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NOTES

Preparation of Oriented Lipid Bilayer on Ultrathin Polymers for Solid-State NMR Analyses of Peptide–Membrane Interactions

SOPHIE AUGÉ, HONORÉ MAZARGUIL, MARIELLE TROPIS, AND ALAIN MILON

Institut de Pharmacologie et de Biologie Structurale, CNRS, 118 rte de Narbonne, 31062 Toulouse, France

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Solid-state NMR is a widely used method for the determination of the orientation and conformation of peptides embedded in a membrane (*1, 2*). For studying various peptides such as gramicidin (*3–8*), melittin (*9*), fd coat protein (*10*), magainin, and M2δ (*11*), ^{15}N chemical shift (*3, 7, 10, 11*), ^{13}C chemical shift (*4, 9*), ^{15}N–^{1}H (*6*) and ^{15}N–^{13}C (*5*) dipolar couplings, and ^{2}H quadrupolar splittings (*6, 8*) have been analyzed. One of the crucial and most difficult steps in solid-state NMR analyses of membrane-bound peptides is very often the preparation of oriented lipid bilayers having a unique plane orientation. One must achieve a good degree of ordering, with a large amount of lipid and peptide, in a sample size which is limited by the receiver coil (typically 1–2 cm long and 5–10 mm in diameter). We present herein new approaches using ultrathin polymers and unilamellar vesicles which improve in several aspects the usual techniques so far employed.

Typically the oriented samples are prepared by spreading an organic solution of the peptide–lipid mixture onto 20–50 small glass coverslips which are then stacked, and by removing the solvent and rehydrating the dry lipid film. The degree of ordering may be improved by drying and rehydrating the sample several times, cycling the temperature from a low to a high value, or simply waiting for several days or weeks (*7, 8, 12, 13*). The organic solvent must be able to solubilize both the lipids and the peptide, and its nature may influence the results (*14*). The amount of lipid per square centimeter is also an important parameter, and typically it should be kept below 2–3 mg/cm^2, although concentrations up to 7.4 mg/cm^2 have been reported (*6*). The quality of the sample may be assessed by optical microscopy (*14*), neutron scattering, ^{31}P NMR (*14*), or deuterium NMR. In the latter two cases, spectra at zero degrees orientation (i.e., with the bilayer normal parallel to the magnetic field) are very sensitive to the presence of unoriented bilayers, which give signals characteristic of a 90° orientation.

We have prepared oriented samples made of ^{15}N-labeled substance P, a neuropeptide involved in nociception (*15*) which interacts with lipid bilayers, a mixture of phospholipids, DMPG and DMPC. In order to spread 5 mg of peptide and lipids, in a 1:45 molar ratio, 110 mg of lipids had to be used. This would lead to a deposit of about 15 mg/cm^2 over the 15 glass coverslips which could be inserted into a 7 mm tube. Therefore, we decided to test the preparation of oriented samples using much thinner polymer films. Two polymer films were tested: a hydrophobic polymer, made of Halar (a copolymer of ethylene and chlorotrifluoroethylene, Goodfellow Inc., Cambridge, UK), and a hydrophilic polycarbonate film (Goodfellow Inc.). Halar and polycarbonate are, respectively, 25 and 2 μm thick compared with 150 μm for glass coverslips, and thus we could easily stack 60 layers of polymer in the NMR tube and deposit 4 mg of lipids per square centimeter.

The ability of these two polymers to induce bilayer formation has been tested using ^{2}H solid-state NMR (*16*). Figure 1 shows ^{2}H NMR spectra obtained from 1-myristoyl-2-(perdeuterio)-myristoyl-*sn*-glycero-3-phosphocholine (DMPC-d_{27}) bilayers on Halar plates. The uniformly aligned samples were positioned in the magnet such that their bilayer normal is perpendicular (A) or parallel (B) to the magnetic field. From these characteristic spectra, it is clear that our sample had a single orientation between Halar plates and was perfectly hydrated. Indeed, the ^{2}H quadrupolar splittings were identical to those of liposomes in excess water. The same result was obtained with the polycarbonate films (result not shown), but Halar is preferable because of its broader chemical compatibility; Halar is stable in acids, bases, and any organic solvents except halogenated ones, whereas polycarbonate film is stable in dilute acid, alcohol, and oils.

Another problem associated with the use of organic solvents is that they may not cosolubilize both the lipids and the peptide. The substance P is an example of this problem. Moreover, for proteins, they may cause denaturation problems and difficulties with the control of buffer, salt concen-

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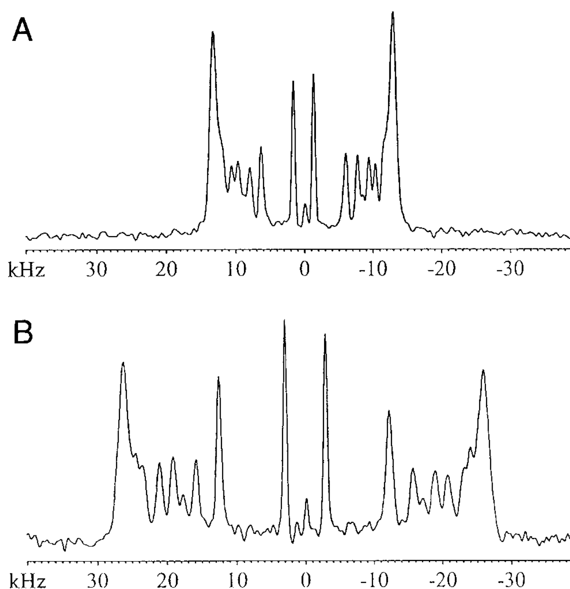

FIG. 1. Solid-state ^{2}H NMR spectra obtained from an oriented bilayer sample containing 5 mg of DMPC-d_{27}. The bilayer normal is perpendicular (A) or parallel (B) to the magnetic field. The lipids were dissolved in isopropanol and spreaded on Halar plates at a density of 0.12 mg/cm^2. After complete solvent evaporation, the plates were stacked inside the NMR tube. Hydration of the lipid was accomplished by placing the tube in a vial containing 1 ml of ^{2}H-depleted water (Aldrich Chemical Co.) at 310 K overnight (*20*). ^{2}H NMR spectra (76.77 MHz) were acquired on a Bruker AMX500 spectrometer. Quadrupolar echoes (*21*) were obtained by using 90° pulses 4.3 μs long, an interpulse delay of 40 μs, a 1 s repetition rate, a sweep width of 500 kHz, and 512 scans. Spectra were recorded at 315 K, i.e., above the lipid phase-transition temperature.

trations, and pH. Therefore a strategy using small unilamellar vesicles (SUVs) would be preferable in some cases. We simply prepared vesicles, mixed them with the protein solution in the desired buffer, and spread the vesicle–protein solution onto the polymer. In order to facilitate the formation of bilayer planes from vesicles, we tested two techniques, the ethanol-induced interdigitation fusion of SUVs, IFV (*17*), and the freeze–thawing procedure (*18*).

With lipids alone, we obtained properly oriented samples in all cases; we then tried to prepare oriented samples with the substance P–lipid mixture, via the IFV technique and Halar films. Five milligrams of substance P was mixed with DMPG/DMPC in a 2/9/81 molar ratio. The oriented membranes were prepared as described in the legend to Fig. 2. In this sample, one problem arose which seems to be specific to the use of Halar film. The samples tend to dehydrate easily, and once dehydrated, the stacked sample can hardly be rehydrated. This may be associated with the very small thickness and hydrophobic character of this material. Good samples are prepared only when the plates are stacked in a water-saturated atmosphere and kept under a humid atmosphere. We used an NMR tube containing a water reservoir as described by Nielsen *et al.* (*19*). Figure 2 shows the spectra obtained with a dehydrated sample (A) and a properly hydrated sample (B). The hydrated sample containing substance P is not perfectly oriented. This problem is due to the presence of substance P as shown in Fig. 3.

Figure 3 illustrates the orientation of different samples monitored by ^{2}H and ^{31}P NMR. The samples A, B and C, D were prepared under the same conditions, via IFV and using Halar film, except that samples A and B do not contain peptide. The sample without peptide is properly oriented (it contains only a few percent of liposomes). The ^{2}H and ^{31}P spectra of the sample with substance P (spectra C and D)

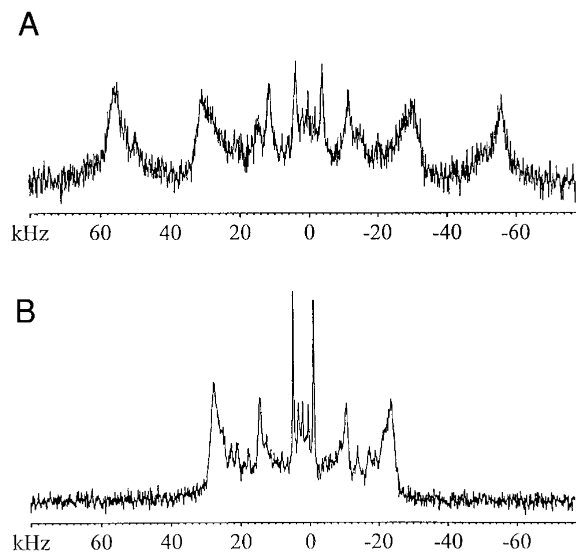

FIG. 2. Solid-state ^{2}H NMR spectra obtained from samples containing substance P, DMPC, and DMPG: stacking of the plates under atmospheric conditions (A) and water-saturated condition (B). The bilayer normal is parallel to the magnetic field and the spectra were recorded at 315 K. The acquisition parameters were identical to those in Fig. 1, except for the number of scans [4096 (A) and 12,288 (B)]. Five milligrams of substance P was solubilized in 250 μl of ^{2}H-depleted water. DMPC/DMPG SUVs were prepared as follows: 11.4 mg of DMPG, 96.8 mg of DMPC, and 5 mg of DMPC-d_{27} in chloroform/methanol (v/v, 1/1) were dried to make a thin film in a round-bottom flask and vacuum dried to remove residual solvent prior to hydration. The phospholipids were suspended in 6 ml of ^{2}H-depleted water and sonicated (at 30 W) for 5 min (50% of time). The SUVs were collected in the supernatant (after centrifugation at 13,400g for 10 min) and the pH was adjusted to 5. Then the lipid and the peptide solutions were mixed in a round-bottom flask, and 4.55 ml of water was evaporated by a gentle stream of N_2 to increase the lipid concentration. Cold ethanol (205 μl) was added to the cold SUVs suspension. After 15 min of incubation at 4°C, 30 μl of solution was spread onto 60 Halar plates (5 × 11 mm). The plates were incubated for 3 hours at 50°C and the water was completely evaporated under vacuum overnight. The plates were then hydrated for 24 hours at 50°C by placing the plates in an atmosphere saturated with ^{2}H-depleted water. The hydrated plates were then stacked inside the NMR tube under atmospheric conditions (A) and in water-saturated conditions (B). To maintain a humid atmosphere within the sample chamber, a plug of absorbent paper wetted by 1 ml of ^{2}H-depleted water was placed inside the reservoir of the NMR tube end cap.

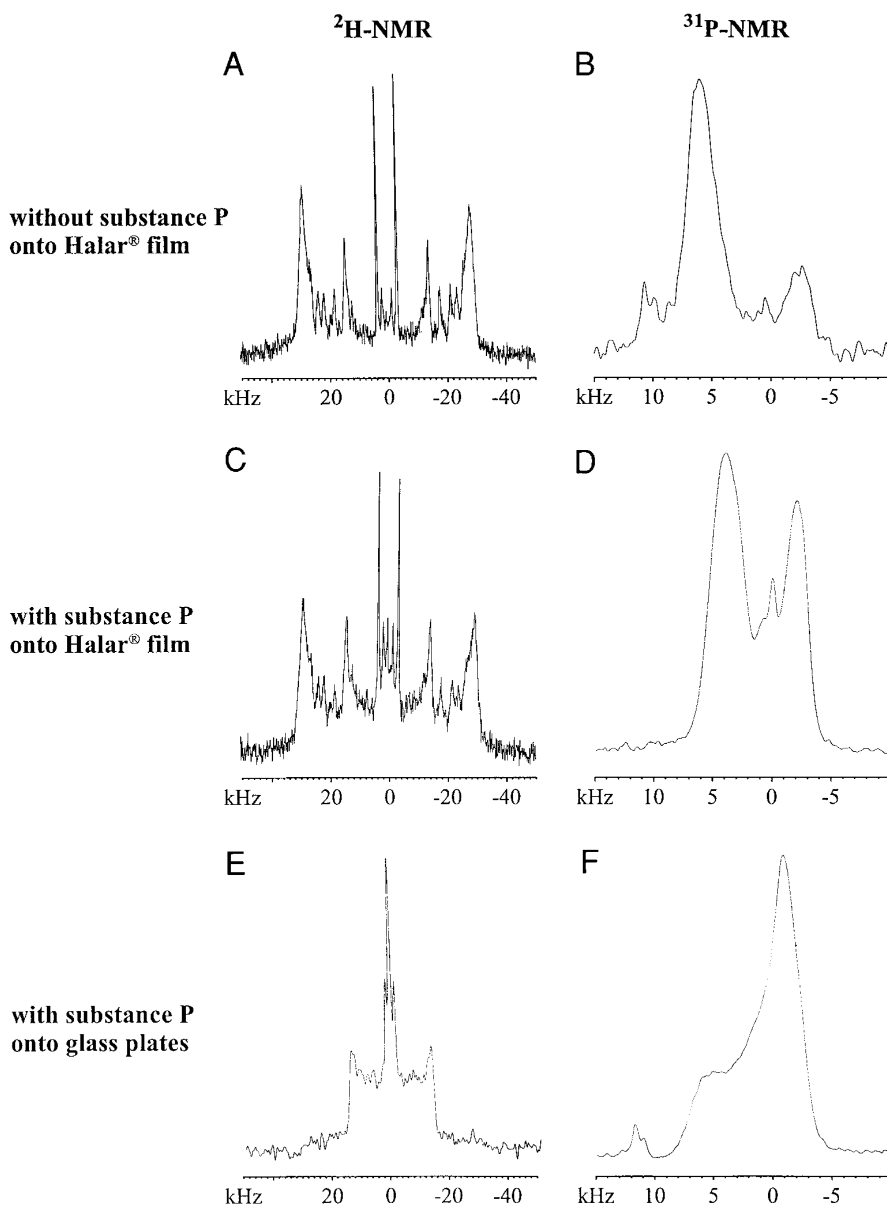

FIG. 3. Solid-state ^{2}H (A, C, and E) and ^{31}P (B, D, and F) NMR spectra obtained from samples containing substance P (C, D, and E, F) or not (A, B). The sample preparations were identical to those of Fig. 2, except for the sample E, F which was prepared with 15 glass plates (5 × 11 mm). The bilayer normal was parallel to the magnetic field and the spectra were recorded at 315 K. The ^{2}H NMR acquisition parameters were identical to those in Fig. 1, except for the number of scans [2048 (A), 8192 (C), and 2048 (E)]. ^{31}P NMR spectra (202.45 MHz) were acquired on a Bruker AMX500 spectrometer. Hahn echoes (*22*) were obtained by using 90° pulses 3 μs long, an interpulse delay of 40 μs, a 1 s repetition rate, a sweep width of 250 kHz, and 2000 scans (B, D) or 8192 scans (F).

show the presence of around 55% of liposomes. This is a characteristic of this peptide, substance P, which prevents good orientation of membranes when incorporated at 2 mol%. However, comparison with spectra E and F (almost no oriented bilayers), in which the same peptide–lipid mixture has been deposited between glass plates by the standard procedure, clearly shows the improvement of the orientation brought by the use of thin polymer films.

This is also confirmed by Fig. 4, showing the ^{15}N NMR spectra of the same sample. It was impossible to observe a ^{15}N signal with an unoriented sample (glass plates), whereas a signal of reasonable quality was observed with the Halar-oriented sample. The comparison of the two spectra shows that the contribution of the unoriented lipids in spectrum C is negligible since no spectrum is observed under the same condition with the unoriented sample (spectrum B).

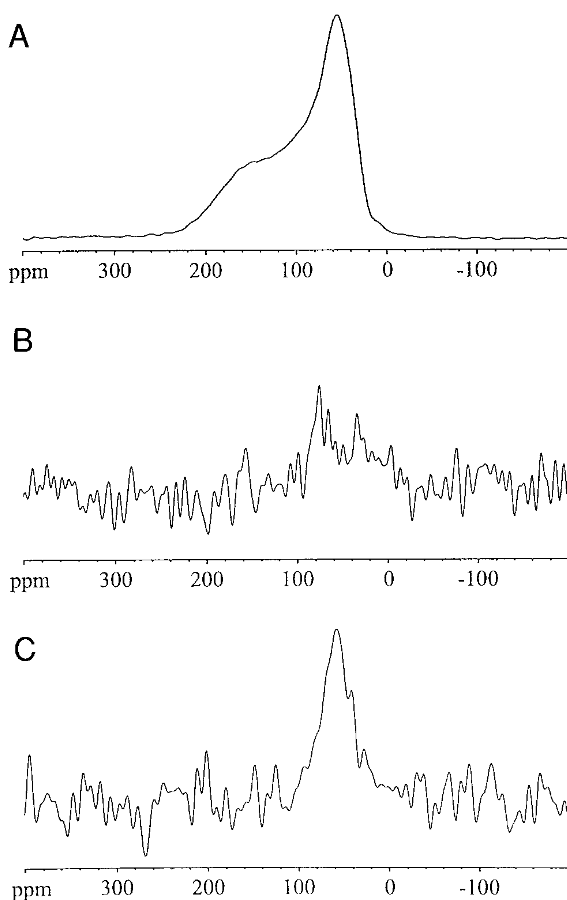

FIG. 4. Solid-state ^{15}N NMR spectra of $[^{15}N]Phe^{8}$-labeled substance P. (A) Anisotropic ^{15}N chemical-shift spectrum of dry peptide powder (58 mg) at 297 K. (B, C) Samples containing substance P in bilayers oriented parallel to the magnetic field at 315 K: (B) bilayers between glass plates (same sample as those in Figs. 3E, 3F); (C) bilayers between Halar plates (same sample as those in Figs. 3C, 3D). Cross polarization (*23*) was obtained by using 90° pulses of 6.1 μs (A) and 5.6 μs (B, C); a mixing time of 1 ms; a cross polarization and decoupling field of 45 kHz; a 6 s recycle delay; a sweep width of 100 kHz (A), 200 kHz (B), and 500 kHz (C); and 13,000 scans (A), 14,745 scans (B), and 12,288 scans (C). The peptide was synthesized on MBHA resin by using t-Boc amino acidchemistry. The t-Boc amino acid of ^{15}N Phe-OH was obtained with Boc-ON (*24*) and commercially available ^{15}N-Phe (Eurisotop, Inc.).

Therefore, ultrathin polymer films made of Halar or polycarbonate represent an alternative to the widely used glass coverslips to prepare oriented membranes with a large area. Combined with the use of IF vesicles, they provide a novel, efficient way of preparing protein–membrane samples for solid-state NMR.

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