membrane interior. However, a much better model of the membrane interface is obtained when detergent micelles (Fig. 1A) or bicelles (Fig. 1C) [100,101] are used to solubilize these peptides and proteins. It has been demonstrated that by matching the hydrophobic diameter of the micelles to the hydrophobic thickness of the membrane protein the resulting spectra are often adequate for high-resolution structural investigations [4], albeit the line widths of NMR resonances of even small peptides are comparatively large when associated with such supramolecular assemblies (15–30 Hz for ^{1}H, Fig. 4).

Despite the extensive use of micelles to mimic the membrane environment, it should be kept in mind that they form highly curved interfaces that can significantly alter the structural features of the polypeptides [102–104]. Therefore an additional improvement is obtained in simulating the morphology of cell membranes by mixing long- and short-chain phospholipids that spontaneously assemble into bicellar structures (Fig. 1C). These encompass a bilayer-like phase formed by the long-chain lipids that is surrounded by a rim of short-chain lipids. By choosing the temperature and the ratio q between short-chain- (typically DHPC) and long-chain lipids (such as DMPC) it is possible to tune the properties and size of the supramolecular assemblies [105]. For example at ratios $q \leq 0.5$ small bicelles form with favorable line width in solution NMR-based structural investigations of peptides and proteins [106–108].

Interesting insights into the effects of membrane curvature and polypeptide structure have been obtained when the conformations obtained in the presence of micelles and bicelles are compared to each other. For instance, in anionic bicelles the 22-residue peptide motilin exhibits a β-turn between residues of Pro-3 and Thr-6 and a straight α-helical structure extending from residue Gly-9 to Lys-20, (Fig. 5A). In the presence of SDS micelles the peptide exhibits a smaller helical content and a bent conformation [109]. The structure of the 33-residues amphipathic peptide dermadistinctin K shows a clear banana shape [70], which correlates well with the micellar curvature (Fig. 5B). Whereas the oriented ^{15}N solid-state NMR spectra of the peptide indicate an alignment approximately parallel to the membrane surface fluorescence spectroscopy shows the partial membrane insertion of Trp-3 [110].

When a peptide segment of the HIV-1 envelope protein gp41 (residues 282–304) in the presence of DHPC micelles or DHPC:DMPC bicelles are compared to each other a pronounced curvature is observed for the peptide in the micellar sample, a feature that is less pronounced and even inverted in direction in the bicellar environment [103]. Finally the transmembrane segment of Integrin β3 (residues 685–727) exhibits high contents of α-helix in the presence of DPC micelles and DHPC:POPC ($q = 0.3$) bicelles [111] (Fig. 5C, D). However, the structures significantly differ between Lys-716 and the C-terminus, which corresponds to the region following the first charged residue at the intracellular side. In addition, the linearity of the helix is disrupted in the micellar solution therefore slightly shortening the helical segment (Fig. 5D).

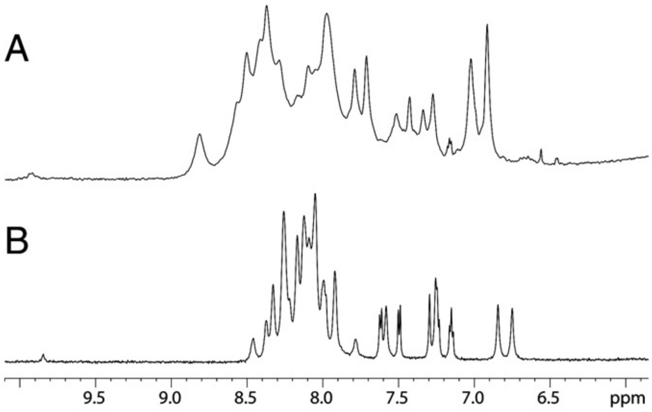

Fig. 4. ^{1}H NMR spectral region encompassing the amide resonance of the cationic antimicrobial peptide dermadistinctin K in the presence of (A) SDS-d_{25} micelles and (B) in a mixture of TFE-d_2/H_2O (50:50, v/v).

Furthermore, by carefully selecting the composition and environmental conditions mixtures of short- and long-chain phospholipids have been made to align with the magnetic field of the NMR spectrometer (Fig. 1D) [112]. In case of DHPC/DMPC an isotropic bicellar phase transforms in a wormlike nematic phase that interacts with the magnetic field once a narrow range of higher temperatures is reached [113]. On the one hand molecules that reside in the aqueous phase of these bicellar suspensions give NMR spectra where small residual dipolar and chemical shift anisotropies (a few Hz) provide valuable additional information for the structure determination of biological macromolecules [114–116]. On the other hand, these effects are considerably more pronounced for molecules strongly interacting with membranes (order parameters in the range of 0.8 and anisotropies of several kHz) and can be investigated by solid-state NMR techniques [117].

6. Combinations of solid-state NMR spectroscopy with other approaches and the influence of dynamics and averaging on solid-state NMR spectra

In order to improve the quality of the resulting structures and/or to accelerate the process of structure determination oriented solid-state NMR spectroscopy has been combined with other approaches such as solution NMR spectroscopy. Studying the conformational characteristics and dynamics of a membrane-associated peptide by solution NMR in an appropriate environment and determining the alignment of the resulting structures relative to the membrane can represent a considerable short cut provided that the structure in the micellar or bicellar environment represents sufficiently well the features of the polypeptide in the bilayer. In such cases it is possible to determine a few angular restraints in order to determine the topology of membrane-associated peptides and proteins relative to the bilayer normal (Fig 2).

For example, in solution NMR investigations the cationic antimicrobial peptide phylloseptin-2 (PS-2) exhibits an amphipathic helical conformation extending from the Ile-5 up to the C-terminal carboxy amide [76]. By reconstituting PS-2 selectively labelled with ^{15}N and ^{2}H into oriented phospholipid bilayers as outlined before, it is possible to define the peptide-membrane topology of the helical domain of this membrane-associated polypeptide in considerable detail (Fig. 2). Other recent examples from our laboratory include the heterodimeric peptide distinctin [59], as well as demastinctin K [70], cathestatin [57], alamethicin, ampullosporin [56], arenicin, chrysophsin [58] or designed peptides [118].

Notably, the line shapes of individual resonances observed in oriented spectra are indicators of the orientational distribution relative to the magnetic field direction of the bonds or the molecule as a whole.

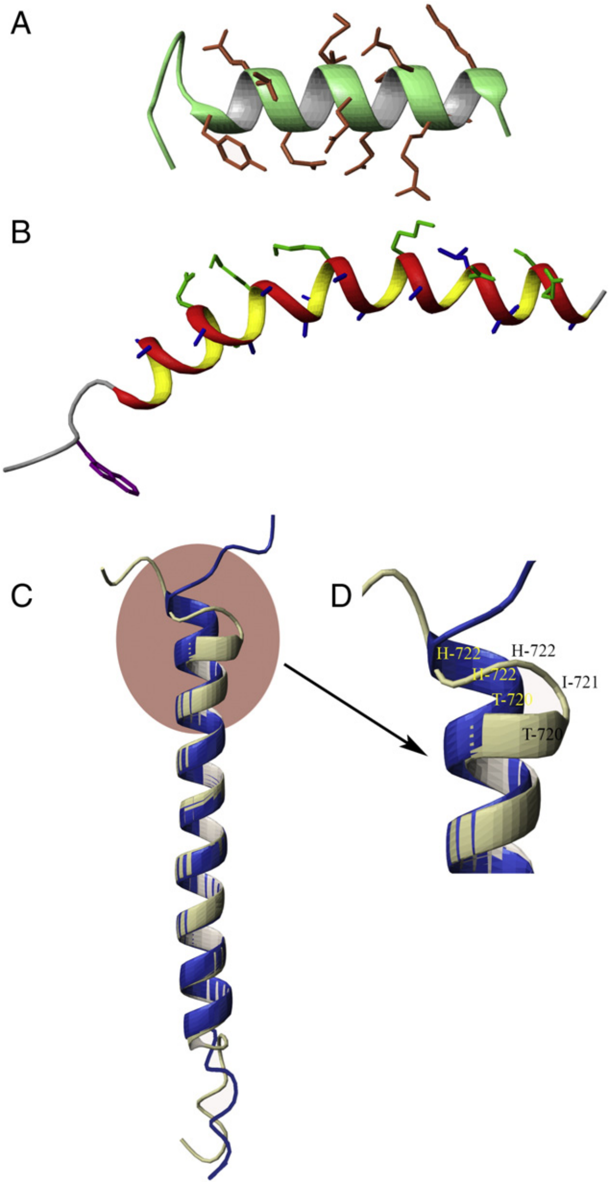

Fig. 5. Three dimensional structures of (A) motilin in the presence of DMPC:DHPC bicelles ($q = 0.5$), (B) of dermadistinctin K in the presence of DPC micelles and (C) structures of the transmembrane segment of Integrin β3 in the presence of presence of DHPC:POPC bicelles ($q = 0.3$) (in dark blue) and DPC micelles (in light grey) superposed between residues of Ile-693 and Lys-716). Panel (D) highlights the main differences between the structures presented in (C). PDB ID codes 1LBJ, 2K9B, 2RMZ and 2RN0.

They thereby represent local or global motions of the polypeptide which can be essential for its functioning and they often provide important information on the heterogeneity of the sample and thus the co-existence of mono- and oligomers, of conformational exchange and/or sample mosaic spread [81]. For example, the structure of the membrane-associated heterodimeric peptide distinctin has been developed and compared to its solution NMR structure, and a careful analysis of the line shapes has revealed the presence of a minor topological population, where chain 1 is rotated around the helix long axis [59]. Whereas chain 2 of this heterodimeric sequence strongly interacts with the membrane and anchors the peptide chain 1 exhibits a much more flexible character.

Furthermore, one-dimensional ^{15}N chemical shift or PISEMA spectra can be used to monitor changes in the helix alignment as a function of lipid composition [119] and gradual tilt angle alterations have been observed for antimicrobial peptides or model sequences as a function of the hydrophobic thickness of the membrane [56,120,121]. In contrast, pronounced changes in topology occur for some peptides when the lipid composition of the membrane is altered. For example, alamethicin changes from a transmembrane to an in-planar alignment when the POPC is replaced by POPE [119]. Major topological changes are also observed when lysine residues were successively inserted into an hydrophobic sequence [90], or when histidines become charged upon acidification of the environment [122].

The deuterium line shape of the alanyl-methyl group is an even more sensitive indicator of the mosaic spread of the alignment vector where reorientation of the $C\alpha–C\beta^2H_3$ group by as little as 1° can translate into changes in the deuterium quadrupole splitting of up to 2 kHz (Fig. 6). This same effect results in pronounced alterations in the deuterium line shape when the labelled peptide exhibits mosaic spread [66] and has been used to considerably refine the alignment of peptides labelled with one ^{15}N and one $^2H_3$ after reconstitution into oriented membranes [59,66,123,124]. The remarkable sensitivity of the deuterium quadrupole splitting has become obvious when the deuterium spectra of a model peptide reconstituted into a series of phosphatidylcholines are recorded, where the ones obtained in DPPC or DOPC exhibiting significant differences [66].

A somewhat different approach was chosen by establishing an algorithm in which conformational and topological restraints from both techniques [73,125] were used during the structure calculations, thereby adding atomistic details to our view of the supramolecular protein–lipid complex [126]. By combining state-of-the art multidimensional solution and oriented solid-state NMR techniques Gianluigi Veglia and co-workers investigated the structure of the membrane-embedded Ca-ATPase regulator sarcolipin. Importantly, by doing so they were able to add considerable refinement to the resulting structures.

Whereas MM and energy minimization were early on used to select the most probable peptide topology among a limited number of orientations that result from the solid-state NMR experimental restraints (e.g. Fig. 2), computational approaches play a more and more important role in the analysis and refinement of such NMR structures. During the years novel algorithms and strategies in combination with the continuous increase in computational power have permitted an extension of the time frame and of the complexity of molecular modelling (MM) and molecular dynamics (MD) calculations. Thereby these theoretical approaches have created models of peptides in lipid bilayers, which deliver a dynamic high-resolution view on the conformations and interactions within membrane environments [127–132] as well as their membrane insertion process [133,134]. The methods develop their full strength when combined with experiment

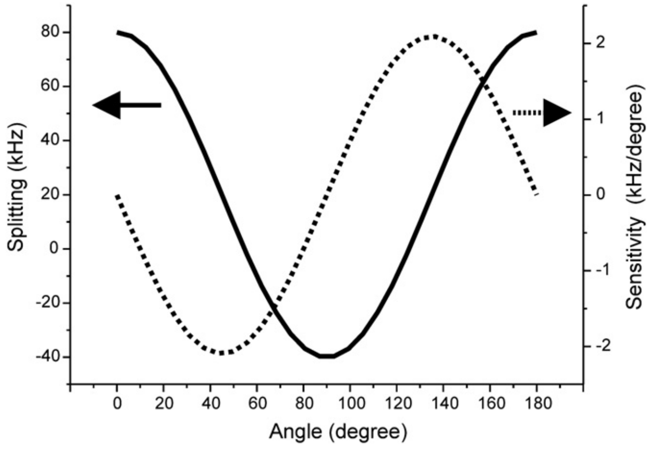

Fig. 6. The figure illustrates the sensitivity of the ^{2}H quadrupolar interactions of $^2H_3$-alanine as a function of the angle of the $C\alpha–C\beta$ bond relative to the magnetic field direction.

where the latter either provide structural constraints that limit the degrees of freedom that need to be explored by the calculations and/or to provide the feed-back necessary to exclude artefacts.

In combination with solid-state NMR measurements MD calculations have been used to better analyze and understand the experimental angular restraints or to resolve ambiguities between complementary techniques. For example, MD calculations show that the tryptophan 9 side chain of membrane-associated gramicidin A occupies two different conformations, and this observation has allowed the reconciliation of differences between the structures of the peptide in detergent micelles and in oriented lipid membranes [135]. Furthermore, by comparing MD and solid-state NMR data the main binding sites for Na^+ within the channel could be identified [136]. Comparative studies were also performed on nuclear localization sequences in their bilayer environment where the helical tilt angles in phospholipid bilayers of different hydrophobic thickness as obtained from solid-state NMR or MD are in good agreement [137]. More recently the team of A. Ramamoorthy has determined the helical tilt angle of the transmembrane segment 2 of the $GABA_A$ channel and used MD simulations to obtain information on the oligomerization and the interaction sites of these protein domains in explicit lipid bilayer membranes [138].

Whereas in the case of WALP model peptides considerable differences in the tilt angle were observed between experiment and previous MD calculations [139,140], simulations using the MARTINI force field [141] show excellent agreement in the relative changes of tilt and rotational pitch angles when single arginines are introduced into the sequence [68]. However, when the absolute topologies are compared to the solid-state NMR data somewhat larger tilt angles are observed in the calculations [68]. The experimentally determined angles for this model compound tend to be 10–30° below those obtained in MD simulations and it has been suggested that this is partially due to averaging around the helix long axis which, if not taken into consideration, results in the underestimation of the tilt derived from such experimental ^2H NMR data [119,140]. Indeed, models in which the peptide dynamics were described by Gaussian distributions that result from orientational variations around the helix main axis (azimuthal fluctuations) and/or explicit variations of the helix tilt (wagging fluctuations) resulted in more consistent results with low RMSD values [140,142]. In addition, the calculations provide additional details with regard to the motions of these peptides in membranes.

This data show, that partial averaging of the anisotropic NMR interactions add an additional layer of complexity to the analysis of oriented solid-state NMR data obtained from membrane-associated polypeptides. In particular the motions can be quite different depending on the site, domain and a variety of environmental parameters such as lipid composition, temperature and hydration. If not considered appropriately, considerable deviations may result when the orientational restraints are deduced from the experimental spectra.

Importantly, molecular dynamics simulations have allowed one to reproduce the averaging of anisotropic NMR parameters that occur in the liquid crystalline membrane system. Numerical and analytical evaluation of the effect that such motions have on the anisotropic solid-state NMR spectra indicates, that rotational diffusion around the helix long axis only marginally affects the ^{15}N chemical shift [119], which therefore remains a robust measure of the tilt angle [8], whereas the ^2H quadrupolar couplings of the alanine $^2H_3$-group are strongly influenced by such motional averaging [84,119]. An all atom MD simulation protocol has been developed, that uses the orientational constraints to establish an additional force field which drives molecular rotations and re-reorientations thus that the time average reproduces the experimental parameters, in particular the spectral alterations that are observed between membranes of different hydrophobic thickness [143]. As these calculations take place *in vacuo* a better image of the dynamics in the membrane can probably be obtained from placing the molecules in an interfacial environment [90,102] or, with the increasing possibilities of modern computers, in a coarse grain or atomistic model of the membrane [141,144]. Furthermore, more recently QC calculations were combined with oriented solid-state NMR to analyze the order parameter of a model peptide in membrane environments [145].

7. Outlook

Solid-state NMR spectroscopy has the demonstrated capacity to determine high-resolution structures, topology, dynamics and lipid interactions of polypeptides when associated with liquid disordered lipid bilayers. The technique has the potential of a full-fledged structure determination also of larger membrane proteins but adds additional details which are important for their functioning such as conformational exchange, mosaicity and motions. The range of applications of this powerful technique could be enhanced when it will be possible to overcome the present limitations imposed by the inherently low sensitivity of NMR, therefore high magnetic fields and dynamic nuclear polarization are very promising developments for membrane protein structural investigations. The technique is particularly powerful when combined with other biophysical and numerical approaches.

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