Review

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Membrane mimetics for solution NMR studies of membrane proteins

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Abstract: Membrane proteins are one of the most challenging and attractive objects in modern structural biology, as they are targets for the majority of medicines. However, studies of membrane proteins are hindered by several obstacles, including their low ability to crystallize, highly dynamic behavior of some of their domains, and need for membrane-like environment. Although solution nuclear magnetic resonance (NMR) is a very powerful technique of structural biology in terms of the amount of provided data, it imposes several limitations on the object under investigation, with the main constraint being related to the size of the object. For this reason, the membrane mimetic has to form particles of small size and simultaneously to properly simulate the bilayer membrane to be applicable for solution NMR spectroscopy. Here we review the recent advances in the field of membrane mimetics for solution NMR studies, discuss the advantages and drawbacks of specific membrane-like environments, and formulate the criteria for the selection of proper environment for a particular membrane protein or domain.

Keywords: bicelles; membrane mimetics; micelles; nanodiscs; NMR.

Abbreviations: MP, membrane protein; NMR, nuclear magnetic resonance; TM, transmembrane; p75NTR, p75 neurotrophin receptor; DAGK, diacylglycerol kinase; LPN, lipid-protein nanodisc; SMA, styrene and maleic acid; SMALP, SMA-lipid particle; CMC, critical micelle concentration; TROSY, transverse relaxation optimized spectroscopy; DM, decyl maltoside; DDM, dodecyl maltoside; DPC, dodecylphosphocholine; LDAO, lauryldimethylamine-*N*-oxide; SDS, sodium dodecyl sulfate; LMPG, 1-myristoyl-sn-glycero-3-phospoglycerol; LPPG, 1-palmitoyl-sn-glycero-3-phospoglycerol; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DH7PC, 1,2-diheptanoyl-sn-glycero-3-phosphocholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; TFE, trifluoroethanol.

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1 Introduction

Membrane proteins (MPs) are one of the most attractive objects in modern structural biology. A total of 20–30% of human genome open reading frames encode the MPs [1], and MPs represent the vast majority of drug targets [2]. MPs take part in the development of many severe diseases, including cancer, neurodegenerative and autoimmune disorders, pain syndromes, etc. Altogether, the listed facts highlight the importance of structural studies of MPs. Solving the spatial structures of such proteins would allow the deep understanding of the structure-function relationship for MPs, rational protein engineering, and drug design. By contrast, of 100,000 structures that are available in the Protein Data Bank, only 3% are annotated as MPs, implying that the MPs are underinvestigated from the structural viewpoint for several reasons. Many MPs, including all type I or bitopic proteins, are highly dynamic and often contain intrinsically disordered regions, and it prevents their crystallization and high-resolution studies by cryo-electron microscopy. Recombinant MPs are also very difficult to produce: yields in eukaryotic cells are extremely low [3] and refolding of MPs that are obtained in inclusion bodies of bacterial cells is not straightforward and is often a state-of-the-art task. Some MPs contain both the extracellular and the intracellular globular domains that require the different redox properties of the environment – cysteine residues are engaged in disulfide bridges outside the cell and are reduced in the

cytoplasm. Last, but not the least, MPs require the specific environment to retain their native structure. Almost all conventional approaches of structural biology are not applicable in cells and even in liposomes. Therefore, the special membrane mimetics are necessary, which contain the unnatural components affecting the properties of protein under investigation, and this is especially important in the case of solution nuclear magnetic resonance (NMR) spectroscopy.

Solution NMR is one of the most powerful techniques of structural biology in terms of the amount of provided data. Apart from the determination of high-resolution spatial structures, NMR is used to study the intramolecular mobility of proteins, to monitor the conformational transitions, and to investigate the kinetic and thermodynamic parameters of various processes. However, the wide spread of NMR in structural studies is limited because of the several experimental problems. The major limiting restraint of NMR spectroscopy is the size of the object under investigation. Large molecules tumble slowly in solution, which results in the enhanced transverse relaxation, broad lines, loss of sensitivity, and resolution in NMR spectra. In addition, large molecules contain many nuclei that give rise to the signals in NMR spectra, which, in turn, becomes overcrowded and hard to interpret. This was in part overcome by the recent advances. Transverse relaxation optimized spectroscopy (TROSY) pulse sequences that were developed for the aromatic [4], amide [5, 6], and methyl [7] groups allow to decelerate the transverse relaxation and enhance the sensitivity, whereas the novel techniques of specific labeling of protein methyl groups and other moieties [8–13] simplify the analysis of NMR spectra and abolish the dipole-dipole interactions between protons, which contribute a lot to the transverse relaxation. However, the size of molecules/molecular complexes studied by NMR in solution rarely exceeds 50–70 kDa. Investigation of larger objects is usually a state-of-the-art work [14, 15] and requires the great time and money expenses. Therefore, if an MP needs to be studied by NMR in solution, the membrane mimetic has to form particles that are relatively small and simultaneously be alike the lipid bilayer to adequately model the properties of the real cell membrane. Conventional membrane mimetics that are applicable for solution NMR spectroscopy are well described in several recent reviews [16–26]. For that reason, we will give a brief overview on the types of available membrane mimetics (Figure 1), including the most recent data, and then focus on the main problem of NMR studies of MPs – approaches to the rational selection and optimization of a membrane-like environment for the particular protein.

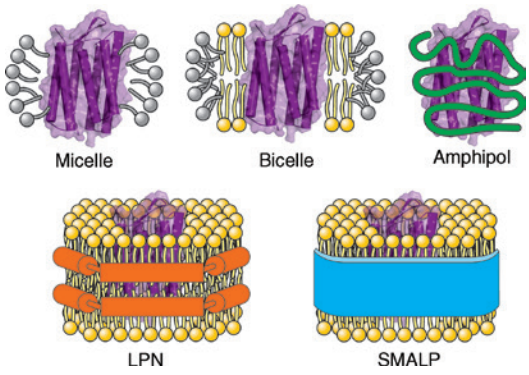

Figure 1: Shape and architecture of particles of membrane mimetics, applicable for the solution NMR spectroscopy. Orange cylinders represent the MSP or other belt-forming protein, blue band is the SMA molecule, and green ribbon is the amphipol. Gray are the molecules of detergent and lipids are shown in yellow.

2 General types of membrane mimetics for solution NMR studies

2.1 Organic solvents

MPs are usually not soluble in water because of the presence of large hydrophobic regions. One of the strategies to shield the hydrophobic parts of the MP is to add up to 100% of one or mixture of organic solvents such as methanol, ethanol, isopropanol, trifluoroethanol (TFE), chloroform, dimethyl sulfoxide, etc. For example, fragments of bacteriorhodopsin were studied in organic solvents by solution NMR [27–29], and the mixture of chloroform-methanol-water (4 : 4 : 1) was shown to mimic the membrane properties for the transmembrane (TM) H+-transporting subunit c of the F1Fo ATP synthase [30]. Although MPs usually adopt the proper secondary structure in such mixtures, the tertiary structure is not formed because of the absence of the expressed interface between the polar and the nonpolar portions of the solution. Therefore, the use of organic solvents is nowadays restricted to the studies of secondary structure of the single-TM or two-span helical proteins [31–33] and small membrane-active peptides [34, 35].

2.2 Detergents

Detergents are historically the first membrane mimetic, which is indeed membrane-like. One of the most important characteristics of detergents is the critical micelle

concentration (CMC). Below the CMC, detergents are soluble in water, whereas above the CMC, the amphipathic properties of detergent molecules cause the formation of aggregates – detergent micelles with the hydrophobic core and hydrophilic outlet. Detergent micelles have a distinct border between the polar and the hydrophobic compartments, which makes the various parts of MPs to interact with one another within the micelle and to form the tertiary structure. Detergents are traditionally called "harsh" and "mild" based on their ability to denature the MPs [36]. Harsh detergents are commonly ionic and are used to dissolve the bacteria inclusion bodies or protein precipitates of other kinds, whereas mild detergents are uncharged, sometimes bear the hydrocarbon moieties, and are used to extract the proteins from the membrane, retaining their native structure and activity. The properties of lipid packing inside the particles and the curved shape of the micelle surface are quite far away from the characteristics of real cell membrane. This may cause the improper folding of the MP and is definitely a disadvantage of this membrane mimetic. By contrast, micelles have a relatively small size (20–100 kDa), which is extremely important for solution NMR spectroscopy. Besides, many detergents are now available in the deuterated form. As we will show in the next section, MPs may retain their native structure in a specific detergent or a mixture of detergents, which makes micelles the most widely used membrane mimetic for the solution NMR studies.

On the dawn of the solution NMR studies, MPs were investigated mainly in harsh detergents, such as sodium dodecyl sulfate (SDS) [37–39], and even now some studies are performed in this mimetic [40–46]. Despite the variety of the detergents that are commercially available, only few are used in solution NMR studies and in mimetic screenings (Figure 1). Very mild detergents, decyl maltoside (DM) and dodecyl maltoside (DDM), can be used to extract the proteins from the membranes in the active form and are taken to investigate the 7-TM proteins, such as bacteriorhodopsin [47, 48] and G-protein-coupled receptors (GPCRs) [49–51], and other helical MPs, e.g. voltage-gated channels [52]. GPCRs are also active in the mixtures of DM and DDM with cholesterol hemisuccinate [53, 54]. These mimetics are known to support the native folding of many proteins but form very large micelles (~70 kDa), which prevents the high-resolution studies in such an environment. Dodecylphosphocholine (DPC) and lauryldimethylamine-*N*-oxide (LDAO) are harsh detergents with small micelles (20–25 kDa) that often maintain the native structure of MPs and provide the good quality of NMR spectra [55–71]. In some cases, the nonconventional analogs of DPC with methylated and hydroxylated fatty chains or with the altered number of carbon atoms, such as FOS-30, FOS-10, or FOS-14, reveal the better performance to dissolve the MPs [72–75]. Anionic lysolipids (1-myristoyl-sn-glycero-3-phospoglycerol [LMPG] and 1-palmitoyl-sn-glycero-3-phospoglycerol [LPPG]) can solubilize the proteins directly from the cell-free reaction precipitates and are often used in NMR studies [32, 76–79]; however, they were shown to cause the improper folding and inactivation of some MPs [19, 80]. In addition, recent studies revealed the prospects of the unnatural micelle-forming short-chain lipid 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DH7PC) as a membrane mimetic for various MPs, including 7-TM [81, 82] and other α-helical proteins [83]. Similar short-chain lipid 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) was often used in the studies of β-barrel bacterial outer MPs [84–88]. In many cases, the best results are obtained in mixed micelles, where the detergents with different length of fatty tails [58, 89–91] and/or charge on the headgroups are combined together [60, 65, 92, 93]. Detergents from the Brij and Tween families and Triton X-100, which are conventionally used for the extraction of MPs from the cell membranes, were never shown to provide the NMR spectra of MPs of reasonable quality [76, 94]. Similarly, no example of the successful use of the bile salt derivatives in micelles, such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), for solution NMR studies of MPs is reported [76]. The use of various detergents for the structure determination by solution NMR is summarized in Figure 2, and properties of common detergents are well described in the review [22].

2.3 Bicelles

Bicelles are one of the most promising mimetic to study the MPs by solution NMR. Bicelles contain the patch of a planar lipid bilayer surrounded by the rim of the detergent [95, 96]. Lipids with various length and saturation of fatty chains [97–99], headgroups [100–104], cholesterol [105], and gangliosides [106] were shown to be capable of bicelle formation alone or in the mixtures with other lipids. This makes bicelles a convenient environment to study the effect of the membrane lipid composition on the structural properties of the MPs and to investigate the specific lipid/protein interactions [107, 108]. In addition, lipid analogs with the ester bonds can be used instead of the conventional phospholipids, to exclude the lipid hydrolysis that can occur at low pH [109]. It is also known that any

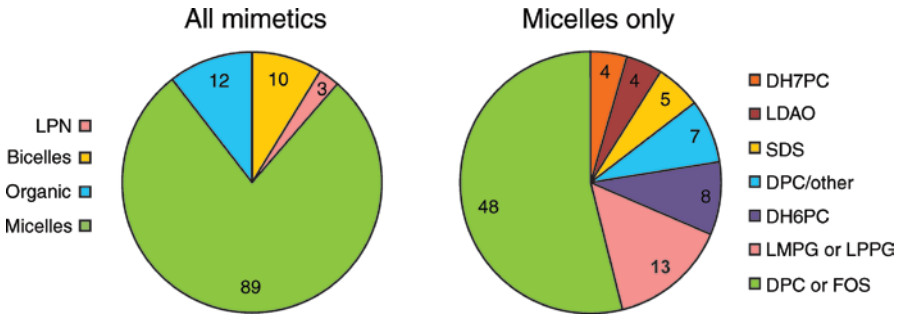

Figure 2: Membrane mimetics for NMR structure determination in solution. Shown is the number of structures in Protein Data Bank (PDB) database determined in various membrane mimetics since 2010. A total of 114 spatial structures were gathered from the websites http://blanco.biomol.uci.edu/mpstruc/ and http://www.drorlist.com/nmr/MPNMR.html and manually found in PDB database among the entries that are annotated as "membrane protein" and are not mentioned on both websites. Left histogram describes the distribution of found structures between the general types of membrane mimetics, blue sector corresponds to the organic solvents. Right histogram describes the use of particular detergents in micelles. FOS-10 (2 structures), FOS-14 (1 structure), and FOS-30 (1 structure) are counted together with DPC. Blue sector corresponds to the mixtures of DPC with other detergent (SDS) or phospholipid.

arbitrary detergent would not always form bicelles, being mixed with lipids. The ability to assemble into the discoidal particles was documented for the DHPC [110, 111], bile salt [112], and its derivatives CHAPS, CHAPSO, and Façade detergents [99, 113, 114]. DH7PC/1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) solution was also called "bicelles" in the NMR structural studies [115]; however, the shape of particles in such a mixture was not characterized. Rim-forming detergents can be polymerized in bicelles to enhance the stability of the MP under investigation [116]. Out of all detergents, CHAPS and CHAPSO are preferred if the MP is sensitive to the presence of the detergents, and DHPC should be selected if the deuteration of fatty chains is required for the needs of the experiment [99].

The size of bicelles can be controlled in quite a wide range, starting from approximately 40 kDa. By varying the lipid/detergent ratio (q), the character of the size dependence is well established for many rim-forming agents [95, 99, 117]. However, it was shown that bicelles increase their size upon dilution and heating [99, 118–120]. The first effect can be excluded keeping in mind that a fraction of the detergent is soluble in the monomeric form [99], whereas the temperature-dependent growth is not observed for bicelles with radii less than 3 nm [99, 119]. Bicelles formed with large q orient spontaneously in strong magnetic fields and are utilized in solid-state NMR spectroscopy of MPs [121] and in solution to measure the residual dipolar couplings of soluble proteins [122]. Small bicelles that are used in solution NMR studies of MPs are not oriented by the magnetic field and are called "isotropic". Lipids in larger bicelles ($q > 0.75$ for DMPC/DHPC) can undergo the phase transition at temperatures, close to the observed for the lipid bilayers [118]. However, the correspondence of the lipid packing parameters to the real bilayer membranes is not established, the mobility of the MPs is enhanced in some kinds of bicelles, and packing of lipids around the protein is not tight [98, 99].

The benefits of bicelles are obvious – they contain the portion of lipid bilayer, can mimic the lipid composition of the cell membrane, and retain the activity and native structure of many MPs [19, 115, 123]. However, the use of bicelles in solution NMR is limited – spatial structure of one β-barrel [96] protein, several dimers of single-TM α-helices [124–132], ArfGTP [133], complex of two cytochrome P-450 subunits [134], and Smr [135] were determined or characterized in this mimetic (Figure 2). In the most recent study, bicelles were successfully utilized to reconstruct and determine the spatial structure of the HIV Env trimer [136]. Altogether, less than 10% of NMR spatial structures of MPs were obtained in bicelles (Figure 2). It may be the consequence of the relatively large minimal size of bicelles and difficulties with the transfer of the MP of interest into bicelles from the detergent that was initially used to extract the MP from cell membranes or to solubilize the MP from the inclusion bodies or cell-free precipitates.

2.4 Lipid-protein nanodiscs

Lipid-protein nanodiscs (LPNs) are, like bicelles, the mimetic that is extremely membrane-like. LPNs were also shown to contain a patch of lipid bilayer surrounded by the belt formed by special proteins. Several belt proteins were suggested for LPNs [137–139], the most widely spread originate from the membrane scaffold protein (MSP), which is a part of the apolipoprotein A-I. MSP consists of

amphipathic a-helices, the nonpolar side of the helices covering the hydrophobic acyl chains of the lipids. The radius of the classic MSP1 LPNs is equal to approximately 5 nm [140, 141]; however, recently the size of LPNs was reduced to 3.5–4.0 nm after the development of shorter MSP versions [55]. LPNs undergo the phase transition at temperature close to the critical temperatures measured for lipid bilayers [140]; however, lipids are packed more tightly in LPNs than in liposomes [141–143] and in bicelles [99], suggesting that the state of the lipid bilayer in LPNs does not correspond to the liquid-crystalline membrane. The most useful advantages of LPNs are their homogeneity and monodispersity, stability to disruption and aggregation, ability for an experimenter to choose among the different lipid compositions to mimic the native membrane, and ability to obtain the suitable thickness of the lipid bilayer [144]. These special properties of the LPNs enable to maintain the stability and integrity of the protein under investigation and reach its high concentrations required for the structural studies by NMR. Compared with bicelles, LPNs are characterized by the absence of detergents, discrete set of possible sizes, and prohibition against the matter exchange between the particles. This prevents the oligomerization of MPs under investigation but makes the studies of interaction between MPs in LPNs impossible.

In the initial works, LPNs were used to study the topology of the membrane-active peptides on the membrane [145–147] and as a reference medium in the detergent screenings [80, 115, 148–150]. The principal possibility of NMR structural studies in large MSP-based LPNs was demonstrated by the works with VDAC-1 channel and CD4 protein [151, 152], and the introduction of smaller LPNs resulted in the structure determination of two β-barrels [153–155] and of the construct, containing the TM and cytoplasmic domains of the p75 neurotrophin receptor (p75NTR) [156]. LPNs formed by the other belt proteins, such as 22A peptide, were also shown to be applicable for NMR structural studies in solution: the complex of cytochrome b_5 and cytochrome P-450 was reconstructed in these LPNs, and high-resolution TROSY spectrum was obtained [157]. The examples of LPN implementations in other fields of structural biology are beyond the scope of this article and can be found in the excellent reviews [20, 158].

Despite many benefits, LPNs have several considerable drawbacks that restrict their applicability in solution NMR studies. The mass of the smallest LPNs composed of truncated variants of MSP with the embedded protein is approximately 60 kDa, which is far too big for conventional NMR techniques and results in the NMR spectra of poor quality for the majority of the MPs [149]. One should use the deuteration of the target protein accompanied by the specific labeling techniques, which, in turn, significantly elaborates the expression protocols and increases the sample cost.

2.5 SMA-lipid particles

The next membrane mimetic is very much like LPNs and contains the portion of lipid bilayer surrounded by the styrene and maleic acid (SMA) copolymer (3 : 1) [159]. SMA is an amphiphilic molecule capable of forming relatively small disk-shaped nanoparticles from lipid vesicles. This novel membrane mimetic was called SMA/lipid particles or SMA-lipid particles (SMALPs). SMALPs were used to solubilize the 7-TM α-helical bacteriorhodopsin [159, 160], the 8-stranded b-barrel lipid A palmitoyltransferase PagP [159], and the function modulator of voltage-gated potassium channels protein KCNE1 [161]. MPs were reported to retain their integrity, stability, and function if incorporated into SMALPs. A particle with an embedded 7-TM protein is ~11 nm in diameter and contains approximately 11 lipid molecules [159]. Thus, SMALPs are monodispersed, thermostable, and soluble nanoparticles applicable for solution NMR investigations. The size of the nanoparticle could be easily controlled by manipulation of the lipid–SMA polymer ratio, as was demonstrated in [162, 163]. One of the most prominent features of SMALPs is their potential ability to solubilize the integral MPs form lipid vesicles or membranes not using the detergents. By contrast, SMALPs have their obvious drawbacks – the minimal size of the SMALP particle is too large and the amount of lipids inside the SMALP is too low because of the high volume of the SMA chains. For unknown reasons, the use of SMALPs in solution NMR studies was not reported; however, this application of the mimetic needs to be tested in the nearest future.

2.6 Amphipols

Amphipols stay separately in this list of membrane mimetics because they do not contain any lipids or detergents. Amphipols are amphipathic polymers that form the coat around MPs with the thickness of 1.5–2.0 nm [164]. The mimetic is characterized by high propensity to stabilize the MP and to prevent its oligomerization [165]. The prospects of amphipols for structural NMR studies is discussed in the recent review [166]. In brief, five MPs, both α-helical and β-structured, were characterized in amphipols to the date by solution NMR spectroscopy [48, 167–176]. These

studies confirm the ability of some kinds of amphipols to retain the native structure and activity of MPs and simultaneously to provide the high-resolution NMR spectra of their TM domains, with the quality, comparable with observed in detergents and bicelles. Some amphipols are available in deuterated form, which is also advantageous in NMR structural studies of MPs in solution.

Amphipols cannot be *a priori* deemed to properly mimic the lipid bilayer because of their unnatural composition; therefore, the biological relevance of the spatial structures obtained in amphipols will always be questioned in the absence of protein activity or other data confirming the native folding of the protein under investigation. However, this also refers to the detergent micelles. It is also noteworthy that the amphipol/MP particles are not monodisperse, and a fraction of the sample needs to be selected to obtain the spectrum of high quality, thus decreasing the effective yield of the protein production [173]. In addition, the use of amphipols is not a universal solution for any arbitrary chosen MP. There are negative results in the literature, when certain MPs could not be reconstituted in amphipols or no NMR spectra of reasonable quality were observed for the MP in amphipol environment [173]. Thus, amphipols may be considered as an alternative to LPNs and other lipid-based mimetics when the MPs of interest are prone to aggregation and inactivation.

3 Experimental protocols for the solubilization of MPs in membrane-like environment

Despite the variety of available membrane mimetics, there are only few approaches to solubilize the MPs. In the most fortunate case, the protein precipitates can be dissolved directly by the aqueous solution of membrane mimetic, such as bicelles or detergents [60, 77]. When the α-helical membrane domain is soluble in the mixture of organic solvents, usually TFE/H_2O or hexafluoroisopropanol/H_2O, the dry powder of lipids or detergents is simply added to such solution. Then the water is added to the mixture until micelles or bicelles are formed, and the solution is lyophilized and redissolved in the aqueous buffer [31, 58]. This option is applicable mainly for the most simple MPs, such as single-helix TM domains of bitopic proteins. MPs can also be extracted by the mild detergents directly from the cell membrane [49] or cotranslationally incorporated into the particles of membrane mimetic during the cell-free reaction [94, 177–179]. The protein of interest, which is already solubilized by the harsh detergent (SDS, LS) or extracted from the membrane, can be transferred to the desired environment using the affine chromatography. MP is usually immobilized on the wax, such as Ni-Sepharose, and then washed by the solution containing the membrane mimetic of choice [180]. Alternatively, mild detergents with low CMC can be exchanged with the detergents of higher solubility using permeable membrane filtering units [181]. LPNs, SMALPs, and amphipols are prepared from the lipid/detergent solutions of the MP under investigation. After the addition of the MSP, SMA, or amphipol, the detergent is removed either by dialysis or by using a special wax capable of absorbing the small hydrophobic molecules [137, 159, 182].

4 Possible criteria for the selection of membrane mimetic, rational approach

The variety of available membrane mimetics raises one of the most essential problems of MP structural studies – the correct and the rational choice of the membrane-like environment for the protein under investigation. In the first decade of the implementation of solution NMR spectroscopy for protein structure determination, the isotope labeling and recombinant protein production were extremely expensive. Therefore, all studies of MPs were performed in the mixtures of organic solvents or in the only cheap detergent that was available in the deuterated form. This also refers to the first solved structure of the MP – gramicidin A [37]. Scientists of those days did not screen the detergents and did not test the activity of the MPs, they just took the only option they had and performed studies in the only model that was available, regardless the possibly nonnative character of the environment. However, now, when isotope labeling is a routine task, one has to formulate the criteria to select the membrane mimetic out of the vast variety of options. The solution may seem simple – choose the environment that is at most like the bilayer membrane, e.g. LPNs or bicelles. However, there are several obstacles on that path. First, as we will show in the next section, in some cases the use of LPNs or bicelles does not result in the proper folding of the MP. Second, both bicelles and LPNs are characterized by the large size of particles in solution, which does not allow the straightforward structure determination. In large particles, the signals in NMR spectra cannot be assigned

using the conventional triple-resonance experiments, and the expensive procedure of the single-point scanning mutagenesis has to be applied for the task [49]. Besides, because the studies of large objects require the deuteration of protein side chains, the spatial structure of the MPs in isotropic bicelles and LPNs cannot be resolved through the conventional NOE approach, the insensitive four-dimensional spectra [57], paramagnetic labeling [77, 183], and use of anisotropic environment are required [184, 185]. Therefore, LPNs, large bicelles, SMALPs, and amphipols are the weapons of the "last chance" and are applied for the structure determination by NMR when other mimetics fail to support the stability, activity, and native folding of the MP under investigation. One would usually like to work in micelles or very small bicelles that do not contain the significant patch of planar bilayer to minimize both time and money expenses. In the next paragraph, we list the possible approaches to select the best detergent for the structural study of the MP under investigation.

The easiest way to screen the membrane mimetics is to monitor the activity of the MP of interest. This can be done for photoactive proteins, such as bacteriorhodopsin, optical rhodopsin, and other similar MPs based on the light absorption spectra [48, 51, 164, 181]. The functionality of the GPCR may be assessed using their ligand/G-protein binding propensity in the model environment [49, 50]. The active state of KcsA potassium channel in SDS was confirmed by the presence of functional tetrameric assembly of the protein in solution [41]. However, these are the exceptional cases; the activity of other MPs cannot be studied that easily. Type I integral MPs, such as receptor tyrosine kinases, require the whole protein to monitor its activity, which is far too large for the NMR studies. The conductance of ion channels cannot be measured in any of the mimetics that are applicable for the solution NMR spectroscopy. Moreover, the absence of the ligand binding or other activity of an MP does not always indicate the improper folding of the protein. There may be subtle differences in the structure that do not affect the overall fold but do prevent the interaction with the ligand. Thus, other criteria apart from the activity of the protein need to be applied for the screening of the membrane mimetics.

The most feasible way to estimate the correctness of the MP folding is to obtain these data directly from the NMR spectra. Such an approach is implemented in the majority of screenings of the membrane mimetics. First, vast detergent screenings relied on the so-called quality of NMR spectra. Krueger-Koplin et al. [76] performed a very wide detergent screening for three different helical MPs in 2004, using dozens of various mimetics. As a merit of the "quality", they used the number of observed signals in NMR spectra and the lifetime of the sample. The reasonable lifetime is necessary to record the long-term NMR spectra, whereas the number of peaks reports on the internal mobility of the MP and determines whether the protein can be investigated in this mimetic. On the basis of the stated criteria, the authors found the anionic lysolipids, such as LPPG, to be the optimal for all three objects. However, nice spectra with narrow peaks of predicted number and long lifetime of the sample mean that the protein does not precipitate in the mimetic of interest and does not experience the slow motions on the microsecond-to-millisecond timescale but says nothing about the folding. In the later work, Girvin et al. [19, 123] found that Smr binds its ligands only in DMPC/DHPC bicelles, but not in the LPPG, which was, this part refers to LPPG selected for the protein in the detergent screening. For that reason, the dispersion of signals in NMR spectra also needs to be estimated, especially for the helical MPs. If the folding is incorrect, and TM helices are not in the tight contact, the dispersion of signals is low, whereas in case of the tight packing of helices with specific contacts, outlying cross peaks should appear. Such a criterion was used by Zhang et al. [72] in their broad screening of membrane mimetics for the OmpX protein. In addition, the use of the signal dispersion was demonstrated clearly on the example of the voltage sensor of the KvAp potassium channel [80]. Although very good spectra were obtained for the protein in anionic lysolipids, the proper folding of the sensor was observed only in the zwitterionic DPC and DPC/LDAO mixture, where the "quality" of spectrum was lower but the dispersion of signals was much higher.

Nonetheless, even the high dispersion of signals does not ensure the proper folding of the MP. Recently, with the introduction of LPNs, it became possible to measure the NMR spectra in the most native environment, provided by the LPNs, and select the micelles or bicelles based on the similarity of NMR spectra in mimetics with small particles and in bilayer-containing system. This approach was first suggested by Shenkarev et al. in 2010 for the KvAp voltage sensor, and since then, it is actively used in screenings [48, 80, 115, 148]. Similarly, if the MP contains soluble domains, one can record the NMR spectra of separate globular domain and compare them with the spectra of the full-size protein in various membrane mimetics [148, 156].

All these listed techniques use the screening approach, but it would be useful to select rationally the membrane mimetic for the specific protein. To do this, one needs to understand clearly what the variables of the membrane mimetics are and how they influence

the quality of NMR spectra and folding of the MP under investigation. The work by Columbus et al. [89] demonstrated that the hydrophobic thickness of membrane mimetics is one of the main determinants of the protein structure and behavior. They took the detergents of various nature and charge to dissolve the model two-helical protein, TM0026. As a result, the appearance of NMR spectra of the protein was shown to be dependent mainly on the effective hydrophobic length of detergent fatty chains; mixtures of two different detergents yielded spectra identical to the other detergents with the hydrophobic length, corresponding to the average length of mixed components. Thus, instead of the wide screening, one could choose first the short-chain detergent and then titrate the sample by the long-chain molecules until the quality of spectra is the best. This principle was utilized in the studies of BniP3 mitochondrial protein: titration of the initial sample in DPC by the long-chain phospholipid DPPC allowed to exclude the unwanted minor conformation of the protein [58]. Mixtures of detergents of various lengths (LMPG/DH7PC, DPC/FOS-16) were used in the screening for the best conditions for the YgaP bacterial protein [90, 91, 148] and to study the effect of the hydrophobic mismatch on the spatial structure of the TLR3 dimeric TM domain [186]. Apart from the length of the detergent chains, charge on the headgroup is also an important factor. For many proteins, the best functionality can be obtained in nonionic detergents, such as DM, DDM, and Facade-EM [48, 49, 181], but in some cases, the addition of charged molecules to the zwitterionic micelles may significantly improve the stability of sample and quality of NMR spectra. In particular, the addition of small amounts of anionic SDS to DPC micelles improved the spectra of the FGFR3 TM domain [65], zz dimer [92], and DAP12 complex [93]. Moreover, the mixture of cationic LDAO with DPC provided the best quality of NMR spectra and the proper folding of the KvAp voltage sensor [80]. It is also necessary to keep in mind the overall concentration of membrane mimetic and lipid-to-protein ratio. At low lipid-to-protein ratio, MPs may start to self-associate, which results in the signal broadening, presence of several sets of signals, and worse overall quality of spectra [187]. By contrast, at a high concentration of membrane mimetic, the rotational diffusion of protein is decelerated, which also leads to the loss of sensitivity and broad signals in NMR spectra [187]. Therefore, the equilibrium between detergent/lipid and protein concentrations needs to be maintained. The size of LPNs and bicelles is also an important parameter for the optimization. Particles of large size decrease the quality of NMR spectra because of the enhanced transverse relaxation, whereas in small particles, the amount of lipids may not be enough to surround the MP, which causes the protein/rim interactions and results in the NMR spectra of poor quality [55]. With all aforesaid, it is clear that the rational selection and "fine tuning" of the membrane mimetic is not a dream and can be performed in the present state of things in solution NMR spectroscopy. All possible approaches and criteria to select the proper environment for an MP of interest are summarized on Figure 3.

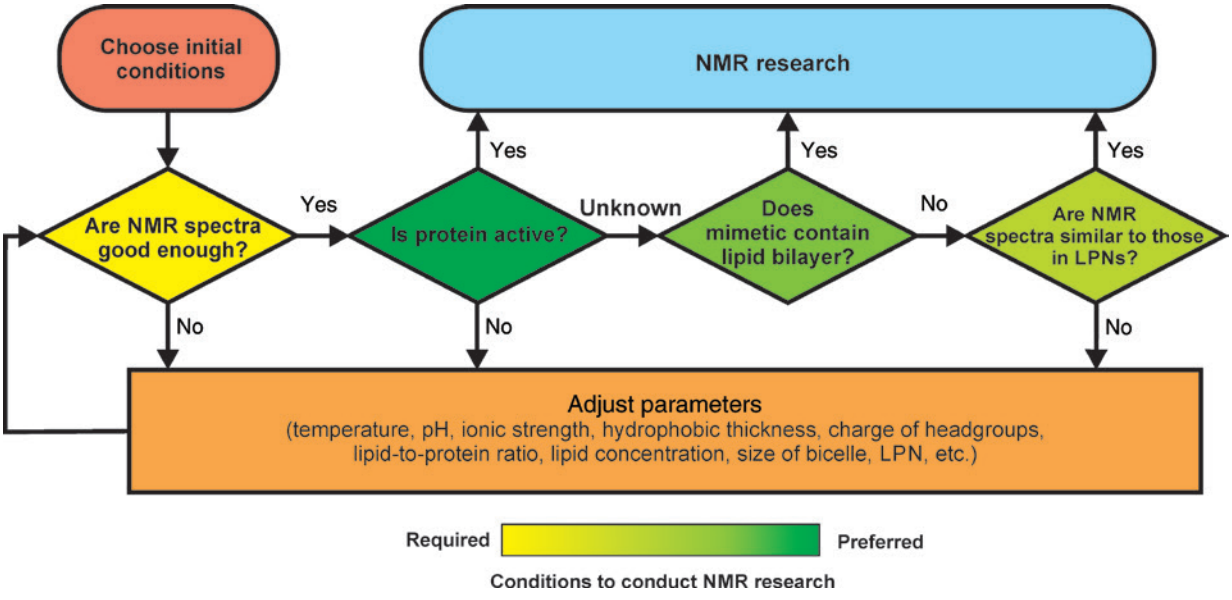

Figure 3: Flowchart, describing the approaches and criteria used to find the proper membrane-like mimetic for an MP of interest.

5 Effect of the mimetic on the structure of integral MPs

To estimate the relevance of data and to interpret the results obtained in solution NMR studies of MPs in membrane mimetics, it is necessary to understand the influence of membrane-like environment on the spatial structure, dynamic behavior, and activity of MPs. On the basis of our experience, every second publication of NMR spatial structure determined in micelles meets the criticism of the reviewers, which is focused on the model character and nonnative properties of the mimetic. By contrast, X-ray structures of MPs are considered as etalons and are thought to correspond to the real state of things in the cell membrane. In addition it is thought that the structure obtained in micelles is always less relevant than the structure of the same protein, which was determined in the environment of bicelles and LPNs. However, both statements are incorrect. We need to point out that all contemporary spatial structures of MPs are determined in the model environment – micelles, bicelles, LPNs, crystals in case of X-ray investigations, and liposomes in case of solid-state NMR studies. All of the listed mimetics are quite far away from the cell membrane, which is especially obvious in case of crystals that contain the negligible amounts of lipids. The exact environment of a protein inside the cell membrane is never known. There is a lot of data regarding the lipid composition of inner and outer leaflets of cell membranes of various kinds [21], but the real membrane has a mosaic structure [188] and contains various microdomains with the special "liquid-ordered" phase of lipid bilayer. Many MPs are active inside such domains; in some cases, the migration between the liquid membrane and the ordered microdomains may be a part of the activation mechanism [189]. Membrane microdomains are thicker than the liquid membrane, have a specific composition, and are usually enriched by sphingolipids and cholesterol [190]. The environment of the membrane microdomain cannot be reproduced by any mimetic with small particles, and even cannot be modeled adequately in liposomes. The real membrane is also rich in surface-associated proteins that can distort the bilayer. Besides, the MP also has its own influence on the surrounding lipids: it can recruit and bind the specific lipid molecules and affect the thickness and packing of lipids in the membrane [191, 192]. Thus, the use of various mimetics may sample the different states of the cell membrane and allow obtaining the different functional states of the MP (i.e. active, inactive, transition state, folding intermediate, etc.). Micelles are believed to adapt their shape to the protein under investigation and, in some cases, may provide even a more physiological environment than bilayer-containing particles because the choice of lipids with the incorrect length of fatty chains in bicelles or LPNs may affect the structure of the MP. The possibility of such errors was illustrated by the recent study of the M2 protein from the influenza virus, which adopted different conformations, depending on the thickness and charge of the bilayer [193]. It is essential to understand what kind of distortion can be introduced by the detergents into the spatial structure of the MP under investigation, to use micelles with necessary care, and to ensure the native state of the protein in such an environment. Several recent studies shed light on the effect of membrane mimetics and crystallization on the spatial structure of various proteins. These studies may help us to understand the influence of the mimetic on the structure of MPs and to judge on the relevance of the obtained structures.

At most, the effect of detergents is pronounced in the case of peripheral MPs, juxtamembrane regions, and water-soluble domains of MPs. The HIV-1 membrane-binding envelope protein was shown to form the curved helix in DHPC micelles and straight helix in DMPC/DHPC bicelles even at q as low as 0.25 [194]. This allowed authors to conclude that the absence of planar bilayer in detergent micelles can distort the structure of protein associated with the membrane surface. On the contrary, the juxtamembrane JMA regions of the human EGFR and HER2 receptors, which are believed to be helical in the context of full-length proteins, formed short amphipathic α-helices in the environment of DPC micelles and were highly mobile and disordered inside the bicelles of various size and composition [131, 195, 196]. Thus, in these particular cases, micelles could provide the more native environment than bicelles because of their ability to adapt the shape of particles to the properties of the incorporated protein. Harsh detergents that are used in both micelles and bicelles often cause the improper folding of the soluble domains of various MPs. The rhodanese domain of the YgaP protein appeared to be misfolded in DPC and DH7PC micelles, whereas the correct structure of the domain was observed in the LMPG/DH7PC mixtures at low excess of the detergents and in DMPC/MSP LPNs [148]. Similarly, the soluble "death domain" of the p75NTR was not folded properly in DPC micelles and DMPC/DHPC bicelles, whereas in various LPNs and in DMPC/CHAPS mixtures, the conformation of the domain was indistinguishable from the structure determined for the isolated "death domain" in solution [156]. Thus, micelles and harsh detergents should be applied very carefully for the MPs with structured juxtamembrane regions and globular

cytoplasmic or extracellular domains. LPNs and bicelles containing the mild detergents, such as CHAPS and CHAPSO, should be considered instead.

The slightly different picture is observed for the integral helical and β-barrel proteins without the structured extramembrane domains. The "canonic" integral MP, glycophorin A, a strongly dimeric single-span protein, was investigated in a variety of mimetics, including DPC micelles [56], DMPC/DHPC bicelles [197], bilayers [198], and crystals [199], and no substantial differences between the determined spatial structures was observed (Figure 4A). Conformation of the similar strong dimer of the single-helix mitochondrial Bnip3 protein was determined in bicelles and DPC/DPPC mixture [58, 124] and appeared to be identical within the experimental error. By contrast, the weakly dimerizing TM domain of HER2 receptor adopted completely different conformations in DMPC/DHPC bicelles and DPC micelles [126, 196]. Thus, if the energy of interactions that promote the folding of the helical MP is high enough, the protein structure will not be dependent on the environment, whereas for many proteins, the change in the nature of the membrane mimetic and even the altered length of lipids in the bilayer may lead to the changes in spatial structure. At most instructive are the studies of the voltage sensor of KvAp voltage-gated channel. The protein was misfolded in lysolipids, such as LPPG, whereas the native structure of the domain was observed in LPNs and, surprisingly, in completely unnatural detergents, such as the short-chain lipid DH7PC [83] and cationic detergent LDAO [60]. The spatial structure of the protein in detergents was almost identical to the structure observed for the protein, crystallized from the β-octyl-glycoside in complex with the antibody fragment [200]. This example demonstrates the falseness of the assumption that the proper structure is always adopted by

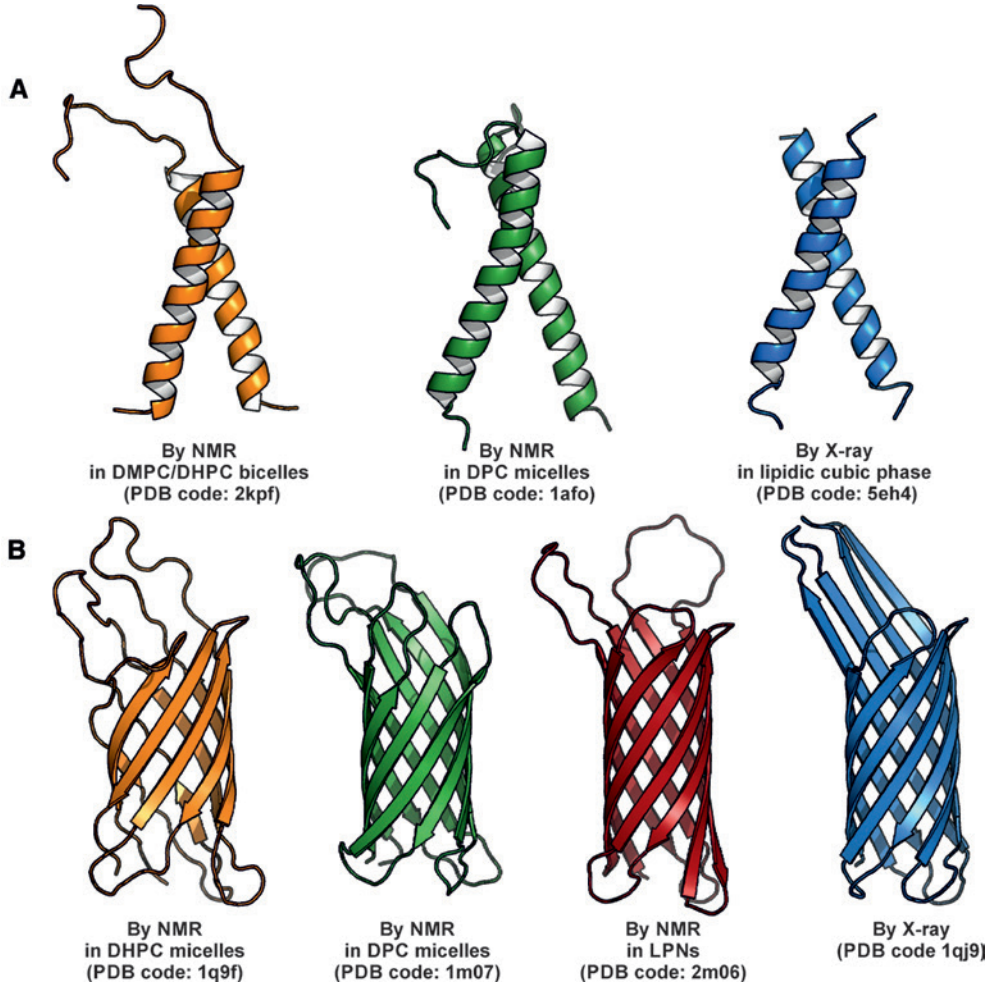

Figure 4: Spatial structures of MPs in various membrane mimetics. (A) Spatial structures of glycophorin A determined in DPC micelles [56, 197], DMPC/DHPC $q = 0.25$ bicelles [197], and in lipidic cubic phase [199]. The structure in lipid bilayers [198] was not deposited to the PDB and is therefore not shown. (B) Spatial structures of OmpX determined in DHPC [85] and DPC [55] micelles, LPNs [153], and crystals with n-octyltetraoxyethylene [201].

an MP in the environment that we ourselves deem as more native. LPPG and LMPG much more resemble the lipids of the real cell membrane than DH7PC and LDAO but do not support the folding of the voltage sensor. In the past few years, there appeared several other studies comparing the structure of the MP in detergents to the conformation of the protein in bilayer-containing mimetics. All these studies reveal that for many proteins, there is a micellar environment that supports the native folding of the membrane domain. In particular, "unnatural" LDAO micelles were shown to maintain the native state of the BamA insertase as well as DMPC/DH7PC mixtures and LPNs [115]. Bacteriorhodopsin, which is inactive in the majority of detergents, folds properly in DDM micelles as well as in the amphipols and DMPC/MSP LPNs [48]. Finally, KcSA ion channel can be assembled into the tetrameric complex in SDS micelles [41].

Another instructive example is provided by the studies of the β-barrel bacterial outer MP OmpX (Figure 4B). The spatial structure of OmpX was determined in two different detergents: DHPC [85] and DPC [55], small DMPC/MSP LPNs [153], and crystals with *n*-octyltetraoxyethylene [201]. Assuming that the correct fold of the protein is observed in LPNs, both detergents and crystallization affect the folding of the protein. DHPC and, to a certain extent, DPC disturb the β-sheets, strands become shorter, and interstrand loops become longer than in LPNs. By contrast, crystallization reveals the adverse effect – the OmpX structure is stabilized excessively; all interstrand loops that are mobile in solution become fixed and are now parts of the β-sheet structure. These data demonstrated clearly that the X-ray structures may not be considered as a perfect etalon. Crystallization results in the unnatural protein-protein contacts that affect the mobile and unstructured regions of the protein, making them more rigid and even resulting in the formation of the regular secondary structure. For that reason, it is still unknown whether the NMR-derived structure in DPC micelles [63] or the X-ray structure [202] correspond to the native state of the diacylglycerol kinase (DAGK) – the structures are completely different, but the nature of the observed discrepancy is unknown. Moreover, the solid-state NMR study in real *Escherichia coli* membranes [203] reveals the secondary structure of DAGK, which is different substantially from both the X-ray and solution NMR data.

6 Conclusions

To sum up, here we review the variety of membrane mimetics, applicable for the solution NMR spectroscopy. We formulate the criteria that are used to select the appropriate environment for the MP in mimetic screening and show that the rational approach to the selection and adjustment of the mimetic is possible. On the basis of the recent structural studies of same MPs in different environment, we suggest that none of the structural data should be approached with prejudice, regardless of the nature of membrane-like environment. If due care is taken, the use of detergent micelles can result in the native folding of the MP. By contrast, the protein may be folded improperly even in large bilayer-containing particles. For that reason, all spatial structures of MPs obtained in solution either by NMR or by Cryo-EM need to be validated with functional assays, mutagenesis, or other independent experiments. However, this also refers to the X-ray structures: crystallization may as likely disturb the native folding of the MP as the use of other model membrane-like media.

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