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Abstract

Solid-state NMR spectroscopy has been developed for the investigation of membrane-associated polypeptides and remains one of the few techniques to reveal high-resolution structural information in liquid-disordered phospholipid bilayers. In particular, oriented samples have been used to investigate the structure, dynamics and topology of membrane polypeptides. Much of the previous solid-state NMR work has been developed and performed on peptides but the technique is constantly expanding towards larger membrane proteins. Here, a number of protocols are presented describing among other the reconstitution of membrane proteins into oriented membranes, monitoring membrane alignment by ^{31}P solid-state NMR spectroscopy, investigations of the protein by one- and two-dimensional ^{15}N solid-state NMR and measurements of the lipid order parameters using ^2H solid-state NMR spectroscopy. Using such methods solid-state NMR spectroscopy has revealed a detailed picture of the ensemble of both lipids and proteins and their mutual interdependence in the bilayer environment.

Keywords

(separated by '-')

Membrane reconstitution - Oriented bilayer - Bicelle - Helix topology -
Membrane protein structure - Detergent - Surface alignment -
Transmembrane orientation - Protein insertion - Channel - PISEMA -
Separated local field spectroscopy - Cross-polarization - Lee-Goldburg
decoupling

Solid-State NMR Approaches to Study Protein Structure and Protein–Lipid Interactions

Christopher Aisenbrey, Evgeniy S. Salnikov, Jesus Raya, Matthias Michalek, and Burkhard Bechinger

Abstract

Solid-state NMR spectroscopy has been developed for the investigation of membrane-associated polypeptides and remains one of the few techniques to reveal high-resolution structural information in liquid-disordered phospholipid bilayers. In particular, oriented samples have been used to investigate the structure, dynamics and topology of membrane polypeptides. Much of the previous solid-state NMR work has been developed and performed on peptides but the technique is constantly expanding towards larger membrane proteins. Here, a number of protocols are presented describing among other the reconstitution of membrane proteins into oriented membranes, monitoring membrane alignment by ^{31}P solid-state NMR spectroscopy, investigations of the protein by one- and two-dimensional ^{15}N solid-state NMR and measurements of the lipid order parameters using ^2H solid-state NMR spectroscopy. Using such methods solid-state NMR spectroscopy has revealed a detailed picture of the ensemble of both lipids and proteins and their mutual interdependence in the bilayer environment.

Key words Membrane reconstitution, Oriented bilayer, Bicelle, Helix topology, Membrane protein structure, Detergent, Surface alignment, Transmembrane orientation, Protein insertion, Channel, PISEMA, Separated local field spectroscopy, Cross-polarization, Lee–Goldburg decoupling

1 Introduction

Membrane proteins constitute about 30% of the expressed reading frames [1, 2], are extremely important in cellular function and of major importance in biomedical and pharmaceutical research. However, their structural investigation lags much behind that of soluble proteins due to problems of obtaining the quantitative amounts of proteins needed for structural investigations 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 conformation, a requirement for high-resolution X-ray diffraction techniques. For example,

G-protein coupled receptors (GPCR) are of major importance in pharmaceutical and medical research and the last few years have shown good progress in their structural analysis [3]. Nevertheless, revealing their functionally important dynamics in membrane environments remains a major challenge [4]. Notably, for our understanding of their functional states it is essential that membrane proteins are reconstituted and investigated in liquid crystalline bilayers [5].

Solid-state NMR spectroscopy provides an interesting alternative route for protein structure determination also within large complexes including membrane polypeptides [6–12]. Whereas in solution fast diffusion and motional averaging assure that the NMR spectra exhibit narrow lines and high resolution, the anisotropy of NMR interactions remains apparent in the spectra taken from solids or from molecules or complexes whose size prevents efficient isotropic averaging. Therefore in static solid or semi-solid samples the chemical shift, dipolar interactions and quadrupolar splittings are all dependent on the molecular alignment relative to the magnetic field direction, and the anisotropy of nuclear interactions is a predominant feature of the NMR spectra also of membrane-bound proteins [13]. Whereas under magic angle spinning conditions the spectral appearance is related to the isotropic chemical shift spectra observed in solution, here we present the analysis of oriented samples, which contain valuable structural information as the chemical shifts (e.g., ^{15}N , ^{13}C , ^1H), quadrupolar (e.g., ^2H , ^{14}N) and dipolar couplings (e.g., ^{15}N - ^{13}C , ^1H - ^{15}N) all depend on the alignment of bonds and molecules relative to the magnetic field direction. This approach requires that polypeptides are reconstituted into macroscopically oriented membranes, and the resulting samples inserted in the magnetic field of the NMR spectrometer, in the simplest case with the bilayer-normal parallel to the magnetic field direction.

Clearly the reconstitution of the proteins into phospholipids bilayers is a critical step before the analysis by NMR spectroscopic methods can take place. Therefore, Subheading 3.1 provides a detailed step-by-step protocol for the membrane reconstitution of proteins that are soluble in aqueous buffer but also capable to insert into the membrane. A number of alternative pathways is described including the use of detergents thus that the approach can be extended to other membrane proteins. In particular, **Notes 2**, **6** and **10** (Subheading 4), respectively, give indications how to proceed with other lipid bilayers and buffers, or which detergents are commonly used to extract and solubilize membrane proteins.

From such samples, solid-state NMR spectra are obtained that provide orientation-dependent information by revealing ^{15}N and ^{31}P chemical shifts as well as ^{15}N - ^1H dipolar couplings (Subheadings 3.2.1–3.2.3). This chapter therefore complements previous protocols where the investigation of synthetic peptides by ^{15}N

and ^2H solid-state NMR spectroscopy was described [14]. In Sub- 81
heading 3.2.2 the procedure to record oriented ^{15}N chemical shift 82
solid-state NMR spectra is presented in detail. These spectra allow 83
one to simply “read out” the membrane alignment of α -helical 84
peptides that have been labeled with ^{15}N at one of the amide 85
bonds [15, 16]. At membrane alignments where the normal is 86
parallel to the magnetic field direction transmembrane α -helical 87
peptides exhibit ^{15}N resonances in the 200 ppm range whereas 88
they resonate at <100 ppm when oriented parallel to the mem- 89
brane surface [15]. In a related manner, the dipolar coupling is 90
 $K \times (3\cos^2\Theta - 1)$, where Θ is the angle between the N-H vector 91
and the magnetic field direction (parallel to the bilayer normal if 92
oriented correspondingly) and for the amide nitrogen K is typically 93
 -10 kHz [15]. As the N-H vectors are close to collinear with the 94
helix long axis values around 20 kHz (ignoring the negative sign, 95
which cannot be determined easily) indicate transmembrane align- 96
ments, whereas the splitting is zero at the magic angle and close to 97
10 kHz for in planar alignments. Therefore, this parameter provides 98
related information to ^{15}N chemical shift measurements from the 99
same helix [17]. When both values are correlated from a large 100
number of residues of the same helix the resulting pattern is indica- 101
tive of the tilt and pitch angles as well as reveals kinks or other 102
irregular features. Other parameters that can be used for structural 103
analysis are ^{13}C chemical shift or the ^2H quadrupolar splittings of 104
methyl-deuterated alanines [6, 17, 18] and the corresponding 105
protocols can be found in a previous publication of this series [14]. 106

Solid-state NMR spectroscopy is also used to monitor the 107
macroscopic phase properties of phospholipids, membrane thin- 108
ning and the formation of pore structures involving the phospho- 109
lipids. First, ^{31}P solid-state NMR spectra have been used to 110
monitor the macroscopic phase properties of phospholipid mem- 111
branes as bilayers, hexagonal, cubic and isotropic phases exhibit 112
distinct spectral line shapes [19]. Second, oriented ^{31}P and ^2H 113
solid-state NMR spectra have been analyzed for membrane thin- 114
ning and toroidal pore assemblies [20, 21] and the sample prepara- 115
tion and NMR measurements are presented in Subheadings 3.1 and 116
3.2.1, respectively. 117

Third, ^2H solid-state NMR spectroscopy of deuterium-labeled 118
lipids allows one to monitor the order parameter of the fatty acyl 119
chains in a segment-dependent manner and thereby also the mem- 120
brane thickness, the protein penetration depth, and the resulting 121
membrane disruptive properties of polypeptides [22, 23]. By mixing 122
deuterated lipids with non-deuterated ones it is also possible to 123
monitor interactions of the protein with specific membrane compo- 124
nents using the same procedures [24]. The protocols necessary for 125
such investigations are provided in Subheading 3.2.4. Lastly, a simi- 126
lar approach can be used on head-group deuterated lipids to monitor 127
electrostatic interactions at the membrane interface [25, 26]. 128

A major advantage of solid-state NMR spectroscopy is its capacity to work with bilayers in their native liquid disordered state. However, the technique is also relatively insensitive when compared to other types of spectroscopy. Therefore, it is worthwhile to consider the sensitivity of the measurements described here and the requirements for isotopic labeling. On the one hand the ^{31}P nucleus is present in every phospholipid at 100% natural abundance and compared to many other heteronuclei exhibits a relatively high sensitivity. Therefore, one of the most straightforward measurements discussed here is the analysis of the alignment and macroscopic phase properties of the membranes by ^{31}P solid-state NMR and many of these measurements can be performed in time scales of minutes (e.g. [20, 27, 28]). On the other hand, recording the ^{15}N (or ^2H) solid-state NMR spectra of polypeptides reconstituted into membranes is more difficult as the proteins are diluted in the membrane and in water. In addition, in oriented membranes much of the space within the coil volume is either occupied by glass plates or the conditions for the formation of oriented bicelles require a high content of aqueous buffer [29]. Furthermore, the spins typically observed (^{15}N , ^2H) are relatively insensitive when compared to ^1H or ^{31}P . Therefore, quantitative amounts of uniformly or selectively labeled proteins have to be prepared usually by a combination of molecular genetics, bacterial overexpression, and biochemical methods [30–32]. A typical sample encompasses milligram amounts of proteins, labeled with ^{15}N in 100–200 mg of lipid and even though signal enhancement techniques such as cross-polarization are used the measurements can take several hours (one-dimensional experiments) to days (two-dimensional experiments). In this context it is noteworthy that dynamic nuclear polarization (DNP)/solid-state NMR equipment has been developed to boost the sensitivity of NMR spectroscopy by orders of magnitude thereby reducing the acquisition times of spectra from days to minutes [33–35], a method that is under continuous development especially when investigations of membrane proteins are of concern [11, 36, 37].

2 Materials

2.1 Materials Needed for the Preparation of Samples of Membrane Proteins Reconstituted into Oriented Lipid Bilayers

1. Disposable glass tubes with conical ends ≥ 10 mL (e.g., ~ 15 mm $\times$ 100 mm), glass pipettes with long tip for organic solvents and standard glass pipettes for aqueous solutions, 250 μL Hamilton syringe, glass petri dishes with lid (diameter about 10 cm), watchmaker forceps one with pointed and one with flat tips (Outils Dumont SA, Montignez—Switzerland), Teflon tape, plastic foil that goes with an impulse sealer also used in kitchens (e.g., TEW Electrical Heating Equipment, Taipei, Taiwan).

2. Sterile filtered water. Solvents and reagents of analytical grade: buffers, salts, sodium azide. To dissolve lipids dichloromethane or chloroform, methanol or other organic solvents (*see Note 1*). 174
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3. Buffer stock solution used to dissolve the protein (e.g., 200 mM Tris, pH 7.4). 178
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4. Protein sample in buffer. 180
5. Sodium azide (NaN_3) solution (0.02% w/v) for the resuspension of the protein–lipid pellet after ultracentrifugation. 181
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6. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and depending on the desired lipid composition other phospholipids (Avanti Polar Lipids, Birmingham, AL), cholesterol (Sigma-Aldrich) (*see Note 2*). In order to perform the ^2H solid-state NMR experiments described in Subheading 3.2.4 a number of different phospholipids deuterated at various head group and/or fatty acyl chain positions are commercially available (e.g., Avanti Polar Lipids, Birmingham, AL). 183
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7. Lipid extruder (e.g., Avanti[®] Mini-Extruder, Avanti lipids, Alabaster, AL, USA), with polycarbonate filters (100 or 200 nm) and/or tip sonicator (e.g., Sonoplus, Bandelin electronic GmbH & Co. KG, Berlin, Germany). 191
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8. Centrifuge (MiniSpin[®], Eppendorf, AG, Hamburg, Germany) for the removal of metal particles after sonication. 195
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9. Dialysis chamber with pore size (MWCO) of 3.5–10 kDa and a sample volume of 3–12 or 0.5–3 mL (e.g., Slide-A-Lyzer Dialysis Cassette, Thermo Scientific, Rockford, IL) and syringes (e.g., 5 mL syringe, Terumo, Leuven, Belgium) equipped with an injection needle (0.8 × 4 mm Sterican, Braun, Melsungen, Germany or 0.4 × 2 mm Neolus, Terumo, Leuven, Belgium) to insert the sample into the chamber in order to desalt the protein–lipid suspension. 197
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10. Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) for the effective detergent removal from the protein sample after reconstitution. 205
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11. Centrifugal concentrator tubes (e.g., Macrosep[®] Centrifugal Devices, Pall, Port Washington, NY), MWCO (molecular weight cutoff) typically 3 kDa (big enough for easy diffusion of buffer (and detergent), small enough to retain the protein). 208
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12. To equilibrate the atmosphere at 93% r.h. a saturated solution of KNO_3 (for analysis min 99% Merck Darmstadt) is used in a closed chamber (*see Note 3*). 212
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13. Nitrogen gas (bottled or prepared by evaporation of liquid nitrogen) with sensitive pressure gauge, connected to a silicon tube which finishes with a Pasteur pipette. 215
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Ultrathin glass plates that fit your NMR coil (thickness 00 from Marienfeld, Lauda-Königshofen, Germany). Before preparing the sample test if a stack of glass plates sealed and wrapped in plastic film fits the coil (cf. Subheading “Application of Lipid Bilayers onto Solid Supports for Alignment”). Custom-made glass plates of dimensions 11×20 , 9×20 , 8.5×20 , 8×12 or 6×10 mm have been used. If you have the choice 9×20 is about half the prize when compared to 8.5×20 . However, the width of the glass plates should match the coil dimensions, leaving some space for wrapping. The length of the glass plates should not exceed the length of the coil or of the proton cage in case of an e-free probe (*see Note 4*).

Alternatively, supports made of polymers such as high-density polyethylene (Goodfellow, Cambridge UK) and of sapphire plates have been used for DNP/solid-state NMR measurements where glass plates interfere with these measurements [36]. These have the additional advantage of being thinner thereby allowing more membrane material to enter the coil, to have better heat conductivities and by being flexible to be also suitable for magic angle oriented sample spinning [38].

14. High vacuum with cold trap to remove traces of organic solvent (e.g., lyophilizer).
15. Ultracentrifuge to fractionize lipid bound protein from unbound protein staying in solution (e.g., Optima L-100 XP, Beckman Coulter, Palo Alto, CA).

2.2 Materials Needed for NMR Spectroscopy

1. Access to a solid-state NMR spectrometer (at least two channels except for ^2H) with the corresponding probes: that is, for the experiments described here static $^{31}\text{P}\{^1\text{H}\}$, $^{15}\text{N}\{^1\text{H}\}$, and/or ^2H . For better sensitivity during the investigation of peptides in membranes flat-coil NMR probes have been developed [39–41] and are commercially available from several manufacturers (e.g., Bruker, Rheinstetten, Germany; Doty Scientific, Columbia, SC). Preferably NMR probe heads that avoid strong electric fields at the interior of the coil are used as they assure less heat intake and thereby improved sample stability (*see Note 4*). For the investigation of lipids by $^{31}\text{P}\{^1\text{H}\}$ or ^2H solid-state NMR or for highly concentrated polypeptide samples by $^{15}\text{N}\{^1\text{H}\}$ (or $^{13}\text{C}\{^1\text{H}\}$) solid-state NMR, where smaller overall quantities of sample are required (e.g., a ^{31}P NMR signal can already be obtained from 5 mg phospholipids oriented between a pair of glass plates) conventional solenoidal coil shapes have also been used.

2. Solid-state NMR references: ^{15}N -ammoniumchloride or ^{15}N -ammoniumsulfate for $^{15}\text{N}\{^1\text{H}\}$ experiments, 85% H_3PO_4 for $^{31}\text{P}\{^1\text{H}\}$ experiments and water enriched with $^2\text{H}_2\text{O}$ for ^2H NMR.
3. For DNP/solid-state NMR measurements additional hardware is required [36, 42]. Furthermore, biradicals such as TOTA-POL or AMUPOL need to be added to the sample [37] (*see Note 5*).

3 Methods

3.1 Reconstitution of Proteins into Oriented Lipid Bilayers

An oriented solid-state NMR sample is typically composed of a few milligram of protein in about 50–200 mg of lipid oriented between glass plates, the exact amounts depending on finding a compromise between a good “dilution” of the protein in the membrane and consequently good bilayer alignment, good signal intensity which is correlated to the sensitivity (magnetic field strength) of the NMR spectrometer and the maximum sample size which is related to the volume of the NMR coil (typically in the range $10 \times 8 \times 3$ mm, cf. [36]). Also, to keep the protein functional or to test the effects of the membrane composition it may be desired that samples of variable lipid composition are prepared. Therefore, it is notably that a large variety of bilayers with different composition have been oriented mechanically between glass slides [23, 43]. As long as liquid disordered bilayers form uniaxial alignment has been possible.

In the following a preparation scheme for different lipid mixtures is therefore provided. Whereas during the reconstitution procedure peptides can often be mixed with lipids in organic solvents [14, 44] this should in general be avoided for proteins. Therefore, the protocols presented here consists in preparing phospholipid vesicles (Subheading “Preparation of Lipid Vesicles”), followed by membrane reconstitution of the protein (Subheading “Membrane Reconstitution of the Protein”), removal of detergent/buffer exchange (Subheading “Removal of Detergent” or “Reducing the Volume of the Protein–Lipid Sample After Dialysis”) and orienting the resulting vesicles along the surface of small cover glasses/polyethylene film (Subheading 3.1.2). Alternatively, bicelles that orient in the magnetic field of the NMR spectrometer are prepared (Subheading “Formation of Oriented Lipid Bicelles that Orient in the Magnetic Field”). The full protocol is summarized in the flow chart shown in Fig. 1.

Fig. 1 Flow chart for the reconstitution of proteins into oriented lipid bilayers. For DNP/solid-state NMR experiments the addition of biradicals needs to be considered at some step during the preparations (see **Note 5**)

3.1.1 Reconstitution of Proteins into Lipid Vesicles

Preparation of Lipid Vesicles

1. Prepare a homogenous mixture of the lipids organic solvents (at ~50 mg/mL) in glass tubes of approximate dimensions ~15 mm × 100 mm. Depending on the lipid composition chloroform, dichloromethane, or mixtures of one of the two solvents with methanol 2:1 v/v have been used (*see Note 1*).
2. The organic solution is gently evaporated in a hood using a stream of nitrogen in such a manner to create a film of the lipid on the inner surface of the test tube. Pasteur pipettes attached to a soft silicone tube can be used for this purpose and can be easily replaced by fresh ones to avoid cross-contamination of samples. Before exposing the solution to the stream of nitrogen carefully adjust the gas flow as turbulences may cause spray dispersion and the loss of sample. Warming the test tube in a small beaker of warm water helps in the steady evaporation of the solvent. If dichloromethane is used warming by the hand is sufficient.
3. Remove the remaining traces of organic solvents by high vacuum during a few hours or best overnight. This can be achieved, for example, in a lyophilizer.
4. Hydrate the lipid film with of the buffer also used to dissolve the protein to obtain a lipid concentration ≤ 10 mM (7–8 mg/mL) (*see Note 6*) and close the tube with Parafilm. Leave for about an hour or longer followed by vortexing. The addition of 3–4 glass beads of about 2 mm in diameter helps in transferring the film into a suspension of multilamellar vesicles.
5. To homogenize the samples, submit to ≥ 3 freeze (using dry ice in isopropanol) or a -20 °C freeze–thaw (using lukewarm water)–vortex cycles (*see Note 7*).

Preparation of Unilamellar Vesicles by Extrusion

Unilamellar vesicles of a defined outer diameter in the range 100–1000 nm can be produced by extrusion through a polycarbonate film that has a specific pore size. The extrusion device for a few milliliter amounts of solution typically consists of two syringes and a holder supporting the polycarbonate films.

1. Place two polycarbonate films in the holder onto a droplet of water, which has been positioned into the holder with the help of one syringe.
2. Wash the device with buffer. When the membranes are intact pressing the syringes requires certain strength.
3. Place the lipid suspension in one of the syringes and press the suspension through the polycarbonate film for 21 times (*see Note 8*).

<i>Preparation of Small Unilamellar Vesicles by Sonication</i>	Alternatively, small unilamellar vesicles of a size that is typically <100 nm can be produced by sonication. This section thereby replaces Subheading “Preparation of Unilamellar Vesicles by Extrusion.”	348 349 350 351
	1. Position the sonicator tip centrally into the lipid suspension and the sample tube in an ice bath for cooling. Avoid direct contact of the sonication tip and the surface of the tube as both the tip and the glass tube may be damaged.	352 353 354 355
	2. Sonication is performed at intervals that avoid sample heating until the suspension clears. The time varies between 10 and 60 min depending on the lipid composition and the salt concentration.	356 357 358 359
	3. After sonication particles that tend to come off the metal tip of the sonicator are removed by centrifugation in a tabletop centrifuge at $10,000 \times g$ for 10 min (e.g., the highest speed on an Eppendorf-centrifuge, MiniSpin [®]) (<i>see Note 9</i>).	360 361 362 363 364
<i>Membrane Reconstitution of the Protein</i>	In the simplest case the lipid vesicle suspension and the protein solution are mixed and the protein spontaneously inserts into the lipid membranes. This is the case for membrane-active colicin domains, diphtheria T-domain as well as several members of the Bcl-2 family of proteins and the protocols described here follow our work on these proteins [30, 32, 45]. The buffers involved in this step have to be adapted to the requirements of the protein and may for example involve triggering of membrane insertion by a decrease in pH [45, 46] (<i>see Note 6</i>).	365AU4 366 367 368 369 370 371 372 373 374
<i>Reconstitution of Water-Soluble Proteins into Lipid Vesicles</i>		
<i>Reconstitution of Detergent-Solubilized Proteins into Lipid Vesicles</i>	Many membrane proteins need to be solubilized and stabilized by detergent during the extraction and purification steps. In these cases, the protein–detergent solution and lipid bilayer suspension are mixed and the detergent molecules are removed by one of the methods outlined below. Which detergent and which of the methods of its removal works best for a given protein has to be tested or the information may be available in the literature (<i>see Note 10</i>). Two alternative procedures for removing the detergents after protein reconstitution into liposomes are given here in Subheadings “Detergent Removal Using Dialysis Tubes” and “Detergent Removal Using Micro Beads.”	375 376 377 378 379 380 381 382 383 384 385 386
<i>Removal of Detergent</i>	1. The detergent molecules are removed in a dialysis chamber with pore size (MWCO) of 3.5–10 kDa with a sample volume of 3–12 or 0.5–3 mL. The pore size should be sufficiently small to avoid loss of the protein but large enough to allow efficient exchange of buffer and detergent. The sample (some milliliters) is placed into the dialysis chamber by a syringe equipped with	387 388 389 390 391 392
<i>Detergent Removal Using Dialysis Tubes</i>		

an infusion needle following the instructions of the manufacturer.

2. The closed dialysis tube is placed into a large beaker (of volume >200 times the sample volume). The liquid in the beaker is constantly agitated by a magnetic stirrer and placed at for 12–16 h typically at 4 °C or at room temperature, respectively (depending on the phase transition temperature of the lipids and the requirements of the protein sample).
3. The buffer is replaced every 4 h.

Detergent Removal Using Micro Beads

The hydrophobic interaction of detergents like CHAPS and octyl-glucoside with Bio-Beads is an alternative procedure for the effective removal of detergents after reconstitution of proteins into model membranes [47].

1. Detergent-containing solutions are incubated with for instance Bio-Beads SM-2 (BioRad Laboratories, Richmond, CA) for 1 h with gentle shaking at room temperature and consequently eluted to get a detergent-free sample.
2. This procedure is repeated several times, as some less hydrophobic detergents like CHAPS associate less well with the beads [48].

3. The sample can be concentrated as in [AU5](#)
Note 11 describes the usage of beads for the reconstitution of membrane proteins in a unique orientation.

Reducing the Volume of the Protein–Lipid Sample After Dialysis

In order to reduce the sample volume of the protein–lipid suspension the sample is ultracentrifuged after the dialysis step.

1. The suspension is placed into 25 × 89 mm polycarbonate tube (Beckman, Palo Alto, CA) and centrifuged at >100,000 × *g* for 5 h, 4 °C.
2. Consequently, the supernatant is separated from the pellet and tested for protein remaining in solution, for instance by UV-absorption at 280 nm.
3. The pellet is washed and resuspended in sterile filtered water, complemented with 0.02% sodium azide (w/w) before being spread on glass plates (Subheading 3.1.2). This assures that only lipid-associated protein contributes to the solid-state NMR measurements and the salt concentration in the sample is reduced which is advantageous for the NMR spectroscopic performance (cf. Subheading 3.2).

3.1.2 Formation of Oriented Lipid Bilayers on Solid Supports

A lipid vesicles suspension which is depleted of its bulk water and equilibrated at defined relative humidity spontaneously forms stacks of oriented bilayers on glass plates or other solid or polymer supports. However, samples of proteins reconstituted in lipid

	vesicles are typically of several milliliters and contain salt, which	438
	would concentrate during the drying process and, therefore,	439
	should be removed before the sample is stacked. This can be	440
	achieved by dialysis and/or ultracentrifugation (cf. Subheading	441
	“Removal of Detergent/Buffer Exchange”). Alternatively, a con-	442
	centration step followed by some washing steps can be used to	443
	reduce the salt concentration.	444
		445
Concentration of the Samples	The samples can be easily concentrated by centrifugation devices	445
	where the solvent passes a filter of defined molecular size, but larger	446
	macromolecules or aggregates are retained. Different systems are	447
	available and they usually come with instructions how to use them.	448
	In our hands devices where the membrane runs parallel to the	449
	centrifugal forces work well as this arrangement avoids that the	450
	biological material precipitates onto and clogs the filter (e.g.,	451
	Macrosep [®] Centrifugal Devices). These devices can also be used	452
	for washings and buffer exchange. However, it should be kept in	453
	mind that with each centrifugation (which takes several hours) only	454
	about 90% of the buffer has exchanged and that the volume inside	455
	the membranes remains inaccessible. The final volume should be	456
	in the range of 0.5 mL. For DNP/solid-state measurements <i>see</i>	457
	Note 5 .	458
		459
Application of Lipid Bilayers onto Solid Supports for Alignment	This part of the protocol is illustrated in [14].	460
	1. Carefully place about 30 clean ultrathin microscope cover	461
	glasses side by side on two or three glass petri dishes. Anal-	462
	ogous procedures apply for other solid supports such as HDPE	463
	films, which are also a good alternative for DNP/solid-state	464
	NMR experiments where glass plates have shown to be unsuit-	465
	able (<i>see Note 5</i>). Use fine watch maker forceps to manipulate	466
	the glass plates. Apply equal quantities as small elongated dro-	467
	ples onto the central areas of all but one glass plates using a	468
	Hamilton syringe. Keep one glass plate on the side to be used	469
	during the stacking of the plates (step 3). Let the sample dry in	470
	air until most of the solvent has evaporated. Should dehydra-	471
	tion be harmful to your protein perform this step in a more	472
	controlled fashion for example in a chamber of defined relative	473
	humidity (<i>see Note 3</i>). Should the volume be too large to allow	474
	for the stable application onto the glass plates in a single round,	475
	it is possible to apply part of it, dry off some of the liquid and	476
	reapply another droplet onto the same region of the glass plate.	477
	2. After the sample has been equally distributed and the bulk	478
	solvent evaporated in air the samples are placed in a closed	479
	chamber of 93% r.h. (e.g., desiccator or a closed plastic box)	480
	and left to equilibrate for typically about 1–3 days. This humid-	481
	ity is established in contact with a saturated solution of KNO ₃ .	482
	Other hydration conditions are also possible (<i>see Note 3</i>).	483

3. Once the lipid films become translucent and soft (*see Note 3*) the glass plates are stacked on top of each other, with one empty glass plate used to cover the remaining surface. Membrane alignment can be improved by shearing with delicate pressure and small movements. The stack is stabilized by wrapping with Teflon tape and the whole sample sealed in a plastic wrapping using an impulse sealer. The volume of the sample should be fit tightly into the NMR coil (cf. Subheading 3.2.1, step 6).

Formation of Oriented Lipid Bicelles that Orient in the Magnetic Field

Instead of preparing mechanically oriented samples it is also possible to align membranes by their magnetic anisotropy and the interactions with the magnetic field of the spectrometer provided that specific sample conditions (water content, lipid composition, the presence of short chain lipids, detergents or of amphipathic peptides, temperature, salt) are met. This section thereby replaces Subheading “Application of Lipid Bilayers onto Solid Supports for Alignment.”

Different bicellar compositions have been published (e.g., [29, 49, 50]) and a representative protocol for DMPC/DHPC bicelles is described here: The concentrated protein–membrane sample prepared in Subheading “Concentration of the Samples” (with a volume fitting into NMR measurement tube, that is, typically ~200 μL or less) is transferred into the NMR tube containing the correct amount of dry DHPC. Stable bicelles that self-align in the magnetic field are obtained for ratios of long-to-short lipid >2.5 (the ratio of 3.2 is used for the sample shown in Fig. 2e). Thereafter the sample is heated to 50 $^{\circ}\text{C}$ /chilled on ice (0 $^{\circ}\text{C}$)/vortexed several times. Once equilibrated the DMPC/DHPC bicelle sample is viscous at 50 $^{\circ}\text{C}$, but fluid and transparent at 0 $^{\circ}\text{C}$. Therefore, chose this low temperature to transfer the sample into the NMR measurement tube, which are tightly sealed with a silicone stopper.

By using a transparent NMR tube, it is possible to remove air bubbles during the sealing process. For that purpose, the silicone stopper is initially penetrated with a hollow needle. When the stopper is pushed on the top of the bicelle sample air can escape. The needle is removed thereafter.

3.2 Solid-State NMR Measurements

In this paragraph, the setup procedure for four types of NMR measurements is described. However, be aware of the precautions that have to be taken in the proximity of the high-field magnet and due to the high operating voltage of the electronic equipment. Because of these potential dangers the beginner should receive a detailed hands-on introduction into the use of a solid-state NMR spectrometer as well as into the language of the user’s interface. Furthermore, it is assumed that the pulse programs have been established and tested. Some simple setup procedures and pulse

Fig. 2 Typical line shapes of proton-decoupled ^{31}P solid-state NMR experiments from oriented samples of liquid disordered phosphatidylcholine bilayers. **(a)** Nonoriented sample where the powder pattern line shape reflects the functionality of the chemical shift interactions as well as the distribution of molecules relative to B_0 . The chemical shift range of about 45 ppm is typical for liquid disordered phosphatidylcholine bilayers. **(b)** Well-aligned sample where nearly all phospholipid oriented in a narrow range relative to B_0 . **(c)** A small portion of nonoriented sample is superimposed on oriented phospholipids. **(d)** Mixture of phosphatidylcholine (PC) and phosphatidylserine (PS). The two main intensities around 30 and 40 ppm indicate that most lipids are well aligned albeit PC and PS exhibit different chemical shifts and chemical shift anisotropies. A small fraction of phospholipid resonates at values up to -20 ppm indicative of a limited amount of orientational or of conformational

sequences such as standard ^1H acquisition, Hartmann–Hahn matching or cross-polarization are also explained in an applications-oriented manner in ref. 51. Furthermore, ref. 14 describes some additional ^2H and $^{31}\text{P}\{^1\text{H}\}$ solid-state NMR measurements of oriented or non-oriented samples on membrane-associated peptides as well as magic angle sample spinning experiments, which may be also useful in this context.

3.2.1 Verification of Sample Alignment by Proton-Decoupled ^{31}P Solid-State NMR Spectroscopy

1. Insert a sample of 85% H_3PO_4 into the center of the NMR coil. Close probe head and insert the probe into the NMR magnet. Connect the ^{31}P (X) and the ^1H electronic circuits of the probes to the respective electrical connections with incoming RF pulses from the console and outgoing signal from the sample via filters and the preamplifier that covers the ^{31}P Larmor frequency.
2. Tune and match the probe (^{31}P and ^1H electronic circuits).
3. Using a simple pulse-acquisition sequence vary the pulse length to determine the maximal and the zero intensities (90° and 180° pulses, respectively). During this setup procedure using an isotropic liquid the ^1H decoupling is unnecessary and can even heat and damage the sample and/or probe head as long acquisition times are used to fully record the free induction decay. Adjust the carrier frequency to have the signal close to the center of your spectral window and all the other parameters according to Table 1. Adjust the receiver gain to avoid saturation and good sensitivity at the same time. Using a short pulse adjust and save the phases to have a purely positive signal intensity. Calibrate the chemical shift scale with the maximum of the phosphoric acid signal at 0 ppm. Adjust the power of the pulses to have a 90° pulse in the range of 5 μs . Once these parameters have been determined for a spectrometer and probe they provide a valuable starting point for future setup procedures, which should then require only minor readjustments.
4. Change the spectrometer settings to acquire the ^1H signal. Adjust the power levels of the ^1H channel to have a 90° pulse length $\leq 7 \mu\text{s}$ and the ^1H carrier frequency using a procedure analogous to the previous step. This step is required only once

Fig. 2 (continued) heterogeneity at the level of the phospholipid head groups. Possible morphologies as well as the dynamics explaining such line shapes are discussed in [21]. (e) Bicellar mixture of DMPC, DMPG, and DHPC short-chain lipids (2.4:0.8:1 molar ratio). The intensities at -15 to -10 ppm are indicative that the bilayer made predominantly from DMPC and DMPG orients with the normal perpendicular to the magnetic field direction. The close to isotropic signal intensity at -1 ppm is mostly from the DHPC lipid which forms the rim and undergoes fast realignment

Table 1
Typical parameters for proton-decoupled ³¹P Hahn-echo experiments

Experiment	Proton-decoupled ³¹ P Hahn-echo experiment
Reference sample	85% phosphoric acid
Parameters for optimization	³¹ P 90° and 180° pulses (power level, duration) ¹ H decoupling field (power level)/corresponding ¹ H 90° pulse ³¹ P carrier frequency, ¹ H carrier frequency
Typical ³¹ P B ₁ field for ³¹ P 90° and 180° pulses	50 kHz (corresponding to a 90° pulse of 5 μs)
Typical ¹ H B ₁ field for decoupling	>30 kHz (<8.5 μs)
Recycle delay	1.5 s for phospholipids in a fluid bilayer environment; 5 s for phosphoric acid
Interpulse delay (τ)	40 μs

in a while to measure or control the ¹H decoupling field strength and the ¹H frequency.

- Using the ³¹P and ¹H power levels determined in **steps 3** and **4** adjust the durations of the 90°-τ-180°-τ-(acquisition with ¹H decoupling) pulse program [52]. Adjust the 90° and 180° pulses as determined and all the other parameters according to Table 1. Using the reference sample the performance of the pulse program can be tested by adding a full phase cycle (16 scans). Check the FID for being additive from scan to scan, for the absence of artifacts in its beginning (probe ringing may require longer τ), or spikes (reduction of the decoupling power and/or cleaning the coil may help).
- Stably insert the sample into the center of the NMR coil (*see Note 4*). Reconnect the probe, tune and match (**steps 1** and **2**) for each new sample and after equilibrating to a new temperature. Use the same parameters as before (Table 1) but reduce the recycle delay to 1.5–3 s. Readjust the receiver gain for the lipid sample for maximal sensitivity without saturating the receiver. For a sample containing 100 mg phospholipid the signal should become available with the first scans, a spectrum with good signal-to-noise in a few minutes.

By inspecting (and simulating) the resulting proton-decoupled ³¹P solid-state NMR experiments verify the morphology, alignment and integrity of your lipid bilayer before and after the ¹⁵N NMR measurements (Fig. 2).

3.2.2 Proton-Decoupled
 ^{15}N Cross-polarization
 Solid-State NMR
 Spectroscopy of Membrane
 Samples

The setup procedure for a one-dimensional cross-polarization pulse sequence is described albeit with settings that already aim at being sufficiently close for the two-dimensional correlation experiment (separated local field spectroscopy) described in Subheading 3.2.3.

1. Insert a sample of $^{15}\text{NH}_4\text{Cl}$ (or $(^{15}\text{NH}_4)_2\text{SO}_4$) into the center of the NMR coil. Close probe head and insert the probe into the NMR magnet. Connect the ^{15}N (X) and the ^1H electronic circuits of the probes to the respective electrical connections with incoming radiofrequency pulses from the console and outgoing signal from the sample via filters and the preamplifier that covers the ^{15}N Larmor frequency.
2. Tune and match the probe (^{15}N and ^1H electronic circuits).
3. To acquire ^1H signals using a simple pulse-acquisition sequence use a short pulse (e.g., 2 μs) to adjust and save the phases to have a purely positive signal intensity. Adjust the receiver gain to avoid saturation and good sensitivity at the same time and the carrier frequency to have the signal close to the center of your spectral window. Keep a note of this frequency which should also be used later during cross-polarization and decoupling. The spectrum is relatively broad, but the maximum gives a good initial value for the proton carrier frequency (*see Note 12*). After these adjustments verify the phase settings. Then vary the pulse length to determine the maximal and the zero intensities (90° and 180° pulses, respectively) and adjust the power of the pulses to have a 90° pulse in the range of 4 μs . Once these parameters have been determined for a spectrometer and probe they provide a valuable starting point for future setup procedures, which should then require only minor readjustments (*see Notes 13 and 14*).
4. Switch to the acquisition of the ^{15}N signal. It is recommended to use or program a cross-polarization sequence [51] with the possibility to vary the power levels of the 90° ^1H pulse, the ^1H and ^{15}N contact pulses and the ^1H decoupling independently (cf. the different CP pulse schemes shown in Fig. 3). Keep the ^1H power level fixed as determined in **step 3** for all intervals (90° pulse, contact time and decoupling during acquisition), set the length of the 90° ^1H pulse as determined, chose a contact time of ≤ 5 ms and vary the power level of the ^{15}N channel to obtain the maximal intensity. Once you obtain the first signal correct and save the phases for a positive ^{15}N signal, calibrate the chemical shift scale with the maximum of the ammonium peak of $^{15}\text{NH}_4\text{Cl}$ at 39.3 ppm [53], and set the ^{15}N carrier frequency close to 0 ppm (*see Note 15*). In case major corrections of the frequencies had to be made check and readjust the power levels for maximal signal intensity as well as the phases. At all times adjust the receiver gain to avoid saturation and good sensitivity at the same time.

Fig. 3 (a) PISEMA pulse sequence. (b) Proton nutation pulse sequence with frequency offset of the ^1H pulse by $+\Delta\nu$ or $-\Delta\nu$, respectively. (c) ^{15}N nutation experiment. The cross-polarization (CP) and decoupling steps involve a frequency that is well-centered with respect to the ^1H spectrum ("on-resonance"). The prime in panel **b** indicates that at this stage the offset is arbitrary, whereas during Lee–Goldburg decoupling it has a well-defined value (Figs. **a** and **4**)

5. Once the cross-polarization conditions have been optimized verify that the 90° ^1H pulse provides indeed the maximal ^{15}N signal intensity within the cross-polarization pulse sequence. The ^{15}N signal should be zero when a 180° ^1H pulse is applied. Once all these parameters have been determined for a spectrometer and probe they provide a valuable starting point for

future setup procedures, which should then require only minor corrections (*see* **Notes 13** and **14**). By always comparing the noise level and the signal intensity with previous settings using the same reference sample it is possible to keep track of the performance of the probe and the spectrometer.

6. Using the reference sample the performance of the pulse program can be tested by adding a full phase cycle (eight scans). Check the growing free induction decay for being additive from scan to scan.
7. Remove the reference sample and stably insert the membrane sample into the center of the NMR coil, tune and match the ^{15}N and ^1H channels for each new sample.
8. Switch to the acquisition of ^1H . Record the ^1H NMR spectrum of the sample and adjust the ^1H power levels to obtain the same $90^\circ/180^\circ$ pulse length as determined in **step 5**. Depending on the content of water and salt significant increases in power may be necessary (typically by 1–3 dB) to obtain the same probe performance (i.e., effective B_1 field) (*see* **Notes 4** and **14**).

Switch to the cross-polarization pulse program and the spectrometer to the acquisition of ^{15}N . Adjust the following parameters by keeping the previous ^{15}N —but by using the new ^1H power levels: 90° ^1H pulse as determined in **steps 5** and **8**, contact time 0.4–1 ms, spectral width 40 kHz, and an acquisition time of 1–3 ms (long enough to avoid truncation of the FID, but short enough to avoid excessive sample heating during ^1H decoupling). Adjust the receiver gain to avoid saturation and good sensitivity at the same time. Use the chemical shift calibration of **step 4**. Check the first acquisitions (mostly noise) for the absence of artifacts in its beginning and spikes throughout the FID (reduction of the decoupling power and/or cleaning the coil may help). During acquisition the sample should be cooled with a stream of air, which can be humidified by bubbling through a gas wash bottle provided that acquisition is at $\geq$ room temperature (if the temperature of the water bath is above that of the connecting lines and probe water condensation will occur at the colder sites). It is recommended to check the tuning of the ^1H channel after a few hours of signal averaging as water loss or redistribution within the sample can occur due to RF heating (*see* **Notes 4** and **5**) (Table 2).

3.2.3 Two-Dimensional ^{15}N Solid-State NMR Spectra of Oriented Membrane Samples Correlating the ^{15}N Chemical Shift and the ^{15}N – ^1H Dipolar Coupling

This family of experiments reveals from the labeled amide sites the ^{15}N chemical shift as well as the ^{15}N – ^1H dipolar coupling [54], both parameters being orientation dependent [17]. The two-dimensional experiment presented here has been shown to provide additional spectral resolution when prelabeled polypeptides are investigated (Fig. 3a). It consists of two parts: a cross-polarization experiment (Subheading 3.2.2) and a SEMA pulse train during

Table 2
Typical parameters for proton-decoupled ^1H - ^{15}N cross-polarization experiments

Experiment	Standard cross-polarization (CP) experiment
Reference sample	^{15}N -ammonium chloride or ^{15}N -ammonium sulfate
Parameter for optimization	Proton 90° pulse (power level, duration) ^{15}N - ^1H CP condition (power level ^1H and ^{15}N) ^{15}N carrier frequency, ^1H carrier frequency
Typical ^1H B_1 field for the initial 90° pulse	62.5 kHz (corresponding to a 90° pulse of 4 μs)
Typical ^1H B_1 field for CP	62.5 kHz (4 μs)
CP time for amides	300–800 μs
Recycle delay	3 s for amides (this time could in principle be shortened but for protecting the sample from excessive heating due to RF irradiation), >5 s for ammonium salts

the t_1 evolution period (Subheading “Adjustment of the SEMA Pulse Train”). Following the flow of events described in Fig. 5 the corresponding settings are optimized step by step and should start with the simple one-dimensional cross-polarization setup described in Subheading 3.2.2 using for example a ^{15}N -ammonium salt as a reference sample.

Adjustment of the SEMA Pulse Train

Before setting up the SEMA pulse train the B_1 field for the SEMA pulse train has to be adjusted and calibrated (*see* **Note 16**). As the B_1^{eff} field determines the width of the spectral window in the t_1 dimension a minimum of about 50 kHz is required for B_1^{eff} field to cover well the dipolar ^{15}N - ^1H coupling in the amide bonds of peptides and proteins (≤ 20 kHz; [15]) (*see* **Note 17**). Aiming at an efficient B_1 field of 65 kHz seems reasonable and this value will be adjusted during the following two-dimensional nutation experiments (Subheading “Adjustment of the SEMA Pulse Train”).

Determination of the Power Level for the Proton B_1^{eff} Field

1. Before starting this optimization step the nitrogen spectrum should be calibrated following Subheading 3.2.2, **step 4** and the nitrogen carrier frequency should thereafter not be altered. Although the position of the proton carrier will be verified and adjusted during this experiment a good starting value will significantly accelerate the optimization steps (cf. Subheading 3.2.2, **step 3**). The CP conditions of the ^1H nutation experiment (Fig. 3b) are also taken from the previous settings (Subheading 3.2.2).
2. The effective B_1^{eff} field is measured from a nutation experiment (Fig. 3b) where the initial proton pulse is incremented in a

systematic manner during t_1 in steps of $4\ \mu\text{s}$ for a spectral width of 250 kHz up to typically 0.25 ms for a resolution of 4 kHz. The carrier frequency of this initial pulse is fixed to either $\nu_0 + B_1^{\text{eff}} \times \cos(54.74^\circ)$ or $\nu_0 - B_1^{\text{eff}} \times \cos(54.74^\circ)$, respectively, where ν_0 is the center frequency and B_1^{eff} the *intended* effective B_1 field (which should match the ^{15}N B_1 during t_1 , Fig. 3a). The detection is performed on the ^{15}N channel after cross-polarization to avoid the necessity for analysis of the broad ^1H signal of the protons. The resulting oscillation of the ^{15}N cross-polarization signal (Fig. 3b) reflects the nutation frequency of the ^1H dipolar coupled magnetization around the magic angle during t_1 (Fig. 3a) and is easily measured after Fourier transformation in t_2 and t_1 . The power level of this initial ^1H pulse is adjusted until the intended effective B_1 field is observed in the nutation experiment (Fig. 3b). In order to specifically detect the nutation frequency of the directly bonded amide protons and avoid offset miscalibrations the contact time must be set equal to a small value, ca. 50–100 μs .

3. The proton center frequency is then verified and corrected by comparing a nutation experiment with *positive* and *negative* frequency offset of the initial proton irradiation period (both experiments should give similar B_1^{eff} if the center frequency is set correctly). If this is not the case modify the center frequency and go back to **step 2**. Perform **steps 2** and **3** in an iterative manner where adjustments are made until a match is obtained. Note, that the CP conditions should be verified after modifications of the center frequency (*see* **Notes 14** and **18**) (Table 3)

Determination of Power Level for the Nitrogen B_1 Field

The B_1 field for nitrogen is measured by a ^{15}N -nutation experiment (Fig. 3c). It consists of a CP experiment followed by an incremented ^{15}N irradiation period. The experiment consists in adjusting the power level of the ^{15}N irradiation until the nutation of this nucleus

Table 3
Typical parameters for a ^1H nutation experiment with a frequency offset detected through ^1H - ^{15}N cross-polarization

Experiment	^1H nutation CP experiment (Fig. 3b)	t.2
Sample	^{15}N ammonium chloride or sulfate	t.3
Parameter for optimization	^1H power level for requested B_1^{eff} field ^1H center frequency (<i>see</i> Note 18)	t.4
Typical ^1H B_1^{eff} field	65 kHz (corresponding to a frequency offset of 37.528 kHz)	t.5
Typical ^1H B_1 field for CP	62.5 kHz (4 μs)	t.6
CP time	50–100 μs	t.7

Table 4
Typical parameters for a ^{15}N nutation experiment detected through ^1H - ^{15}N cross-polarization

Experiment	^{15}N nutation CP experiment (Fig. 3c)
Sample	^{15}N ammonium chloride or sulfate
Parameter for optimization	^{15}N power level for requested B_1 field
Typical ^{15}N B_1 field (matching the ^1H B_1^{eff} under off-resonance irradiation)	65 kHz
Typical ^1H B_1 field for CP	62.5 kHz (4 μs)
CP time	400 μs

matches B_1^{eff} of the ^1H (cf. Subheading “Determination of the Power Level for the Proton B_1^{eff} Field”); thus, Hartmann–Hahn matching is achieved during the SEMA pulse train (t_1 period in Fig. 3a) (Table 4).

Assembly into the PISEMA
Pulse Sequence and
Verification

To assemble the building blocks with the parameters adjusted in the previous sections the PISEMA pulse-sequence is first tested on a reference sample. Ideally, to this end a monocrystal of [^{15}N]-Ac-Leu, [^{15}N]-Ac-Gly, or another N-terminally protected amino acid is available. The free induction decay of the ^{15}N signal should be clearly visible at least until 5 ms of Lee–Goldburg decoupling period (i.e., the 325th 1D slice at $B_1^{\text{eff}} = 65$ kHz). This assures that the parameters for homonuclear decoupling were determined correctly. New pulse sequence should be tested with a powder of molecule representing a peptide bond, that is, an N-terminally protected amino acid such as [^{15}N]-Ac-Leu, [^{15}N]-Ac-Gly, etc. With such a compound, one should obtain a well-known “butterfly” spectrum with correct dipolar couplings. This allows verification of the correctness of the data processing protocol. The number of scans should be enough to unambiguously see the signal in nearly each 1D spectrum (typically 4000 scans for membrane samples, which results in days of acquisition, *see* **Note 14**). After acquiring of the first few 1D spectra oscillations of the signal should become apparent. For membrane samples the ^1H power levels can be adjusted with higher precision by using one-pulse ^1H nutation experiment (Subheading “Determination of the Power Level for the Proton B_1^{eff} Field” and **Note 18**). To compensate for B_1 losses in wet samples it can be advantageous to test and fine-tune the PISEMA experiment on a fully hydrated biological sample such as magnetically oriented filamentous bacteriophage preparations (*see* **Notes 4** and **14**).

3.2.4 ^2H Solid-State NMR Spectra of Oriented or Non-oriented Samples

The experiments described here allow one to monitor a variety of physical properties of the membrane in the presence or in the absence of protein. These experiments require that during the preparation of membranes deuterated lipids are used and depending on the deuteration pattern different properties of the membranes can be revealed. For example, by incorporating fatty-acyl chain deuterated phospholipids the lipid order parameter is monitored as a function of lipid composition, label position, temperature, buffer and/or the presence of protein. The ^2H spectra therefore reveal membrane disruptive properties of proteins and peptides [21, 55]. By mixing deuterated and nondeuterated lipid species selective interactions of the polypeptides with one lipid over the other are detected [24]. Finally, the ^2H spectra of lipids deuterated at the lipid head groups have been calibrated and used for the investigations of electrostatic interactions at the level of the bilayer interface [25, 26]. The measurements can be performed on nonoriented (usage of the pellet that result from Subheading 3.1.1) or oriented samples (procedures Subheadings 3.1.1 and 3.1.2). The protocol is also provided and illustrated in [14].

1. Insert a sample of deuterated water into the center of the NMR coil. Close the probe head and insert the probe into the NMR magnet. Connect the ^2H (X-channel) electronic circuit of the probe to the console. This line carries incoming radio frequency (RF) pulses from the console to the probe and outgoing signal from the sample via the preamplifier (that is tuned to the ^2H Larmor frequency) to the detector.
2. Tune and match the probe for the ^2H carrier frequency.
3. Using a simple pulse-acquisition sequence vary the pulse length to determine the maximal and the zero intensities (90° and 180° pulses, respectively). Adjust the carrier frequency to have the signal close to the center of your spectral window and all the other parameters according to Table 5. Adjust the receiver

Table 5
Typical parameters for ^2H -echo experiments

Experiment	^2H echo experiment	
Reference sample	Deuterium-enriched water	
Parameters for optimization	^2H 90° (power level, duration) ^2H carrier frequency	
Typical ^1H B_1 field for ^2H 90° pulses	>50 kHz (corresponding to a 90° pulse of <5 μs)	
Recycle delay	0.3 s for phospholipids in a bilayer environment; 5 s for deuterated water	
Interpulse delay (τ)	40 μs	

- gain to avoid saturation and good sensitivity at the same time. 816
 Using a short pulse adjust and save the phases to have a purely 817
 positive signal intensity (Lorentzian). Calibrate the chemical 818
 shift scale by setting the maximum of the $^2\text{H}_2\text{O}$ signal to 0 Hz. 819
 Adjust the power of the pulses to have a 90° pulse in the range 820
 of $\leq 5 \mu\text{s}$. Once these parameters have been determined for a 821
 spectrometer and probe they provide a valuable starting point 822
 for future setup procedures, which should then require only 823
 minor readjustments. 824
4. Using the ^2H power levels determined in **step 3** adjust the 825
 durations of a $90_x\text{-}\tau\text{-}90_y\text{-}\tau$ -acquisition pulse program 826
 [56]. Adjust the 90° pulse as determined and all the other 827
 parameters according to Table 5. Using the reference sample 828
 the performance of the pulse program can be tested by adding a 829
 full phase cycle (eight scans). Check that the oscillations (free 830
 induction decay—FID) recorded from each repetition add to 831
 the overall signal intensity, for the absence of artifacts in its 832
 beginning (probe ringing may require longer τ) or of spikes 833
 (cleaning the coil may help). With some spectrometers, an 834
 installed lock unit may interfere with the signal. This problem 835
 can be solved by reducing/turning off the lock power. 836
 5. Stably insert the membrane sample into the center of the NMR 837
 coil. Reconnect the probe, tune and match (**steps 1 and 2**) for 838
 each new sample and after equilibrating to a new temperature. 839
 Use the parameters determined before (Table 5). Readjust the 840
 receiver gain for the lipid sample to obtain the maximal sensi- 841
 tivity without saturating the receiver. The signal of deuterated 842
 lipids should become visible within minutes, a spectrum with 843
 reasonable signal-to-noise in about 0.5–1 h. Note that the 844
 signal from natural abundance deuterium in water may show 845
 up first and can be reduced by using deuterium-depleted water 846
 for sample hydration. 847
 848

4 Notes

1. The solubility of lipids in chloroform and dichloromethane are 850
 very similar. Therefore, preference should be given to the latter 851
 as it evaporates more easily, has less of an effect on the environ- 852
 ment and is less toxic. To dissolve lipids and peptides solvents 853
 such as 2,2,2-trifluoro ethanol hexafluoroisopropanol, formic 854
 acid, and/or MilliQ water are commonly used [14]. However, 855
 the use of any of such solvents to complex proteins can cause 856
 irreversible denaturation. 857
2. Often it is desired to test the effect of lipid composition on 858
 the membrane protein–lipid interactions. On the one hand 859
 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) 860

represent well the phospholipid composition of many biological membranes as the choline head group is abundant in the bilayers of higher organisms. The composition of these membranes is furthermore mirrored by a saturated fatty acyl chain at position 1 and an unsaturated chain at position 2 and POPC represents well their average hydrophobic thickness. Notably, whereas some polypeptides exhibit stable structural properties in a variety of different lipid membranes [43] others are quite sensitive to even small changes in lipid composition [57]. Furthermore, the negative charges of eukaryotic membranes are often represented by phosphatidylserine, and these membranes are also rich in cholesterol. On the other hand, a simple bacterial membrane mimic could be phosphatidylethanolamine/phosphatidylglycerol 3:1. Notably, however, many of these lipid components exhibit low solubility in alcohols. Therefore, dichloromethane (or chloroform) provides an alternative for most long-chain lipids (e.g., see the catalog information by Avanti Polar Lipids, Alabaster, AL).

3. The equilibration period for hydration ranges from a few hours up to a few days. Hydration can be accelerated by passing through a vapor steam or by adding microliter amounts of water per glass plate. Diverse equilibration conditions are obtained by contact of the atmosphere with saturated solutions of LiCl (15% r.h.), K_2CO_3 (44% r.h.), NaBr (57% r.h.), NaCl (75% r.h.), KCl (86% r.h.), KNO_3 (93% r.h.) or pure water (100% r.h.) [58]. Extended equilibration and storage at 100% r.h. should be avoided as this promotes microbial growth. Hydration chambers can be easily prepared by filling a plastic box (e.g., lunch or plastic box) with salt and some water. An inverted petri dish serves as a platform to avoid the direct contact of the salt solution with the sample.
4. Usually solid-state NMR experiments are set up with the help of standard samples with high signal intensity. By doing so it is assumed that the parameters do not change between the standard and the sample of interest. However, with the advent of high-field NMR spectrometers the proton B_1 fields tend to be attenuated in wet samples (i.e., hydrated lipid bilayers). This can be corrected by readjusting the proton parameters through a direct measurement of the proton parameters on the sample itself [14]. With linear amplifiers the correction increments (measured in dB) should be the same for all of the power levels. If this is not the case the initial pulse and the cross-polarization need to be adjusted and the decoupling power should remain sufficiently strong (≥ 50 kHz). Depending on the instrumentation it may be easier to use the same power levels for all the periods of proton irradiation.

Furthermore, conventional probes tend to heat the sample upon radiofrequency irradiation, which leads to the loss of water and changes of the proton settings during long acquisitions. In such cases and in order to enhance sample stability the following modifications help (individually or in combination): reduction of the CP period (0.3 ms often provides more signal intensity than 1 ms), acquisition times and power levels of decoupling as short/small as possible (e.g., 1.5 ms, B_1 less than 50 kHz), increased recycle delays. Furthermore, the recent development of probe heads where the coil center is depleted of the electric-field (e-free) has the advantage to reduce heating of the sample. In addition, the proton-tuning and -parameters are barely dependent on the water content of the sample. This makes e-free probes an ideal tool for more sophisticated NMR experiments like SEMA, where a precise adjustment of the proton parameters is essential.

5. For DNP/solid-state measurements biradicals need to be added to the sample [37]. This has been achieved in different manners depending on the detailed sample conditions, the solid-state NMR approach used and the polypeptide under investigations. For MAS experiments the highest enhancements have been obtained when soaking proteoliposome pellets with “DNP juice,” a biradical solution containing deuterated solvent and a high amount of the cryoprotectant glycerol [59, 60]. However, no bulk water is present in oriented membrane stacks. Therefore, the addition of cryoprotectants is not necessary and only interferes with membrane alignment. In these cases, the concentrated dispersion after reconstitution of the protein into the membranes (Subheading “Concentration of the Samples”) is transferred (by centrifugation) into a tube containing the dry powder of a water soluble biradical, such as TOTAPOL or AMUPOL [37]. A final concentration of 10–15 mM (calculated by taking into consideration the total sample volume) has been found optimal (i.e., typically ~0.3 mg of biradical for 30 μ L volume or for 30 mg of lipids) [37]. After a few freeze–thaw–vortexing cycles the mixture can be applied to polyethylene films and treated similar to the usual glass plate sample (*see* Subheading “Application of Lipid Bilayers onto Solid Supports for Alignment”). Note, because of their low heat conductivity glass plate samples have been found unsuitable for DNP experiments and polymer films and sapphire plates have been introduced for static oriented DNP/solid-state NMR measurements [36] or to magic angle oriented sample spinning on rolled up polymer cylinders [61]. When oriented membrane samples encompassing peptides have been prepared the biradicals were applied early on when dissolving the lipids and the peptides in organic solvent

[37, 41, 61] but this is not feasible for most membrane proteins.

In analogy to DNP experiments under MAS conditions, deuterated lipids and/or hydration with D₂O/H₂O vapor have been tested for oriented DNP/solid-state NMR samples, however, for samples hydrated at 93% r.h. so far the DNP enhancement were found unaffected by deuteration [37]. Because bicelles orient in the magnetic field only in a limited temperature range [29] these have to our knowledge so far not been investigated by DNP solid-state NMR.

The CP contact times under DNP conditions (100 K) range tend to be somewhat shorter [37, 41]. The ¹H T₁ relaxation times are dependent on the type and concentration of the biradical and the temperature. For samples with TOTA-POL or bTbk typical values are ~1.2 s, for AMUPol or PyPol-C16 ~2.3 s [37]. Nevertheless, a 3 s recycle delay is typically maintained.

6. A wide variety of buffers can be used including Tris, HEPES, or citrate buffer. However, the interference of the buffer with the NMR experiments should be considered. For example, phosphate buffers result in large isotropic peaks hampering the analysis of ³¹P NMR powder patterns of phospholipids, water contains a significant amount of deuterium (which can be reduced by using deuterium-depleted water) or other buffers cause background signals in ¹³C NMR experiments. Furthermore, high salt concentrations interfere with the tuning of NMR probes and the radio frequency irradiation during NMR spectroscopy (*see Note 4*). Furthermore, extreme pH values cause the hydrolysis of ester bonds and thereby lipid degradation. Long-term stability of lipids can be assured by pH 6–7 [62], reduced hydration and/or storage in the freezer. When oriented samples are prepared at <100% r.h. it should further be considered that buffer and salt are concentrated into the small intermembrane space where the residual water molecules interact with the adjacent membranes. We have noted that the presence of buffer and salts often degrades the quality of the membrane alignment (e.g., [63]).
7. For freezing the lipid samples liquid nitrogen is also used. However, by doing so, we have observed the occurrence of isotropic signal intensities in ³¹P NMR spectra of pure lipids which may be related to the speed of freezing and the corresponding ice/glass formation. Therefore, slow freezing in a –20 °C freezer seems a better choice. The effect of freeze–thaw cycles on different membranes has been analyzed previously [64].
8. For extrusion, the lipid concentration should not exceed a concentration of 10 mM (7–8 mg/mL). Furthermore, lipids

t.1 **Table 6**
t.2 **Detergents used for membrane protein solubilization and their physical properties**

t.2	Detergent	MW (g/mol)	Aggregation number	CMC (mM)	Charge	Dialyzable	Micelle MW
t.3	CHAPS	615	10	7.4–10	Zwitterionic	Yes	6149
t.4	CHAPSO	631	11	8–10	Zwitterionic	Yes	6940
t.5	DDM	511	110–140	0.18	Neutral	Yes	70,000
t.6	DPC	351	38–56	0.9–1.1	Zwitterionic	Yes	19,000
t.7	OG	292	27–100	23.2–25	Neutral	Yes	8000–29,000
t.8	OTG	308	92–114	7.6	Neutral	Yes	18,000
t.9	SDS	288	63	1.4–8	Anionic	Yes	18,000
t.10	C12E8	539	90–110	0.07–0.10	Neutral	Yes	54,000

t.11 *CHAPS* 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate [68–70], *CHAPSO* 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate [70, 71], *DDM* dodecylmaltoside [72], *DPC* dodecylphosphocholine [73–75], *OG* octyl- β -glucopyranoside [76–78], *OTG* octyl- β -thio-glucopyranoside [79, 80], *SDS* sodium dodecyl-sulfate [81], *C12E8*: n-dodecyl octaethylene glycol monoether. Reference [82] provides an extensive overview over many more detergents used for protein reconstitution

with a phase transition temperature above room temperature might require relatively high forces for extrusion.

9. The relatively small diameter of the sonicated vesicle imply relatively high curvature of the bilayers, which might influence the interaction to membrane proteins and the small radius makes sedimentation by centrifugation very difficult.

10. Table 6 provides some basic physical parameters for a number of detergents used for the purification and/or structural analysis of membrane polypeptides. These data give some first indication of their suitability for the reconstitution protocol of a given protein.

11. Beads are also used to control the orientation of proteins reconstituted into lipid bilayers, like proton channels [65]. Here, a recombinantly expressed protein contains a long histidine tag (His₁₀), which is used for immobilized metal affinity-chromatography interactions with commercially available nickel-NTA silica beads (Qiagen Inc., Valencia, CA). Following the manufacturer’s instructions, the recombinant proteins are bound and immobilized on nickel beads via the decahistidine tag. Thereafter the protein–bead suspension is reconstituted into lipid vesicles by the detergent dialysis method. In doing so the part of the protein directly linked to the histidine tag faces the outer membrane of the double layer and is thereby available for interaction with the ligands in the bulk solution. In cases where further investigations of protein interactions are planned by NMR spectroscopy, the

paramagnetic nickel, leading to line broadening, should be exchanged with diamagnetic Zn^{2+} ions. To do so, nickel-beads are washed with a 0.3 mM EDTA solution to remove the nickel ion and consequently washed with water. Finally, incubation with 100 mM zinc sulfate solution recharges the nitrilotriacetic acid (NTA) matrix, which then is used as described before.

12. If the ^1H line width of the ammonium salt hampers the accurate determination of the ^1H frequency a water or a humid membrane sample can be used giving sharper line width and allowing a more accurate frequency setting. The ^1H frequencies determined in Subheading 3.2.1 should be close to the one determined in this context.
13. Once the approximate settings of ^1H power and pulse length have been obtained with the probe and the same type of samples **step 3** can be omitted as these parameters can be optimized in further experiments by first adjusting the Hartmann–Hahn matching conditions (**step 4**) and then by variation of the initial ^1H pulse (**step 5**). A pulse program that allows for the independent modification of the proton power levels during the various phases of the experiment (90° pulse, CP, decoupling) makes this task easier as the level of the 90° pulse can be changed without affecting the CP matching condition. At the Hartmann–Hahn matching condition of static samples the X and the ^1H spins precess at the same speed in the rotating frames defined by the respective B_1 fields (i.e., $\gamma_X B_{1X} = \gamma_H B_{1H}$) and the precession frequency is calculated from the 90° -pulse length (t_p) by $\nu_1 = 1/4t_p$.
14. Once the approximate setting for NMR experiments are known it may be possible to adjust CP power levels and pulse durations on the membrane samples themselves provided that there is enough signal intensity to obtain reasonable spectra within a few scans. This can be the case for sensitive nuclei such as ^1H and ^{31}P , or for ^{15}N proteins which are uniformly labeled and reconstituted into well oriented membranes. Beneficial to the signal-to-noise ratio are higher magnetic fields of the spectrometer, better electronics, the peaks are usually sharper for oriented bicelles when compared to mechanically oriented samples, and the cross-polarization efficiency is increased for strong dipolar couplings such as they occur in transmembrane protein domains and immobile sites. Furthermore, much higher signal intensities can be obtained under DNP conditions even though with the suppression of averaging motions at low temperatures the line widths tend to increase [37, 41].

For samples with high water and/or salt content, such as bicelles, the RF efficiencies tend to be attenuated by 10–20% when compared to crystalline samples. In such cases it is

advantageous to adjust the ^1H and ^{15}N pulses and the CP conditions on the sample, whenever possible. Furthermore, the water line (4.7 ppm) can be used to determine the ^1H offset frequencies of amides (which occur at 7–10 ppm depending on the ^1H - ^{15}N orientation relative to the magnetic field, that is, the helical tilt angle and the bicelle alignment (cf. Fig. 12 in [66])).

15. The range of nearly constant excitation covers a spectral range of $1/4t_p$ where t_p is the pulse length. Therefore, by adjusting the ^{15}N carrier frequency in this manner positions it close to the chemical shift range of the amide and amine resonances. At the same time by placing the carrier outside the spectral range eventual artifacts at the position of the irradiation that could potentially be confused with spectral resonances are avoided.
16. The SEMA pulse provides a spin lock of the ^1H magnetization along the magic angle, thereby removing ^1H - ^1H dipolar interactions. At the same time the ^{15}N power level should be adjusted to satisfy the Hartmann–Hahn matching condition. It allows the magnetization to travel between ^{15}N and surrounding protons but not within the ^1H network thereby increasing the life time of the ^{15}N signal. The first condition is fulfilled by off-resonance ^1H irradiation ($\Delta\nu = B_{\text{eff}} \times \cos 54.7^\circ$) to direct the vectorial addition of B_1 field and $\Delta\nu$ along the magic angle ($B_1 = B_1^{\text{eff}} \times \sin 54.7^\circ = B_1^{\text{eff}} \times 0.816$ which also provides the scaling factor for the dipolar interaction). The second condition consists in matching the ^{15}N B_1 field (i.e., the power level) to the B_1^{eff} field of the protons.

Specific PISEMA variants exist for better sensitivity and resolution depending on the application [54, 66, 67]. Furthermore, it should be noted that it can be easier to adjust the correct ^1H and ^{15}N frequencies (*see Note 13*) when PISEMA of the in-planar and transmembrane region are recorded separately, thereby avoiding artifacts.

17. Generally, it is considered that the spectral quality is ameliorated with higher B_1 fields although we have found, using ^{15}N , ^{13}C -labeled crystals, that above a certain threshold, artifacts tend to appear possibly due to limitations of the probe to take up the power properly in combination with the long duration of the SEMA pulse trains.
18. In real samples different types of protons exist and these interact with each other and with the magnetic field in an anisotropic manner resulting in broad ^1H spectra (e.g., *see* Fig. 1A in reference [13]). During the SEMA sequence the protons which are in strong dipolar contact with the ^{15}N nuclei (e.g., the amide protons in the case of backbone-labeled polypeptides) are decoupled from the ^1H - ^1H interaction network. The

Fig. 4 (a) Calculation of the effective proton B_1 field (B_1^{eff}) during the SEMA pulse train. Whereas the B_1 field is controlled by the pulse power level the frequency offset $+\Delta\nu$ and $-\Delta\nu$ relative to the center frequency is generated by the pulse synthesizer. The center frequency has to match the resonance frequency of the nuclei. The symmetry for positive and negative offsets ($+\Delta\nu$ and $-\Delta\nu$, respectively) is only given if ν_0 , the center frequency of the protons, is well adjusted. Note that here the “strength of the B_1^{eff} field” is indicated by the resulting nutation frequency (in Hz). Typical result of the two-dimensional nutation experiment for the proton channel (Fig. 3b)

optimal ^1H center frequency is determined empirically using a reference sample (Subheading “Determination of the Power Level for the Proton B_1^{eff} Field”) which ideally matches as closely as possible the situation of the oriented membrane samples. In practice, crystals of N-terminally protected amino acids and/or oriented bacteriophages are used (*see also* Note 4).

A precise measurement of the B_1 nutation frequency requires a relatively sharp nutation peak (Fig. 4). This is only the case when B_1^{eff} is close to the magic angle and the dipolar interactions are averaged. This also means that the nutation signal provides a good indicator for the Lee–Goldburg conditions.

If the two nutation frequencies at positive and negative offset (B_{1+}^{eff} and B_{1-}^{eff} , respectively) are different the correction term for the center frequency ($\delta\nu_0$) can be calculated from the respective B_{1+}^{eff} and B_{1-}^{eff} frequencies using the relationship

$$\delta\nu_0 = \frac{(B_{1+}^{\text{eff}})^2 - (B_{1-}^{\text{eff}})^2}{4\Delta\nu}$$
Note that in this equation the “strength of the B_1^{eff} fields” are represented by the resulting nutation frequency (in Hz). An additional nutation experiment should be used to verify the correct setting, but the iterative approach described in Subheading “Determination of the Power Level for the Proton B_1^{eff} Field” (steps 2 and 3) may become unnecessary.

3.2.2.

Parameter for optimization	Preliminary ^1H Center Frequency
Sample	^{15}N Ammoniumchlorid or humid sample
Experiment	^1H pulse(90°) — aquisition

Aim	Proton-Decoupled ^{15}N Cross-Polarization Solid-State NMR Spectroscopy
Sample	^{15}N Ammoniumchlorid or ^{15}N -acetyl-amino acid (mono-crystal)
Parameter for optimization	^1H 90° Pulse (Power Level, Duration) ^{15}N - ^1H CP Condition

3.2.3.

Aim	Determination of the Power Level for the Proton B_1^{eff} Field
Sample	^{15}N Ammoniumchlorid or ^{15}N -acetyl-amino acid (mono-crystal)
Experiment	^1H nutation CP experiment Figure 3b

Aim	Determination of the Power Level for the Nitrogen B_1 Field
Sample	^{15}N Ammoniumchlorid or ^{15}N -acetyl-amino acid (mono-crystal)
Experiment	^{15}N nutation CP experiment Figure 3c

Aim	Assembly into the PISEMA Pulse Sequence and Verification
Sample	^{15}N -acetyl-amino acid (mono-crystal), ($\text{U-}^{15}\text{N}$)-filamentous bacteriophage (magnetically oriented)
Experiment	PISEMA experiment Figure 3a

Fig. 5 Flowchart summarizing the setup protocol for the PISEMA experiment

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