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Review

# Choosing membrane mimetics for NMR structural studies of transmembrane proteins

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ABSTRACT

The native environment of membrane proteins is complex and scientists have felt the need to simplify it to reduce the number of varying parameters. However, experimental problems can also arise from oversimplification which contributes to why membrane proteins are under-represented in the protein structure databank and why they were difficult to study by nuclear magnetic resonance (NMR) spectroscopy. Technological progress now allows dealing with more complex models and, in the context of NMR studies, an incredibly large number of membrane mimetics options are available. This review provides a guide to the selection of the appropriate model membrane system for membrane protein study by NMR, depending on the protein and on the type of information that is looked for. Beside bilayers (of various shapes, sizes and lamellarity), bicelles (aligned or isotropic) and detergent micelles, this review will also describe the most recent membrane mimetics such as amphipols, nanodiscs and reverse micelles. Solution and solid-state NMR will be covered as well as more exotic techniques such as DNP and MAOSS.



**Contents**



*Abbreviations:* AAO, anodic aluminium oxide; c7-DHPC, 1,2-heptanoyl-1-*sn*-glycero-3-phosphocholine; CMC, critical micelle concentration; CMT, critical micelle temperature; CTAB, cetyl trimethylammonium bromide; DBBPC, 1-dodecanoyl-2-(4-(4-biphenyl)butanoyl)-*sn*-glycero-3-phosphocholine; DDM, dodecylmaltoside; DHAB, dihexadecyldimethylammonium bromide; DHPC, 1,2-hexanoyl-1-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DM, decylmaltoside; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphate; DMPC, 1,2-myristoyl-1-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol); DMPE, 1,2-myristoyl-1-*sn*-glycero-3-phosphoethanolamine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine; DMSO, dimethyl sulfoxide; DNP, dynamic nuclear polarization; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DOPE, 1,2-oleoyl-*sn*-glycero-3-phosphoethanolamine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine); DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DPC, *n*-dodecylphosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; GUV, giant unilamellar vesicle; HDL, high-density lipoprotein; HIMSELF, heteronuclear isotropic mixing leading to spin exchange via the local field; HSQC, heteronuclear single quantum coherence; LOPG, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-rac-glycerol); LPPG, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-rac-glycerol); LUV, large unilamellar vesicle; MAOSS, magic angle oriented sample spinning; MAS, magic-angle spinning; MLV, multilamellar vesicle; MP, membrane protein; MSP, membrane scaffold proteins; NLP, nanolipoprotein; NMDA, *N*-methyl-D-aspartate; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser effect spectroscopy; OG, octylglucoside; PDC, protein–detergent complex; PEEK, polyetheretherketone; PET, polyethelene terephtalate; PISEMA, polarization inversion spin exchange at magic angle; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol); PSC, protein–surfactant complex; PSPC, 1-palmitoyl-2-stearoyl-1-*sn*-glycero-3-phosphocholine; $R^2$, rotational resonance; REDOR, rotational echo double resonance; SDS, sodium dodecylsulfate; SUV, small unilamllar vesicle; TBBPC, 1-tetradecanoyl-2-(4-(4-biphenyl)butanoyl)-*sn*-glycero-3-phosphocholine; TFE, 2,2,2-trifluoroethanol; $T_m$, melting temperature; TROSY, transverse relaxation optimized spectroscopy

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## 1. Introduction

Biological membranes are complex natural barriers which isolate the content of the cells from the extracellular environment while controlling exchanges with the external milieu. They also compartmentalize a variety of cellular organelles such as the endoplasmic reticulum (ER), mitochondria, chloroplast, Golgi apparatus, lysosome and nucleus. The total surface area of internal eukaryote cell membranes is of 7000 $\mu m^2$, i.e., ten times that of the plasma membrane, highlighting the important biological role of organelle membranes [1]. Biomembranes consist of phospholipid bilayers (mostly replaced by glycolipids in algae and plant chloroplasts), which may also contain cholesterol and are spanned by membrane proteins (MPs). Cholesterol is not found in prokaryotes and is replaced in yeasts, fungi, algae and plants by ergosterol, sitosterol and lanosterol [2]. The lipid composition of biomembranes varies in the different cell types, organelles and organisms as illustrated in Table 1. These differences can be explained by the functions of the membranes and organelles. For example, cholesterol and phospholipids are synthesized in the ER, explaining its high membrane phospholipid content [3]. The lipid composition may even vary between two monolayers of a bilayer and within different regions of a bilayer as evidenced by the hypothesized "lipid rafts" [4]. The variability in membrane composition is also observable between Gram (−) and Gram (+) bacteria. While both bacteria have an inner phospholipid membrane with comparable composition, Gram (−) have an additional outer membrane, of which the external monolayer is enriched in lipopolysaccharides [5]. Although not entirely understood, the incredible diversity of lipids in membranes, and its conservation, is an indication that they must play specific roles in biological processes.

The membrane and its constituents are involved in essential biochemical processes such as molecular transport, signalling, catalysis, cell–cell interactions and fusion. The protein content of membranes is variable and depends on the cell type and activity. For instance, human erythrocytes contain 40% of lipids and 60% of proteins while myelin is composed of 80% of lipids and 20% of proteins in weight [6,7]. MPs are divided into three classes according to their interaction with the membrane, namely intrinsic transmembrane proteins spanning the membrane, peripheral proteins which do not interact with the membrane core, and lipid-anchored proteins covalently bound to one or several lipid molecules [1].

Because one-third of the human genes codes for MPs [8], their structure–function relationship is of great interest considering their involvement in many pathologies. For example, mutations in the vasopressin receptor and cystic fibrosis transmembrane conductance

**Table 1**
Lipid composition of selected biomembranes expressed in average weight% of total lipids except for Peas, *Chlamydomonas reinhardtii* and *Aspergillus niger* membranes where the composition is in mol% of total lipids.

| Cell/Organelle | PC | PE | PS | PG | PI | SM | CL | Ste | Other |
|---|---|---|---|---|---|---|---|---|---|
| *Eukaryotes* | | | | | | | | | |
| Erythrocyte (human) [7,31,262] | 20 | 16 | 5 | 0 | 0 | 20 | 0 | 23 | |
| Erythrocyte (rat) [262] | 34 | 11 | 1 | 0 | 0 | 16 | 0 | 28 | |
| Myelin (rat brain) [263] | 11 | 14 | 7 | 0 | 0 | 6 | 0 | 22 | |
| Rough endoplasmic reticulum (rat liver) [262,263] | 58 | 18 | 3 | 0 | 9 | 4 | 0 | 5 | |
| Mitochondria (rat liver) [262,263] | | | | | | | | | |
| Inner | 43 | 25 | 1 | 2 | 6 | 3 | 17 | 3 | |
| Outer | 47 | 22 | 3 | 3 | 12 | 6 | 4 | 3 | |
| Golgi apparatus (rat liver) [262,263] | 43 | 16 | 5 | 0 | 8 | 11 | 0 | 8 | |
| Nuclear membranes (rat liver) [262,263] | 55 | 20 | 4 | 0 | 9 | 3 | 0 | 9 | |
| Pea root plasma membranes (plant) [264] | 57 | 38 | 0 | 0 | 0 | 0 | 0 | 5[a] | |
| Pea leave chloroplast membranes (plant) [265] | 7 | 0 | 0 | 6 | 1 | 0 | 0 | 2 | 84 (GL) |
| *Chlamydomonas reinhardtii* (alga) [266] | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 3[b] | 56 (GL) |
| *Aspergillus niger* (fungus) [267] | 37 | 32 | 4 | 0 | 3 | 4 | 6 | 7[b] | |
| *Candida albicans* (yeast) [268] | 32 | 21 | 3 | 0 | 4 | 0 | 0 | 13[b] | |
| *Prokaryotes* | | | | | | | | | |
| *Escherichia coli* (Gram-negative bacteria) [5,269] | | | | | | | | | |
| Inner | 0 | 60 | 0 | 33 | 0 | 0 | 7 | 0 | |
| Outer | 0 | 61 | 0 | 13 | 0 | 0 | 1 | 0 | 25 (LPS) |
| *Bacillus subtilis* (Gram-positive bacteria) [270] | 0 | 13 | 0 | 77 | 0 | 0 | 4 | 0 | 6 (GL) |

PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, PG = phosphatidylglycerol, PI = phosphatidylinositol, SM = sphingomyelin, CL = cardiolipin, GL = galactolipids (monogalactosyldiacylglycerol digalactosyldiacylglycerol, sulfoquinovosyldiacylglycerol), LPS = lipopolysaccharides, Ste = sterols (mostly cholesterol).
[a] Mostly sitosterol.
[b] Mostly ergosterol.

regulator (CFTR) protein are respectively responsible for X-linked nephrogenic diabetes insipidus (NDI) [9] and cystic fibrosis [10]. A defect on aquaporin AQP0 in lens fiber cells is involved in congenital cataracts [11], and there is evidence that channels of the TRP (transient receptor potential) superfamily are involved in kidney disorders, cancer and hypertension [12–14]. Sometimes, a single mutation in the transmembrane part of a protein prevents dimerization and causes various cancers as in the case of the tyrosine kinase receptor family [15]. Therefore, several MPs garner the attention of the pharmaceutical industry. For example the transactivation of the epidermal growth factor receptor (EGFR) is being studied in order to prevent the progression of chronic kidney disease [16], and the calcitonin gene related peptide (CGRP) receptor is targeted to treat migraine headaches [17]. Since voltage-gated sodium channels are expressed in different cancers, they are becoming targets for drug design [18], and so are G-protein-coupled receptors (GPCRs) [19].

The arduous crystallization as well as the difficulty to obtain enough quantities of pure and active MPs has limited the number of known three-dimensional structures which account for only 3% of the identified three-dimensional (3D) structures of proteins [20]. Nuclear magnetic resonance (NMR) is a unique tool to determine MP structure and dynamics in native-like conditions [21]. NMR can tackle samples that are solid or liquid, viscous or fluid, oriented or isotropic, static or spinning, cold or warm, etc. It is a non-invasive technique that can give access to very accurate local distances, orientations or dynamics, the docking of biomolecules, but also the full high-resolution 3D structure of a protein by measuring a wealth of structural constraints through 2D or 3D spectra. Beside protons, NMR mostly exploits $^{13}C$, $^{15}N$ and $^{2}H$ when studying MPs. Since those isotopes are not naturally abundant, proteins have to be isotopically enriched, either uniformly or specifically [22–24].

The structural complexity of biological membranes, however, constitutes a challenge for NMR. This obliges the reconstitution of MPs in membrane mimetics which are generally prepared using lipids or detergents. The composition of these mimetics needs to be chosen carefully as it strongly influences several physical properties such as shape, curvature, thickness, lateral pressure, dielectric constant and hydration [25]. It can also modulate the structure and activity of MPs including ion channels [26–28]. A variety of membrane mimetics are available for NMR studies of MPs which can be selected according to the experiment to be performed. This review article first describes crucial parameters that should be examined in order to choose the suitable model. It then presents the numerous options available depending on the type of NMR study to be performed. For each membrane mimetic, a thorough description of the surfactant and of the protein–surfactant complex (PSC) will be given, as well as general guidelines for making the complex, a discussion on the advantages and drawbacks of each approach, and several recent examples of successful NMR applications in the study of MPs. The reader is referred to the list of abbreviations for complete nomenclature of the lipids and detergents.

## 2. Parameters dictating the choice of a model membrane

Because MPs are very hydrophobic molecules and peptide chemical synthesis yields decrease with length, larger proteins must be over-expressed in host organisms such as *Escherichia coli* or yeasts, or by the newly designed cell-free expression system, preferably with an affinity tag for purification [24,29–31]. During its expression, an MP is either targeted to the membranes of the expressing organism or into inclusion bodies (or protein precipitates). Subsequently, these proteins need to be purified, re-natured and reconstituted so they can regain a native and active structure. Because MPs are hydrophobic molecules they need to be transferred to membrane mimetics in order to be manipulated in solution, which is a very delicate step. The membrane mimetics should meet two criteria: first, it should reflect as closely as possible the natural environment of the protein to reproduce the MP–environment interactions and allow the protein to achieve its native state and, at the same time, it must lead to well-resolved NMR spectra. The parameters to be considered when choosing a membrane mimetic for the NMR study of a MP can therefore be divided into biological and technical requirements that will be examined below.

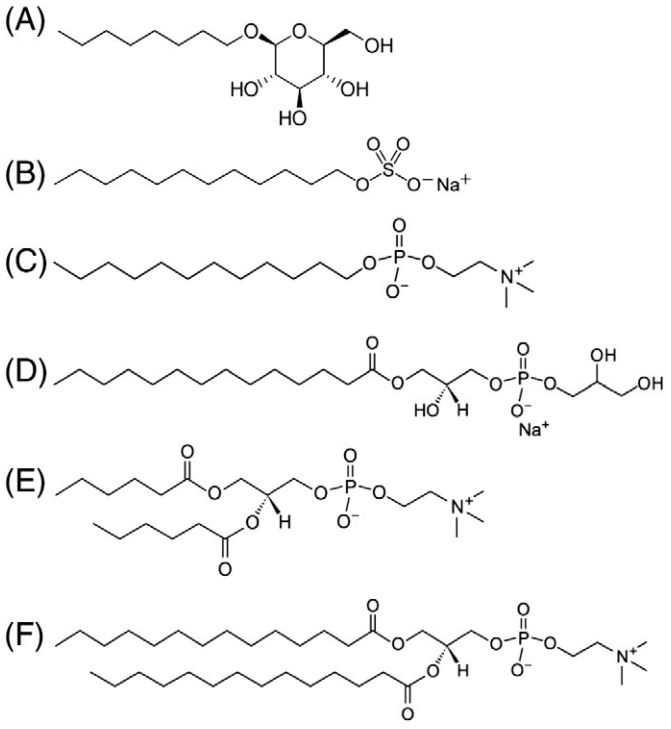


**Fig. 1.** Molecular structure of commonly used surfactants for the preparation of membrane mimetics: (A) octyglucoside (OG), (B) sodium dodecylsulfate (SDS), (C) *n*-dodecylphosphocholine (DPC), (D) lyso-myristoylphosphatidylglycerol (LMPG), (E) dihexanoylphosphatidylcholine (DHPC), (F) dimyristoyl-phosphatidylcholine (DMPC).

### 2.1. Biological requirements

Membrane mimetics are prepared by self-association of surfactants in aqueous solution. Surfactants can be divided into two large classes, i.e., those which form micelles (called detergents) and those which form bilayers (i.e., lipids). While detergents can be used to solubilize MPs by extracting them directly from their native membrane, the incorporation of MPs into lipid bilayers requires an additional reconstitution step. To some extent, a detergent micelle will never fully mimic a biomembrane which is primarily a lipid bilayer [25]. Nevertheless, the selection of surfactants should as much as possible take into account the physical characteristics of the biomembrane to be mimicked. To begin with, biological membranes are lipid bilayers ~4 nm thick in a liquid crystalline phase. Therefore an ideal model should be morphologically similar at accessible temperatures since changes in the membrane fluidity will affect the equilibrium distribution of the different conformers [25]. The structures of commonly used surfactants are shown in Fig. 1.

As discussed previously, inter-specific and inter-organelle membrane compositions vary. Cholesterol, for example, is not found in prokaryote membranes, and PC is the most abundant phospholipid in eukaryote cell membranes while PE is dominant in many bacterial strains. The nature of the lipids (charge, acyl chain length, insaturations) composing a biological membrane is important because it dictates the propensity to form a bilayer, as well as the membrane thickness, curvature, fluidity or stiffness [32,33]. For instance, cholesterol, which rigidifies the lipid acyl chains, tunes the membrane fluidity. The balance between the mechanical properties of the biomembrane as determined by the nature of its lipid content can

also have a significant importance on protein structure and function [26,34]. In consequence, the model membrane thickness, curvature and stiffness can modulate the folding, function and equilibrium of MPs [34–36]. For example, the presence of sphingomyelin is required by the pore forming equinatoxin II [37]. The function of the mechanosensitive channel of large conductance (MscL) from *E. coli* was shown to be dictated by its interaction with surrounding lipids depending on their chain lengths or headgroups [38,39]. Similarly, the charge of the membrane constituents counts when studying MPs and peptides which have an affinity for charged lipids, as it is the case for a variety of antimicrobial peptides [40–43]. Likewise, lipid composition can affect the orientation of transmembrane helices in a membrane. Studies show that the tilt of such helices is affected when the model membrane phospholipid chain lengths do not match the hydrophobic length of the protein [44,45]. A review on the interaction of helical transmembrane proteins with membrane lipids can be consulted [46].

The literature shows examples where MP structures differed depending on the composition of the membrane mimetics used in NMR. This stresses the importance of knowing the physico-chemical properties of the mimetics discussed in this review, in order to explain their effect on the protein structure. It should be remembered that biological molecules are dynamic and that sample preparation can trap a molecule in a variety of states that may be "native" or not. Current efforts in structural biology focus on trying to determine the lower populated intermediate states that are thought to be more informative than the more stable, low-energy states that proteins take along a specific mechanism pathway [47]. For example, the structure of phospholamban was studied by NMR in different conditions and gave various 3D structures in bilayers with different thickness and fluidity such as pure POPC bilayers, POPC/POPG bilayers, mechanically aligned DOPE/DOPC bilayers or DPC micelles [48–51]. In such a case, as discussed below, all structures were considered "active" in different relevant states. Similarly, the structure of membrane-bound α-synuclein was shown to be modulated in presence of curved vesicles and micelles or flat bicelles, confirming the importance of the environment upon protein folding into its final structure [52]. The challenge of finding the best membrane mimetics is not specific to NMR structure determination. For example, the influenza virus M2 proton channel was studied in bilayers made of pure lipids of various lengths (DLPC, DMPC, DOPC) as well as in mechanically aligned DOPC/DOPE bilayers and DHPC micelles and gave different results by solution, oriented and magic-angle spinning solid-state NMR techniques [53–58] but also by X-ray crystallography in OG micelles [59]. In this particular case, the structures were not all considered "native-like". Cross and co-workers recently discussed the bias introduced by the crystal packing on the one hand (high pressure and presence of PEG that resulted in an artificial dimer and no pore) and the extremely high curvature of the detergent micelle that left the solid-state NMR structure the most relevant one [25].

The assessment of MP activity, especially in detergent solution, is a challenge for NMR as well as for 2D and 3D crystallization methods [60]. In favorable cases, protein activity could be assessed *in situ* using complementary studies, e.g., by measuring the binding, by isothermal titration calorimetry, of a known ligand requiring a specific protein conformation [61]. In other instances, it is essential to ascertain that the membrane mimetic did not irreversibly *denature* the protein. To exclude such mimetics, it is often possible to reconstitute the MP in a native-like environment and verify the loss of activity.

### 2.2. Experimental requirements

The choice of membrane mimetics to study MPs should also be dictated by the type of information that is searched for and by the NMR experiment to be performed. Parameters such as experiment time, temperature, pH, hydration and surfactant-to-MP ratio must be considered.

The structure determination of MPs requires 2D NMR experiments that are usually performed over long periods of time. Therefore, the stability of membrane mimetics is of great importance as they should maintain the functional state of the protein and limit its conformational flexibility [34]. In solution NMR, for example, Krueger-Koplin et al. [62] have evaluated 25 detergents such as SDS and lysophospholipids using $^{1}H$–$^{15}N$ HSQC experiments. Their study showed that LPPG generally produced high-quality spectra with sample lifetimes greater than 1 month for five proteins tested. Nevertheless, other detergents were also favorable depending on the MP studied. However, the presence of multiple conformations was suspected for certain test proteins in SDS micelles, as suggested by an abnormally high number of cross peaks.

An appropriate membrane mimetic should also have a temperature range of stability covering that of the experiment to be performed – which can be up to 37 °C in some cases while certain solid-state NMR experiments are carried out at sub-zero temperatures [63]. The temperature can affect the quality of the NMR spectra as well as sample life time. In solution NMR, Krueger-Koplin et al. [62] report that the spectral intensities and linewidths of subunit c from *B. pseudofirmus* observed by $^{1}H$–$^{15}N$ HSQC varied between 22 and 48 °C when studied in LOPG and LPPG micelles. Surfactant stability with respect to pH is also desired to avoid potential hydrolysis of ester links (in the case of phospholipids, for example) and subsequent membrane disruption. Models using ether-linked phospholipids have shown improved stability at low pH although with a different phase behaviour [64].

Membranes are hydrated systems, and a minimum hydration value must be maintained in order to ensure that the structure survives. The primary hydration shell of a lipid is composed of about 20 water molecules [65], which imposes a water content of around 50% in weight and no less than 30% for correct swelling. In addition, a high lipid-to-MP ratio should be achieved to provide sufficient hydrophobic environment. A typical ratio used in NMR is between 4:1 and 1:1 (in weight) [24,56,63,66–69]. A ratio of 1:1 is close to the one found in biomembranes and guarantees a high NMR sensitivity and, at the same time, that each protein is surrounded by at least one layer of lipids. Standard functional reconstitutions, on the other hand, are typically done a ratio of 80:1 in weight [70]. In all instances, NMR tubes are filled with large amounts of water and lipids or detergents and very low quantities of protein material.

As was mentioned previously, membrane mimetics can be divided into micelles and bilayers. They can also be classified as oriented *vs.* isotropic systems or according to other physical parameters (size, composition, charge etc.). Since the nature of the sample will dictate the NMR experiment (and *vice versa*), the following sections will be presented according to the most important of the parameters: the correlation time of the protein–surfactant complex (PSC). PSCs are highly dynamic structures with slow, intermediate, and fast local and global dynamics depending on the overall molecular weight of the complex [71]. The weight and shape of this complex, the temperature and viscosity of the milieu will mostly affect the global correlation time τc of the system. Fast-tumbling small PSCs (<100 kDa) can be studied by solution NMR [62,72–77], while slow reorienting aggregates are more amenable to solid-state NMR [78,79]. Protein size and PSC tumbling rate are related but not directly proportional. First, the surfactant molecules contribute to at least half of the molecular weight of a PSC. Second, while a protein such as the light-harvesting complex 2 (LH2) is only 16 kDa small, it organizes in the membrane as a nonamer of 150 kDa and can only be studied by solid-state NMR [80]. While protein size is generally inversely proportional to spectral sensibility, the LH2 nonamer of 150 kDa is actually nine times more sensitive than the 16 kDa monomer since the signals of each monomer add up. In the following section, slow-tumbling membrane systems will be divided into two sections depending on their ability to align in the magnetic field B0 of the NMR spectrometer.

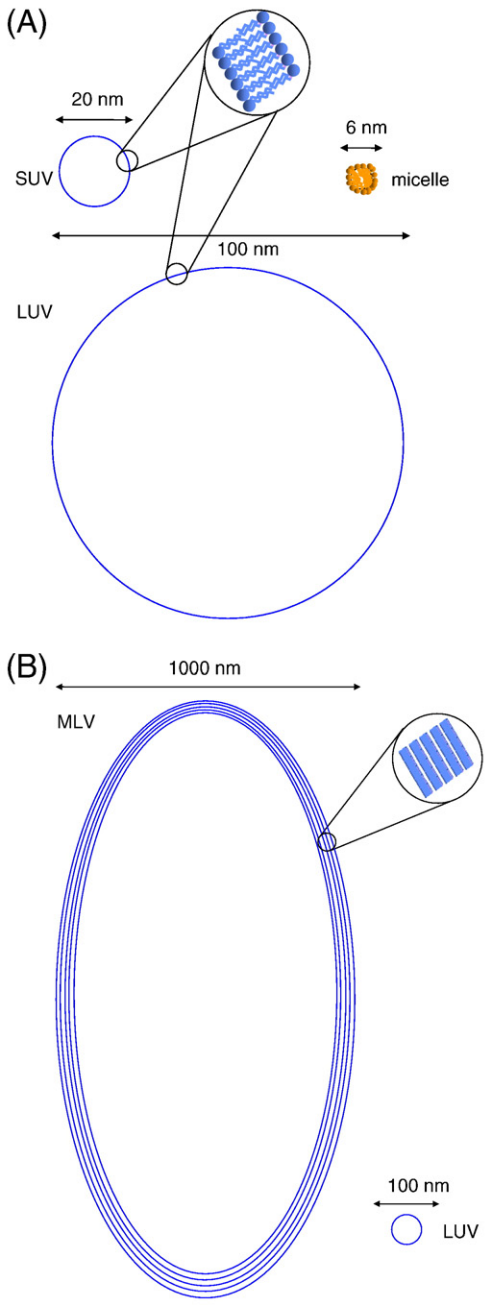


**Fig. 2.** Cartoons depicting several membrane mimetics described in the text. (A) detergent micelle, small unilamellar vesicle (SUV) and large unilamellar vesicle (LUV). (B) large unilamellar vesicle (LUV) and multilamellar vesicle (MLV).

## 3. Slow-tumbling objects

NMR spectroscopy and spectral resolution respectively rely and depend on magnetic interactions that are often orientation-dependent. With objects that are fast-tumbling (under 50 ns correlation time), the effect of such interactions is suppressed and high-resolution spectra can be obtained. In the case of slow-tumbling objects that we will examine in this section, other approaches have to be followed to regain the high spectral resolution—this is the field of solid-state NMR. One such approach is magic-angle spinning (MAS) where the "solid" sample is spun at high rates at an angle of 54.7° with respect to the magnetic field, yielding spectra alike to those obtained in the presence of fast molecular tumbling in solution. A second approach is sample orientation, if possible, where the orientation dependence of the magnetic interactions is fixed to a single value rather than averaged out. Finally, another option for slow-tumbling objects is to retain the orientation dependence of magnetic interactions and extract meaningful data from poorly resolved NMR spectra.

### 3.1. Isotropic membrane systems

#### 3.1.1. Multilamellar vesicles

Lipids in water organize as closed bilayers called vesicles or liposomes. Depending on their preparation, they can have various sizes and lamellarity. Spontaneously, lipids in water form large multilamellar vesicles (MLVs) that are inhomogeneous in composition, but on the order of 1 μm diameter and up to a dozen bilayers [81] (Fig. 2). Very easy to prepare, MLVs can incorporate any type of lipids and can be tailor-made to mimic any biological membrane composition. Nevertheless, MPs do not insert spontaneously into lipid membranes and an additional reconstitution step is necessary [24,82]. Whether produced chemically or biologically, in biomembranes or in inclusion bodies, MPs have to be solubilized to facilitate their manipulation. Some MPs, usually small peptides, are very resistant and can be dissolved in organic solvents with the appropriate lipid mixture and even dried out entirely without losing their ability to fold back to an active structure [42,49,66,83–88]. MLVs containing MPs are obtained by rehydration of such a mixture.

More fragile MPs have to be solubilized using detergent molecules. They can either be studied in detergents (Section 4.1) or be transferred to another environment such as vesicles, supported membranes (Section 3.2.1), bicelles (Section 3.2.2), nanodiscs or amphipols (Section 4.4). The general procedure consists in making ternary complexes of MP, detergent molecules and the new surfactant in which the MP will be reconstituted. The detergent is subsequently removed by one of several methods, the most popular ones being the use of polystyrene beads (biobeads) or simple dialysis until the detergent concentration is below the critical micelle concentration [70].

Although the vesicle multilamellarity is not biologically relevant, the local constraints in the MLV membrane are that of a cell because of the vesicle size and curvature. In addition, this type of sample is advantageous for NMR analysis because it is highly concentrated in lipids and embedded proteins. A typical sample may contain a ratio of 1:1 (in weight) of lipids-to-water, and 4:1 of lipids-to-protein [24]. The multilamellar nature of these objects makes them improper for the study of lipid asymmetry or transport across the membrane since it is difficult to define an exterior and an interior of the vesicle and because of inter-bilayer effects. The large size of these objects and the resulting slow tumbling also make them unsuitable for solution NMR, but they are appropriate for solid-state NMR applications.

NMR spectra of static solids are poorly resolved, but focusing on specifically labelled parts of the molecule, one can still obtain crucial dynamic or structural information. Solid-state $^{2}H$ and $^{31}P$ NMR can be employed to study lipid/protein interactions in MLVs incorporating deuterated lipids [85,89]. Using $^{2}H$ NMR, Jones et al. [66] have measured the orientation and local dynamics of the protein EGF receptor containing deuterated alanines in POPC MLVs, with and without cholesterol.

Spectral resolution can be increased by spinning the sample at the magic angle. In the 1990s, the technology was insufficient for resolving *all* atoms of a protein in a sample, but by specifically labelling some $^{13}C$ and $^{15}N$ atoms, one could measure very accurate distances in a protein. With a technique called Rotational Resonance (R2) and only two $^{13}C$ atoms, Griffin and co-workers could determine the conformation of the small retinal molecule inside a macromolecular complex formed by the 26-kDa MP bacteriorhodopsin, lipids from the bacterial membrane and water [90]. With a similar technique called REDOR, two $^{13}C$ and one $^{15}N$ atoms, Schaefer and co-workers could determine the secondary structure and orientation of the antibacterial peptide magainin in DPPG or DPPG/DPPC MLVs [83]. With three $^{13}C$ and one $^{15}N$ atoms, Separovic and co-workers could

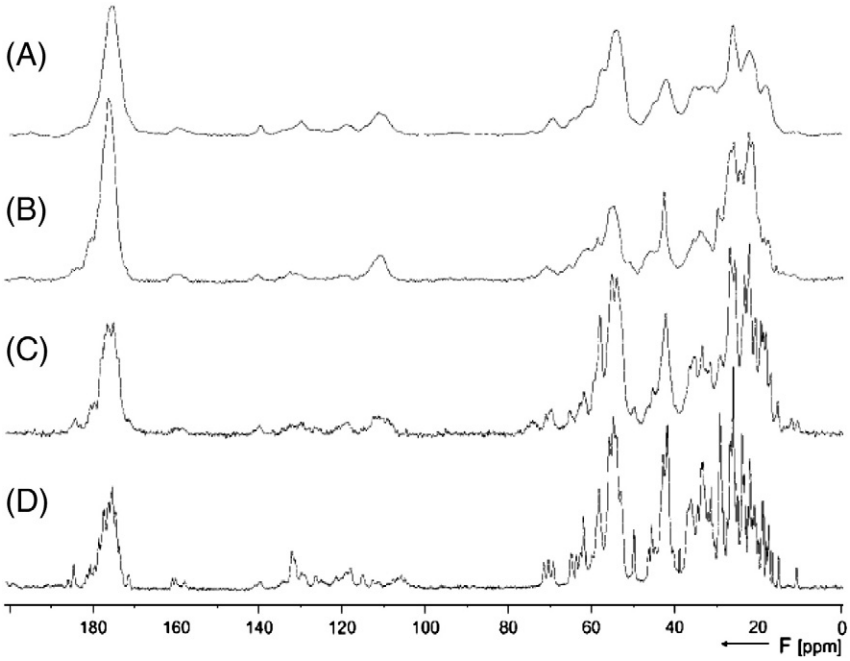


**Fig. 3.** 1D $^{13}C$ solid-state NMR spectra of differently prepared α-spectrin SH3 samples. Sample (A) was entirely lyophilized from aqueous low-salt buffer. Sample (B) was partly lyophilized from aqueous low-salt buffer. Sample (C) was lyophilized from a solution containing poly-ethylene glycol and sucrose. Sample (D) was precipitated from a $(NH_4)_2SO_4$-rich solution. Remark: the proteins in sample (D) are micro-crystallized.
Adapted from [92] with permission.

locate the conformational flexibility of an α-helix of the nicotinic acetylcholine receptor in DSPC or POPC MLVs [86].

Since the seminal work of Oschkinat and co-workers [91], magic-angle spinning NMR has become an important technique that provides new protein structures itemized in the Protein Data Bank (PDB) [21]. MAS-NMR is now tackling samples made of uniformly labelled proteins embedded into hydrated MLVs and has helped to determine a number of 3D structures such as protegrin in POPC MLVs [87] or the Influenza M2 channel in DLPC or DMPC MLVs [56,67]. The membrane environment should provide a native/active conformation for the protein, which would also be an advantage for NMR since a single conformation would give a highly resolved spectrum, whereas any other preparation that may result in a distribution of conformations would compromise the quality of the spectra, as illustrated by Fig. 3 on a soluble protein [92]. Nevertheless, membranes are also rich in slow dynamics and spectra may still suffer from a distribution of active conformations. Temperature is a parameter that can be used to either slow down or accelerate conformational exchange [67,68,93]. Resolution and sensitivity in solid-state NMR is currently insufficient for routine 3D structure determination of uniformly labelled MPs in MLVs. Nevertheless, this area is constantly evolving, especially with the development of Dynamic Nuclear Polarization (DNP) [94] or new isotopic labelling techniques [95].

Several large transmembrane proteins are being assigned in a membrane environment, and the structure is on the way, such as for the phospholamban pentamer in DMPC or DOPC/DOPE MLVs [69], the outer membrane protein G in MLVs made of *E. coli* lipid extracts [68], the potassium channel KcsA in asolectin MLVs [63], the cytochrome *bo*3 oxidase in MLVs made of endogenous *E. coli* lipids [93], the sensory rhodopsin in purple membrane lipids MLVs [96] or the mechano-sensitive channel MscL in DOPC MLVs [24]. DNP is one of the most fascinating recent developments in solid-state NMR for signal enhancement, and one obvious application is the study of MPs. Such an approach is currently being followed by the group of Griffin on frozen bacteriorhodopsin in MLVs with deuterated lipids, water and glycerol used for cryoprotection [94].

*3.1.2. Giant and large unilamellar vesicles*

Several methods exist to make unilamellar vesicles, the most popular ones being reverse-phase evaporation of organic solvents, extrusion through polycarbonate filters or sonication [81]. Most vesicles can be prepared with almost any type of lipids. The major advantage, as mentioned before, is that the inner layer, the outer layer, the interior and the exterior of the liposome are easily defined, and the transport of solutes through a protein can be measured across the membrane. Other properties depend on the liposome diameter that can range from 10 μm (Giant Unilamellar Vesicles or GUVs) down to 100 nm for typical Large Unilamellar Vesicles (LUVs), or even 20 nm for Small Unilamellar Vesicles (SUVs) obtained by sonication.

GUVs and LUVs have a small curvature and, hence, provide a native-like local environment for MPs. They also have large interior volume and, thus, a low membrane concentration in the sample rendering them unfavorable for NMR which is an insensitive technique. For these reasons, GUVs are never used in NMR and LUVs are mostly studied for looking at lipids, but not at MPs. The use of SUVs for the study of MPs by solution NMR will be reviewed below in Section 4.3.

The combined effect of vesicle tumbling (radius $R$, viscosity $\eta$ and rate $1/t_r$) and lateral diffusion (constant $D$ and rate $1/t_d$) can lead to partial or complete averaging of NMR interactions. Assuming that the effective correlation time $t_v$ is described by the following equation [97]:

$$\frac{1}{t_v} = \frac{1}{t_r} + \frac{1}{t_d} = \frac{3kT}{4\pi\eta R^3} + \frac{6D}{R^2} \quad (1)$$

Bloom et al. [97] have shown that lipid diffusion around the vesicle was independent of vesicle size and that high-resolution $^1H$ NMR spectra were obtained when the vesicle tumbling was fast enough, i.e., when the vesicles were sufficiently small.

### 3.2. Oriented membrane systems

Orienting all molecules of a sample in the same direction can be difficult. Luckily, in the case of lipids, there are several cases where they *spontaneously* align in a given direction. Peptides or proteins embedded into the lipid membrane can therefore be oriented as well. We will examine two different types of oriented membrane systems: supported on glass plates or non-supported magnetically aligned bicelles. We will also consider the emerging field of MAOSS which combines orienting *and* spinning of the sample.

#### 3.2.1. Mechanically aligned bilayers

When hydrated with the adequate amount of water, almost all lipids spontaneously align on glass plates with the bilayer normal perpendicular to the plates' plane. Furthermore, when introduced in the magnetic field, additional magnetic alignment significantly improves the lipid mosaic spread (deviation from perfect orientation) to be down to 0.3° [98]. The mechanical alignment of lipid bilayers supported on glass plates is, therefore, a very convenient way to orient a MP in the magnetic field.

Oriented samples are prepared by spreading a lipid/protein dispersion on glass plates. Typically, lipids and proteins are dissolved in an organic solvent but if necessary, proteins can be reconstituted in a hydrated lipid membrane without using solvents. In both cases, the dispersion first has to be significantly dehydrated and the plates are subsequently rehydrated in a chamber for a couple of days. Phospholipid alignment can easily be checked by $^{2}H$ or $^{31}P$ NMR which provide characteristic oriented lipid signals. Each plate is a square of ~1 $cm^2$, and about twenty such plates can be stacked into a sealed NMR tube [99,100].

With the appropriate NMR probe, any orientation of the supported bilayers can be achieved, whereas bicelles (see next section) can only adopt parallel or perpendicular orientations. Glass plate supported membranes can be aligned with almost any lipid, at almost any temperature, whereas bicelles only align in a specific temperature range depending on their composition. Nevertheless, lipid bilayers will align on a glass plate only within a specific hydration range (typically around 40% by weight [99]), which is low compared to native membranes and hardly permits the change of pH or solute concentration once the samples are made. Furthermore, a significant fraction of the sample volume is occupied by the glass plates, thus reducing the sensitivity of the NMR experiments since a typical sample will contain a weight ratio of lipids-to-protein between 3:1 [99] and 160:1 [100], and typically 20:1.

While this system was first used to study lipid dynamics (see, for example, [101]), solid-state NMR of mechanically aligned membranes has generated the first 3D structure of a MP by NMR [99]. This seminal work by the group of Cross has resulted from a systematic $^{15}N$ NMR study of gramicidin A in DMPC membranes started in 1986 [102] where the peptide structure is deduced from each peptide plane orientation determined independently. This approach has been improved by the group of Opella with the development of PISEMA [103–106] and related sequences such as SAMMY [107] or HIMSELF [108] that can be applied to uniformly $^{15}N$-labelled peptides. These sequences provide 2D spectra with patterns that directly depend on helix tilts in the membrane, as shown on Fig. 4 with the peptide tBid [106]. In solid-state NMR, the approach of using oriented membranes on glass plates has been the most efficient, as of today, for providing new MP structures [21]. Since 1993 over half a dozen membrane peptide structures have been determined to high resolution, such as several M2 channels (from the nicotinic acetylcholine receptor, NMDA receptor and influenza A virus), in DMPC or POPC/DOTAP 4:1 membranes, as well as the small Vpu channel from HIV-1 in DOPC/DOPG 9:1 membranes [54,100,109]. Finally, a combination of solid-state NMR on DOPC or DOPC/DOPE 4:1 (molar ratio) membranes aligned on glass plates and solution NMR on MPs solubilized in DPC detergents has allowed the determination of *several* dynamic and active structures of phospholamban, resting and active, compared to the static picture determined in solution only [48,51]. As discussed before regarding the structure determination of the influenza virus M2 proton channel [25], this combination of different NMR techniques – especially if performed in the same membrane environment – or NMR and crystallographic techniques, may well be the best approach to compensate for each technique pitfall.

#### 3.2.2. Magnetically aligned bicelles

Bilayered micelles – or so-called *bicelles*– have been introduced in the 1990s as new membrane mimetics [110,111] and quickly gained popularity considering the numerous advantages they offer for studying membrane peptides and proteins. Their composition and local morphology resemble those of biomembranes – i.e., a planar bilayer of phospholipids – and they have proven their versatility as they can be used in both solution and solid-state NMR. Moreover, bicelles are transparent and, thus, amenable to optical spectroscopy such as circular dichroism [112–117].

Bicelles are composed of long- and short-chain phospholipids, generally DMPC and DHPC [111] although DMPC can be replaced by large fractions of other saturated or unsaturated long-chain lipids with various headgroups, as well as cholesterol, gangliosides and ceramides [118–133]. Thereby bicelles can mimic a variety of biomembranes such as prokaryote, mitochondria, erythrocyte, myelin, neurons and skin [134]. Both lipids can be replaced by PCs with chain length different by one or two carbons [130,135]. DMPC and DHPC can also be replaced by analogs with ether links in lieu of carboxy-ester

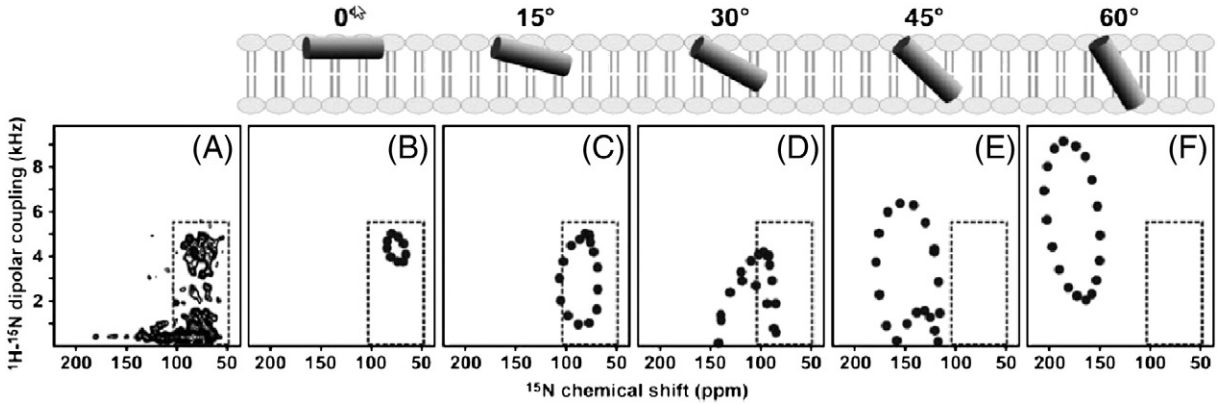


**Fig. 4.** 2D PISEMA NMR spectra (A) and simulations (B–F) of the uniformly $^{15}N$-labeled tBid peptide in oriented lipid bilayers. The helical tilt angle is indicated above each simulation. The experimental spectra are best fitted for helix parallel to the membrane surface, or with a tilt under 20°.
Adapted from [106] with permission.

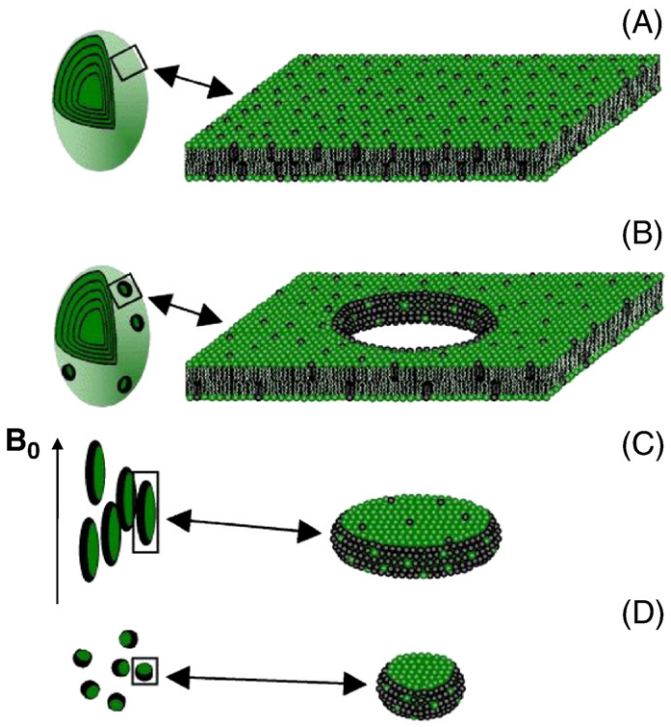


**Fig. 5.** The various morphologies of bicelles: (A) large mixed multilamellar vesicles, (B) perforated multilamellar vesicles, (C) large magnetically aligned bicelles and (D) small isotropic bicelles. Large bicelles align with the normal to their bilayer perpendicular to the external magnetic field B0, as shown on (C).
Adapted from [141] with permission.

bonds to access acidic and basic pH and improve bicelle stability [64,136,137]. Recently, cholesterol sulfate was shown to improve the thermal stability of bicelles and broaden the interval of temperature at which they align [138]. It is also possible to prepare bicelles mixed with non-lipid surfactants such as bile salt analogues [110,139] or Triton X-100 [140].

The morphology of bicelles has been the object of much debate and was extensively studied by several research groups, as reviewed by Marcotte and Auger [134]. However, recent $^{31}$P solid-state NMR studies published by Triba et al. [141] support a model in which the shape is dictated by the long-to-short-chain phospholipid *q* ratio as well as temperature. Bicelles can thus be found as discs, vesicles or perforated vesicles as shown in Fig. 5, and therefore prepared according to the NMR experiment to be performed. Fast-tumbling discoid (isotropic) bicelles are characterized by a low *q* ratio and used in solution NMR studies of MPs, as will be described in Section 4.2, while high-*q*, magnetically self-aligning bicelles are prepared for the orientation measurements of MPs that will be described below. For detailed applications of isotropic and oriented bicelles for the NMR study of membrane-associated surfactants and proteins, the reader is referred to several reviews [134,142,143].

At temperatures above DMPC's gel-to-fluid phase transition (typically>30 °C), long-to-short-chain lipid ratios q>2.3, and lipid concentrations of 3–60% w/v in aqueous solution, bicelles are known to spontaneously align in the magnetic field [141,144–147] with their bilayer normal perpendicular to the direction of the magnetic field, as illustrated in Fig. 5C. The magnetic alignment of bicelles is attributed to the anisotropy of the magnetic susceptibility of the phospholipids, as detailed elsewhere [134]. Although the average bicelle orientation is determined, the distribution and oscillation around this position have an effect on the NMR data [141]. The mosaic spread affects the width and symmetry of resonances, and it is reported around 6° [148]. Oscillations partially average magnetic interactions in molecules embedded in bicelles and additional order parameters between 0.7 and 0.9 therefore have to be included to take this effect into account [114].

Aligned bicelles can be prepared by mixing the phospholipids in the appropriate buffer solution, followed by a series of freeze (liquid nitrogen)/thaw (37 °C)/vortex shaking, leading to a transparent non-viscous sample. Proteins can be co-solubilized with the phospholipids in solvents such as 2,2,2-trifluoroethanol (TFE), followed by vacuum-drying prior to hydration [88,113]. In some cases proteins do not survive organic solubilization or full drying, thus reconstitution protocols have been optimized to incorporate MPs into bicelles without such damaging steps [114]. Typically, a q value of 3 to 3.5 is employed with 20–60% w/v lipids in aqueous solutions [44,84, 108,114,120,149–154]. Details on the preparation of bicelles samples for solid-state NMR study of MPs can be found in De Angelis and Opella [155]. Proteins as big as 350 residues have been successfully incorporated into oriented bicelles, as demonstrated with the G-

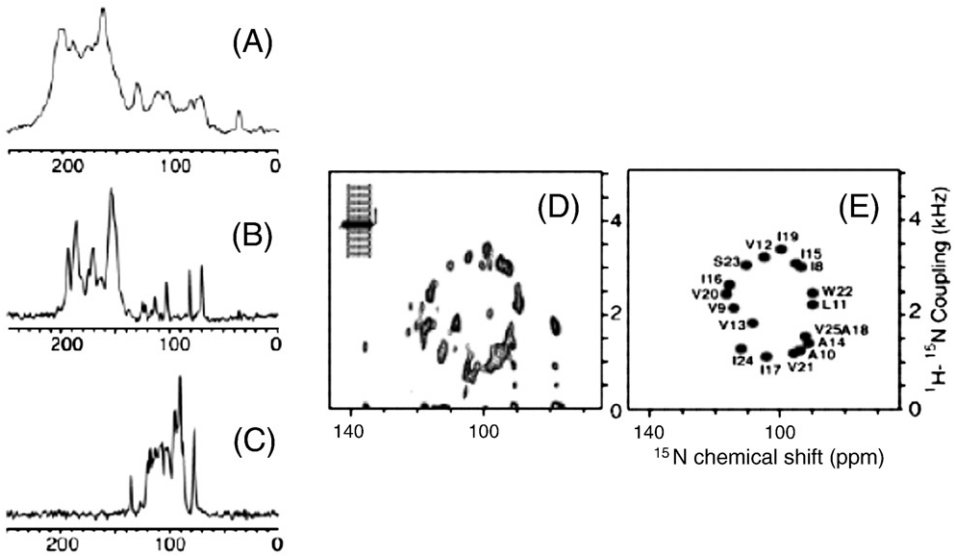


**Fig. 6.** Solid-state NMR spectra of the transmembrane domain of Vpu in lipid bilayers differently aligned: on glass plates (A), in "flipped" bicelles (B) or in "normal" bicelles (C-E). (A-C) are 1D $^{15}$N NMR spectra while (D) is a 2D PISEMA NMR spectrum allowing for resonance assignment (shown in E) and helix tilt determination (determined here to be approximately 30° with respect to the bilayer normal).
Adapted from [151] with permission.

protein-coupled receptor CXCR1 [156]. Fannucci et al. [157] observed that large bicelles preserved the folded structure of the Ton box motif of BtuB, and Czerski and Sanders [158] showed that the integral MP diacylglycerol kinase (DAGK) maintained its catalytic activity when incorporated into DMPC or DPPC bicelles.

DMPC/DHPC bicelles have a thickness of ~4 nm similar to a natural bilayer [159] as well as a planar surface. They are also stable over a wide extent of ionic strengths and pH [111,144], as opposed to acidic bicelles which are more fragile and subject to hydrolysis [120,122]. Aligned bicelles have several advantages over mechanically aligned bilayers: in the correct temperature range, bicelles tend to improve the resolution of $^{15}$N and PISEMA spectra as compared to bilayers mechanically aligned on glass plates as illustrated in Fig. 6A and B with the transmembrane domain of Vpu [151]. The sample is easier to prepare, better hydrated and the volume is large because of the absence of glass in the sample. In addition, MPs expressed in bacteria can be purified either with the bicellar long- or short-chain lipid to which the lipid companion can then be added in order to obtain bicelles [114,156,160]. However, it should be noted that the presence of a protein may affect the orientation of bicelles, as was observed with gramicidin [88].

Aligned bicelles are frequently used to determine the orientation of transmembrane helices with respect to the bilayer normal such as that of antimicrobial peptides but also large transmembrane proteins such as the β-barrel tOmpA and OmpX, as long as their axial diffusion is fast [114,154]. This information – which is complementary to the structure that can be obtained by solid-state NMR, solution NMR, or any other method – can be provided by $^{15}$N or $^{2}$H NMR as the $^{15}$N chemical shift value or the $^{2}$H quadrupolar splitting of specifically labelled residues are indicative of the chemical bond orientation with respect to $B_0$ direction [108,120,150]. Improvement over this approach with PISEMA related sequences has allowed the resonance assignment and helix tilt determination of the Vpu channel from HIV-1, as shown on Fig. 6C–E [151]. Similar approaches have provided the structure of a truncated construct of the mercury transport membrane protein MerF-t [151], as well as the orientation of several proteins such as the cytochrome *b*5 [108], TatAd from *B. subtilis*[153] or the p7 protein of hepatitis C virus [161] in lipid bilayers. Although aligned bicelles are slow-tumbling objects used in solid-state NMR, large bicelles can also be employed in solution NMR to induce residual alignment in water-soluble proteins and obtain residual dipolar couplings used in structure determination, as thoroughly detailed elsewhere [162,163].

Due to their large magnetic susceptibility, the addition of paramagnetic lanthanides flips the orientation of the bicelle normal from perpendicular to parallel to the static magnetic field direction. This effect achieved at very small lanthanides/lipids molar ratios (<0.1) is well detailed by Prosser et al. [164] and will not be discussed further here. The advantage of this method is to double the NMR frequency range of $^{15}$N chemical shifts (Fig. 6B compared to 6C), thus improving the resolution of the amide spectral region, as demonstrated with the fd coat and Vpu proteins [84]. The same effect is observed on $^{2}$H NMR spectra. Another advantage of the parallel alignment is that protein orientation in the membrane becomes independent of its azimuthal orientation and axial diffusion rate. Spectra obtained at 0° and 90° alignments can also help confirming the orientation of proteins such as the large outer MPs from *E. coli*[114,154] or the small MerF-t and Vpu transmembrane α-helices [44,151,152]. The presence of paramagnetic ions may perturb protein activity, shift amino acid resonance frequencies or catalyze lipid hydrolysis, although lipids with chelating headgroups can be used to sequester the lanthanides away from the protein [165]. However the protein moieties within the hydrophobic core of the bicelles should not be affected [84].

In order to avoid the use of lanthanide ions altogether, it is possible to prepare parallel-aligned bicelles by replacing DMPC with phospholipids containing a biphenyl group on one acyl chain, such as DBBPC or TBBPC [166,167]. The large positive magnetic susceptibility of the biphenyl groups is responsible for flipping the bicelle orientation. Although the temperature range where "biphenylated" bicelles align is larger compared to DMPC/DHPC bicelles, the composition domain for which orientation occurs is reduced [166]. These new bicelles have yet to be proven general for the incorporation of MPs, but they have already been successfully applied to the study of the Pf1 coat protein [168].

### 3.2.3. Spinning oriented systems

Aligned bilayers can also be utilized in combination with MAS to improve the resolution, signal-to-noise ratio and sensitivity of the NMR measurement of orientation. This approach introduced as MAOSS (magic angle oriented sample spinning) was proposed by Glaubitz and Watts in 1998 [169]. The original membrane system consists of a series of lipid bilayers deposited on glass discs which are then stacked and placed in a MAS rotor to orient their bilayer normal parallel to the rotor axis, as shown in Fig. 7A. Purple membranes – an hexagonal crystalline lattice of the light-driven proton pump bacteriorhodopsin (BR) from *H. salinarium*– can also be oriented on glass plates and spun [170]. This strategy presents two assets from an NMR point of view. Firstly, high sensitivity in the orientation measurement is achieved since small deviations from the normal result in dramatic spectral changes. Secondly, this orientation allows anisotropic motions such as rotational diffusion to partially average interactions which are predominantly dipolar [71]. Residual interactions can easily be removed even at slow spinning speeds of up to 3 kHz. Mosaic spread values are reported below 20° [171]. Using this approach, high resolution for $^{1}$H NMR resonances can be obtained [169]. MAOSS has also been employed for a $^{2}$H NMR study of photointermediates of specifically deuterated bacteriorhodopsin [172] as well as for the study of the M13 coat protein by $^{13}$C NMR [173]. Interestingly, this method can also be combined with standard solid-state NMR experiments such as REDOR where deviations from the non-oriented case can be analyzed in terms of the targeted molecule orientation [171].

Certain disadvantages are however also associated to this method. For example, under MAS conditions, centrifugal forces induce migration of the sample towards the edges of the plates, thus limiting the spinning rate. Likewise, the heftiness of the glass plates also makes it difficult to spin the sample at high rates, and since glass occupies most of the rotor's volume, only a small amount of protein is actually introduced [174]. More recently Sizun and Bechinger [175] suggested an alternative approach to obtain a bilayer with its normal oriented *perpendicular* to the magnetic field. In such a case, the bilayer alone or with reconstituted peptides is first dissolved in chloroform or TFE then adsorbed onto a thin polymer foil from which the solvent is

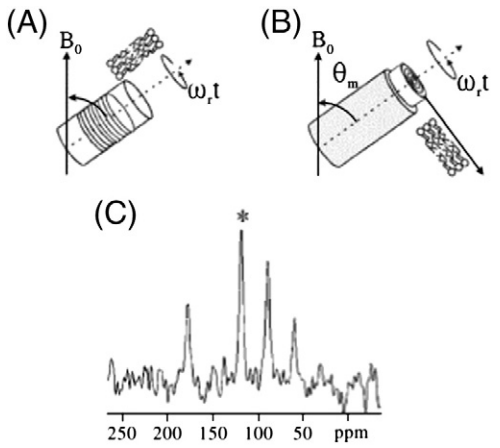


**Fig. 7.** (A) Arrangement of glass plates and rotor used in MAOSS. (B) Arrangement of polymer sheets and rotor used in the alternative MAOSS approach. (C) 1D $^{15}$N MAOSS NMR spectrum of a peptide, obtained using the approach described in (B). Adapted from [175] with permission.

subsequently evaporated under vacuum. The resulting dry polymer sheet is then brought to desired humidity, rolled up and placed with its long axis parallel to the rotor axis (Fig. 7B and 7C) [170,174–177]. Major advantages of this approach are the ease of preparation, the low cost of the required materials, especially since this type of system can be prepared on various types of polymer foils such as polyetheretherketone (PEEK) [174–176], polycarbonate [174] and polyethelene terephthalate (PET) [170,177–179]. In addition, samples can fit in 4-mm rotors and reach high spinning rates of up to 10 kHz as opposed to the limited 3–5 kHz of conventional MAOSS [175]. The reported mosaic spread values are 3.7° [175], below 8° [178] and up to 22° in the case of short-chain lipids [170]. However, in all cases, spinning the sample should increase the quality of the orientation as a result of centrifugal forces [175,180].

Another approach exploits large magnetically aligned bicelles to determine the structure of MP segments in combination with MAS. The high-resolution $^{1}$H NMR spectra obtained can be used to acquire 2D experiments such as NOESY and deduce structurally-relevant information [150].

## 4. Fast-tumbling objects

As mentioned before, high spectral resolution can be obtained in solution NMR with objects that have a correlation time below 50 ns which corresponds to an upper limit of approximately 100 kDa. In some favorable cases, especially with proteins under 30 kDa, a complete interaction map can be obtained and allow for the determination of a 3D molecular structure. With such approaches, solution NMR has elucidated the structures of some 6000 soluble proteins in the past 20 years [181]. In more challenging cases, partial structural and dynamic information can still be obtained, sometimes with the use of specific isotopic labelling schemes [182]. The most common way to study MPs by solution NMR is to solubilize them in detergent micelles [62,72,74,75] but new systems are being developed [73,76,77] and will be described below.

### 4.1. Micelles

Micelles are the most frequently used membrane mimetics for the structure determination of peptides and proteins by solution NMR and have helped in solving the structures of ca. 25 MPs in the past 15 years [21,62,74,76]. These aggregates are formed by self-assembly of amphiphilic molecules in solution in order to minimize the contact of their hydrophobic tails with the aqueous environment (Fig. 2A). Because they are small spherical monolayers with a rough surface, the micelle morphology differs from that of biomembranes. They can also adopt elliptical or rod-like shapes at high detergent concentrations or with weakly polar surfactants [183]. Micelles are formed at a specific detergent-dependent concentration – the critical micelle concentration (CMC). They are also characterized by an aggregation number ($N$) that can be as low as 4 and up to several hundred of molecules [76,183,184]. The molecular weight of a micelle is therefore $N$ times the molecular weight of the detergent monomer. They are on the order of ~3 nm radius, corresponding to a correlation time of ca. 25 ns. The micelle concentration [$M$] is related to the detergent concentration [$D$] by the relation:

$$[M] = ([D] - \mathrm{CMC}) / N \quad (2)$$

Micelle formation also occurs at a critical micelle temperature (CMT). SDS, for example, precipitates at temperatures below 4 °C. Therefore, the incorporation of an MP into micelles as well as the NMR experiments must be performed above the Kraft point, i.e., at the temperature at which a turbid detergent solution becomes clear due to micelle formation.

In the case of the ternary mixture of detergents, MPs and water, the detergent hydrophobic moieties often spontaneously cover the hydrophobic parts of the proteins to form protein–detergent complexes (PDCs) that remain water-soluble. When the protein is small, the complex resembles a micelle in which the protein is embedded. In other cases, the detergent may play the role of a lifebuoy around the MP. As discussed by Strandberg and Ulrich it is assumed that each micelle accommodates only one peptide [41]. The fast tumbling of micelles in solution often averages out orientation-dependant interactions in NMR such as dipolar coupling and chemical shift anisotropy, thus leading to high spectral resolution. Micelles are, thus, well indicated for structure determination of MPs by solution NMR. If possible, perdeuterated detergents should be employed to avoid overlap with the protein resonances on the $^{1}$H NMR spectra. Nevertheless, most modern NMR experiments make use of $^{15}$N- or $^{13}$C-filtered experiments, thus removing the detergent contribution in the spectra, even if it is protonated [185–187].

After purification, the NMR sample needs to be concentrated to obtain a high protein concentration (on the order of 1 mM), but the final detergent concentration increases as well. Detergent concentration can then be reduced, more easily when its CMC is high. In addition to the micelles and PDCs, detergent monomers are present in the solution (at a concentration corresponding to their CMC), hence contributing to the solution viscosity and molecular tumbling rate. Since MPs may precipitate when solubilized *at* the CMC, it is advised to maintain them in solution with detergents *above* the CMC, especially for detergents with high aggregation numbers, so that the micelle concentration [$M$] exceeds the protein concentration [184]. Detergent concentration should be optimized on a case-by-case basis [188].

The properties of PDCs highly depend on the physicochemical properties of the detergents. DPC and SDS micelles are the most commonly used for structure determination of MPs and peptides [41,189,190] and references in [75]. It is important to favor detergents with small aggregation numbers to obtain small, fast-tumbling PDCs and increase the protein signal because of the reduced surfactant proportion. For example, although DDM is a mild detergent that tends to maintain the functionality of MPs, it forms large micelles (~60 kDa) that reduce protein signals in solution NMR both because the micelles tumble slowly – which broadens the protein signals – and because the protein-to-surfactant ratio is smaller [60]. Anionic detergents such as SDS can be more denaturating than the other types, non-ionic micelles being the mildest [191,192]. Zwitterionic detergent micelles such as DPC are used to mimic eukaryote membranes while the negatively charged SDS micelles would resemble bacterial membranes [41].

Unfortunately, no rules apply when searching for the right detergent; however Vinogradova et al. [72] reported that the catalytic activity of DAGK was higher in micelles of medium-chain length detergents such as DPC or DM which therefore should be considered as good models since their low molecular weight contributes little to the overall weight of the PDC. The use of a mild detergent does not guarantee that a protein will be properly folded, though. Mo et al. [193] showed that the helical proteins bacteriorhodopsin and Ste2p from *S. cerivisiae* are found in partially folded states in OG (octylglucoside) and DM micelles, respectively. Comparison with different detergent types reveals that the small multidrug-resistance pump (Smr) adopts a native conformation in bicelles and DM micelles, but not in LPPG and DPC micelles [76]. One risk is that the surfactant covers not only the hydrophobic part of the protein but also its active sites, thus affecting its activity [62,76]. As an illustration the *E. coli* enzyme PagP has no activity in OG and DPC micelles due to detergent binding in the active site, but recovers its activity in CYFOS-7 micelles (a DPC analogue with a chain terminal cyclohexyl group) [187,194]. LPPG micelles often lead to high-quality spectra [62,76]; however, as seen for the protein Smr, it is not necessarily the ideal detergent for a given protein. The spontaneous curvature of micelles does not always accommodate MPs and the activity of enzymes or ion channels can be compromised [25,52,76,194].

**Table 2**
Average characteristics of detergents frequently employed for structural NMR studies of proteins.
Adapted from [31,74,76,183,188]. Details on the protein structure can be found in [21].

| Detergent | Monomer MW (g/mol) | Monomer CMC (mM) | N | Micellar MW (kDa) | Typical NMR conditions | Some MP structures solved by solution NMR[a] |
|---|---|---|---|---|---|---|
| Non-ionic (mild) | | | | | | |
| DDM (C12-DM) | 511 | 0.2 | 140 | 72 | | |
| DM (C10-DM) | 483 | 2 | 80 | 39 | | |
| OG (C8-G) | 292 | 20 | 90 | 26 | 200 mM/40 °C | PagP |
| Zwitterionic | | | | | | |
| DPC (C12-PC or FOS-12) | 352 | 1.5 | 70 | 25 | 200 mM/40 °C | GpA, OmpA, PagP, OmpX, OmpG, PLN, DsbB, i3, DAGK, Rv1761c, CD4 |
| LDAO (C12-DAO) | 229 | 1 | 75 | 17 | 300 mM/40 °C | VDAC |
| c7-DHPC (D7-PC) | 482 | 1.5 | 25 | 12 | 100 mM/50 °C | SRII |
| DHPC (D6-PC) | 454 | 15 | 35 | 16 | 300 mM/40 °C | M2, OmpA, kpOmpA, Pf1 |
| Anionic | | | | | | |
| LPPG (L16-PG) | 507 | 0.02 | 160 | 81 | 200 mM/50 °C | DTF |
| LMPG (L14-PG) | 479 | 0.2 | 55 | 26 | 80 mM/40 °C | KCNE1 |
| SDS (C12-S) | 288 | 3 | 80 | 23 | 500 mM/40 °C | MerF, FXYD1, FXYD4 |
| Mixtures | | | | | | |
| DPC/SDS 5:1[b] | – | – | – | – | 150 mM *DPC*/30 °C | ZZ |
| DHPC/DMPC 4:1[b](isotropic bicelles) | – | – | 200 *DHPC* 50 *DMPC* | 125 | 200 mM *DHPC*/40 °C | Bnip3, ErbB2, EphA1, αIIb-β3 |

[a] Protein abbreviations: αIIb-β3 (Integrin αIIb-β3); Bnip3 (Bnip3 transmembrane domain); CD4 (human CD4); DAGK (diacylglycerol kinase); DTF (G-protein-coupled receptor double transmembrane fragment); EphA1 (EphA1 transmembrane domain); ErbB2 (ErbB2 transmembrane segment); FXYD1 (human FXYD1, Na,K-ATPase regulatory protein); FXYD4 (human FXYD4, CHIF); GpA (human Glycophrin A transmembrane domain); i3 (i3 intracellular loop of the vasopressin V2 receptor); KCNE1 (human KCNE1); M2 (Influenza M2 proton channel); Pf1 (Pf1 major coat protein); PLN (human phospholamban); Rv1761c (Rv1761c from Mycobacterium tuberculosis); SRII (Sensory rhodopsin II); VDAC (human VDAC-1); ZZ (zetazeta transmembrane domain).
[b] mol/mol ratio.

If possible, functional assays should be conducted to identify the best surfactant to be employed [60,72].

The challenge is to find the most appropriate detergent for the structural NMR study that also solubilizes the protein in a native conformation—and no such universal detergent exists. Recently the group of Nietlispach [186] solved the structure of sensory Rhodopsin in c7-DHPC micelles after trying over 15 different detergent molecules and several samples conditions (concentration, temperature, isotopic labelling scheme etc.), showing that NMR of MPs is not yet a routine technique. We have reviewed in Table 2 the main characteristics of commonly used detergents for solution NMR studies of MPs and peptides and typical conditions at which they have been used in structural determination of MPs (detergent concentration and sample temperature). The reader should refer to these conditions for initial screening.

With the progress that has allowed solution NMR to tackle larger objects in solution, a greater number and diversity of structures have recently been solved for MPs solubilized in detergents [21]. Among the ca. 25 MP structures determined this way, over half were obtained on MP solubilized in DPC. SDS was mostly used for small peptides whereas DPC and DHPC were used for larger proteins. From small α-helical proteins [189] and large β-barrels [185,187,195–197], applications have now extended to larger α-helical complexes such as the 30 kDa pentamer of human phospholamban [48], the 20 kDa tetramer of the Influenza M2 proton channel [55], the 18 kDa enzyme DsbB [198], the 43 kDa trimer of diacylglycerol kinase [199] and the 26 kDa sensory rhodopsin II [186]. This last example shows the potential to determine structures of other 7 α-helix complexes such as GPCRs. Because of the potential pharmaceutical applications of GPCRs and the extreme difficulty to crystallize them in an active form, this is the current Holy Grail for solution NMR.

### 4.2. Isotropic bicelles

The composition and preparation of isotropic and aligned bicelles are very similar and the insertion of a MP in bicelles is usually

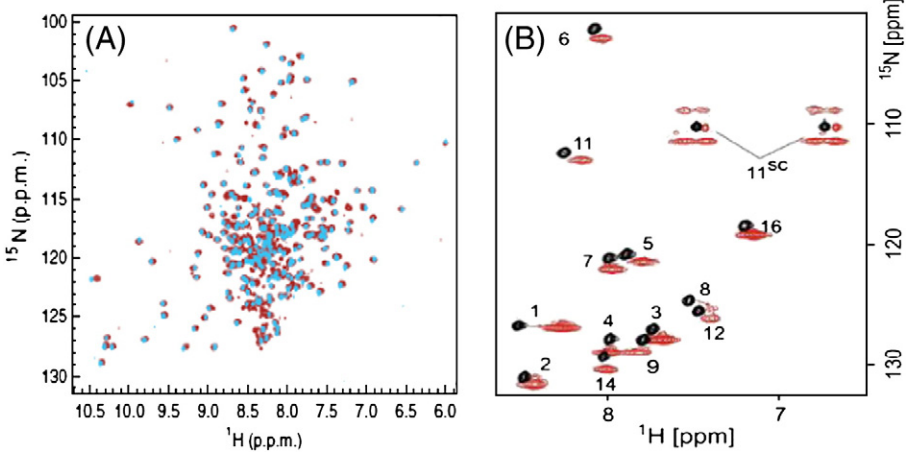


**Fig. 8.** 2D $^{1}$H–$^{15}$N NMR spectra of membrane proteins differently solubilized. (A) Overlay of the phototaxis receptor sensory rhodopsin II in DHPC micelles (cyan) and in isotropic bicelles (red). (B) Overlay of the antiamoebin I in isotropic bicelles (black) and in nanodiscs (red).
Adapted from [186,232] with permission.

performed by co-dissolution of the lipids and protein in aqueous solution when bicelles systems are prepared [112,133,200–203], or in solvents such as chloroform/methanol before evaporation (under a stream of inert gas or vacuum oven) and rehydration [137,204]. For isotropic bicelles, a typical $q$ ratio of 0.25–0.5 is used with a hydration of 85–95% and lipid-to-peptide molar ratios between 25:1 and 100:1. Chain-perdeuterated lipids are usually employed to limit their contribution to the $^1$H signals [112,117,159,200,202,203,205].

Because they offer a small lipid bilayer domain, isotropic bicelles (Fig. 5D) frequently prove advantageous over micelles for the study of MPs or MP segments. Lau et al. [133] showed a distortion of the α-helical conformation of integrin β3 transmembrane domain in micelles as compared to bicelles, while Lindberg et al. [201] observed a different position of penetratin in SDS micelles *vs.* bicelles, which could be forced by the curvature and size of the micelle. Poget et al. [137] showed that bicelles preserve the ligand-binding activity of Smr. They also found that bicelles, over a series of detergent micelles, were the best membrane mimetics for the solution NMR structure determination of Smr and other proteins that must retain their native conformation [76]. Although about twice as large as micelles because they comprise approximately 200 DHPC and 50 DMPC molecules for a total of approximately 125 kDa (for $q = 0.25$) [137,159], isotropic bicelles are very promising mimetics as shown in Fig. 8A which compares 2D spectra of pSRII in bicelles and DHPC micelles. In the future, they should often replace micelles in the study of MPs, helped by progress in solution NMR spectroscopy such as TROSY experiments which have extended the weight limit of protein study to above 100 kDa [47,182,206]. Actually, isotropic bicelles could be seen as just another detergent (DHPC) solubilizing a complex made of a MP and lipids, and we have added this particular mixture to the list of frequently used detergents in solution NMR of MPs (Table 2).

The reader is referred to Prosser et al. [143] for detailed applications of isotropic bicelles for the NMR study of membrane-associated amphiphiles and proteins. Briefly, typical 2D and 3D NMR experiments used for structure determination can be carried out on bicelle-inserted proteins [112,132,133,137,200,203,205,207]. The structure of a variety of MPs or protein transmembrane domains has been elucidated so far in isotropic bicelles, such as integrin β3 transmembrane segment [133,208], the third helix of the *Antennapedia* homeodomain protein of *Drosophila*[201], Smr [137], the transmembrane domain of the apoptotic protein BNip3 [209], the dimeric transmembrane domain of the growth factor receptor ErbB2 [210], and of the receptor tyrosine kinase EphA1 [211], to name a few.

### 4.3. Small unilamellar vesicles

SUVs are the smallest possible vesicles with a diameter of around 30 nm—which is still an order of magnitude larger than a detergent micelle (Fig. 2A). They are formed by sonicating lipid dispersion in water while cooling the sample to avoid degradation from heating. Almost any type of lipids can be used – egg yolk phosphocholine is often preferred – except that vesicle fusion and aggregation occur more slowly with an addition of 10% (molar) of charged lipids (serine headgroup for example). SUVs containing MPs are obtained by sonicating lipid dispersions with MPs reconstituted into them, but only resistant proteins – usually small and globular – will stand such a procedure.

The properties of SUVs are opposite to those of LUVs: while their curvature is very high – imposing an asymmetrical distribution of lipids in the outer and inner monolayers and a strong pressure on MPs – their intermediate size provides very concentrated and moderately fast-tumbling samples, suited for solution NMR. A typical MP sample in SUVs may contain a ratio of 1:10 (in weight) of lipids to water, and 30:1 (molar) of lipids to proteins [212]. Although SUVs combine the advantages of a real lipid bilayer and a system amenable to solution NMR, they also have the inconvenient of being large, diluted and unstable. They have, therefore, seldom been used for MP structure determination by NMR [212]. However, SUVs are often employed in NMR to study water-soluble proteins that are known to interact with lipid membranes [213].

### 4.4. Other systems

#### 4.4.1. Organic solvents

Since MPs are not soluble in water, this whole article reviews membrane mimetics that can surround a protein to make it soluble while retaining its activity. Membrane mimetics and solvent viscosity are two factors that slow down PSC tumbling and, thereby, reduce the spectral resolution and sensitivity in solution NMR. Hydrophobic molecules such as MPs are often soluble in low viscosity organic solvents without the need of additional surfactants, and their correlation time is much smaller in such environment. Pure or mixtures of organic solvents such as methanol, isopropanol, chloroform, TFE or DMSO have been employed. NMR spectra of small membrane peptides in TFE have given results comparable to those solubilized in SDS [214,215]. Other small integral MPs have been studied in mixtures of methanol/chloroform/water which were shown to mimic the membrane environment [216–218]. However, interactions in membranes are a subtle balance between hydrophobic and hydrophilic contacts, and this subtlety is lost in organic solvents. While such milieu will trigger and stabilize helices, it will also disrupt helix–helix contacts and, in most cases, MPs will not retain significant native structure in organic solvents. Often limited to small and helical membrane peptides, this approach has to be carefully validated case by case.

#### 4.4.2. Reverse micelles

As stated before, spectral resolution in solution NMR depends on molecular correlation time which is function of size but also of viscosity. Using apolar low-viscosity fluids (alkanes for example) is an attractive option that requires some optimization of the sample preparation. For instance, if the biological molecule structure is not retained in such an apolar solvent, it first requires to be encapsulated with water in an appropriate reverse micelle [219]. Micelle surfactants have to be screened in order to minimize the volume of the complex. The reverse micelle encapsulation approach for NMR study of MPs is also being developed by the Wand group [220]. The current best option, which was used to assign the NMR spectra of the potassium channel KcsA [220], is to encapsulate the protein in CTAB/DHAB:1/1 and to solubilize the complex in ethane and hexanol under high pressure (300 bar), requiring a specialized commercially available apparatus (Daedalus Innovations). This assembly is shown on Fig. 9A where the protein is surrounded by water (in blue), CTAB and DHAB surfactants (in red), hexanol (in green) and ethane (in grey). This approach is also used to study lipid-anchored proteins that bind to membranes via a covalently attached lipid or fatty acid, such as recoverin or the HIV-1 matrix protein, in their myristoylated form [221]. The development of this complex procedure is motivated by the possibility to push the size limit of proteins that could be studied by solution NMR. Nevertheless, in the case of MPs, reverse micelles suffer from the same problem as detergent micelles, i.e., the non-native environment which may affect their function and stability.

#### 4.4.3. Amphipols

Detergent molecules are used both to extract MPs from their membrane environment and to keep them in solution by covering their hydrophobic parts. Alternative molecules have been designed to keep MPs in solution while preventing detergents to disrupt subunits or cofactors of the protein, occupy its active site, or affect its function [191,222,223]. Developed by the group of Popot, amphipols are among the most promising new membrane mimetics for NMR [222]. They are a family of short amphipathic polymers carrying a large number of hydrophobic chains that can replace detergent molecules as a lifebuoy around the MP with a very low rate of spontaneous

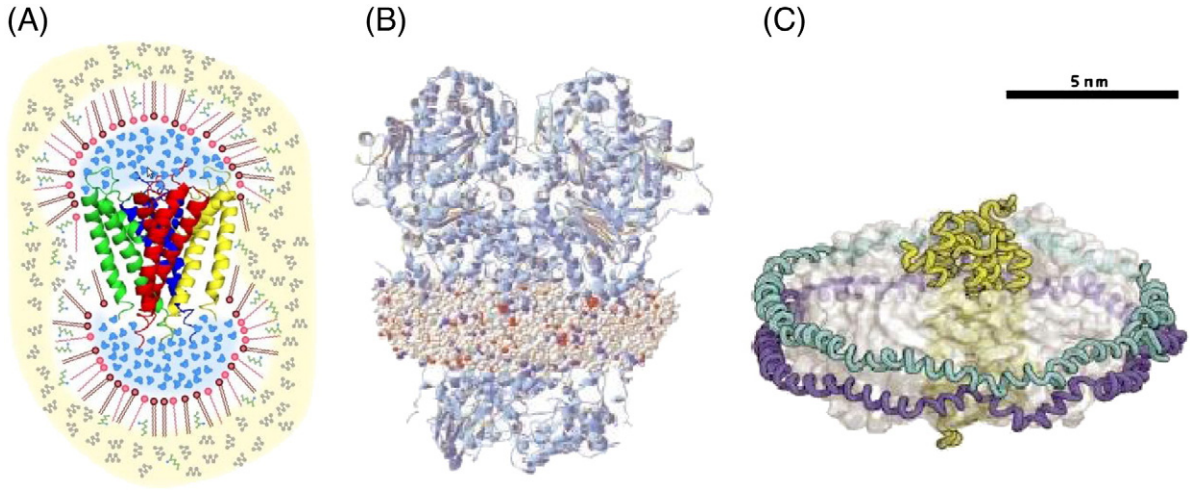


**Fig. 9.** Models of (A) potassium channel KcsA in reverse micelles; (B) cytochrome *bc*1/amphipol complex and (C) rhodopsin/nanodisc complex. The models are represented approximately to the scale given on the top right corner.
Adapted from [220,224] with permission.

desorption and a high affinity for protein transmembrane surfaces (Fig. 9B). The length and charge of amphipols are tunable, but the most common version is composed of a ~70 residue polyacrylate chain in which some (~17) of the carboxylates have been randomly grafted octylamine, and some (~28) with isopropylamine. Carboxylates can also be changed to obtain a zwitterionic, sulfonated or non-ionic version [224]. Since amphipols cannot extract a MP from its membrane, the protein is usually transferred to them from a PDC, just like a reconstitution in lipid membranes: amphipols are added to the PDC and the detergent is removed through the usual procedure (see Section 3.1.1).

The quasi absence of free amphipols reduces the viscosity of the solution and the protein correlation time as compared to the detergent option. Amphipols have also been shown to maintain solubility of a very broad range of MPs such as the mitochondrial complex I, human GPCRs BLT1 and BLT2, bacterial outer MPs OmpA and OmpX, bacteriorhodopsin, the SR calcium ATPase, the nicotinic acetylcholine receptor and, more importantly, to biochemically stabilize them, as compared to detergent solutions [73,224,225]. Deuterated amphipols have been devised for NMR and although the complexes still tumble more slowly than in detergent solutions, they are amenable to solution NMR studies [226–228]. Fig. 10 compares 2D [$^{15}$N,$^{1}$H]-TROSY spectra of OmpX in DHPC micelles and amphipols and shows that the dispersion is identical [227]. While the resolution is slightly worse in amphipols, it was enough for unambiguous resonance assignment of about 60% of the protein residues. An amphipol-trapped GPCR (human BLT2) was also used to determine the structure of its bound ligand (leukotriene B4) [226]. Under these conditions, functional folding yields of 70% were achieved for the GPCR which was shown to be active for over 3 weeks—a duration sufficient to perform all relevant NMR experiments. Comparatively in detergents, BLT2 folding yield never exceeded 4% and its receptor binding capacity dropped after only 10 days [229].

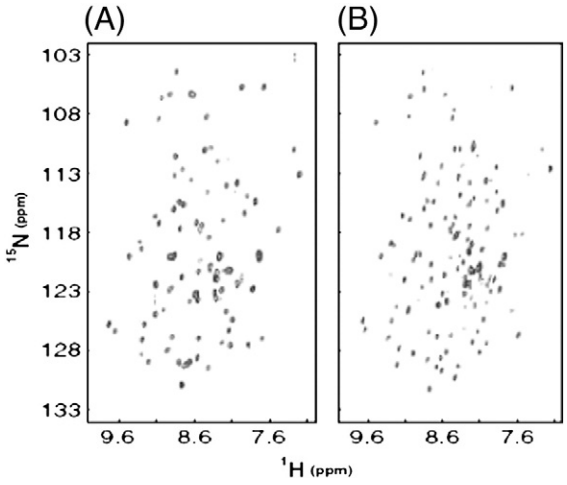


**Fig. 10.** 2D $^{15}$N-$^{1}$H NMR spectra of OmpX in (A) amphipols and (B) in DHPC micelles.
Adapted from [227] with permission.

*4.4.4. Nanolipoproteins (nanodiscs)*

Nanolipoproteins (NLPs) are new membrane systems for MP reconstitution [230–233]. As shown in Fig. 9C, NLPs are phospholipid bilayers encircled by stabilizing amphipathic helical membrane scaffold proteins (MSPs) leading to nanoscale disc-shaped objects also known as *nanodiscs*. A little larger than isotropic bicelles, these water-soluble assemblies have a diameter of ~10 nm and a thickness of ~4 nm equivalent to that of biomembranes. NLPs have several advantages for the NMR structural study of MPs: their size can be varied between 9.5 and 15 nm diameter [233–236] but there is only one protein per particle, they are monodisperse, stable and can be easy diluted, concentrated or dialysed if a change of buffer is required. Because of their flat lipid bilayer, NLPs are a better membrane mimetics compared to micelles which have a strong curvature [232,233]. Because of the stability of the MSP/lipid interaction with temperature, NLPs are also better membrane models, compared to bicelles which are stable only in a small temperature range. A thorough review on NLPs has been recently published [233].

Nanodiscs are prepared by sonicating a purified MSP such as apoliprotein A-1 (apoA-1) with phospholipid vesicles (such as DMPC, DOPC, DPPC, POPC, DMPG, DOPG or gangliosides) at the gel–liquid crystal phase transition of the lipid. They can also be made by mixing phospholipids, with or without cholesterol, with the MSP in a detergent solution (like cholate or deoxycholate) followed by a slow detergent removal via dialysis or biobeads [230,233,237,238]. The detergent solution can also be used to solubilize the integral MP to be incorporated into the discs. Cell-free expression systems containing nanodiscs are commercially available [239] and more technical details on the preparation can be found in Ref. [233]. To avoid signal from the apolipoprotein and considering the size of the MP normally studied, isotopically labelled proteins of interest can be employed [240].

A variety of MPs have been reconstituted in a functional form and studied into nanodiscs, including cytochrome P450s [237,241,242],

bacteriorhodopsin [243], human CD4 [240] and G-protein-coupled receptors [244]. The size of NLPs makes them amenable to solution NMR studies since their correlation time is equivalent to an isotropic rotation of a globular protein of *ca.* 200 kDa [232]. A $^{1}H$–$^{15}N$-TROSY spectrum was collected for the antibiotic peptaibol antiamoebin with amide proton resonances shown to be a little broader to those obtained in isotropic bicelles (Fig. 8B) [232]. The voltage-sensing domain of the KvAP channel (four transmembrane helices) could also be reconstituted into nanodiscs and gave solution NMR fingerprint spectra with broad resonances but with a large amide proton signal dispersion, indicative of a native-like folding [245]. For large nanodisc–MP complexes, MAS solid-state NMR is an alternative as exemplified by the precipitation of the nanodisc/CYP3A4 complex with polyethylene glycol (PEG) that yielded high-quality 2D solid-state NMR spectra of the folded, active protein [246].

## 5. Conclusion

The biomembrane complexity is a challenge for the NMR structural study of MPs; however this review shows that a variety of artificial membranes are available for applications in both the solution and solid states. Systems of almost any size and lamellarity exist, either lipid- or detergent-based, self-orienting or mechanically aligned, and even tunable to experimental requirements such as bicelles. Detergents are most useful in structural studies because of their multiple roles in protocols such as solubilization, purification, transfer, renaturation and reconstitution of MPs. New techniques have been proposed to help the study of MPs such as MAS or reverse micelles at high pressure. Likewise, new molecules like amphipathic polymers and lipid/protein nanoparticles have been developed with the same objective. In search of additional oriented membrane systems with a large surface area, single bilayered nanotubes formed by adsorption into the pores of anodic aluminium oxide discs have been proposed [247–250]. In the recent years, a variety of new membrane mimetics have been used in NMR and protein crystallization such as lipid cubic phases [251–253], bola-amphiphile and diamine hollow cones [254], and porous phospholipid nanoshells [255], to name a few. Their application in structural study of proteins can be expected in a near future.

These efforts for identifying new membrane mimetics have been fruitful in solution NMR since, among the ~30 MP structures solved in the past 15 years, the maximum protein size has raised from around 6 kDa in the first decade, to 43 kDa in the last 2 years. These developments have contributed to solid-state NMR as well since, among the 20 protein structures solved in the last decade, 12 were solved in the past 2 years. Most of them were not MPs, though, but this is clearly the next goal to pursue since solid-state NMR is the only technique that can solve the atomic structure of a MP inside a native-like environment.

Thirty years ago in structural NMR laboratories, SDS was almost the only detergent [256], DMPC almost the only lipid [257], and solid-state NMR could only tackle small peptides [258,259]. Designing new membrane mimetics has not only helped us to better understand the interactions between MPs and their environment but has also allowed to push the limits of NMR in terms of size and complexity of the samples amenable to study. Current samples are often made of mixtures of MPs and several lipids and/or detergents, each providing essential properties to the complex. Consequently, NMR now solves structures of MPs made of several helices, barrels, multimers or protein–drug complexes.

We have often described membrane mimetics as “native like”. The next step will probably be the development of techniques that will allow the study of MPs in their native environment, i.e., *in vivo* biomolecular NMR and structural determination. As always, the first application has come out in the field of soluble proteins [260,261]. The goal is now to also transpose this approach to MPs.

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