Chapter 18

NMR Spectroscopy of Lipid Bilayers

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Abstract

Knowledge of lipid structure and dynamics in a membranous environment is of first importance for deciphering cellular function. Sterols and sphingolipids are key molecules in maintaining membrane integrity and are the building blocks of membrane domains, such as "rafts". Phosphatidyl inositols are crucial in signalling pathways as they are recognition sites at the membrane surface. Other lipids such as Phosphatidylethanolamines, Cardiolipins, or diacylglycerols are essential in fusion processes. It is fundamental to have techniques that can resolve the structure and dynamics of various classes of lipids in a membrane environment. Solid state NMR with its high resolution and wide line facets is a very powerful tool for such determinations. Here it is shown that multinuclear solid state NMR provides information on the nature of the membrane phase (bicelle, lamellar, hexagonal, micelle, cubic, etc.), its dynamics (fluid or gel, or liquid-ordered with cholesterol), and the molecular structure of embedded lipids when using the magic angle sample spinning (MAS) apparatus. Typical examples of relatively simple experiments are shown both with high resolution MAS and wide line NMR of lipids. Relaxation time measurements are also described to measure lipid motional processes from the picosecond to the second timescale.

Key words: Solid state ^{1}H–^{2}H–^{14}N–^{31}P-NMR, Bicelles, Liposomes, Micelles, Gel and fluid phases, Hexagonal phases, Liquid-ordered state, Cholesterol, Sphingolipid, Diacylphosphatidylcholine, Diacylphophatidylethanolamine, Deuterium-labelled lipids, Magic angle sample spinning, Wide line spectra, Relaxation times, Order parameters

1. Introduction

Lipids are one of the building blocks in cell biology and are present in many cellular locations. They may be found as small aggregates allowing their transport in water media or may be embedded in cellular membranes where their dynamics are much more reduced. In this case, they constitute the cement of the bilayer membrane and also play many signalling roles. For instance, cholesterol and sphingolipids are key molecules in maintaining membrane integrity and are the building blocks of membrane liquid-ordered phases,

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also named "rafts" (1, 2). Lipids such as phosphatidylinositol (PtdIns) are essential in signalling pathways as they are recognition sites at the membrane surface (3).

In order to decipher their structure in their native membrane environment, Nuclear Magnetic Resonance is one of the most powerful techniques because it can act in media of reduced dynamics such as membranes or aggregates. NMR relies on the presence of active nuclei in atoms that constitute the lipid molecules. A lipid naturally contains protons (^{1}H), carbon (only the ^{13}C is active, but in low natural abundance), phosphorus (^{31}P), oxygen (only the ^{17}O is magnetically active but has a very low natural abundance) and nitrogen (^{14}N). Some lipids may be chemically labelled with the hydrogen isotope, deuterium (^{2}H), with fluorine (^{19}F), carbon-13 (^{13}C), or with other nuclei of interest for structural biology.

Lipids are not water soluble; as a consequence, their structure and dynamics must be evaluated in a hydrated model membrane state (bicelles, liposomes, bilayers vesicles of various sizes, etc.) (4) or in real membranes (envelopes of living cells) (5).

2. Materials

2.1. Samples, Preparation Tools and Measuring Cells

1. Ninety percent ultra pure water (miliQ-system) + 10% deuterated water ($^{2}H_2O$), v:v (Eurisotop, Saint-Aubin, France).
2. Deuterium-depleted water, $^{1}H_2O$ (Isotec-Sigma-Aldrich, France).
3. Lipids: 1,2-Dipalmitoyl-$^{2}H_{62}$-*sn*-glycero-3-phosphocholine (16:0/16:0 DPPtdCho-$^{2}H_{62}$), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0/14:0 DMPtdCho), 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (6:0/6:0 DCPtdCho), Liver Phosphatidylethanolamine(PtdEth),LiverPhosphatidylcholine (PtdCho), brain Sphingomyelin (SM) and cholesterol are from Avanti Polar Lipids (USA). 1-Tetradecanoyl-2-(4-(4-biphenyl)butanoyl)-*sn*-glycero-3-phosphocholine (14:0/BB TBBPtdCho) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine $^{2}H_{72}$ (14:0/14:0 DMPtdCho-$^{2}H_{72}$) were synthesized in the laboratory (6, 7).
4. Table top centrifuge Eppendorf (VWR, France).
5. Two millilitre micro centrifuge tubes (VWR, France).
6. MS2 Minishaker (VWR, France).
7. Water bath, thermostated at 50°C.
8. Liquid nitrogen, 50 ml in a 500 ml Dewar.
9. Zirconia (ZrO_2) 4 mm diameter magic angle spinning (MAS) rotor of HR-MAS type (50 µl) and of CP-MAS type (100 µl) (Cortec, France).

2.2. NMR Instrumentation for ^{31}P-NMR

1. NMR Spectrometer equipped for solid state: Bruker Avance 300 operating at 121.5 MHz (Bruker, Wissembourg, France).
2. NMR Probe: ^{1}H/X, 4 mm CP-MAS probe tuned at 121.5 MHz (^{31}P channel) and 300.13 MHz (^{1}H channel); equipped with temperature regulation (thermocouple and heater).
3. PC station with TopSpin software v2.0 (Bruker, Wissembourg, France).
4. Bruker BCU (air cooling) and VT (variable temperature) units to control sample temperature using dry airflow.

2.3. NMR Instrumentation for ^{2}H-NMR

1. NMR Spectrometer equipped for solid state: Bruker Avance 500 operating at 76.8 MHz (Bruker, Wissembourg, France).
2. NMR Probe: Low Gamma ^{1}H/X, 4 mm CP-MAS probe tuned at 76.8 MHz; equipped with temperature regulation (thermocouple and heater).
3. PC station with TopSpin software v2.1 (Bruker, Wissembourg, France).
4. Bruker BCU (air cooling) and VT (variable temperature) units to control sample temperature using dry airflow.

2.4. NMR Instrumentation for ^{14}N-NMR

1. NMR Spectrometer equipped for solid state: Bruker Avance 500 operating at 36.1 MHz (Bruker, Wissembourg, France).
2. NMR Probe: PE triple ^{1}H–X–Y CP probe tuned at 36.1 MHz on X channel; 50 Ω on the Y and ^{1}H channels; equipped with temperature regulation (thermocouple and heater). Homemade 4 mm horizontal coil.
3. PC station with TopSpin software v2.1 (Bruker, Wissembourg, France).
4. Bruker BCU (air cooling) and VT (variable temperature) units to control sample temperature using dry airflow.

2.5. NMR Instrumentation for HR-MAS ^{1}H-NMR

1. NMR Spectrometer equipped for solid state: Bruker Avance 500 operating at 500.16 MHz (Bruker, Wissembourg, France).
2. NMR Probe: ^{1}H/^{13}C, ^{2}H-lock, z-gradient, 4 mm HR-MAS probe tuned at 500.16 MHz, equipped with temperature regulation (thermocouple and heater).
3. PC station with TopSpin software v2.1 (Bruker, Wissembourg, France).
4. Bruker BCU (air cooling) and VT (variable temperature) units to control temperature on sample using dry airflow.
5. Bruker pneumatic MAS unit to control sample rotation at the magic angle.

2.6. Data Analysis

1. PC computer loaded with Topspin 2.1 software (Bruker, Wissembourg, France) and Origin software V7.5 (OriginLab Corporation, Massachusetts, USA).
2. NMR Friend V1.2 plug-in software to import and treat NMR spectra (see Note 1) on Origin software developed by Sébastien Buchoux, UMR5248 CNRS-University Bordeaux 1, France (see Note 2).

3. Methods

The membrane that defines the cell entity or the cell organelles is by its very nature a medium that is half way between a liquid and a solid. This state called soft matter is by definition a liquid crystalline medium whose anisotropic properties are essential for membrane function and cells. Molecules there embedded, such as lipids, proteins, drugs may undergo many dynamic processes such as lateral diffusion in the bilayer plane, rotational diffusion around the bilayer normal or transverse diffusion from one membrane leaflet to the other. They may also group as ordered patches in the membrane plane that are named "rafts" to picture the rigidity of these membrane domains that swim in a "sea" of more fluid lipids and proteins. Understanding structure and dynamics of membrane components will provide insight towards deciphering complex biological processes such as cell fusion, trafficking, apoptosis, energetic, signal transduction, etc.

Solid state NMR is the only non-destructive, non-invasive and quantitative spectroscopy that can measure membrane structure and dynamics. There are two data categories that can be obtained using NMR, spectra and relaxation times. Spectra are in turn of two types "wide line" and "high resolution". Wide line NMR (spectra may span several hundredths of kilohertz) can be sensitive to membrane macroscopic orientation and symmetry (Fig. 1) and molecular motions (Fig. 2). It is a useful tool to determine the nature of membrane phases (bicelles, lamellar, hexagonal, isotropic (micelles or cubic), etc.) (8, 9) and track membrane dynamics (membrane fluidity, fusion, gel (solid-ordered), liquid-ordered, and fluid (liquid-disordered) states). The well-known regulating effect of cholesterol on membrane phases (increasing fluidity of solid-ordered phases and decreasing that of liquid-disordered phases) can easily be monitored by using phospholipids or sterols that are deuterated (2, 10–12). Analysis of spectral moments or quadrupolar splittings allows describing membrane dynamics either globally (bilayer/molecule fluctuations) or locally by measuring the reorientation of individual chain segments in the bilayer core (Fig. 2). By taking advantage of

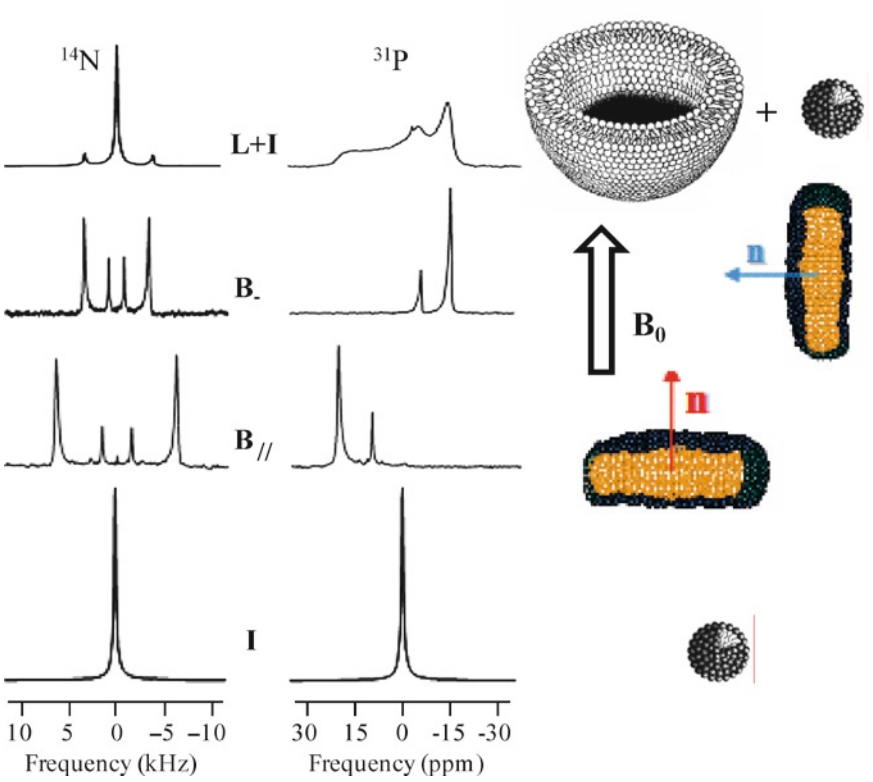

Fig. 1. Nitrogen-14 and Phosphorus-31 solid state NMR spectra of lipids embedded in different membrane phases. *Top row*: spectra obtained for a mixture of large micrometric liposomes and nanometric micelles made of phosphatidylcholine lipids. *Middle-top row*: spectra of DMPtdCho/DCPtdCho bicelles in 80% D_2O oriented such that the normal to the disc (400 nm) plane is perpendicular to the magnetic field direction, B_0. *Middle-bottom row*: spectra of TBBPtdCho/DCPtdCho bicelles (biphenyl bicelles) in 80% D_2O oriented such that the normal to the disc (800 nm) plane is parallel to the magnetic field direction, B_0. *Bottom row*: spectra of phopholipid micelles (nm size). On the *right hand side* are represented *cartoons* to picture the nature of the lipid systems. *L*, *I*, *B* and $B_{//}$, B_0, and *n* stand respectively for lamellar, isotropic, bicelle normal perpendicular and parallel, magnetic field, and bilayer normal.

magnetic field orientational dependence, wide line NMR spectra can also probe average orientations of molecules embedded in membranes (membrane topology) (10, 11, 13) when used in conjunction with X-rays or neutrons structural information. The 3D structure of molecules in membranes is also obtained by making use of MAS, a technique by which the sample is rapidly spun at an angle of 54.7° with respect to the magnetic field (Fig. 4d) leading to pseudo "high-resolution" spectra (sharp lines of less than 1 Hz width), as in the liquid state, the sample being still in the membranous "liquid-crystalline" state (Fig. 4). All multidimensional NMR techniques used in solution NMR can hence be applied bringing assignment of resonances to molecular structure (Table 1, Fig. 4). Relaxation times (T_1 and T_2) are obtained by making use of specific pulse sequences (see Note 10) and bring

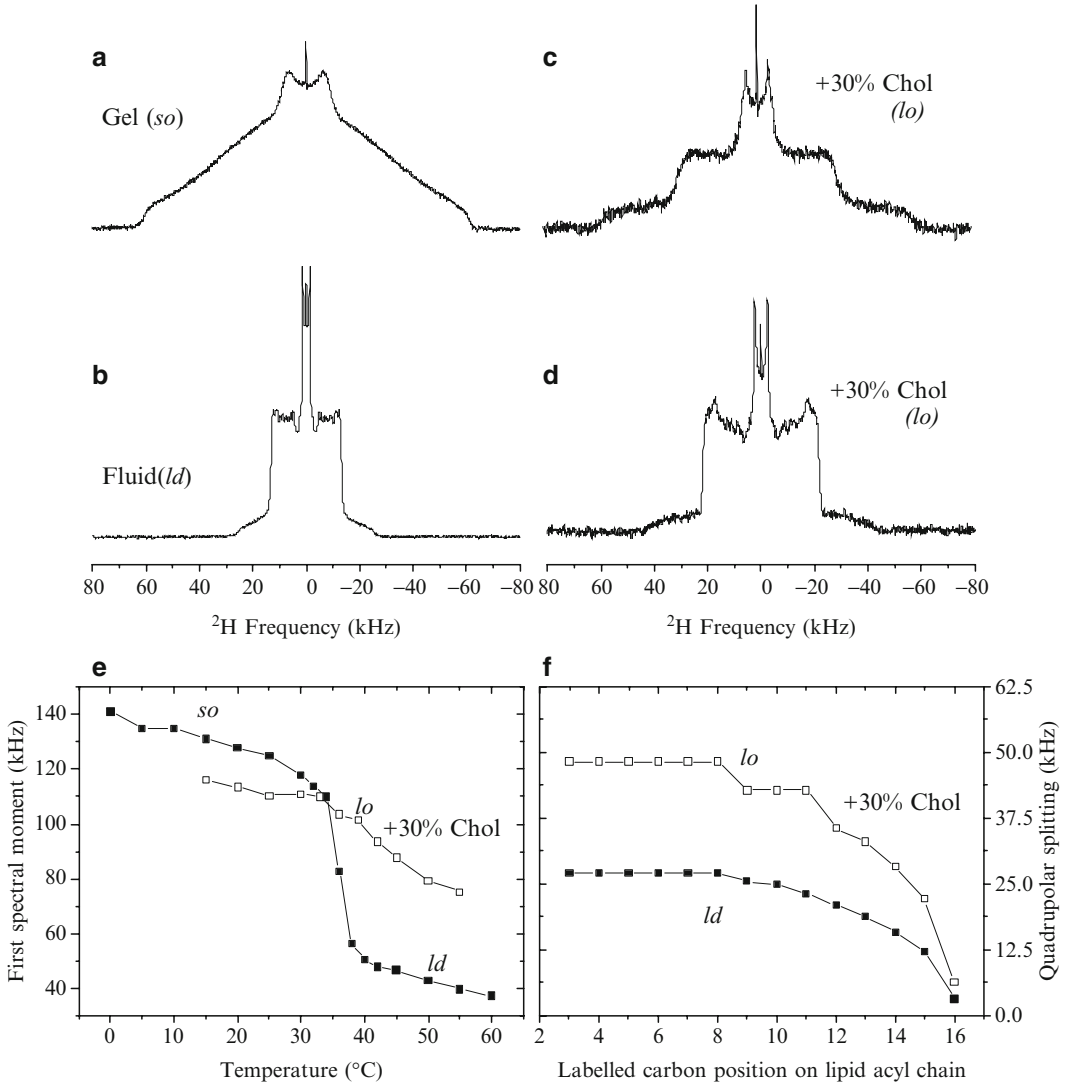

Fig. 2. Solid-state wide line ^{2}H-NMR spectra of DPPtdCho-$^2H_{62}$ with and without cholesterol dispersed in water (liposomes). (**a**) DPPtdCho, $T = 10°C$, gel (*so*) phase; (**b**) DPPtdCho, $T = 40°C$, fluid (*ld*) phase; (**c**) DPPtdCho/cholesterol (2/1 molar), $T = 10°C$, *lo* phase; (**d**) DPPtdCho/cholesterol (2/1 molar), $T = 40°C$, *lo* phase. (**e**) First spectral moment as a function of temperature for DPPtdCho (*filled symbol*) and DPPtdCho/cholesterol (*empty symbol*) spectra. (**f**) Quadrupolar splittings for DPPtdCho acyl chains at 40°C in the presence (*empty symbols*) and absence (*filled symbols*) of cholesterol.

values ranging from microseconds to minutes (Fig. 5). Analysis of relaxation times in the frame of proper motional modes leads to the calculation of speed of molecular motion and activation energies. This allows describing membrane dynamics from the atomic level where intra-molecular motions dominate (nano-to-picoseconds timescale), to the cell level where membrane hydrodynamic modes of motion play an important role (seconds time scale) (14–16).

3.1. Determination of Lipid Phase Nature (Lamellar (Liposomes), Oriented-Bicelle, Isotropic) Using Wide Line ^{31}P-NMR

1. Appropriate amounts of phospholipids are weighed (see Note 11), and a suitable volume of water is added to obtain a lipid hydration (water mass/total mass) 80–95% (w/w). Lipid concentration is of the order 10–200 mM. For bicelle samples, 14:0/BB TBBPtdCho and 14:0/14:0 DMPtdCho represent about 80 mol% of the total lipid, 6:0/6:0 DCPtdCho the remaining 20%.
2. The hydrated sample is then vigorously shaken in a vortex mixer, frozen in liquid nitrogen, and heated to 50°C for 10 min in a water bath. This cycle of liposome (multilamellar vesicles of micrometre size) or bicelle (bilayer discs of 400–800 nm diameters) formation is repeated three to five times until a homogeneous preparation is obtained at room temperature.
3. The resulting sample (may appear translucent when in bicelle phase) is transferred (see Note 12) into a 4-mm NMR ZrO_2 rotor (100 µl) that is placed in the magnetic field. The measuring probe is properly tuned to 121.5 MHz (^{31}P channel) and 300.13 MHz (^{1}H channel) and the space homogeneity ("shimming") is adjusted at best.
4. Using the variable temperature set up (VT and BCU units), samples are allowed to equilibrate 15–30 min at a given temperature before the time dependent NMR signal (FID) is acquired; the temperature is regulated to ±1°C (see Note 9).
5. ^{31}P-NMR spectroscopy is performed at 121.5 MHz. FID are acquired using a phase-cycled Hahn-echo pulse sequence with gated broad-band proton decoupling (17). Typical acquisition parameters are as follows: $\pi/2$ pulse width (P1) of 14.5 µs (see Notes 3 and 10) spectral window (SW) of 32 kHz, interpulse delay (D6) of 50 µs and a repetition delay (D1) of 5 s (see Note 4). Typically, 512 scans are recorded in 42 min (see Note 5).
6. Processing parameters: noise filtering of the FID with an exponential window (see Note 7), characterized by the Lorentzian line-broadening factor (LB = 10–50 Hz, see Note 6), is used. Fourier Transformation is applied to get the spectrum, and its phase is adjusted to correct for unavoidable time delays in acquisition. Reference is made to 85% H_3PO_4 (0 ppm). The width of the spectrum is measured and used to calculate the chemical shift anisotropy (CSA).
7. The spectrum (Fig. 1, ^{31}P column, top) is characteristic of a non-oriented spectrum (broad and major axially symmetric pattern of 45 ppm CSA) superimposed on a small isotropic line (0 ppm). This is typical of a mixture of micrometre liposomes and nanometre micelles (sketches on the right).

Table 1
^{1}H chemical shifts of liposomal lipids in H_2O/D_2O (90/10). Ambient temperature, reference TSP (0 ppm). The sample is spun at 7 kHz at the magic angle

	N	Sphingomyelin	Liver PtdEth	Liver PtdCho
Head group	α	nd	3.97	3.99
	β	nd	3.22	3.66
	γ	3.66	7.87	3.21
Backbone	g1	nd	nd	nd
	g2	5.31	5.29	5.31
	g3	4.00	4.03	4.27
Fatty acyl chains	1	5.71		
	1′			
	2	5.71	2.40	2.28
	2′	2.22/2.31	nd	2.28
	3	2.02	1.57	1.57
	3′	1.60	nd	1.57
	4	1.29	1.24	1.25
	4′	1.29	2.00	1.25
	5	1.29	1.24	1.25
	5′	1.29	nd	1.25
	6	1.29	1.24	1.25
	6′	1.29	nd	1.25
	7	1.29	1.24	1.25
	7′	1.29	2.75	1.25
	8	1.29	1.24	1.25
	8′	1.29	nd	2.02
	9	1.29	1.24	1.25
	9′	1.29	nd	nd
	10	1.29	1.24	1.25
	10′	1.29	2.75	nd
	11	1.29	1.24	1.25
	11′	1.29	nd	2.77
	12	1.29	1.24	1.25

(continued)

Table 1 (continued)

N	Sphingomyelin	Liver PtdEth	Liver PtdCho
12′	1.29	nd	nd
13	1.29	1.24	1.25
13′	1.29	2.75	nd
14	1.29	1.24	1.25
14′	1.29	nd	2.02
15	0.88	1.24	1.25
15′	1.29	nd	1.25
16		1.24	1.25
16′	1.29	2.00	1.25
17		1.24	1.25
17′	1.29	1.24	1.25
18		0.85	0.86
18′	0.88	1.24	0.86
19			
19′		1.24	
20′		0.85	

nd not determined. Numbers separated by "/" (e.g. 2.22/2.31) indicate that there are two lines of indicated chemical shifts

8. The spectrum (Fig. 1, ^{31}P column, middle-top) is much narrower and made of two sharp lines representing 14:0/14:0 DMPtdCho (major, –12 ppm) and 6:0/6:0 DCPtdCho (minor, –4 ppm) in a bicelle (disc) structure with the bilayer normal oriented at 90° with respect to the magnetic field direction (sketches on the right).
9. The spectrum (Fig. 1, ^{31}P column, middle-bottom) is also made of two sharp lines representing 14:0/BB TBBPtdCho (major, 20 ppm) and 6:0/6:0 DCPtdCho (minor, 8 ppm) in a bicelle (disc) structure with the bilayer normal oriented at 0° with respect to the magnetic field direction (sketch on the right).
10. The spectrum (Fig. 1, ^{31}P column, bottom) is characteristic of a phase with isotropic symmetry (cubic, micelles, etc.): a single, very intense sharp line appears and is centred at ca. 0 ppm.

3.2. Determination of Lipid Phase Nature (Lamellar (Liposomes), Oriented-Bicelle, Isotropic) Using Wide Line ^{14}N-NMR

1. Step 1 as in Subheading 3.1.
2. Step 2 as in Subheading 3.1.
3. The resulting sample (may appear translucent when in bicelle phase) is transferred into a 4-mm NMR ZrO_2 rotor (100 µl) that is placed in the magnetic field. The measuring probe is properly tuned to 36.1 MHz (X-channel).
4. Step 4 as in Subheading 3.1.
5. ^{14}N-NMR spectroscopy is performed at 36.1 MHz. NMR spectra are acquired using a quadrupolar echo pulse sequence (18). Typical acquisition parameters are as follows: spectral window of 100 kHz; $\pi/2$ pulse width 10 µs (see Note 13), and interpulse delay of 200 µs. A recycle delay of 0.2 s is used. Typically, 40,000 scans are accumulated in 2.2 h.
6. Processing parameters: noise filtering of the FID with an exponential window, characterized by the Lorentzian line-broadening factor (LB = 100 Hz), is used. Fourier Transformation is applied to get the spectrum, and its phase is adjusted to correct for unavoidable time delays in acquisition. The spectrum centre is set to 0 ppm.
7. The spectrum (Fig. 1, ^{14}N column, top) is characteristic of a non-oriented spectrum (broad axially symmetric powder pattern of ca. 9 kHz) superimposed on a isotropic line (0 Hz). This is typical of a mixture of micrometre liposomes and nanometre micelles (sketches on the right).
8. The spectrum (Fig. 1, ^{14}N column, middle-top) is made of two sets of sharp doublets representing 14:0/14:0 DMPtdCho (major, ca. 7 kHz splitting) and 6:0/6:0 DCPtdCho (minor, ca. 1.5 kHz splitting) in a bicelle (disc) structure with the bilayer normal oriented at 90° with respect to the magnetic field direction (sketches on the right).
9. The spectrum (Fig. 1, ^{14}N column, middle-bottom) is also made of two sets of sharp doublets representing 14:0/BB TBBPtdCho (major, ca. 13 kHz Splitting) and 6:0/6:0 DCPtdCho (minor, ca. 3 kHz splitting) in a bicelle (disc) structure with the bilayer normal oriented at 0° with respect to the magnetic field direction (sketch on the right).
10. The spectrum (Fig. 1, ^{14}N column, bottom) is characteristic of a phase with isotropic symmetry (cubic, micelles, etc.): a single, very intense sharp line appears and is centred at ca. 0 Hz.

3.3. Determination of Lipid Dynamics in Non-oriented Multilamellar Bilayers (Gel, Fluid, Liquid-Ordered) Using Wide Line ^{2}H-NMR

1. Appropriate amounts of phospholipids are weighed to reach a final concentration of ca. 10–200 mM (see Note 11).
2. A suitable volume of deuterium-depleted water, $^{1}H_2O$ is added to obtain a lipid hydration of 80–90% (v/w). Hydration is defined as the mass of water over the total mass of the system (phospholipids and water).

3. The hydrated sample is then vigorously shaken in a vortex mixer, frozen in liquid nitrogen, and heated to 50°C for 10 min in a water bath. This cycle of liposome (multilamellar vesicles) formation is repeated three to five times until a milky dispersion is obtained at room temperature.
4. The resulting dispersion is transferred into a 4-mm NMR ZrO_2 rotor (100 µl) that is placed in the magnetic field (see Note 12). The measuring probe is properly tuned to 76.77 MHz (^{2}H Channel), and the space homogeneity is adjusted at best.
5. As step 4 in Subheading 3.1.
6. ^{2}H NMR spectroscopy is performed at 76.77 MHz. NMR spectra are acquired using a solid quadrupolar echo pulse sequence (18). Typical acquisition parameters are as follows: spectral window of 500 kHz, $\pi/2$ pulse width 3 µs (see Note 13), and interpulse delay of 30 µs. A recycle delay of 1.5 s is used. Typically, 512–1,024 scans are accumulated in 10–20 min.
7. Processing parameters: noise filtering of the FID with an exponential window, characterized by the Lorentzian line-broadening factor (LB = 50–300 Hz), is used. Fourier Transformation is applied to get the spectrum, and its phase is adjusted to correct for unavoidable time delays in acquisition. The peak-to-peak separation, called the quadrupolar splitting, that can be in principle obtained for non-equivalent C–^{2}H bonds, can be measured on some spectra. Spectral moments can also be used to estimate width and shape changes in spectra (14, 19).
8. The spectrum at 10°C for DPPtdCho-$^2H_{62}$ liposomes (Fig. 2a) is characteristic of a gel phase where the quadrupolar interaction occurring for each C–^{2}H bond (there are 62 deuterium labelled positions on the acyl chains of DPPtdCho-$^2H_{62}$) reveals non-axial symmetry due to the very restricted chain dynamics (solid-ordered phase, *so*) and to the symmetry of the phase. No peak-to-peak measurement can be measured on such a spectrum, except for the central broad doublet assigned to the chain-end deuterated methyl groups. The first moment is calculated using a routine built in the Origin software.
9. The spectrum obtained at 40°C for DPPtdCho-$^2H_{62}$ liposomes (Fig. 2b) is much narrower along the frequency axis and shows a shape characteristic of a lamellar fluid phase with axial symmetry (liquid-disordered phase, *ld*). The overall spectrum is nonetheless complex because it represents the superposition of all spectra coming from all C–^{2}H bonds. Several peak-to-peak separations, quadrupolar splittings, can be measured and plotted as a function of the labelled carbon position along the acyl chain (Fig. 2f). Spectral deconvolution

to get oriented-like spectra (20, 21) can be applied here for more resolution (see Note 2).

10. The spectrum at 10°C for DPPtdCho-$^{2}H_{62}$/cholesterol (2/1) liposomes (Fig. 2c) is characteristic of a liquid-ordered phase (*lo*) where the quadrupolar interaction occurring for each C–^{2}H bond reveals axial symmetry due to the axial diffusion of lipids in the membrane.
11. The spectrum at 40°C for DPPtdCho-$^{2}H_{62}$/cholesterol (2/1) liposomes (Fig. 2d) is characteristic of a liquid-ordered phase with greater dynamics than at 10°C (spectrum of smaller width). Several peak-to-peak separations, quadrupolar splittings, can be measured and plotted as a function of the labelled carbon position along the acyl chain (Fig. 2f).
12. Spectra were recorded as a function of temperature, the first moment calculated and plotted against temperature (Fig. 2e). Elevated values are found for gel phase temperatures and depict an ordered rigid state (*so*) whereas smaller values are found above the gel-to-fluid phase transition temperature (ca. 37°C) for pure DPPtdCho-$^{2}H_{62}$ liposomes and depict a disordered lamellar fluid phase (*ld*). In case of added cholesterol, the transition is nearly smoothed out demonstrating the regulating effect of cholesterol: it increases the fluidity of ordered phases and decreases that of fluid phases: this is the liquid-ordered state (*lo*).
13. Peak-to-peak separation, so-called quadrupolar splittings, measured for individual C–^{2}H bonds report on local space and time angular fluctuations (order parameters, see Note 14). When plotted against labelled acyl chain position, they report on local dynamics (order/disorder) (Fig. 2f). The greatest the quadrupolar splitting, the highest the order (less bond fluctuations). C–^{2}H bonds close to the glycerol backbone (positions 2–8) are much more ordered than those near the bilayer centre (positions 12–16).

3.4. Determination of Lipid Molecular Structure in a Membrane Environment Using ^{1}H HR-MAS (Fig. 3)

1. Appropriate amounts of phospholipids are weighed to reach a final concentration of ca. 1–10 mM (see Note 11).
2. A suitable volume of ultra pure water mixed with deuterated water (90:10, v:v) containing traces of TSP (trimethylsilylpropionic acid) as a reference (0 ppm) is added to obtain a lipid hydration of 80–95% (v/w). Hydration is defined as the mass of water over the total mass of the system (phospholipids and water).
3. The hydrated sample is then vigorously shaken in a vortex mixer, frozen in liquid nitrogen, and heated to 50°C for 10 min in a water bath. This cycle of liposome (multilamellar vesicles) formation is repeated three to five times until a milky dispersion is obtained at room temperature.

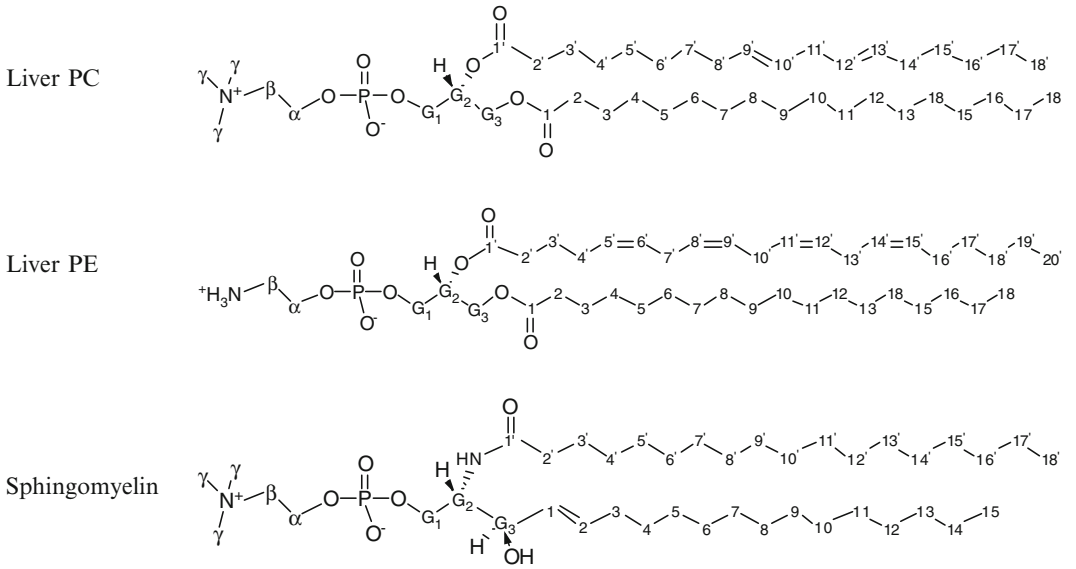

Fig. 3. Molecular structures of selected lipids with numbering for Table 1.

4. The resulting dispersion is transferred into a 4-mm NMR ZrO_2 rotor for "High-Resolution MAS NMR" (50 µl) that is placed in the magnetic field.
5. Speed for MAS is set to 7–10 kHz using the pneumatic unit device. Using the variable temperature set up (VT and BCU units), samples are allowed to equilibrate 15–30 min at 30°C before the time dependent NMR signal (FID) is acquired; the temperature is regulated to ±1°C. The measuring probe is properly tuned to 500.16 MHz and the space and time homogeneity is adjusted by a deuterium "lock" system using the deuterated solvent as a reference.
6. ^{1}H-NMR spectroscopy is performed at 500.16 MHz. NMR spectra are acquired using a single pulse sequence with water suppression (22). Typical acquisition parameters are a pulse duration (P1) of 5.5 µs, a time domain (TD) set to 32 K points, a spectral width (SW) of 20 ppm and a relaxation delay (D1) of 3 s. The number of scans is set to 16. These conditions determine a total acquisition time of 90 s for the time dependent signal (FID).
7. Processing parameters: noise filtering with an exponential window of the FID, characterized by the Lorentzian line-broadening factor (LB = 0.3 Hz), is used. This noise filtering generally improves the signal-to-noise ratio, but at the cost of resolution. Fourier Transformation is applied to get the spectrum, and its phase is adjusted to correct for unavoidable time delays in acquisition (Fig. 4). Referencing (TSP rightmost resonance is set to 0 ppm), peak picking (measure of chemical

shifts in parts per million), and peak integration (measure line areas) are part of the relevant processing parameters.

8. The spectrum obtained in the absence of rotation (Fig. 4c) is characteristic of an unresolved proton spectrum of a membrane phase. Very little peak assignment can be made on such a spectrum.
9. The spectrum obtained for a MAS speed of 10 kHz (Fig. 4b) shows a resolution that is similar to that obtained in liquids (organic solution, Fig. 4a), the sample being still under the form of liposomes. Referencing, peak and peak integration can now be applied.
10. The strategy for structure elucidation is the same as for lipids in organic solvent solution; 2D-NMR experiments (COSY,

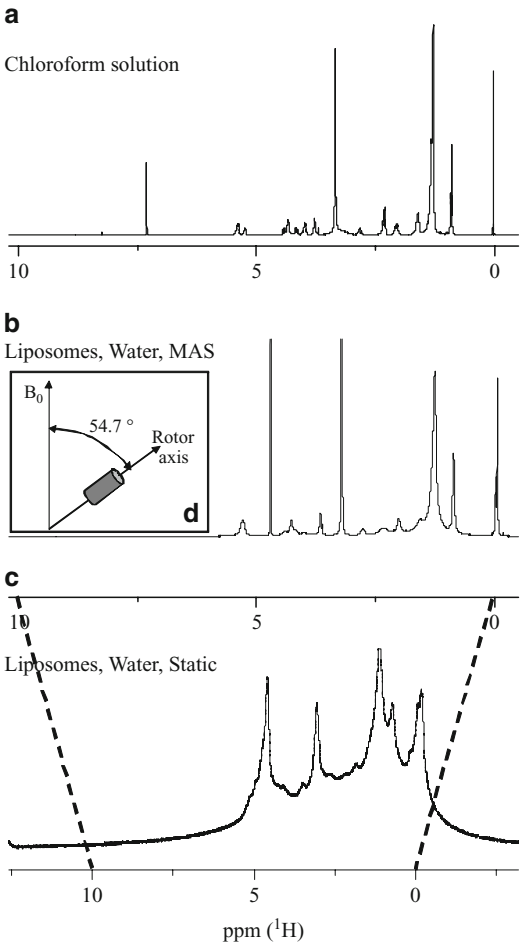

Fig. 4. ^{1}H-NMR spectra of Liver PtdCho. (**a**) Lipid dissolved in $CDCl_3$ solution. (**b**) Solid state "high-resolution" magic angle sample spinning (sketch in inset **d**) of lipid dispersed in water containing 10% D_2O, MAS speed = 10 kHz. Water suppression was used during signal acquisition. (**c**) Spectrum without spinning. Note the change in *x*-scale from (**b**) to (**c**).

TOCSY) are often needed for complete assignment. Table 1 reports the structural assignment of ^{1}H chemical shifts for selected lipids (Fig. 3) under liposomal form, i.e., as water dispersions (see Note 8).

3.5. Determination of Lipid Internal Dynamics in Oriented Bilayers Using ^{2}H-NMR Spin-Lattice Relaxation Time Measurements

1. Appropriate amounts of phospholipids are weighed to obtain a bicelle composition (ca. 80 mol% of 14:0/14:0 DMPtdCho-$^{2}H_{72}$ and 20 mol% of 6:0/6:0 DCPtdCho (23)) and dissolved in organic solvent ($CHCl_3$/MeOH), the solvent is evaporated, water is added, the sample shaken in a vortex mixer and lyophilized to remove solvent traces.
2. A suitable volume of deuterium-depleted water is added to obtain a lipid hydration (water mass/total mass) 80–95% (w/w). Total lipid concentration is of the order of 200–50 mM.
3. The hydrated sample is then vigorously shaken in a vortex mixer, frozen in liquid nitrogen, and heated to 50°C for 10 min in a water bath. This cycle of bicelle (bilayer discs of 400 nm diameter) formation is repeated three to five times until a homogeneous translucent preparation is obtained at room temperature.
4. The resulting dispersion is transferred into a 4-mm NMR ZrO_2 rotor (100 µl) that is placed in the magnetic field (see Note 12). The measuring probe is properly tuned to 76.77 MHz (^{2}H channel) and the space homogeneity is adjusted at best.
5. As step 4 in Subheading 3.1.
6. ^{2}H NMR spectroscopy is performed at 76.77 MHz. A series of ca. 30 NMR signals are acquired using an inversion recovery quadrupolar echo pulse sequence. Typical acquisition parameters are as follows: spectral window of 250 kHz, $\pi/2$ and π pulse widths of 3 and 6 µs (see Note 13), interpulse delay in the echo sequence of 30 µs, variable delays, t_1, between the inverting π pulse and the first $\pi/2$ pulse of the echo sequence ranging between 1 and 1,500 ms. A recycle delay of 1.5 s is used and typically, 256 scans are accumulated per spectrum in 6 min. The whole experiment lasts for 4.5 h.
7. Processing parameters: noise filtering of all FIDs with the same exponential window, characterized by a Lorentzian line-broadening factor (LB) of 50 Hz. Fourier Transformation is applied to get the spectrum series. The zero order phase correction is adjusted on the last spectrum of the series and applied to all spectra (Fig. 5c).
8. Quadrupolar splitting can easily be measured on oriented spectra and attributed to labelled carbon positions in the molecular structure (Fig. 5b) using previous assignments (6).

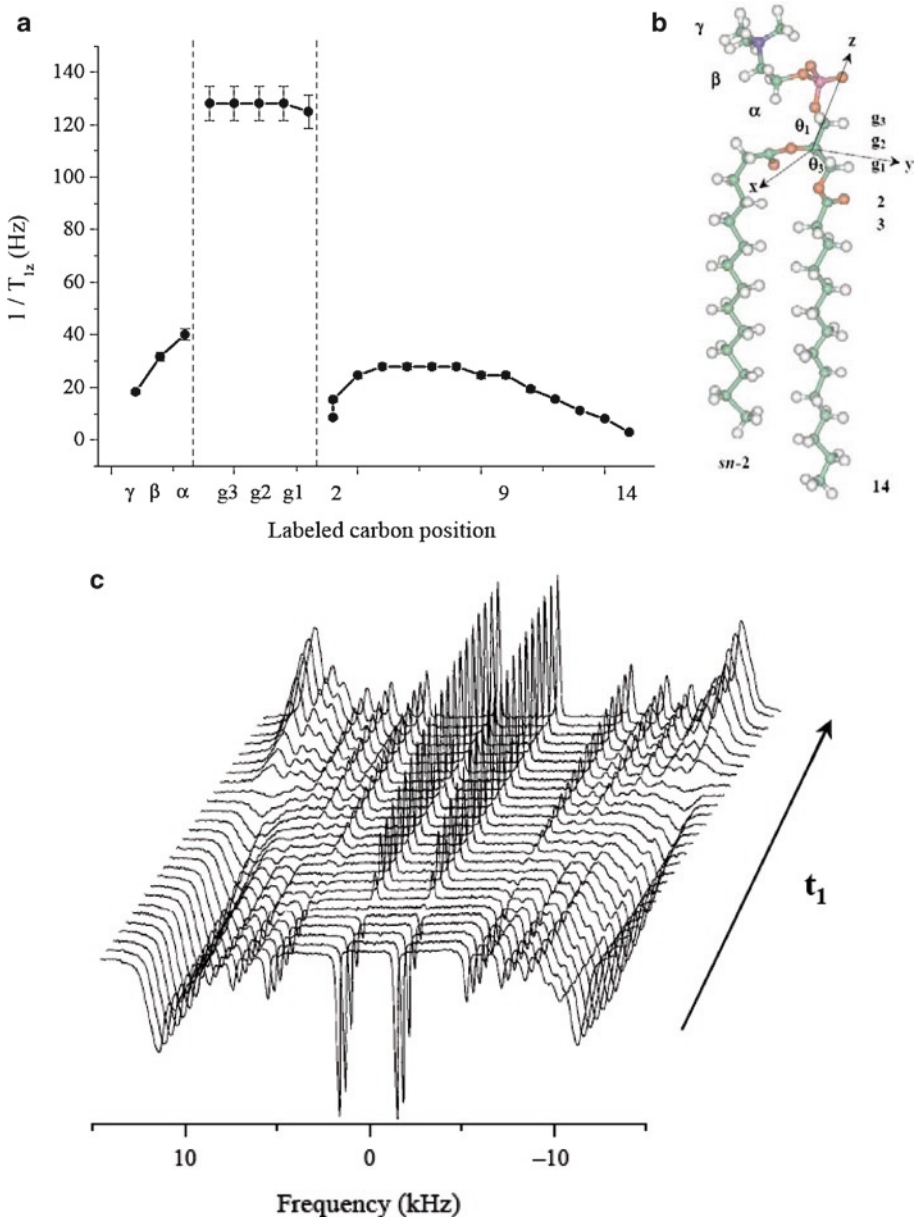

Fig. 5. ^{2}H-NMR relaxation of DMPtdCho-$^{2}H_{72}$ in DMPtdCho/DCPtdCho bicelles hydrated at 80% with $^{1}H_{2}O$. (**a**) Rate of spin-lattice relaxation ($1/T_{1z}$) as a function of labelled carbon position. (**b**) DMPtdCho-$^{2}H_{72}$ molecule showing carbon numbering. (**c**) Stack of Fourier transformed NMR signals after an inversion recovery quadrupolar echo sequence. *X*-axis is the classical frequency axis (kHz) and the t_1 axis is that of delays between the inverting pulse and the first pulse of the echo sequence. Analysis of such data yields (**a**).

The area of each individual splitting is determined using a Bruker routine, and the area variation as function of t_1 is fitted against the proper equation for inversion-recovery sequence in the Bruker package to obtain the individual spin lattice relaxation time T_1 (see Note 15).

9. Spin-lattice relaxation times measured for individual C–^{2}H bonds report on molecular motions occurring at the nanosecond timescale. When plotted against labelled carbon position, they report on local dynamics (correlation times for segment or molecular motions) (Fig. 5a). The relaxation rate (inverse of T_1) is the largest for C–^{2}H bonds of the glycerol backbone (positions g1, g2, g3) and is much smaller for head group (α, β, γ) or chain (2–14) positions. This indicates that the glycerol backbone possesses a very restricted motional freedom and can be considered as a semi-rigid "kneecap" separating two very fluid zones, the phosphocholine head group facing the water medium and the acyl chains constituting the oily bilayer interior.

4. Notes

1. Data may also be treated on the computer that drives the spectrometer. Most spectrometer-linked computers are now PCs.
2. Spectral moment calculations or spectral de-Pake-ing may not be available on computers driving spectrometers. They can nonetheless be implemented in PC by obtaining the source code from the authors (14, 21).
3. P1 is determined with a routine provided on all spectrometers (POPT on Bruker software). It must be determined especially for samples with different solvents (because of the different dielectric constants that may modify the P1 value). Note that elevated salt concentration may alter the P1 duration.
4. Relaxation delays are also determined using a routine provided on spectrometers, which allows calculation of spin lattice relaxation time, T_1. D1 is usually set to $5 \times T_1$ for optimum relaxation delay.
5. The number of scans is chosen such that the signal-to-noise ratio is about or greater than 50.
6. Line broadening is set in order to filter spectral noise without marked modification of peak line width.
7. More sophisticated window filtering can be used as they are available on most spectrometer software.
8. Many groups can be identified by their ^{1}H chemical shifts using chemical shift tables (24) and coupling constants (multiplet separation). Two-Dimensional NMR experiments (COSY, TOCSY) are sometimes needed for complete assignment.
9. Experiments may sometimes be run overnight implementing a temperature variation from low to high temperatures and back to low to detect possible sample hysteresis.

10. A pulse sequence is a series of radiofrequency impulsions separated by specific delays, which allow selecting one or several magnetic or electric molecular interactions to be observed. The time dependent signals (FID) that are detected following a pulse sequence must be Fourier Transformed to obtain spectral data.
11. In case of lipid mixtures (DPPC/Cholesterol), lipids are dissolved in organic solvent ($CHCl_3$/MeOH), the solvent is evaporated, water is added, the sample shaken in a vortex mixer and lyophilized to remove solvent traces All these experiments can be run with DPPtdCho-$^2H_{31}$ (less expensive) instead of DPPtdCho-$^2H_{62}$, the numbers of scans must then be increased by a factor 2.
12. In order to facilitate the transfer into the rotor, it may be wise to vary (increase or decrease) the temperature to be in a fluid state. The sample may then be easily poured into the rotor.
13. The pulse must be very short to acquire very wide spectra (hundredths of kilohertz). Using longer pulse widths will lead to intensity loss in the outer verges of the spectrum and hence errors in spectral moment calculation.
14. Order parameters, *S*, represent a time and space average of molecules or of molecular bonds with respect to the bilayer normal, *z*, in an axially symmetric situation, as in lipid bilayers. The *SZ* order parameter may vary from 1 (M_1 of ca. 150 kHz or C–D_2 quadrupolar splitting of 62.5 kHz) for fully rigid solid state systems to 0 for liquid systems.
15. $M_z(t_1) = M_z(t_1 = \infty) \times (1 - 2 \times \exp(-t_1/T_1 z))$ where $M_z(t_1)$ is the *z*-magnetization at time t_1 obtained by straight integration of individual lines; $M_z(t_1 = \infty)$ is the Boltzmann equilibrium magnetization; t_1 is the delay between the inverting π pulse and the first $\pi/2$ pulse of the echo sequence and T_{1z} is the spin-lattice (longitudinal) NMR relaxation time.

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