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# Solid-state NMR for the study of membrane systems: The use of anisotropic interactions

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**Summary** — The use of solid-state nuclear magnetic resonance (NMR) as a tool to determine the structure of membrane molecules is reviewed with a particular emphasis on techniques that provide information on orientation or order. Experiments reported here have been performed in membranes, rather than in micelles or organic solvents. Several ways to prepare and handle the samples are discussed, like sample orientation and magic-angle spinning (MAS). Results concerning lipids, membrane peptides and proteins are included, as well as a discussion regarding the potential of such methods and their pitfalls (© Société française de biochimie et biologie moléculaire / Elsevier, Paris).

**membrane / solid-state NMR / protein structure / orientation / lipids**

## Introduction: scope of this review

Accurate structure determination of biological macromolecules is imperative for understanding of their function. Besides radiocrystallography, nuclear magnetic resonance (NMR) is one of the most versatile techniques used to determine structures of biological macromolecules. NMR can be used to obtain three-dimensional structures of relatively large proteins in solution [1–6] and even of membrane proteins in organic solvents or micellar systems [7]. With proteins embedded in lipid bilayers, on the other hand, radiocrystallography and NMR fail to determine structures at atomic resolution, though for different reasons. In the former case the difficulty comes from the absence of crystals, while in the latter case it is due to severe line broadening (fig 1).

Spectral manifestations of anisotropic interactions are usually averaged to zero in solution by fast and isotropic motion. In solids, on the other hand, the distribution of orientations is only subject to partial averaging. Often spectral lines overlap and specific residues are generally not resolvable. Membranes, however, are not real solids. In a fluid membrane, proteins and lipids generally undergo fast rotational motions around the normal bilayer. Furthermore, residues are not immobile within a protein or lipid: most lipid or protein segments undergo fast motion in a limited angular domain. Average orientations can be determined and order parameters express the amplitudes of these local motions. Thus, NMR of membrane samples is useful and can provide a dynamic picture of local molecular motions. It can be used in favorable cases for structure refinement based on orientational constraints.

Several recent reviews have been devoted to extensive descriptions of NMR methods that allow, at least in principle, complete structure determination of membrane proteins [8–15]. Generally, these techniques are based on the determination of relative or absolute orientations of molecular segments in membranes (fig 2). New solid-state NMR techniques have proven to be capable of measuring internuclear distances of a few angstroms within molecules embedded inside the membrane. It should be stressed that while these techniques have proven to be useful in lipid studies, only a few structures of membrane peptides were determined completely by solid-state NMR so far. We would like to review briefly the theoretical background underlying NMR of membranes. Through recent examples, we will afterwards indicate the current stage and the directions taken in this field. We show that the large anisotropic interactions in membranes can be either suppressed by techniques such as magic angle sample spinning (MAS) or exploited to investigate membrane organization and structure.

* Correspondence and reprints.

*Abbreviations:* NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; CSA, chemical shift anisotropy; QCC, quadrupolar coupling constant; MAS, magic-angle spinning; SLF, separated local field; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; MLV, large multilamellar vesicles; DISTINCT, dipolar sine term by indirect-coupling transformation; DROSS, dipolar recoupling on-axis with scaling and shape preservation; DMPC, dimyristoylphosphatidylcholine; INEPT, insensitive nuclei enhanced by polarization transfer; CP, cross polarization; $R^2$, rotational resonance; REDOR, rotational echo double resonance.

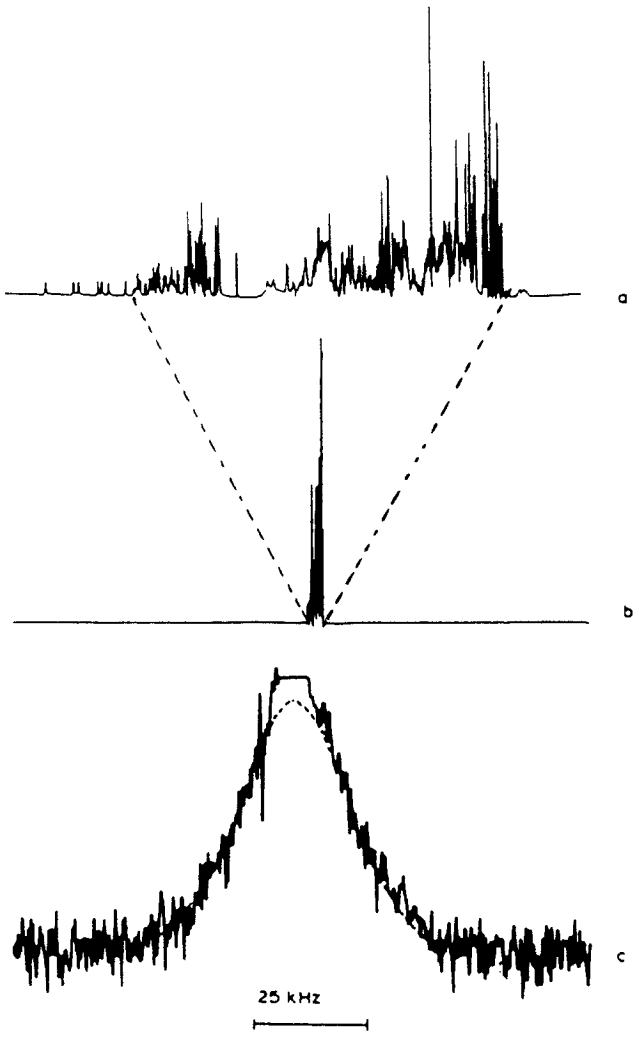


**Fig 1.** Illustration of the different nature of $^{1}$H-NMR spectra of proteins in water and in membranes. **a.** The 400 MHz $^{1}$H-NMR spectrum of bovine pancreatic inhibitor in aqueous solution exhibits an enormous number of sharp lines over a range of a few kHz. **b.** Same spectrum drawn at the scale of (**c**). **c.** The $^{1}$H-NMR spectrum of rhodopsin reconstituted in perdeuterated phospholipids exhibits a featureless single line approximately 25 kHz wide which is due to non-averaged proton-proton dipolar interactions. The sharp features appearing in spectrum (**c**) are due to noise (adapted from [91]).

## Solid-state NMR and membrane samples: theoretical background

Among nuclear spins that are observable by NMR and useful for biological investigations, $^{1}$H, $^{14}$N, $^{19}$F and $^{31}$P are naturally 100% abundant. Others ($^{2}$H, $^{13}$C, $^{15}$N, $^{17}$O, etc) require isotopic labeling or methods that enhance the sensitivity of the NMR experiment. An NMR spectrum results from the interaction of the observed nuclear spin with its magnetic environment. Typically, the nuclear spin interacts with the external magnetic field (Zeeman interaction), with its electronic environment (chemical shift, quadrupolar coupling, etc) and with neighboring nuclei (dipolar and scalar couplings, etc). Some interactions are isotropic while others are anisotropic, like throughspace dipolar couplings, the chemical shift or the quadrupolar interaction of spin $I > 1/2$ nuclei ($^{14}$N and $^{2}$H). As stated previously, spectral manifestations of anisotropic interactions can be averaged to zero by fast isotropic motion of molecules in solution. This occurs when the rotational correlation time is typically 20 ns or less. In that case, a typical NMR spectrum is determined only by isotropic chemical shifts and scalar couplings. It consists of highly resolved lines that can be assigned to different nuclei and their neighbors. Further experiments allow one to determine distances between nuclei through the nuclear Overhauser effect (NOE) [1–6]. Regardless of signal-to-noise considerations, applications of solution-state NMR to proteins have an upper limit corresponding to a molecular mass of about 60 kDa [4–6]. Membrane molecules are motionally restricted and their NMR is usually dominated by anisotropic interactions. For $^{13}$C, $^{15}$N and $^{31}$P spectra, the chemical shift anisotropy is important, while for $^{2}$H and $^{14}$N spectra, the quadrupolar interaction is dominant. Finally, the anisotropy of the dipolar interactions predominates in $^{1}$H spectra but also plays a role for other spins.

### *Chemical shift anisotropy (CSA)*

Electrons around a nucleus interact with the applied magnetic field so that the Zeeman interaction is disturbed and the resonance is slightly shifted. If the electron cloud around the nucleus is anisotropic then so is the interaction. In a solid, where different molecules have different orientations with respect to the external field, the same nucleus on different molecules may have differently shifted resonances and the total signal will be a broad line. A typical chemical shift anisotropy pattern may extend over as much as 100 ppm for $^{13}$C (*ie* 10 kHz at a magnetic field strength of 9 T) and about 200 ppm for $^{31}$P. Some functional groups are more asymmetric than others: a carbonyl carbon will have a more pronounced anisotropy than a methyl carbon for example.

The CSA can be represented by a $3 \times 3$ tensor. In a particular molecule-fixed frame, this tensor is diagonal. The three non-zero elements, called principal components, can be read directly from the NMR spectrum and are usually determined from measurements on polycrystalline samples. This has been done in many different samples including all amino acids [16].

The difference between the spectrum of a polycrystalline powder and the spectrum of a membrane results from orientational order and dynamic effects. For example, the partial averaging of the $^{31}$P-CSA by rapid anistropic motion of membrane phospholipids transforms the static chemical

shift tensor of the phosphorus into an effective average chemical shift tensor with axial symmetry. The broad spectrum observed with unoriented phospholipid bilayers has a maximum splitting, $\Delta\sigma$, which corresponds to $\Delta\sigma = \sigma_{//} - \sigma_{\perp}$, where $\sigma_{//}$ is the chemical shift associated with bilayers oriented parallel to the applied magnetic field $B_o$ and $\sigma_{\perp}$ is associated with bilayers oriented perpendicularly to $B_o$. $\Delta\sigma$ is easy to measure and reflects qualitatively the viscosity of the membrane. However, because the CSA tensor of an immobilized phosphate has no axial symmetry, the spectroscopic parameter $\Delta\sigma$ cannot be related to a single order parameter describing the motion of a phospholipid head group and the spectrum cannot be interpreted rigorously in a simple manner. For this reason deuterium NMR, which gives a more direct relationship to a single order parameter through the anisotropy of the quadrupolar interaction, is often preferred over $^{31}P$ in spite of its lower sensitivity.

*Quadrupolar interaction*

Nuclear spins with I > 1/2 possess an electric quadrupolar moment capable of interacting with the electric field gradient generated by the surrounding electrons. This anisotropic term often dominates the Zeeman interaction. One of the most useful nuclei in biophysics with I = 1 is $^2H$ which gives rise to two transitions. In the case of a $^2H$ bound to a carbon atom, the quadrupolar splitting depends on the angle $\alpha$ between the C-$^2H$ bond and the external magnetic field according to the following formula:

$$\Delta\nu_Q(\alpha) = \frac{3}{2}\chi_Q\left(\frac{3\cos^2\alpha - 1}{2}\right) \qquad (1)$$

$\chi_Q$ is the quadrupolar coupling constant which can be determined from measurements in powder samples; its value is on the order of 170 kHz for a C-$^2H$ bond [17]. Note that the two lines collapse if the angle $\alpha$ is equal to 54.7° (the 'magic' angle). In a powder, every orientation gives rise to a doublet and the sum of all doublets results in a so-called Pake pattern that can be calculated by integration over all $\alpha$ angles. A Pake pattern is also obtained with lipid dispersions. However, due to the fast anisotropic motions of C-$^2H$ residues, the splitting between the two maxima in a membrane is, in general, reduced by about an order of magnitude with respect to crystalline samples. In the case of fluid membranes, the molecular rotation around the bilayer normal is fast, so that the effective averaged symmetry axis of the C-$^2H$ residues from a lipid or a transmembrane peptide is parallel to the bilayer normal. As a result the splitting $\Delta\nu_Q$ is a function of the angle $\beta$ between the normal to the bilayer and the direction of the magnetic field:

$$\Delta\nu_Q(\beta) = \chi_Q\left\langle\frac{3\cos^2\theta - 1}{2}\right\rangle\left(\frac{3\cos^2\beta - 1}{2}\right) \qquad (2)$$

where $\theta$ is the instantaneous angle between the C-$^2H$ bond and the bilayer normal. The term between angular brackets represents a time average over the rapid fluctuations of $\theta$

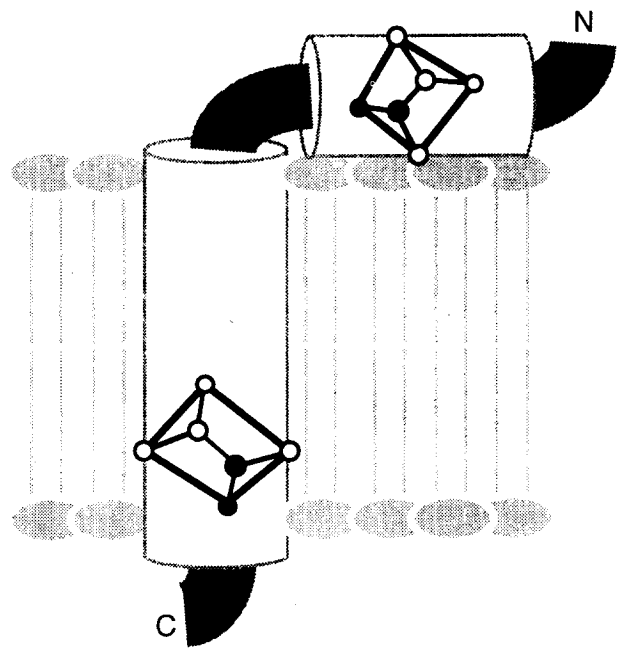


**Fig 2.** The orientation of the peptide planes for Leu-14 and Leu-41 of fd coat protein in phospholipid bilayers, as determined from the $^1H$-$^{15}N$ heteronuclear coupling and $^{15}N$ chemical shift derived from three-dimensional correlation spectra of oriented samples of uniformly $^{15}N$ labeled fd coat protein in phospholid bilayers [15].

and is called the segmental order parameter $S_{CD}$ (see fig 3 for the definition of $\alpha$, $\beta$ and $\theta$). If the bilayer normal wobbles around an average value $\beta_o$, $S_{CD}$ can be decomposed in the following fashion:

$$S_{CD} = \left\langle\frac{3\cos^2\beta - 1}{2}\right\rangle\left\langle\frac{3\cos^2\theta - 1}{2}\right\rangle = S_{wob}\,S_{fluc} \qquad (3)$$

In practice, it is often easy to measure the splitting associated with $\beta = 90°$ for giant unoriented vesicles. Note that different dynamic regimes will affect the lineshapes differently: For slow motion (correlation times larger than $1/\chi_Q$) the line shape will be distorted but the linewidth will remain essentially constant. This is the solid-state NMR regime where simulations of the lineshape provide orientational information. Only one line will be present in the case of fast motion (extreme narrowing limit), as is observed with sonicated membrane vesicles. In such systems, the line width will decrease with decreasing correlation times [17–22].

If the rapid motion of the C-$^2H$ bond does not have an axial symmetry, for example within an immobilized protein, the expression of $\Delta\nu_Q$ is more complicated and involves a complete set of order parameters. In the case of $^{14}N$ which also has a spin I = 1; similar expressions can be written although they are a little more complicated [8]. They will not be discussed here.

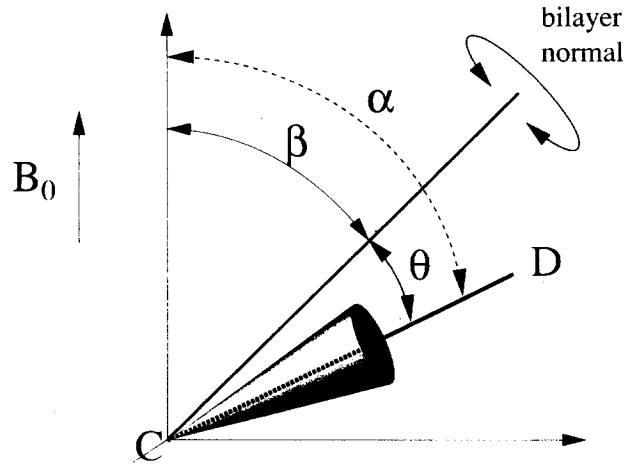


**Fig 3.** Definition of the angles α, β and θ used to describe the local motions of a CD bond around the bilayer normal. α is the angle between the CD bond and the direction of the magnetic field $B_0$; β is the angle between the local bilayer normal and $B_0$; θ is the fluctuating angle between the CD bond and the bilayer normal.

It is clear that the measurement of $^2H$ quadrupole splittings allows one to obtain in certain cases an important dynamic parameter $S_{CD}$. On the other hand, the interpretation of a spectral parameter which relates to the average position of residues undergoing independent or coupled motions will be model-dependent. In other words, the structural information cannot be inferred directly from the spectroscopic data. In the most general case, one would need to determine the whole order matrix, which contains nine elements (of which five are independent) to obtain a complete average structure of a molecule. With incomplete data, one can only determine probabilities and boundaries of conformations, in a model-independent fashion [10, 23].

These limitations explain why segmental order parameters in lipids obtained from $^2H$ NMR are often interpreted in elusive terms, *eg* as 'probabilities of being in the *trans* conformation' [17–19, 24]. We have also seen that a small value of an order parameter could be due either to an angle close to the magic angle in a well-ordered molecular segment or to a wide range of angles in a very disordered region. Both examples have been found in lipids.

*Dipolar interaction*

Unlike the CSA and the quadrupolar interaction, dipolar interactions occur between two nuclei with spins greater than zero. Each nucleus with a dipolar coupling to another nucleus with I = 1/2 gives rise to a doublet. The splitting between the two maxima has a similar form as the quadrupolar splitting:

$$\Delta\nu_D(\alpha) = \frac{\mu_0}{4\pi}\frac{\gamma_i\gamma_j}{r_{ij}^3}\frac{h}{2\pi^2}\left(\frac{3\cos^2\alpha-1}{2}\right) \quad (4)$$

where $\gamma_i$ and $\gamma_j$ are the gyromagnetic ratios characteristic of the nuclei, h is Planck's constant, $r_{ij}$ is the internuclear distance, $\mu_0$ is the permeability of vacuum and α is the angle between the vector connecting the two nuclei and the direction of the external magnetic field. A Pake pattern is again obtained if all orientations are present. If the nuclei *i* and *j* are at a fixed distance but θ is allowed to fluctuate rapidly around an average value $\alpha_0$, one should rewrite equation (4) as follows:

$$\Delta\nu_D(\alpha_0) = \frac{\mu_0}{4\pi}\frac{\gamma_i\gamma_j}{r_{ij}^3}\frac{h}{2\pi^2}\left\langle\frac{3\cos^2\alpha-1}{2}\right\rangle \quad (5)$$

Obviously, the term between brackets depends on a probability distribution. Knowledge of $\Delta\nu_D$ is very valuable since it reflects the distance, the orientation and the relative motion of a pair of nuclei. Two of these parameters have to be determined independently in order to deduce the third parameter. By cooling the sample, the fluctuations of α around $\alpha_0$ can be minimized, at the risk of determining a non-physiological structure. Distances between bound nuclei can be determined from neutron diffraction measurements [25] although these can be misleading since NMR is sensitive in different manner to averaging of vibrational motions [26].

$^{13}C$-$^1H$ dipolar couplings are similar in form to the $^2H$ quadrupolar couplings and therefore similar precaution should be taken with regard to dynamic or structural interpretation. Overall, $^{13}C$-$^1H$ dipolar couplings are approximately one order of magnitude smaller than $^2H$ quadrupolar couplings, which displace the regime over which the motion can be considered to be slow but also reduces the precision with which α can be measured. There are many cases where $\Delta\nu_D$ is very small and difficult to measure: when the distance r is large, when the motion is fast, when α is close to the magic angle, or when the observed nuclei have small gyromagnetic ratios. As a consequence, intermolecular couplings are rarely observable. Theoretical treatment is not easy when a nucleus interacts with many other nuclei, which is typically the case with abundant protons. In the case where these abundant couplings are undesirable, they can be removed by homo- or heteronuclear decoupling.

*The effect of sample orientation*

When working with membrane molecules, one can orient the membrane bilayer mechanically or magnetically in such a way that the bilayer normal has the same orientation throughout the whole sample. Mechanical orientation of bilayers can be achieved by spreading suitably hydrated lipids between thin glass plates. The orientation of the glass plates with respect to the external magnetic field can be controlled

by the experimenter [27]. More satisfactory methods for magnetic orientation of lipid bilayers have recently been developed. They consist in mixing the desired lipid with other molecules that have a large anisotropic susceptibility. The bilayers generated in this fashion, called 'bicelles', spontaneously orient, either parallel or perpendicular to the external magnetic field, depending on the sign of the anisotropy (fig 4) [28–30].

In an oriented sample, the CSA of a given nucleus leads to a single line with a chemical shift that reflects the sample orientation. Similarly, $\beta_0$ can be imposed by the sample orientation, so that the quadrupolar and dipolar interactions are easier to measure. For example, if the macroscopic orientation of the bilayer normal is at 90° with respect to the direction of the magnetic field, the quadrupole splitting can be can be rewritten as:

$$\Delta\nu_Q = \frac{3}{4}\chi_Q S_{CD} \qquad (6)$$

The lineshapes of oriented samples can be obtained with isotropic samples by spectral deconvolution, also called 'de-Pake-ing' [31]. This technique is particularly useful in the case of perdeuterated lipids since it allows one to obtain a whole set of order parameters with a single sample, provided the signal-to-noise ratio is sufficient. Sample orientation not only results in a simplification of the spectra, but also increases the signal-to-noise ratio, since broad lines associated with anisotropic interactions are transformed into narrow lines. To interpret the dynamics, it is sometimes possible to assume, for simplicity, that $\beta$ is a constant, thereby neglecting any fluctuations of the director axis (peptide wobble or membrane undulation, for example), but such approximations should be checked and justified.

*The effect of magic angle spinning*

In addition to sample orientation, another method has been developed to reduce the effect of anisotropic interactions and to increase the signal-to-noise ratio. It consists of placing the sample in a rotor spinning about an axis tilted at the magic angle, thereby giving all anisotropic interactions an additional time dependence. Depending on the spinning speed, interactions that are defined as 'non-homogeneous' can be partly or totally suppressed [32]. Today's technology allows magic angle spinning (MAS) at spinning frequencies of 10 kHz in a routine fashion and 30 kHz with special equipment. Combining sample orientation and MAS by slowly spinning oriented samples is an idea that has been successfully put into practice by Glaubitz and Watts [33].

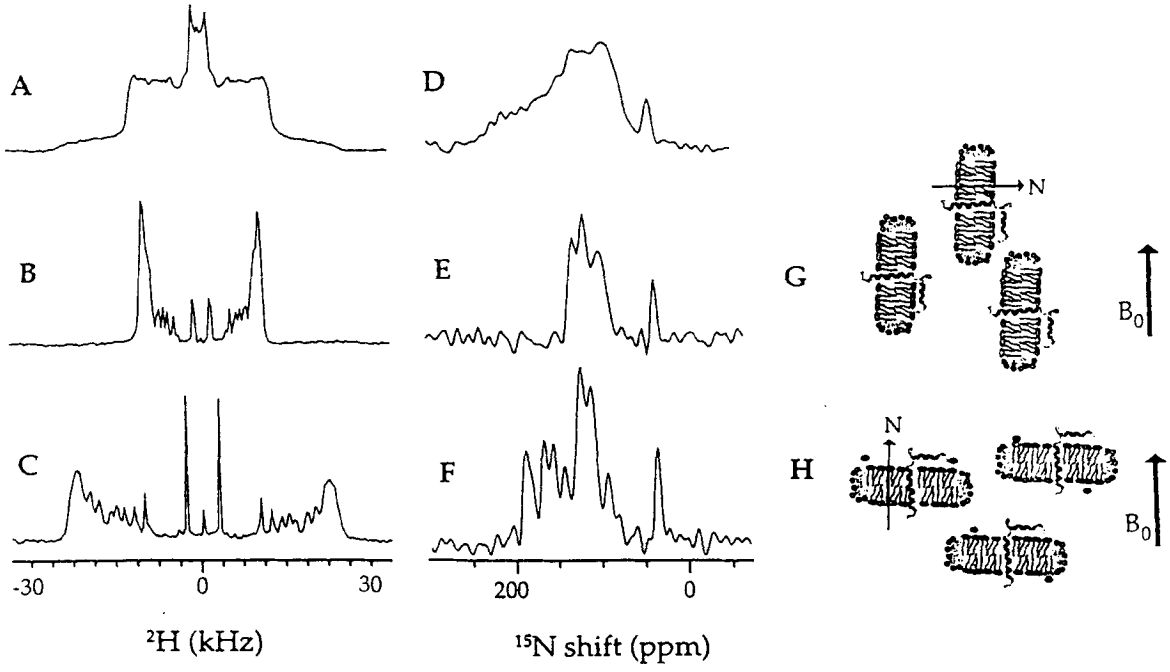


**Fig 4.** NMR spectra of samples containing lipids with perdeuterated acyl chains and a uniformly $^{15}$N-labeled transmembrane protein (fd coat protein). Spectra were recorded either with isotropic samples (**A** and **D**) or bicelles that are oriented with the bilayer normal parallel (**B** and **E**) or perpendicular (**C** and **F**) with respect to the applied magnetic field. Samples (**C**) and (**F**) contain a small percentage of lanthanide ions ($TmCl_3^{3+}$) which change the magnetic properties of the samples and orient the bicelles so that the bilayer normal is parallel to the applied magnetic field. **G, H.** The hypothesized cross-sections of the DMPC/DHPC bicelles containing the coat protein. The directions of the bilayer normal (*N*) and magnetic field ($B_0$) are indicated. (From [49]).

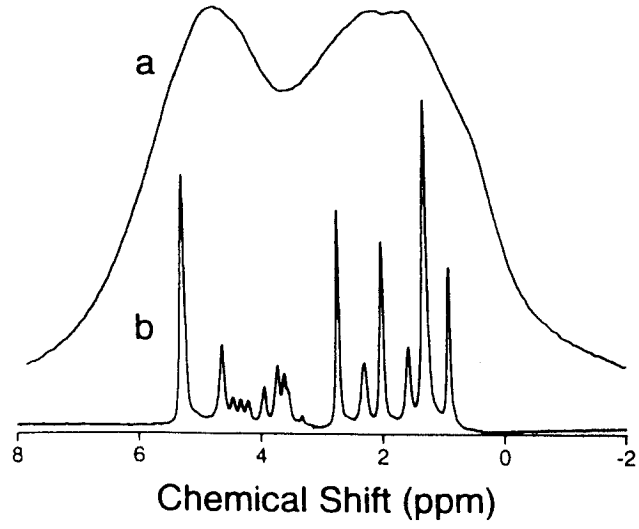


**Fig 5.** $^1$H-NMR spectrum of a suspension of glycolipid (monogalactosyl diacyldiglyceride) in $D_20$. Static spectrum (a); MAS spectrum (b) (spinning speed 6.6 kHz) (from [65]).

The CSA is an non-homogeneous interaction and will be averaged to zero if the spinning frequency is greater than the width of the static line, leaving a single isotropic line. The quadrupolar interaction is also non-homogeneous but can be averaged out only at very high spinning speeds that are difficult to achieve. Heteronuclear dipolar interactions are non-homogeneous but homonuclear dipolar interactions are generally homogeneous and cannot be averaged out by spinning at the magic angle. Lipids and some membrane peptides are rare exceptions to this rule, since fast axial diffusion renders all dipolar interactions non-homogeneous so that they can be averaged by MAS [34, 35] (see fig 5).

In certain cases, MAS allows one to remove the effects of CSA and dipolar interactions, which increases the spectral resolution and the signal-to-noise ratio. High resolution spectra obtained in solids with orientation or MAS sometimes allow the measurement of J couplings, which were previously unobservable in solids, and which can provide additional structural constraints. However, MAS is mostly useful in our quest for orientational information if it can be combined with other methods that reintroduce anisotropic interactions. This is generally performed by synchronizing sample spinning and suitable radio-frequency pulse sequences [36]. Alternatively, spinning about an axis that is deliberately slightly off the magic angle reintroduces all interactions, scaled down by a known factor [37]. By using multi-dimensional techniques and by combining high-resolution and broad-band NMR, one can separate and assign overlapping lines. This technique is called 'separated local field' (SLF) and some examples will be given below.

*Relaxation measurements*

The anisotropic character of magnetic interactions is one of the major causes of spin relaxation. As in the case of soluble proteins the longitudinal relaxation, $T_1$, provides information on fast motions, *ie* with a time scale in the range of nanoseconds, while transverse relaxation, $T_2$, is sensitive to slower motions. Although the relaxation parameters are anisotropic, they are difficult to interpret in terms of orientation and, hence, of structures. They have mostly been used to obtain dynamic information. Relaxation of $^2$H, for example, can help to characterize motions in lipids and proteins on very different timescales [17–22]. When specific residues can be assigned either by chemical labeling, sample orientation or by MAS, $T_2$ and $T_1$ values can be determined for specific atoms. Conventional techniques developed for liquid (inversion recovery and spin echoes) are applicable. For example Trouard *et al* [38] have analyzed the relaxation data at each carbon position of perdeuterated phospholipids for oriented sample in the liquid crystalline state. They found a remarkable parallel between the plots of $T_1$ values or $S_{CD}$ values as a function of carbon position along the acyl chains. Prosser and Davis, on the other hand, studied whole body motions of gramicidin A in lipid bilayers and deduced from deuterium relaxation measurements correlation times for the diffusion of the peptide around its helix axis and for the wobbling motions [39]. Finally several groups have measured variations of $T_1$ and $T_2$ of lipids as a function of increasing presence of integral proteins. They deduced a partial immobilization of the lipids by the proteins [22]. In spite of these investigations, the work carried out on NMR relaxation rates in membrane proteins is much less advanced than it is with soluble proteins. We shall not discuss this subject any further in this review.

## Examples of applications of solid state NMR to membranes

In the following we shall not attempt to give a comprehensive review of all applications of solid state techniques to the investigation of membranes. Our goal is rather to give some representative examples. We should also like to emphasize those techniques that appear to be most likely to have important future applications.

*$^{13}C$ and $^{15}N$ chemical shift anisotropy*

The local orientations of amino acid residues can in principle be deduced from the knowledge of the chemical shift tensors. This has allowed the determination of secondary structure elements and of conformational information in bacteriorhodopsin (molecular mass of 26 kDa), and in other isotopically labeled membrane proteins using MAS NMR spectroscopy [14, 40, 41].

This task is greatly simplified when the molecule itself is oriented. For example, an extensive study of gramicidin A has been carried out by the group of Cross on mechanically oriented lipid membranes. The residual CSA's of each of the 15 amide $^{15}N$ nuclei have been measured, using a different isotopically labeled sample for nearly each measurement. Together with $^{15}N$-$^{13}C$, $^{15}N$-$^{2}H$ and $^{15}N$-$^{1}H$ dipolar couplings, this has led to the first high-resolution structure of a transmembrane peptide measured by solid-state NMR [42]. $^{13}C$ CSA's of gramicidin or melittin carbonyls have also been measured by Cornell and coworkers [27, 43, 44]. Once the secondary structure of a peptide segment is known with relatively good accuracy, it is sufficient to determine only one CSA to deduce if a peptide, or part of it, forms an integral part of a membrane or if it is merely peripheral (fig 2). Similar strategies have been adopted by the group of Opella [8, 11, 15] to determine or refine the structure of other membrane peptides such as the M2 segment of the acetylcholine receptor (23 residues), magainin (23 residues), the Pf1 coat protein (46 residues) and the fd coat protein (50 residues, molecular mass of 5.5 kDa).

Using magnetically oriented lipid bilayers (bicelles), the groups of Sanders and Prestegard and collaborators have measured several $^{13}C$ and $^{15}N$ CSA's of lipids and of some membrane proteins [45–48]. Recently, Howard and Opella [49] have also measured $^{15}N$ CSA's of the fd coat protein using bicelles of the 'first generation' (*ie* with bilayer normal perpendicular to the field) or the 'second generation' (normal parallel to the field). Figure 4 compares $^{2}H$ spectra of lipids and $^{15}N$ spectra of fd coat protein that were obtained in unoriented bilayers (A, D), bicelles of the first generation (B, E, G) and bicelles of the second generation (C, F, H). One can notice the increasing resolution starting from a full $^{2}H$ Pake pattern or $^{15}N$ CSA pattern. This new tool for membrane studies seems to be very promising and is constantly improving.

### *$^{31}P$ chemical shift anisotropy*

Because phospholipids contain a phosphorus-31 nucleus with a natural abundance of 100%, many studies on natural membranes or lipid suspensions were carried out by $^{31}P$ NMR with oriented and unoriented static samples. The maximum splitting $\Delta\sigma$ is used as an indicator of the bilayer viscosity and of lipid perturbation by intrinsic or peripheral proteins. For reasons that were indicated in the theoretical section (see above), spectra cannot be interpreted in a simple manner and accurate headgroup conformation is difficult to measure [50]. Lipids that will form a hexagonal phase rather than a lamellar phase will have different motional restrictions and their $^{31}P$ CSA's will therefore be averaged differently. Similarly, micelles or small vesicles will exhibit different patterns. Thus $^{31}P$-NMR is a useful tool for lipid phase determination [51].

High resolution $^{31}P$ NMR can also be performed with lipid membranes using either small size vesicles or large size liposomes observed with MAS [52]. A specific example is detailed in the following lines. As shown initially by the group of Roberts [53], the isotropic chemical shift of $^{31}P$ in phosphatidic acid (PA) is sensitive to the local pH and indirectly to membrane curvature. With sonicated vesicles (SUV's) with an average diameter of 20 nm, the $^{31}P$ spec-

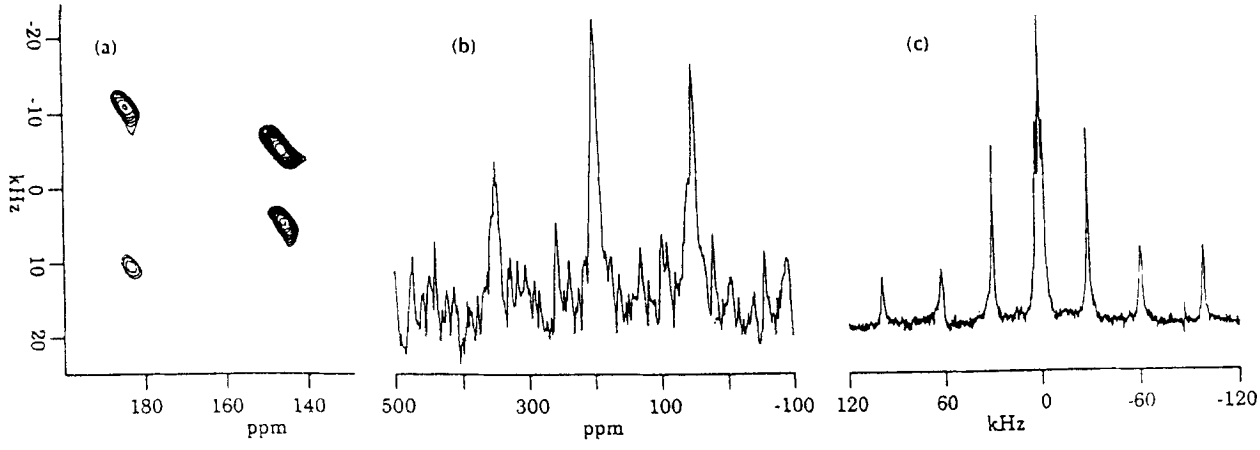


**Fig 6.** Examples of spectra that can provide orientational constraints for hydrated lipid bilayer preparations containing gramicidin A aligned between glass plates and oriented with the bilayer normal (and the channel axis) parallel to the applied magnetic field. **a.** 2D $^{15}N$-$^{1}H$ separated local field spectrum of selectively $^{15}N$ labeled $^{15}N_{\alpha,\,\varepsilon}$-Trp9 gramicidin A. **b.** $^{15}N$ spectrum of selectively $^{15}N$ and non-selectively $^{2}H$ labeled $^{15}N$-Ala5 gramicidin A displaying a 1:1:1 triplet driving from $^{15}N$-$^{2}H$ dipolar coupling. **c.** $^{2}H$ spectrum of selectively deuterated $d_5$ Trp15 gramicidin A shows three well resolved splittings. The two smaller splittings are from the two carbon-bound deuterons in the indole ring that are in a para configuration to each other (from [11]).

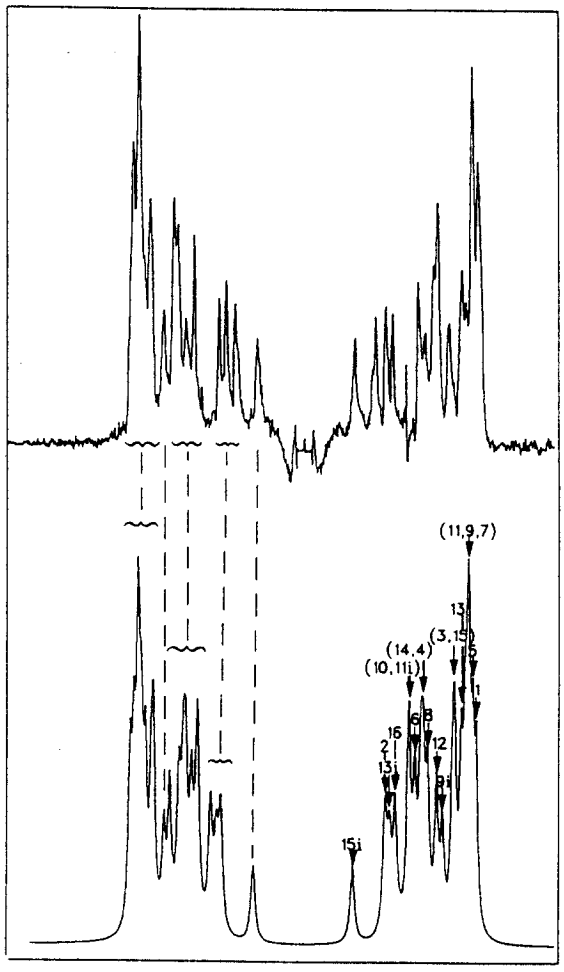


**Fig 7. Top.** $^{2}$H-NMR spectrum of $^{2}$H exchange-labeled gramicidin D in oriented DLPC multilayers ($\beta = 90°$) at 65°C where the lipid-to-peptide ratio is 15. **Bottom.** Theoretical fast motion limit $^{2}$H-NMR spectrum of oriented exchange-labeled gramicidin D, derived from the Arseniev model. The peaks have been labeled according to the residue from which they originate; a suffix 'i' after the residue number indicates indole moieties (otherwise the numbers assigned to the given peaks indicate the residue number of the amide moieties) (from [56]).

trum is composed of narrow lines. Vesicles containing a suitable percentage of PA give two lines associated with the inner and outer leaflets of these highly curved vesicles. These experiments can be extrapolated to larger vesicles provided the CSA is averaged to zero by magic angle spinning. However, for intermediate size vesicles, with a diameter of about 100 nm (LUV's), there is an interesting spectroscopic problem referred to as 'incoherent averaging'. Indeed, the vesicles are not small enough to average out the CSA by spontaneous vesicle tumbling, yet the motion of the vesicles interferes with the averaging by the rotor. High resolution can be obtained for MLV's with MAS at reasonable spinning speed or for SUV's in static samples. In contrast, the rate of LUV tumbling is not fast enough to provide high resolution and interferes with MAS averaging of the $^{31}$P CSA. This problem can be overcome by increasing the viscosity of the solution with glycerol, so that MAS can restore the high resolution $^{31}$P spectrum [54].

*Quadrupolar interactions*

Several reviews have been published that summarize the wealth of information obtained by $^{2}$H NMR in the last three decades for the determination of average lipid structures, conformation, organization, dynamics and order parameters. Systems studied include dispersions of one or several deuterated lipids, lipid dispersions with membrane proteins and even biological membranes, either oriented or not [17–22]. Today, the same techniques are successfully applied to new lipid systems such as bicelles (fig 4A–C) [28, 29, 49].

$^{2}$H NMR is also being used to determine local orientation, and thereby provide structural constraints, in transmembrane peptides such as gramicidin [11, 55, 56] or in proteins such as bacteriorhodopsin [57]. In figure 6c, the local orientation of a tryptophan side chain is deduced from the observation of $^{2}$H splittings in specifically deuterated $d_5$ Trp15 gramicidin in oriented lipid bilayers [11, 55]. In figure 7, nearly every amide and indole N-$^{2}$H resonance in gramicidin embedded in oriented lipid bilayers are observed, assigned and simulated in such a way that global structural and orientational constraints can be deduced [56]. As mentioned earlier, protein dynamics have also been studied through $^{2}$H NMR relaxation and lineshape studies but fall outside of the scope of this review [20–22, 38, 39]. Similar information could theoretically be obtained through $^{14}$N NMR but has so far only been applied to single crystals of small peptides [8].

*Dipolar interactions in lipids*

Dipolar couplings between two isotopically labeled spins separated by a known distance can provide orientational information. Occasionally, dipolar couplings between many spins in unlabeled materials have also allowed the determination of order parameters. For example, studies of the $^{1}$H lineshape resulting from $^{1}$H-$^{1}$H dipolar interactions in a static lipid dispersion, and more recently in MAS experiments, have provided insight regarding lipid dynamics [58–60].

Many dipolar couplings have recently been measured in lipids, mostly corroborating the results obtained by $^{2}$H NMR without the need for isotopic labeling. Some $^{1}$H-$^{13}$C, $^{31}$P-$^{13}$C and $^{13}$C-$^{13}$C couplings have been measured directly in spectra obtained with magnetically oriented bicelles [45, 47, 61]. 2D NMR on dispersions that were either static or spinning off the magic angle have provided more $^{1}$H-$^{1}$H and $^{1}$H-$^{31}$P couplings between atoms in lipid head-groups [62].

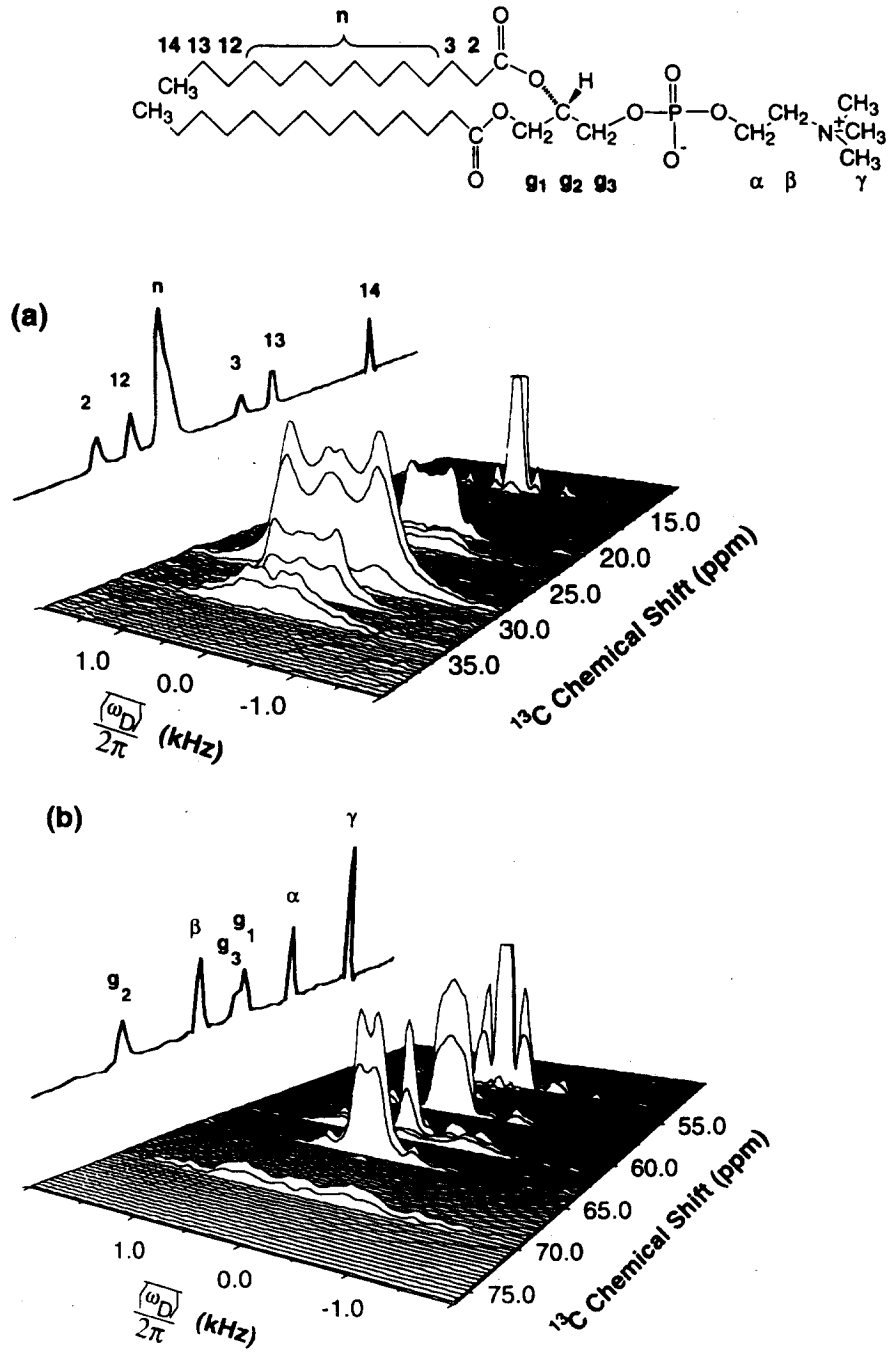


**Fig 8.** 2D $^{13}C$-$^{1}H$ DROSS spectrum of unlabeled dimyristoylphosphatidylcholine in suspension in water at 30°C. **a.** Acyl chain region and head group (**b**) and glycerol region. The powder pattern in the $\omega_D$ dimension reveals the extent of order for each of the carbon-13 sites, separated according to their isotropic chemical shifts (from [64]).

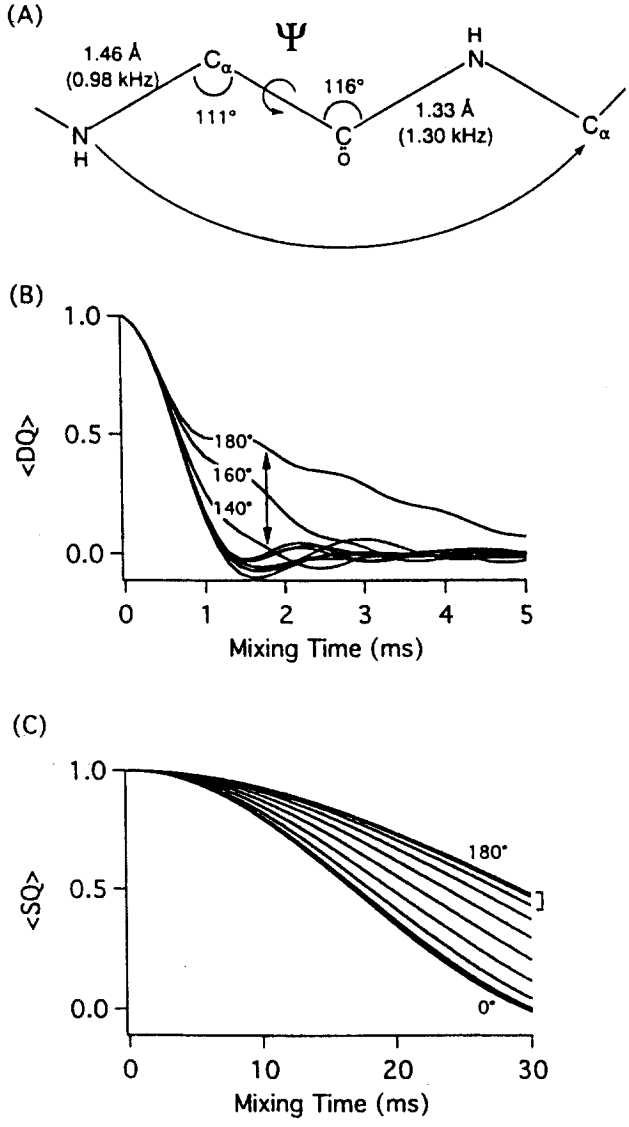


**Fig 9.** Simulated dephasing curves as a function of the NCCN torsion angle ψ. **A.** Peptide backbone geometry. **B.** Simulated dephasing of double-quantum coherence as a function of ψ. For comparison, **C** displays the calculated REDOR curves (single-quantum dephasing) for the N-$C_\alpha$ internuclear distance indicated in **A** as a function of ψ (from [80]).

The wide range of $^1$H-$^{13}$C coupling constants encountered in lipids (on the order of 500 Hz to 5 kHz) is such that none of the known recoupling sequences have been used until a few years ago. Two different experiments have recently been developed for the purpose of measuring all recoupled $^1$H-$^{13}$C couplings in lipids at once, separating every one of them according to their $^{13}$C isotropic chemical shift. The first one is called DISTINCT (dipolar sine term by indirect-coupling transformation) and involves changing the angle of the rotor during the pulse sequence [63]. Two measurements are necessary since the coupling obtained depends either on J + χD or J - χD, J and D being the scalar and dipolar couplings and χ being a scaling factor. The second one is called DROSS (dipolar recoupling on-axis with scaling and shape preservation) and allows one to recouple the $^1$H-$^{13}$C interaction with rf pulses while the rotor spins in a conventional magic-angle probe [64]. This leads to an independent determination of D and J. As opposed to measurements of the quadrupolar interaction which cannot differentiate between α and -α, it can be shown that measurement of dipolar couplings by DISTINCT or DROSS allows the determination of α with its sign. Figure 8 shows the DROSS spectrum of natural abundance dimyristoylphosphatidylcholine (DMPC) where a Pake pattern is obtained for each resolved carbon site, thus allowing the measurement of the $^1$H-$^{13}$C couplings and order parameters. One may notice the wide Pake patterns, corresponding to a high degree of order for the glycerol backbone and for the acyl chains, while the order of the $g_1$ glycerol and the terminal methyl group (line 14) are much less pronounced. Much narrower Pake patterns are obtained for the highly disordered lipid headgroup (lines α, β, γ).

High resolution obtained by MAS in lipids is such that J couplings can be measured and can be used to transfer magnetization, using the INEPT pulse sequence (insensitive nuclei enhanced by polarization transfer) like in solution NMR [65]. Use of INEPT has allowed one to resolve additional $^1$H-$^{13}$C couplings in lipids for bonds oriented near the magic angle (see $g_1$ line in fig 8) [64].

*Dipolar interactions in peptides and proteins*

Many techniques have been developed to measure dipolar interactions in membrane peptides or proteins to deduce intermolecular distances. Most successful have been the techniques that combine MAS and recoupling of the desired interaction by synchronous rf pulses [36]. $R^2$ (rotational resonance) and REDOR (rotational echo double resonance) are the most widely used pulse sequences for structure refinement of peptides [66–69] or proteins such as bacteriorhodopsin [70] in membranes. Since couplings are smaller when the distance is larger, it is important to devise pulse sequences that can measure couplings as small as 25 Hz (corresponding to a static sample with a $^{13}$C-$^{15}$N distance of 5 Å). New sequences with increased efficiency have recently been developed [71] and progress in structure determination has been reviewed recently [13, 14]. The measurement of dipolar couplings between directly bound nuclei, where the distance r is known, with the purpose of deducing orientation and dynamics of membrane peptide segments, has also been performed, although to a lesser extent. Using MAS and REDOR, Hing and Schaeffer have measured a $^{13}$C-$^{15}$N coupling in gramicidin in powders and

in lipid bilayers and have deduced the preferred conformation of labeled $^{13}C$-$^{15}N$ peptide bonds [72].

Using mechanically or magnetically oriented samples, different groups have measured $^{1}H$-$^{13}C$, $^{1}H$-$^{14}N$, $^{1}H$-$^{15}N$, $^{2}H$-$^{15}N$, $^{13}C$-$^{13}C$ and $^{13}C$-$^{15}N$ couplings in peptides or proteins in lipid environments with growing success in their ability to deduce bond orientations [8, 10, 11, 15, 42, 47–49, 55]. For example, in figure 6a, a 2D SLF spectrum allows the measurement of two different $^{15}N$-$^{1}H$ couplings and, in figure 6b, a 1D $^{15}N$ spectrum allows the measurement of one $^{15}N$-$^{2}H$ coupling in selectively labeled samples of gramicidin in oriented lipid bilayers [11]. As explained previously, this information makes it possible to define the orientation of each peptide plane with respect to the magnetic field and, eventually, to determine the entire peptide backbone structure.

*Cross-correlation effects*

When two interactions take place at the same time, measuring the relative orientation of two tensors rather than the absolute orientation of one of them seems to be an attractive alternative. It could be particularly helpful to determine secondary structures of proteins by measuring the torsion angles $\phi$ and $\psi$ of the polypeptide backbone. This approach has recently been developed by Schmidt-Rohr in a static non-oriented sample [73]. One can determine the relative orientations of two $^{13}C$ CSA tensors, two dipolar tensors or a $^{13}CO$ CSA tensor and the tensor of the adjacent $^{13}C$-$^{1}H$ dipolar interaction, by studying cross-correlation effects. Such methods have been transposed to MAS-NMR with an obvious gain in sensitivity [74–77] and have been applied to the direct measurement of a torsion angle in the chromophore of the membrane protein rhodopsin [78]. Other methods are being developed with the purpose of measuring the torsion angles $\phi$ [79] and $\psi$ [80, 81]. Figure 9 shows the diagram of the peptide bond together with the simulated dephasing curves as a function of $\psi$. The accuracy of the 'cross-correlated' method is greater than the more classical REDOR method.

## Conclusions and perspectives

As we have seen in this article, the characteristic properties of lipids allow the development of many original and efficient solid-state NMR techniques for determination of molecular structures in membranes. The liquid-crystalline nature of membranes strongly favors the use of a reference frame which facilitates orientational studies of molecular segments. The study of anisotropic interactions will be greatly simplified by the use of oriented samples, which can be prepared either by mechanical or magnetic orientation. A property that is unique to lipids and small peptides is fast axial diffusion which renders homonuclear dipolar interactions non-homogeneous and provides high-resolution $^{1}H$ spectra by magic angle spinning NMR.

Higher magnetic fields, isotopic labeling and faster rotors have increased the signal-to-noise ratio and the spectral resolution and have allowed solid-state NMR to compete with solution NMR and measure distances in solids by using multi-dimensional spectra [82], J-couplings [46, 65, 83] and high-resolution $^{1}H$ NMR [33, 35]. Furthermore, solid-state NMR techniques are applicable to objects independently of their size and could, theoretically, solve the structure of very large membrane proteins. In addition, the development of new tools such as bicelles helps to blur even further the separation between liquid and solid-state NMR.

Solution-state NMR is increasingly inspired by solid-state NMR experiments. Measuring effects of residual anisotropic interactions allows one to determine the orientation of molecular segments in solution. Residual dipolar couplings have first been observed as a perturbation of J-couplings at high fields and have been recently measured in paramagnetic proteins [84], in DNA [85] and in diamagnetic proteins, with [86] or without [6, 87] the use of bicelles. Residual $^{15}N$ CSA [6, 88] and $^{2}H$ quadrupole couplings [88] in proteins have also been investigated. Finally, torsion angles of a protein in solution can also be measured directly using cross-correlated dipolar relaxation effect which provides an alternative to the traditional measurements of NOE's and J-couplings [89, 90].

Let us repeat here that, although the orientational approach is very powerful for structure determination, it suffers from similar problems as the distance approach in the following ways: 1) out of the three unknowns (distance, orientation and dynamics) two always have to be assumed before determining the third; 2) angles and distances obtained from these approaches are only averaged values; 3) the three parameters vary independently and may have opposite effects, so that small order parameters can result from radically different combinations of the three variables; and 4) with incomplete data, all one can determine is boundaries of conformations. Nevertheless, this approach has proven to be extremely efficient for the determination of structures and dynamics of lipids and it is now rivaling with the distance approach. With the advent of direct protein-backbone torsion-angle measurements, the orientation determination by solid-state NMR might become an approach of choice for membrane molecules in the next decade.

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