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Review

Lipid Nanodiscs for High-Resolution NMR Studies of Membrane Proteins

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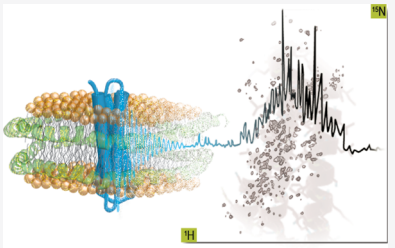
ABSTRACT: Membrane proteins (MPs) play essential roles in numerous cellular processes. Because around 70% of the currently marketed drugs target MPs, a detailed understanding of their structure, binding properties, and functional dynamics in a physiologically relevant environment is crucial for a more detailed understanding of this important protein class. We here summarize the benefits of using lipid nanodiscs for NMR structural investigations and provide a detailed overview of the currently used lipid nanodisc systems as well as their applications in solution-state NMR. Despite the increasing use of other structural methods for the structure determination of MPs in lipid nanodiscs, solution NMR turns out to be a versatile tool to probe a wide range of MP features, ranging from the structure determination of small to medium-sized MPs to probing ligand and partner protein binding as well as functionally relevant dynamical signatures in a lipid nanodisc setting. We will expand on these topics by discussing recent NMR studies with lipid nanodiscs and work out a key workflow for optimizing the nanodisc incorporation of an MP for subsequent NMR investigations. With this, we hope to provide a comprehensive background to enable an informed assessment of the applicability of lipid nanodiscs for NMR studies of a particular MP of interest.



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1. INTRODUCTION

Membrane proteins (MPs) are essential players in a large number of cellular processes including intra- and intercellular signaling, transport of metabolites and proteins across the lipid-bilayer membrane, enzymatic reactions, membrane

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fission−fusion, cell−cell interaction, and energy production.[1−5] About 30% of the human proteome is MPs,[6,7] which, due to their strategic location at membrane interfaces, account for 70% of the targets of currently marketed drugs,[8] emphasizing the importance of MPs in human health. Despite an ever-growing body of information on MPs on a cellular level, the acquisition of structural data of this protein class turns out to be a major bottleneck in obtaining a deeper understanding on a molecular and atomistic level. This can be readily seen by looking at the amount of MP structures in the protein data bank, where only ∼5% of all entries are classified as MPs. In addition to enhancing our knowledge of this protein class on a mechanistic level, high-resolution structures of MPs in particular are helpful to guide rational approaches in drug design.[8−13]

MPs are diverse and can be classified as integral and peripheral MPs. While integral membrane proteins are exposed to both sides of the membrane by traversing it with one or more transmembrane secondary structure elements (α-helices or multiple β-strands), peripheral membrane proteins are associated with one side of the membrane surface via noncovalent interactions, covalently bound aliphatic membrane anchors, or transmembrane helices.[14−17] Unlike soluble proteins, MPs require the presence of a lipid-bilayer environment, often composed of a large variety of individual membrane lipids. The lipid composition of cellular membranes varies among different membrane compartments, individual bilayer leaflets, and even locally on the same leaflet.[18−20] Lipid-bilayer membranes have the unique property of adopting different structural states, depending on the lipid structure, composition of the membrane, temperature, pressure, ionic strength, and pH.[21−23] The most relevant states are the lamellar liquid-crystalline phase, where lipid molecules are disordered and flexible, allowing high inter- and intramolecular motions, and the gel phase, where the lipid hydrocarbon chains are in a more rigid, extended, and trans conformation, restricting motions of the lipids[24] as well as the embedded MPs.[25−27] Bulk biological membranes under ambient temperatures and pressure only exist in the liquid-crystalline phase because their heterogeneity prevents the formation of a gel phase.[28] This feature is essential for allowing conformational changes of MPs to occur,[29] even though this is unlikely to be the main regulatory mechanism for MP function.[30] Apart from the bulk liquid-crystalline properties of a biological membrane, ordered gel-like patches, called lipid rafts, can exist predominantly in the cell membrane.[23,31−33] Lipid rafts are formed by liquid−liquid phase separation of lipids driven by lipid−lipid and protein−lipid interactions. These lateral lipid domains are rich in cholesterol, leading to a stiffening of the membrane as well as to the clustering of various integral and anchored MPs, facilitating their interactions. Not only the phase properties but also the physical properties of membranes such as bilayer thickness, compression, curvature, and bending can affect the conformational landscape and function of MPs.[19,34] In brief, conformation and function of MPs may be regulated by this complex, local, macrostructural, collective behavior nonspecifically or through specific direct chemical interactions between lipids and proteins.[19,21,34−36] In contrast, the presence of MPs can also affect and alter the local behavior and shape of lipid bilayers by perturbing the surrounding lipid molecules.[35,37−39] This multifaceted, reciprocal relationship between membranes and MPs and their hydrophobic nature introduce multiple layers of complexity in studying the structure and function of MPs. In addition to a generally low protein-production level of MPs as well as their high tendency to form aggregates during overexpression,[40−42] the selection of a suitable membrane-mimetic environment is essential for preparing MPs for structural studies.

1.1. Membrane Mimetics for Structural Studies of Membrane Proteins

Over the last few decades, a large selection of membrane mimetics has been developed.[43−48] Among those, detergent micelles can be considered as the most common and easy-to-use systems for MP research.[49] Although a large variety of detergents is available with a wide range of physical properties, detergents generally represent the least native membrane environment and might interfere with the MP structure, leading to reduced stability or even MP misfolding.[48] Furthermore, detergents often bind to functionally important hydrophobic cavities and may cause dissociation of partner proteins and structurally important lipids.[25,50,51] Thus, to improve the native properties of membrane mimetics, lipid/detergent bicelles, amphipols, phospholipid nanodiscs, and liposomes have been used for structural and functional studies of MPs, as discussed in recent reviews.[43,44,52−54] Among these more advanced membrane mimetics, lipid nanodiscs and liposomes provide the most native-like membrane system due to the presence of a detergent-free lipid environment of a desired lipid composition. While liposomes are the system of choice for biochemical and biophysical assays[55−62] as well as solid-state NMR,[63−66] nanodiscs, due to their controllable size and homogeneity,[67,68] gained considerable popularity for structural studies by cryogenic electron microscopy (cryo-EM)[67−77] and solution-state NMR.[78−83]

In this Review, we will provide a comprehensive overview of the characteristic features of the existing lipid nanodisc systems and their use for the investigation of MP structure and dynamics by solution-state NMR. We will compare the advantages and drawbacks of the lipid nanodisc systems that are currently used and actively developed for NMR applications and highlight the advantages of nanodiscs over traditional detergent micellar systems. Finally, we will present a selection of recent examples on the application of lipid nanodiscs for solution-state NMR and highlight the specific insights obtained in these studies that contribute to a better mechanistic and functional understanding of MPs that cannot be obtained by other structural methods.

1.2. Drawbacks of Detergent Micelles for Structural Studies of Membrane Proteins

Detergents are amphiphilic molecules with water-soluble polar headgroups and hydrophobic hydrocarbon groups, similar to the properties of lipids. However, their solubility in water is much higher, and they cannot form bilayers due to their conical molecular shapes. Instead, they tend to form micelles above the critical micelle concentration (CMC). In contrast, many lipid molecules have a roughly cylindrical overall shape because the cross sections of the polar headgroup and the hydrophobic tails are similar in size, leading to the formation of bilayer structures above the CMC. While the CMC value for detergents is in the micromolar to millimolar range, this value is in the nanomolar range or less for lipids.[84−86] Detergents can be used in a relatively straightforward manner for the solubilization of lipid membranes and the extraction of MPs.[49] A large variety of different detergents is available. However, a suitable detergent for a given MP system needs to

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be screened individually.[48,87,88] Structurally, detergents are classified as ionic (cationic or anionic), zwitterionic, and nonionic. Nonionic detergents, such as *n*-dodecyl β-D-maltoside (DDM) and Triton X-100, are considered milder compared to ionic ones such as cholate and sodium dodecyl sulfate (SDS), while zwitterionic detergents like 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and Fos-cholines are roughly in between the two latter classes in terms of solubilization strength and harshness.[84,89] Commonly used detergents in MP research are listed in Table 1 together with their CMC and micellar molecular weights.

Table 1. Commonly Used Detergents for MP Solubilization for Nanodisc Incorporation[68,84,89]

type	detergent	CMC[a] (mM)	micelle size (kDa)
ionic	sodium cholate[90]	9−15	0.9−1.3
	sodium deoxycholate[91]	2−6	1.2−5
	cetyltrimethylammonium bromide (CTAB)	1	62
	N-lauroylsarcosine[92]	14.6	0.6
	SDS[93]	7−10	18
zwitterionic	LysoPC	0.007	92
	CHAPS[94]	6	6.15
	dodecylphosphocholine (DPC)[78]	1.5	19
	lauryldimethylamine oxide (LDAO)[95]	1−2	17
nonionic	digitonin[96]	<0.5	70
	Triton X-100[97]	0.2−0.9	80
	CYMAL-5	2.4−5	32.6
	CYMAL-6	0.56	32
	n-dodecyl-β-D-maltoside (DDM)[98]	0.15	50
	decyl-β-D-maltoside (DM)[99]	1.6	
	octyl-β-D-glucopyranoside[100]	23−25	25

[a]CMC at 20−25 °C.

Mild detergents are generally preferred for multipass integral MPs because intra- and intermolecular protein−protein interactions are largely retained, preserving the structural integrity and consequently the functionality of MPs and their complexes while lipid−lipid and lipid−protein interactions are dissolved. Consequently, harsher detergents may interfere with the MP fold, disrupting their 3D structure. However, the definition of a harsh or mild detergent is always dependent on the MP of interest. In the case of MP complexes, a harsh detergent might dissociate individual subunits. In line with this notion, it has been reported that individual subunits of an MP complex even require different detergent-solubilization conditions.[13,101] Furthermore, if an MP needs to be extracted from the membrane, the lipid composition of a particular membrane is another factor in choosing a suitable detergent, simply because not all membranes can be solubilized with all types of detergents.[102,103] For instance, the inner membrane, but not the outer membrane, of Gram-negative bacteria can be solubilized with Triton X-100.[104,105] Thus, it can be concluded that the selection process for finding a convenient detergent—taking into account the required solubilization conditions, MP stability issues, and compatibility with downstream applications for an MP of interest—is a time-consuming trial-and-error problem[48,106,107] and requires considerable experience in handling MPs. For high-quality solution-state NMR experiments, detergents that form relatively small micelles (20−25 kDa) are preferred, limiting the number of suitable systems in this process.[47]

Despite these difficulties, it is possible to find a suitable detergent for a particular MP that fulfills all mentioned criteria. However, even a good detergent cannot perfectly mimic a real lipid-bilayer membrane, leading to problems and limitations when it comes to studying labile MPs and their complexes. In addition, when surrounded by a detergent micelle, MPs may not be able to sample the conformational space required for proper functionality. Moreover, the presence of detergents may result in reduced thermal stability, misfolding, or complete unfolding.[48,50,51,108] Detergents may insert into functionally important hydrophobic cavities and remove structurally important lipid molecules from the MP.[25,50,51] When it comes to complexes between different MPs, it is very likely that a detergent that has been optimized for one MP is not suitable for the other one, and vice versa. This discrepancy is even more pronounced when complexes between MPs and soluble partner proteins, which are often highly sensitive to detergents, are investigated. Because of the various reasons mentioned earlier,[50,86,109−112] even the mildest detergents can be considered harsher than a true lipid-bilayer membrane. Therefore, efforts have been made to design lipid-based membrane mimetics, such as phospholipid bicelles, a mixture of phospholipids and a detergent or short-chain lipid,[113−115] where a patch of lipid bilayer is stabilized by a rim formed by the short-chain lipid or detergent. The size of bicelles can be fine-tuned by adjusting the ratio (q = [lipid]/[detergent]) of the two components, which is very convenient for solution-state NMR experiments. In fact, it has been found that bicelles with a q-value of 0.5 are small enough for solution-state NMR and appear to provide a sufficiently large planar lipid-bilayer surface to enclose small- to medium-sized MPs.[116] However, even though this system represents a large improvement as compared to pure detergent micelles, the presence of a detergent or a rather harsh short-chain lipid can still be disadvantageous in cases where detergents need to be avoided.

A readily available and detergent-free membrane system is liposomes, which provide a native-like environment for MPs. However, unilamellar liposomes are quite large (>50 nm) and heterogeneous, rendering solution-state NMR experiments of incorporated MPs difficult if not impossible. To improve this situation, the property of amphipathic proteins, peptides, and polymers has been exploited to replace the short-chain lipids in bicelles for a more native component. Such detergent-free lipid particles have been generally termed lipid nanodiscs. In the next section, we will introduce the most common nanodisc systems and will describe their suitability for solution-state NMR studies of MPs.

2. LIPID NANODISCS—A LIPID-BASED AND NATIVE-LIKE MEMBRANE MIMETIC

Lipid nanodiscs were first introduced almost 20 years ago by the lab of Steven Sligar based on truncated versions of human apolipoproteinA-I (ApoA-I[117,118]) or membrane scaffold protein (MSP). In lipid nanodiscs, lipid molecules assemble into a patch of a lipid-bilayer membrane that is surrounded and stabilized by amphipathic molecules such as lipid-binding proteins, peptides, polymers, or nucleic acids, generating nanometer-scale, discoidal lipid-bilayer particles[43,52,119−124] (Figure 1a). This versatile membrane mimetic gained

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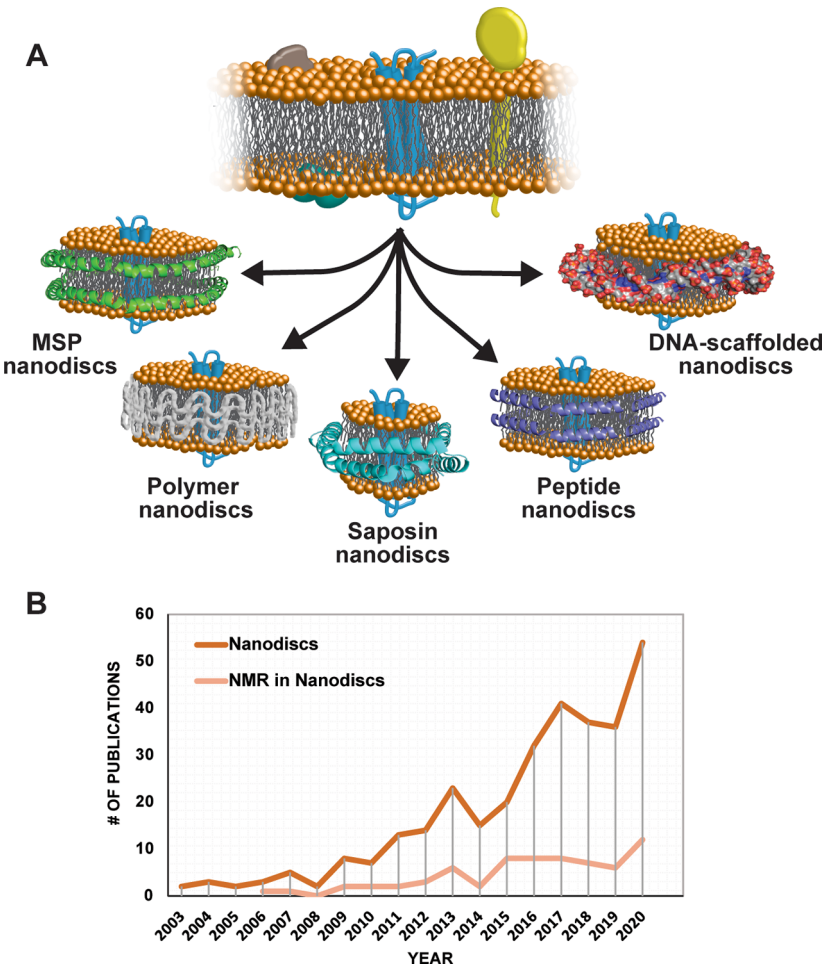

Figure 1. Commonly used lipid nanodisc systems for NMR studies. (A) Lipid-based nanodisc systems can be used to mimic a native membrane environment for NMR structural studies. (B) Number of publications on the search terms "membrane protein" and "nanodiscs" with or without "NMR".

increasing popularity for the structural and functional investigation of MPs in the past almost two decades, as evident from the rising number of publications per year on this topic (Figure 1b). In the next section, we will provide a compilation of all currently available lipid nanodisc systems and briefly summarize their use in NMR.

2.1. Membrane Scaffold Protein Nanodiscs

MSP nanodiscs were designed based on apolipoproteinA-I (ApoA-I), which is the main protein component of human high-density lipid (HDL) particles required for the transport of lipids and cholesterol in the bloodstream.[125,126] MSP proteins fold into amphipathic α-helical structures, and two copies of the MSP protein wrap around the discoidal lipid-bilayer patch in an antiparallel orientation, stabilizing the hydrophobic rim of the membrane (Figure 2A). The diameter of MSP nanodiscs depends on the length of the MSP protein construct used for assembly. The initial MSP1 construct was designed by removal of the N-terminal globular domain (the first 43 residues) of ApoA-I. MSP1 comprised 10 amphipathic α-helical segments, generating nanodiscs of ∼10 nm in diameter.[127] A further removal of the next 20 N-terminal amino acids resulted in the generation of the most commonly used MSP construct, MSP1D1. The size of MSP1D1 nanodiscs was very similar to that of MSP1 discs, suggesting that the deleted peptide stretch is not involved in lipid binding and nanodisc formation.[90] Engineering of MSP1D1 by internal deletions[78] or insertions[90] of helical segments and N-[90] or C-terminal truncations[128] allowed the design of nanodiscs with various diameters commonly ranging from 6.3 to 12.8 nm. A schematic representation of MSP proteins for the assembly of nanodisc of small to medium size is shown in Figure 2B.

As mentioned earlier, MSP1D1 assembles into nanodiscs that are 10 nm in diameter, corresponding to a molecular mass of ∼150 kDa, representing a considerable challenge for NMR studies. Thus, to enable NMR structural studies of MPs in nanodiscs, the use of smaller particles is crucial. Among a larger set of truncated MSP variants, deletions of helices 4 and 5 (MSP1D1ΔH4−5) or H4 or H5 alone (MSP1D1ΔH4 and MSP1D1ΔH5) resulted in stable nanodiscs with diameters of about 7.1 and 8.4 nm (66 and 95 kDa mass), respectively.[78] MSP1D1ΔH5 was successfully used for the NMR structure determination of the MSP protein in empty nanodiscs,[129] the bacterial outer-membrane protein OmpX,[78,79] and the outer-membrane protein Ail of the plague-causing pathogen *Y. pestis*.[83,130,131] Among the C-terminally truncated constructs, D7MSP1D1, a truncated MSP1D1 variant lacking the helices after helix 7, formed nanodiscs that are 7 nm in diameter. However, further truncations resulted in larger partticles, possibly due to the formation of nanodiscs consisting of more than two MSP copies.[128]

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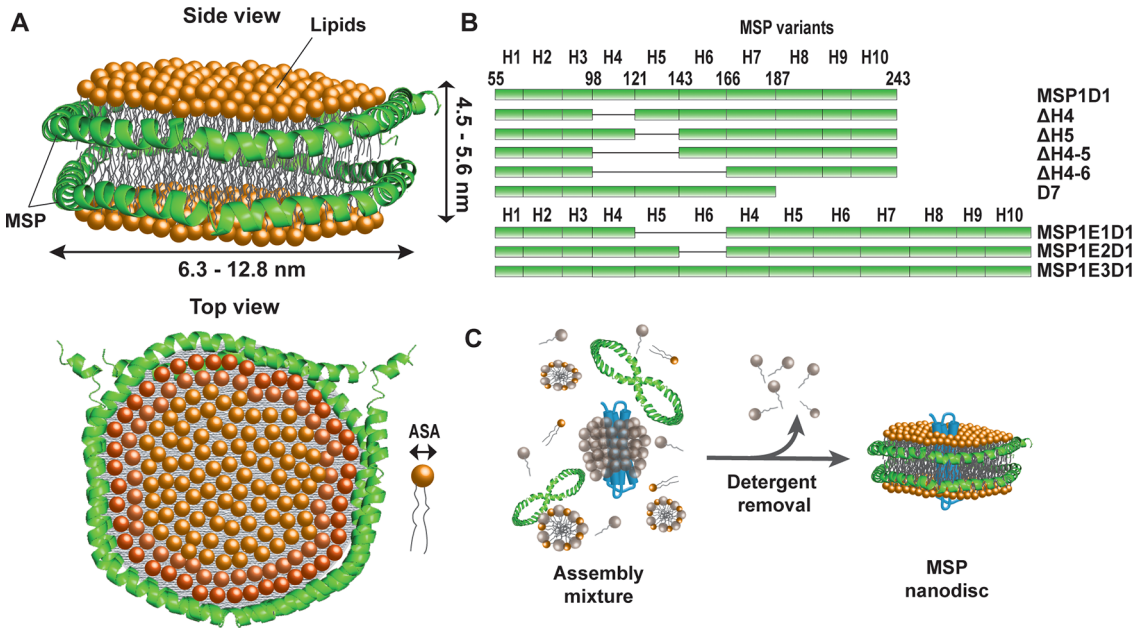

Figure 2. MSP lipid nanodiscs. (A) Structure of MSP lipid nanodiscs with two copies of the MSP (green) surrounding a patch of lipid-bilayer membrane of tunable size (orange). (B) Available MSP constructs that have been used for solution-NMR studies. In particular, the truncated versions are attractive for NMR due to their smaller size. (C) Nanodisc assembly is typically done with a mixture of detergent, lipids, and the MSP and the to-be-inserted MP, followed by removal of the detergent, which initiates the self-assembly process.

More recently, the introduction of covalently circularized MSP versions by Nasr et al.[132] using a SortaseA-mediated protein-ligation approach resulted in more stable and homogeneous nanodiscs with defined sizes. This was enabled by the formation of a covalent bond between the two termini of the MSP protein. In this study, MSP circularization was achieved by the addition of a SortaseA-recognition sequence (LPXTG) to the C-terminus of the MSP and the exposure of at least one glycine residue at the N-terminus, which could be done by treatment with TEV protease.[133] Because the yield of circularized MSP strongly depends on the completeness of the ligation reaction as well as the amount of unwanted oligomeric MSP products, this step could be further optimized by the addition of detergents to the ligation reaction[134] or by using engineered and more-soluble MSP variants.[135] Recently, it was shown that the efficiency of the SortaseA-dependent ligation method can be increased by using SortaseA variants with enhanced activities.[136] In a complementary approach, MSP circularization could be achieved in living *E. coli* cells using a split-intein-based method,[67] where DnaE C- and N-split inteins from *Nostoc punctiforme*[137] were fused to the N- and C-termini of the MSP construct, respectively. This method enabled the direct purification of circularized MSPs from *E. coli* culture at high yields without the need for a downstream enzymatic ligation step. Even very short MSP variants (MSP1D1ΔH4−5 and ΔH4−6) that are suitable for NMR could be obtained in a circularized form. MSP variants and their diameters, which are suitable for solution NMR, are listed in Table 2.

MSP nanodiscs are formed via a self-assembly process by the gradual removal of detergents from a solubilized mixture of the MSP variant, phospholipids, and the MP of interest using hydrophobic adsorbent media, such as Biobeads SM-2 and Amberlite XAD-2, or by dialysis.[81,117,138] Alternatively, nanodisc reconstitution can be directly initiated by washing off the detergent while the MP is still bound to an affinity resin.[128]

Table 2. Suitable MSP Variants for Solution-NMR Measurements and Their Diameters

construct	nanodisc diameter (nm)	ref
MSP1D1 (D10)	9.7	90
MSP1D1ΔH4	8.2	78
MSP1D1ΔH5	8.4	78
MSP1D1ΔH4−5	7.1	78
MSP1D1ΔH4−6	6.3	78
MSP1D1ΔH8−10 (D7)	7	128
cMSP1D1 (cNW11)	11	67, 132
cMSPΔH4−6	7.6	67
cMSPΔH4−5	8.7	67
cMSPΔH5 (cNW9)	8.5	67, 132
MSP1E1D1	10.5	90
MSP1E2D1	11.3	90
MSP1E3D1	12.8	90

The self-assembly process is thermodynamically reversible and starts when the detergent concentration drops below the CMC. Further reduction in the amount of the detergent results in the coalescence of MSP, lipids, and the MP and ultimately in the formation of nanodisc particles.[139] If required, additives such as specific substrates, metal ions, or cosolvents can be supplemented to the assembly mixture at amounts that are compatible with the assembly reaction.[52] The stoichiometry between the MSP and the lipids is defined by the size of the final nanodisc and the accessible surface area (ASA) of the used lipids and can be reliably estimated using geometric considerations to obtain monodisperse nanodisc populations.[68,90] While too few lipids cause deformations in the nanodisc structure,[140−142] an excess of lipids results in larger particles with a broad size distribution[90] or even lipid aggregates. In addition, the assembly conditions need to be adapted for each MP of interest, in particular considering the size and oligomeric state of the MP. When reconstituting MPs,

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the amount of lipid molecules that will be replaced by the MP must be subtracted from the amount of lipid molecules required to assemble nanodiscs without the MP. In this case, the surface areas of the lipid molecules and the transmembrane part of the MP from a known structure or a prediction based on the number of transmembrane segments can be considered a good starting point for further optimization.

Furthermore, the lipid composition should be adapted to the specific MP of interest. Most commonly, lipids with a phosphatidylcholine (PC) headgroup, which constitute the major portion of the plasma membrane, are used for nanodisc preparations. However, some MPs might require anionic lipids such as phosphatidylglycerols (PG), phosphatidylethanolamines (PE), and phosphatidylserines (PS).[143] Commonly used phospholipids for nanodisc preparation, their phase-transition temperatures, and their surface areas are shown in Table 3. In addition to the use of pure lipids and their defined

Table 3. Commonly Used Lipids for Nanodisc Preparations, Their Phase-Transition Temperatures (T_m), and Their Surface Areas (SA)

lipid	T_m (°C)[68,90]	SA (Å^2)
DLPC	−2	63.2[155]
DMPC	24	57[156]
DOPC	−17	72.4[157]
DPPC	41	52[156]
POPC	−2	69[156]
DLPG	−3	65.6[158]
DMPG	23	65.1[158]
DOPG	−18	70.8[158]
POPG	−2	66.1[158]
DOPE	−16	60[159]
POPE	25	56.6[159]
DMPS	35	40.8[160]
DOPS	−11	64.1[160]
POPS	14	62.7[161]
DOPA	−4	53[162]

mixtures, commercially available natural lipid blends extracted from different sources such as *E. coli*, chicken egg, yeast, soybean, and brain can be used for nanodisc assembly.[144−148] Alternatively, assembly of nanodiscs is also possible directly by addition of an MSP protein to detergent-solubilized membranes of cells of the heterologous expression systems or native cells or tissues.[149−153] Even though this method reduces the time of exposure to detergents, it requires a high amount of MSP protein.[143] In addition to the possibility to incorporate defined lipids, other important components of a biological membrane, such as cholesterol, can be added.[138] Among other MPs in the cell membrane, G-protein-coupled receptors (GPCRs) have been shown to be more stable in the presence of cholesterol.[154]

The possibility to include lipids with various different headgroups and acyl-chain lengths allows for the optimization of the lipid-bilayer properties for any MP of interest. Some MPs might require the presence of anionic lipids for their stability and function.[143,163] In addition to the lipid headgroups, the thickness of the lipid bilayer can be adjusted by choosing lipids bearing longer or shorter acyl-chain lengths. This aspect is highly relevant because hydrophobic mismatch between the lipid-bilayer thickness and the hydrophobic region of the MP may cause defects in the nanodisc assembly[27] and might affect the functionality of the MP. In extreme mismatch conditions, exclusion of MPs from the bilayer[164] or nonbilayer assemblies by the lipids[165,166] may occur. As a recent example, the activity of the membrane-bound protease, FtsH, has been shown to be strongly influenced by the lipid-bilayer thickness in nanodiscs.[167]

Lipid molecules in nanodiscs show similar, but slightly higher, phase transitions as compared to pure lipid systems.[168,169] To allow for efficient mixing of the components in the nanodisc-assembly mixture, the temperature must be kept above the liquid-crystalline phase-transition temperature.[43,138] Most likely, the minimization of lamellar lipid-phase interactions at this temperature facilitates the homogeneous formation of lipid−detergent micelles and the efficient incorporation of the lipids into the nanodisc during the self-assembly reaction. In line with this assumption, the formation of a lamellar phase was associated with poor nanodisc-assembly yields and the formation of lipid-less particles.[117] Interestingly, the biophysical properties of lipid molecules are not homogeneous within a nanodisc particle but depend on the position in the bilayer. Lipids in the central region of the nanodisc are less dynamic compared to the peripheral MSP-bound lipids and even form a thicker bilayer region.[170−172] Because of the presence of a lower number of lipids in small nanodiscs, these effects are more pronounced than in larger nanodiscs, where MSP-binding effects are weaker.[171] Recently, MSP nanodiscs were shown to exchange monomeric lipid molecules between different particles, and the observed exchange rate was reduced if supercharged MSP variants were used, possibly due to enhanced repulsion between the nanodisc particles or increased electrostatic interactions between the MSP belt and the lipid headgroups.[173] These engineered MSP variants harbor a larger degree of charges at positions oriented toward the solvent, leading to favorable features such as increased stability and decreased aggregation and fusion tendencies.[135,173]

In summary, if MSP nanodiscs are used as a membrane-mimetic system for an MP of interest, essential parameters such as MSP construct, amount and type of lipid molecules, specific supplements required for the MP of interest, and assembly conditions need to be carefully optimized. With this, a native-like lipid-membrane environment for MPs can be established, leading to marked improvements in MP thermodynamics and long-term stability. Furthermore, the native-like environment enables the MP to adopt a wider and more realistic conformational space, which is important for executing functional (allosteric) structural changes upon binding to ligands or partner proteins.

2.2. Polymer Nanodiscs

In addition to protein-based nanodisc-membrane scaffolds, amphipathic styrene−maleic acid (SMA) copolymers have been described to form discoidal lipid particles, also called SMALPs or Lipodisq.[174,175] Remarkably, these polymers have the ability to solubilize membranes efficiently and thus directly extract MPs into SMALPs together with the bound lipid molecules[174−178] from cellular membrane fractions. Moreover, the bilayer patch within SMALPs is reported to retain the composition and physical properties of the parent membranes, and the solubilization yield is independent of the lipid content of the membranes.[179,180] Because detergent solubilization is not required for the formation of SMALPs, unwanted effects of detergents on the MP structure can be avoided.[181] Hydro-

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phobicity and solubilization properties of SMA copolymers can be adjusted by changing the ratio of styrene to maleic acid during the synthesis of the polymers.[182] Typically, a styrene-to-maleic acid ratio is chosen between 2:1 and 3:1.[183] The solubilization kinetics also depends on the polymer chain length, where shorter polymers appear to solubilize faster than the longer ones.[184] Similar to other nanodisc systems, SMA polymers form lipid particles by wrapping around the hydrophobic rim of the lipid bilayer, stabilizing it via the hydrophobic properties of the aromatic styrene phenyl rings, while the negatively charged maleic acid moiety mediates the water solubility of these nanodiscs.[180]

Even though SMA copolymers offer impressive advantages, they also have limitations to be considered. Because of the protonation of the maleic acid carboxylate moiety, classical SMA polymers are not soluble at acidic pH.[179] Therefore, experiments need to be performed above pH 6.5, preferably above 7.0. Related to this issue, the maleic acid carboxylate moieties are good metal ion chelators, leading to incompatibility with the presence of divalent metal ions, such as Mg^{2+} and Ca^{2+}. Chemical-modification efforts of the maleimide carboxylate have addressed this limitation, resulting in various SMA derivatives. Zwitterionic SMA (zSMA), positively charged SMA quaternary ammonium (SMA-QA), DIBMA (diisobutylene/maleic acid), polystyrene-*co*-maleimide (SMI), SMA-ethanolamine (SMA-EA), SMA-ethylenediamine (SMA-ED), and the covalently modifiable cysteamine derivative (SMA-SH) show better solubilities at lower pH and are compatible with divalent metal ions.[119,124,185−189] DIBMA, in which polystyrene is replaced by aliphatic diisobuthylene units, offers the advantage that the polymer does not adsorb UV light, which facilitates the UV quantification of the inserted MP.[188] More recently, a nonionic, inulin-based polysaccharide, which is stable at a wide range of pH values and compatible with the presence of divalent metal ions, was reported to form nanodiscs and also did not form electrostatic interactions with the inserted MP.[190] However, these synthetic copolymers, like any other polymer, are polydisperse. Polydispersity of polymers is defined by the polydispersity index (PDI), which is ∼2.5 for SMA, which indicates that SMA polymers have a quite broad distribution of polymer chain lengths with the difference between the longest and the shortest polymers being >1 order of magnitude.[178] In contrast to the well-understood size of the monodisperse MSP nanodiscs, the diameter of SMALPs is less clear. Likely parameters are the MP content[179] and the ratio of the SMA polymer to lipids.[185,191]

Taken together, SMALPs provide great advantages for the handling and structural biology applications of MPs at more native conditions because no detergent is required for MP extraction.[192] Especially the increasing number of reports where SMALPs have been used for cryo-EM studies shows their convenience for structural studies following direct solubilization from different membrane sources such as bacteria, yeast, insect, and mammalian cells.[193−202] Although SMALPs have been shown to be suitable for solid-state NMR[203,204] as well, the successful application of solution-state NMR with an integral MP in polymer nanodiscs seems to be limited, most likely by their size inhomogeneity.[43] In addition to their direct usage as a membrane mimetic, SMA polymers have been reported to be suitable as a carrier system for subsequent lipid cubic-phase crystallization and X-ray diffraction of MPs.[205]

2.3. Saposin Nanodiscs

Another protein-based system to generate nanodiscs utilizes the lipid-binding properties of the saposin family proteins (saposins A−D). Saposins are small, lipid-binding proteins involved in lysosomal-degradation pathways of sphingolipids.[206] They are composed of four amphipathic α-helical segments in which helices 1, 4 and 2, 3 are linked via disulfide bridges forming a V-shaped hairpin structure, as evident from the crystal structures of individual saposins.[207−210] Although different saposins display different affinities for different lipids, SapA seems to be less restrictive compared to the other members. This feature makes SapA a suitable candidate for nanodisc assembly using a wide range of lipids.[211] In the absence of lipids at neutral pH, the hydrophobic surfaces of the helices in SapA collapse into a hydrophobic core forming the closed conformation. The addition of lipids or detergents at low pH results in the open conformation, enabling the formation of discoidal lipid particles.[210] However, stable SapA nanodiscs also can be formed by the gradual removal of detergents at neutral pH.[212−214] In contrast to MSP nanodiscs, one SapA monomer is sufficient to cover the entire hydrophobic thickness of the lipid bilayer. The hydrophobic rim of a lipid nanodisc is saturated by multiple SapA monomers that are arranged in a head-to-tail orientation. At least two SapA molecules are required to form the smallest version of a saposin nanodisc. The size of these nanodiscs can be tuned by changing the molar ratio of lipids to SapA, leading to larger discs composed of three or more monomers.[211,215]

The increasing number of successful applications of saposin nanodiscs in cryo-EM[213,216−219] shows its suitability for the structural analysis of MPs in addition to biophysical and biochemical applications.[211,220−222] However, the applications in solution-state NMR are very sparse with only one available study where a 2D-TROSY spectrum of ^{15}N-labeled OmpX could be obtained in SapA nanodiscs containing 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) lipids.[212] However, the quality of the OmpX 2D-NMR spectrum is markedly lower than the one in MSP nanodiscs.[78] This scenario might be caused by the rather low lipid content in SapA nanodiscs, as shown in a recent cryo-EM structure,[213] which could also lead to unwanted protein−protein interactions between SapA and the incorporated MP.[81] Additionally, due to the absence of a continuous belt around the particle, SapA nanodiscs are likely to offer a less-stringent control of MP oligomerization.

2.4. Peptide Nanodiscs

Small amphipathic peptides, most commonly the ApoA-I-derived α-helical peptide 18A (Ac-DWLKAFYDKVAEKL-KEAF-NH_2), have been shown to form discoidal lipid particles[223] similar to MSP nanodiscs. While one side of the peptide is mainly composed of hydrophobic residues for stabilization of the hydrophobic rim of the bilayer, charged residues dominate the other side of the peptide with positively charged residues at the polar/nonpolar boundary and negatively charged residues toward the center.[224] Positively charged residues interact with negatively charged lipid headgroups, stabilizing the rim of the discoidal particles. This property allows these peptides to penetrate into the bilayer, solubilizing membranes directly without the need for detergents.[225] At the rim of the bilayer, two peptides oriented in an antiparallel manner are required to provide a hydrophobic match, as is the case for MSP nanodiscs.[224,226,227]

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Similar to polymer and saposin nanodiscs, the size of nanodiscs can be adjusted by changing the molar ratio of lipid to peptide.[228,229] However, the length of the peptide, as well as its bendability for longer peptides, seems to be an important factor for the adjustability of the lipid-to-peptide ratio.[230] Because they do not form a continuous belt around the lipid bilayer, peptide nanodiscs are polydisperse,[230,231] dynamic, and relatively instable. Exchange of lipids and cargo between nanodiscs can take place during collisions.[223] However, it has been shown that stability problems can be resolved by the use of chemically modified bifunctional peptides that can covalently self-polymerize after nanodisc assembly.[229] Recently, it was reported that peptide nanodiscs change their biophysical properties and increase their size above the phase-transition temperature of the lipids due to translocation of the peptides into the liquid-crystalline bilayer phase of the membrane.[232]

Because these peptides have the ability to solubilize membranes, they can replace detergents as an intermediate platform to extract MPs for subsequent incorporation into MSP nanodiscs.[231] Similarly, they can be used to profile the MP interactome in a more native-like environment without the need for detergents.[233] By using peptides for MP extraction, unwanted effects of detergents are avoided even though the requirement for custom peptide synthesis is rather costly. Interestingly, it has been shown that peptide nanodiscs ∼30 nm in diameter (termed "macrodiscs") align with the magnetic field and can serve as an alignment medium for the measurement of residual dipolar couplings (RDCs) (see section 3.1) of MPs in the absence of detergents.[228]

2.5. DNA-Scaffolded Nanodiscs

The concept of generating nanoscale molecular shapes using DNA, also called "DNA origami",[234] has been known for about 15 years. The high specificity of the base pairing in DNA allows the design of specific 2D and 3D shapes.[234,235] With the same concept, nanodisc scaffolds made of DNA can be constructed. In 2018, two different approaches were published for making nanodiscs using DNA strands. In one approach, a DNA barrel scaffold was hybridized to noncircularized MSPs to generate larger discs up to ∼70 nm diameter via lipid supplementation.[236] The DNA scaffold ensured positioning of protein-based MSP scaffolds rather than stabilizing the lipid bilayer directly. However, because these scaffolds were designed to generate very large nanodiscs, which are convenient to study larger assemblies such as MP oligomers and early stages of viral entry, they are not compatible with solution-state NMR and, thus, not in the scope of this Review. Thus, the interested reader is referred to recent reviews.[121,237]

The other approach is based on a more direct method, which requires selective alkylation of DNA, also called DNA-encircled lipid bilayers (DEBs).[238] Unlike the latter approach, the hydrophobic rim of the bilayer is stabilized directly by alkylated DNA without the need for a protein scaffold. The size of DEBs is strictly dependent on the length of the DNA strand. A 147-nucleotide-long DNA circle results in nanodiscs of ∼14.7 nm in diameter.[238] A recent molecular dynamics simulation study suggests that covering the inner surface of the DNA scaffold with a high number of shorter alkyl chains will provide better bilayer properties compared to longer but fewer alkyl chains.[239] Additionally, neutralization of charges on the DNA scaffold seems to be an important improvement for these nanodiscs,[239] probably due to electrostatic repulsion between the lipid headgroups and the backbone of the DNA. Supplementation with divalent cations might be an alternative solution for this.[240] Despite the absence of reports using this method in the structural analysis of MPs so far, the presence of DNA as a scaffold material might be advantageous for structural applications due to their high homogeneity and the wide range of design opportunities as well as possible chemical modifications.

2.6. Comparison of the Currently Used Nanodisc Systems

For solution-NMR studies, the most important requirements for the lipid nanodisc system are (long-term) stability, homogeneity, and an as-small-as-possible size, while allowing for some layers of lipids between the scaffold and the MP. A particular MP can be already markedly stabilized by a suitable membrane environment containing a realistic lipid composition, regardless of the scaffold material. However, the size and homogeneity of nanodisc samples can be controlled directly by the choice of scaffold material. Interestingly, the available successful applications of nanodiscs for solution NMR show that there is a predominant preference for MSP nanodiscs over the alternative scaffolds mentioned in section 2. Considering the properties of all of these systems, this preference is quite intriguing and needs to be discussed.

MSP nanodiscs are highly versatile regarding the incorporated lipid composition. Compared to other systems, MSP nanodiscs are more stable and homogeneous and allow for a rather strict control of the nanodisc size simply governed by the length of the used MSP proteins. The continuous belt around the lipid bilayer restricts the rate of lipid and MP exchange between individual nanodiscs. This feature is even more pronounced in the circular versions of the MSPs. Furthermore, the oligomeric state of an MP can be controlled by the use of MSP nanodiscs of defined and stable sizes.[67,132] Thus, due to all of these properties, MSPs can be used to assemble homogeneous, stable, and size-variable nanodisc particles for long-term NMR experiments at elevated temperatures.

Polymer, saposin, and peptide nanodiscs are less rigid, but the size of the nanodisc particles is less homogeneous.[211,215,230,231,241] This is caused by a discontinuous saposin or peptide belt around the lipid nanodisc or by the polydispersity of the used polymers. Although the average size of these nanodiscs can be adjusted by changing the molar ratio of scaffold material and lipid molecules, the size control is less well understood than with MSP nanodiscs. Moreover, the rate of molecular exchange between individual nanodisc particles is higher, mediated by monomer-diffusion exchange and/or fast collisional transfer.[223,242] Other points to consider are the more difficult production and handling properties of SapA proteins and the relatively low stability of peptide nanodiscs. Thus, the resulting inhomogeneity and stability issues are most likely the main limitations for these nanodisc systems to be more widely used for high-resolution NMR spectroscopy.

DEBs[238] might be a good alternative to MSP scaffolds. Similarly, the continuous belt around the lipid bilayer with a defined length of circular DNA resembles MSP nanodiscs in terms of stability and homogeneity. However, despite the vast design opportunities enabled by using DNA, this technology requires the chemical synthesis of the DNA scaffold as well as its alkylation, which is more expensive and time-consuming as compared to other nanodisc systems, especially considering the

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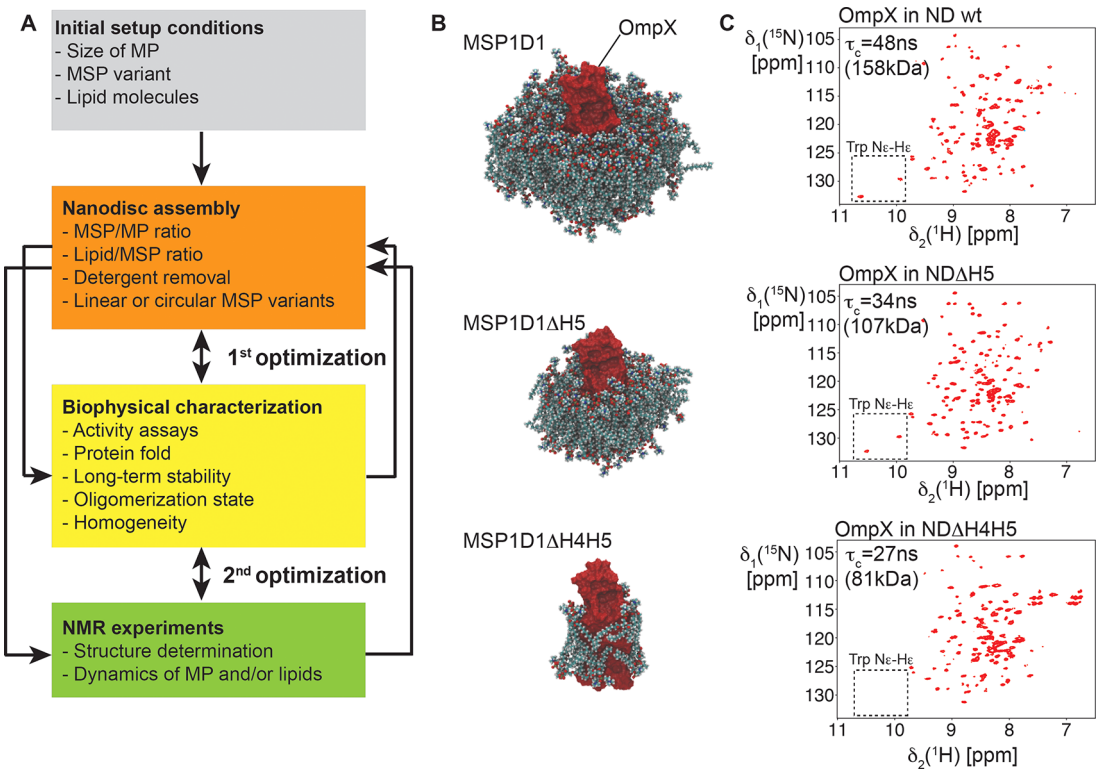

Figure 3. Workflow for the optimization of nanodisc assembly and effect of nanodisc size on NMR spectral quality. (b, c) Reproduced with permission from ref 78. Copyright 2013 American Chemical Society.

high amount of sample material that is required for NMR spectroscopy.

Currently, MSP nanodiscs can be considered the most widely used system for biochemical, biophysical, and structural studies by NMR and electron microscopy, which is supported by a large body of publications involving MSP nanodiscs. Therefore, this Review is focused on this particular nanodisc system with a strong focus on high-resolution NMR studies in solution.

2.7. Practical Considerations of MSP Nanodisc Assembly for NMR

Important parameters with MSP nanodiscs for NMR structural investigations are their size homogeneity, structural integrity, and long-term stability. As mentioned earlier, the size homogeneity and stability can be heavily improved by the use of circularized MSP nanodiscs.[67,132,135] In particular, the very small linear MSP nanodiscs (<8 nm in diameter) tend to disassemble over time and form larger particles.[78] This problem has been addressed by the use of small circularized MSP nanodiscs.[67]

However, even without the use of circularization, most linear MSPs form homogeneous nanodiscs if careful optimization of the assembly conditions is performed. For empty nanodiscs, this mostly requires the optimization of the MSP-to-lipid ratio, taking into account the diameter of the nanodisc (Table 2) as well as the surface area of the used lipids (Table 3). This situation becomes a bit more complicated if an MP needs to be inserted, requiring a more systematic screening procedure as outlined in Figure 3a.

As a first step, the approximate size of the folded MP needs to be estimated. This is a rather trivial task if the structure of the MP is already known. If this is not the case, homology modeling and secondary structure prediction can be used instead. For example, a single transmembrane helix (TMH) has a cross section of ∼6−10 Å, occupying a surface area of ∼80 Å^2, whereas a 6-TMH protein would take up ∼500 Å^2. Ideally, the outer surface of the MP should be surrounded by at least two layers of lipids, resulting in a fairly good estimation of the smallest-possible inner diameter of the assembled nanodisc that can be selected for further trials. At this step, the type of lipids that promote the structural integrity or activity of the MP needs to be considered, too. Lipid blends can be used for nanodisc assembly, but the heterogeneity of the acyl chains should be kept at a minimum because this could lead to reduced NMR spectral quality.

The next step in this procedure is the optimization of the molar ratios of lipid-to-MSP and MSP-to-MP. Because both ratios affect each other, both values need to be considered simultaneously. For an MP that tends to form unspecific dimers or oligomers, a high ratio of MSP-to-MP is recommended because the resulting excess of empty nanodiscs will facilitate the incorporation of a monomer. Furthermore, the lipid-to-MSP ratio needs to be adjusted from the values for empty nanodiscs because the MP occupies considerable space in the nanodisc. However, this adjustment becomes less important if the ratio between MSP and MP is high because only a small fraction of loaded nanodiscs is present in this scenario. Smaller nanodiscs are generally more selective for a monomer because there is simply no space for accommodating the dimeric species. This selectivity can be further enhanced by using covalently circularized MSP nanodiscs, due to their more stable size.[67,132] In addition to the mentioned ratios of nanodisc components, the speed and method for detergent removal also play an important role for successful nanodisc formation. Typically, hydrophobic beads are used for this purpose because this is the most straightforward method.

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However, in some cases, small MPs or TMHs interact with the beads, leading to a strong reduction in the nanodisc-assembly yield. In these cases, or if detergent removal has to take place relatively slowly, dialysis of the assembly mixture is recommended. Finally, if a suitable nanodisc stability or homogeneity cannot be achieved with linear MSP nanodiscs, the use of the circularized counterparts might be beneficial. Using the VDAC1 anion channel, small circularized MSP nanodiscs, and suitable assembly conditions, it has been shown that homogeneous oligomeric states, which could be analyzed by NMR, can be selected.[132]

In parallel to the mentioned optimization procedure, the outcome of the nanodisc assembly, as well as the structural and functional state of the MP, has to be determined by biophysical and biochemical experiments. This is typically done by size-exclusion chromatography, where a single symmetric peak would indicate successful assembly. Ideally, this step should be followed by a biochemical-activity assay with the nanodisc-incorporated MP. However, in many cases, such assays are not available, rendering necessary biophysical experiments on the folding state of the protein, which can be done by thermal melting experiments by, e.g., circular dichroism spectroscopy, fluorescence spectroscopy, or differential scanning calorimetry. In these experiments, a high thermal melting point, as well as a cooperative unfolding transition, is desirable. The combination of these parameters indicates a stable and compactly folded MP structure. Long-term stability that is required for subsequent NMR experiments at high particle concentrations of >200 μM can be assayed by incubation at the required temperature followed by repeated thermal melting experiments and/or size-exclusion chromatography runs. Finally, the oligomeric state of the MP in the nanodisc preparation needs to be monitored in order to optimize the assembly conditions to obtain a sample with a homogeneous oligomeric state. This can be done by multi-angle light scattering (MALS) and mass spectrometric or chemical cross-linking protocols.[243−245] More recently, fusion protein strategies were used to determine the number of MPs in nanodiscs, ranging from larger integral MPs to single TMHs.[246−248]

Once a stable and homogeneous nanodisc sample is at hand, the final validation step is to use an isotope-labeled (typically *U*-^{2}H,^{15}N) MP for the assembly reaction and read out the spectral quality and rotational correlation time (τc) at different temperatures or in suitable nanodiscs of different sizes by 2D-[^{1}H,^{15}N]-TROSY[249] and 1D-[^{1}H,^{15}N]-TRACT[250] experiments, respectively. In general, the NMR spectral quality improves with temperature, unless the stability of the nanodisc or MP is not sufficiently high. Furthermore, the size of the nanodisc should be chosen to be as small as possible. Thus, the spectral quality of the 2D-NMR spectrum of a given MP needs to be assessed in different nanodiscs (Figure 3b and c).[78] Ideally, the spectral quality, as well as the rotational correlation time, improves with smaller nanodisc size. However, in cases where the nanodisc is too small to accommodate several layers of lipids between the MP and the MSP, the transient contact between the MP and the MSP leads to reduced spectral quality and in particular to the disappearance of tryptophane side-chain resonances (Figure 3c). In the case of the bacterial outer-membrane protein X (OmpX), a nanodisc size of 8 nm (with MSP1D1ΔH5) was found to be optimal in terms of spectral quality and long-term stability.[78] In the next sections, we will provide an overview of solution-NMR studies of MPs in MSP nanodiscs and highlight the broad and versatile applications that become possible with this type of nanodisc, and in particular with smaller nanodiscs.

3. SOLUTION-NMR STUDIES OF MPS IN NANODISCS

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique to obtain structural and dynamical insights on biomolecules at atomic resolution.[251−253] In contrast to other structural techniques, solution-state NMR can probe structural and dynamical features of biomolecules in a native soluble form.[251−253] This also holds true for MPs, where the properties and fluidity of the membrane environment are important factors for functionality.[46,54,63,241,254] However, due to the inherent physical limitations of the method, structure determination by solution-state NMR is currently limited to an approximate size range of 40−50 kDa.[255−259] Nonetheless, it has been shown that solution-state NMR can be used to probe dynamics and conformational features of large (membrane) proteins[241] up to 100 kDa to 1 MDa in size, utilizing specific methyl labeling of the proteins.[260,261] With such a strategy, NMR can be very efficiently used in a complementary manner in concert with X-ray crystallography and cryo-EM. The specialty of NMR is the structure determination of small and dynamic MPs, probing the dynamical features of MPs and monitoring the structural and dynamical effects of ligand binding. In the latter two cases, high-resolution structural information is often already available and beneficial for data analysis.

3.1. Isotope-Labeling Schemes to Overcome the Size Limitation of NMR

The molecular weight of the protein system under investigation is a critical parameter for solution-state NMR. The generally slow rotational-tumbling properties of MPs and the resulting faster transverse (T_2) relaxation rates lead to marked line broadening and consequently to poor spectral resolution in combination with an increased number of overlapping signals. Isotope labeling of proteins with ^{13}C and ^{15}N in combination with high-level deuteration (^{2}H)[262] can remedy this situation together with more sensitive NMR instruments operating at high magnetic fields, cryogenic probes, and advanced pulse sequences, such as transverse relaxation optimized spectrometry (TROSY).[249,252,263,264] TROSY experiments benefit from the cancellation between dipolar coupling and chemical-shift anisotropy-induced transverse relaxation pathways, leading to sharper NMR line widths, especially at high magnetic fields.[249,265−267] In addition, the sensitivity and resolution of multidimensional NMR experiments can be further improved by nonuniform sampling (NUS) strategies.[268−273]

Because a membrane mimetic is required for MPs, the apparent size of the MP particle is further increased, depending on the dimensions of the chosen membrane system. An easy means to increase the rotational-tumbling rate and improve the spectral quality is to acquire NMR spectra at elevated temperatures.[255,274] However, this strategy is only possible if the inherent MP stability is high enough—a parameter that can be significantly supported by choosing an appropriate membrane mimetic, such as a lipid nanodisc that supports long-term stability at elevated temperatures.

Despite massive improvements in all technical aspects of the NMR spectrometer setup as well as advanced pulse programs in the last few decades, the most noticeable enhancement in the NMR spectral quality of large protein systems can be

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achieved by applying advanced isotope-labeling methods of the protein sample. The most suitable isotope-labeling scheme for large proteins is selective methyl-group labeling in a perdeuterated background, pioneered by the lab of Lewis Kay.[261,275,276] The free rotation of methyl groups around their symmetry axis provides intense peaks even if the overall tumbling rates are slower for the overall system.[275,277] Thus, protonated and ^{13}C-labeled methyl groups can serve as excellent probes to detect conformational changes using NMR chemical-shift information as well as for extracting dynamical features of proteins that are well above 100 kDa in size. Because perdeuteration is required for NMR studies of large proteins,[262] the selective introduction of proton-containing methyl sites is particularly essential for the NMR-based structure determination of α-helical MPs, where side-chain contacts define the tertiary structure. The six naturally occurring methyl-bearing amino acids (Ala, Leu, Val, Ile, Thr, and Met) account for up to 45% of the amino acids in the TMHs of MPs,[278] providing a multitude of distance information in multidimensional nuclear Overhauser effect spectroscopy (NOESY) spectra if present in an NMR-visible form. Methyl labeling can be done in an amino-acid-specific manner, using specifically isotope-labeled amino acid precursors for the labeling of leucine-$\delta_{1/2}$, isoleucine-δ_1, and valine-$\gamma_{1/2}$ methyl groups, either in a nonspecific,[279−281] stereospecific (pro-S or pro-R),[282−285] or even stereoselective form within a single methyl group (SAIL method[286,287]). Alanine,[288,289] threonine,[290,291] and methionine[292−294] can be added to the culture medium in the desired labeling pattern. The main limitation of this high-level isotope-labeling methodology is that it can only be fully realized in bacterial hosts that tolerate 100% D_2O in the growth medium, such as *E. coli*, or in a cell-free setup.[295] Even if the NMR spectral quality can be markedly improved by these protocols, the NMR resonance assignment of the methyl signals might still be challenging because the large molecular weight of the MPs often prevents the establishment of connectivities between the side chains and the protein backbone using through-bond coherence transfer NMR experiments.[296] Nuclear Overhauser effect (NOE) measurements have turned out to be helpful for the resonance assignment of methyl groups if a structural model of the protein is available.[277,297−299] However, if only single methyl assignments need to be obtained, as is often the case for methionine residues, site-directed mutagenesis and the readout of the resulting spectral changes in a 2D-methyl TROSY experiment turned out to be the method of choice,[300,301] which has been also applied to MPs, such as GPCRs.[302,303]

An alternative strategy to obtain site-specific information at high sensitivity is ^{19}F-labeling. ^{19}F can be incorporated into protein structures either post-translationally by chemical modification of amino acid side chains such via thiol groups, ϵ-amino groups, and hydroxyl groups or by supplementing growth medium with fluorinated versions of amino acids such as Phe, Tyr, Trp, Met, and Leu.[54,304−306] Furthermore, the introduction of methyl groups by modification of cysteine sulfhydryl groups by ^{13}C-methyl methanethiosulfonate (MMTS) is another cost-efficient way to introduce methyl labels at specific positions of large soluble proteins as well as MPs without the need for expensive isotope labeling of the protein itself.[307,308] This portfolio of isotope-labeling methods provides the necessary toolset for the production of suitable MP samples for the successful conduction of high-resolution NMR experiments of MPs.

3.2. NMR Methods To Obtain Long-Range Structural Information of MPs

Because of the inherent sparsity of NOE contacts in an almost completely deuterated MP, other NMR-based distance restraints are often required to obtain high-resolution structural information of sufficient quality. Paramagnetic relaxation enhancement (PRE)[309,310] and residual dipolar couplings (RDCs)[311,312] are used for MPs to obtain additional and orthogonal structural information. Compared to NOE information, the PRE effect is rather long-range with detectable distances of up to 25 Å.[313,314] However, this method requires the introduction of a single nitroxide spin-label at defined positions in the protein, which is most commonly achieved by the production of single-cysteine variants, which can be difficult due to stability issues caused by the removal of cysteine residues in the protein. Furthermore, due to the flexibility of the attached spin-label, the error in the obtained distances is rather large.[313] In addition to direct spin-labeling of the MP, paramagnetically labeled lipids have been used to map the location of MPs in the membrane.[68,313,315−318] RDCs provide orientational information on bond vectors relative to the external magnetic field.[311,312] In solution, this effect can only be measured if the protein is partially restricted in its rotational-tumbling motion, which can be achieved by the addition of an anisotropic alignment medium.[319] Thereby, valuable information, independent of the existence of pairwise protein−proton contacts, can be obtained for improving the quality of NMR structures of MPs.[79,320] However, many commonly used alignment media cannot be used for MPs, and this situation is further complicated if detergents are used as a membrane mimetic. Alignment of MPs for the subsequent measurement of RDCs has been reported with Pf1 phages (using nanodiscs),[79,129,320] DNA nanotubes,[321,322] and bicelles[323,324] as well as with covalently attached lanthanide tags.[325−327] However, the suitability of the particular alignment medium for a given MP needs to be tested in each case. The use of lipid nanodiscs as a membrane mimetic markedly simplifies this selection process because the assembled nanodisc-MP particles behave like a soluble protein and do not heavily interfere with the alignment medium.

3.3. Membrane Protein Studies in Nanodiscs by Solution NMR

With the introduction of lipid-bilayer nanodiscs as a novel membrane mimetic, their use in structural studies of MPs by solution NMR became possible. In particular, their small and defined size rendered these particles very attractive for solution NMR with the additional benefit of providing a detergent-free planar lipid-bilayer environment.

3.3.1. Initial Low-Resolution Nanodisc-NMR Studies with Polytopic MPs. The rather small size of lipid nanodiscs as well as their good size homogeneity quickly attracted the attention of the NMR community. Since the introduction of MSP nanodiscs in 2004, a series of polytopic MPs that have been previously investigated in detergent micelles by NMR have been incorporated into nanodiscs.

Initial work on MPs in nanodiscs by solution NMR has been reported with a membrane-active peptide, antiamoebin-I. This fungus-derived antibiotic was reconstituted in high-density lipoprotein (HDL) particles (MSP lipid nanodiscs)[328] and analyzed by 2D-TROSY NMR experiments, emphasizing the

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applicability of nanodiscs for integral MPs. Shortly later, a 70-amino-acid truncated and mutated version of CD4 (CD4mut), a core T cell receptor, composed of the TMH segment and the cytoplasmic domain of CD4, showed similar 2D-^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra in MSP nanodiscs filled with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids and in DPC micelles.[329] Even though these examples report on relatively small peptides and proteins, it did not take a long time to extend this list to polytopic, integral MPs in lipid nanodiscs.

In a study from the Wagner lab, a 2D-[^{15}N,^{1}H]-TROSY spectrum of the 19-stranded β-barrel human mitochondrial voltage-dependent anion channel (VDAC1) in MSP1D1 nanodiscs containing DMPC lipids showed nicely resolved but slightly broader peaks compared to LDAO micelles[95,329] Although, due to the different environment provided by the lipid bilayer, the peak positions in nanodiscs changed enough to prevent a direct transfer of resonance assignments, the overall similar NMR spectral signature and the site-specific binding properties of NADH indicated that the protein was correctly folded. Moreover, the spectral quality[95] appeared to be promising for conducting multidimensional NMR experiments to determine the structure and dynamics of VDAC1 in this more-physiological membrane environment. A similar study was published later by the same group with human VDAC2,[330] which has 68% sequence identity to VDAC1. VDAC2 was more stable in MSP1D1 nanodiscs than in detergent micelles, allowing the acquisition of 3D-[^{1}H,^{1}H]-NOESY−^{15}N-TROSY and 3D-tr-HNCA experiments at 40 °C, demonstrating the feasibility of backbone resonance assignments in the lipid bilayer for VDAC2. However, backbone assignments for VDAC1 and VDAC2 in nanodiscs have not been obtained so far. On the other hand, a transfer of assignments for an *E. coli* outer-membrane protein, OmpA, possessing a β-barrel fold, was possible because the 2D-TROSY spectra of OmpA in MSP1D1 nanodiscs and in Fos-10 micelles were quite comparable.[331] In the case of BamA, the β-barrel subunit of the bacterial β-barrel insertase complex, 2D-[^{15}N,^{1}H]-TROSY spectra in MSP1D1 nanodiscs, LDAO micelles, and DMPC/DH$_7$PC bicelles showed similar resonance positions for the flexible loops and lid region. Importantly, the POTRA5 domain of BamA was found to be unstructured in solution in the absence of detergents, in contrast to available crystal structures.[332] For the mentioned MP systems where an in-depth NMR characterization is already available in detergent micelles, a transfer of NMR resonance assignments is clearly the most straightforward strategy to obtain resonance assignments in nanodiscs without the need for challenging multidimensional NMR experiments. A detergent-titration method using a stepwise addition of the detergent to an MP in lipid nanodiscs was shown to be useful in a few reported cases[333,334] but might not be applicable in a wider context.

Not only β-barrel but also α-helical MPs have been investigated in lipid nanodiscs. One of the first attempts to record an NMR spectrum of a multipass α-helical MP in nanodiscs was performed on ^{15}N-labeled KcsA, a tetrameric K$^+$ channel from *Streptomyces lividans*, which is composed of N- and C-terminal α-helical soluble domains connected by two TMH segments.[335] Although the large tetrameric structure of KcsA does not seem to be compatible with solution NMR, specific labeling using methyl-protonation in a deuterated background provided information on the conformational states of the C-terminal domain of KcsA in MSP1D1 nanodiscs.[336] Therein, the chosen membrane mimetic affected the conformational equilibrium of this channel, as revealed by methyl-TROSY experiments.[336,337] More recently, KcsA was reported to be found as dimers of tetramers when solubilized in polymer nanodiscs.[338] The TROSY spectrum of an α-helical voltage-sensing domain (VSD) of another K$^+$ channel, KvAP, containing four TMH segments, in DMPG MSP1D1 nanodiscs was used as a reference medium for guiding the screening procedure for suitable detergents.[339] The aim of this study was not to conduct a detailed NMR analysis in nanodiscs but rather to find a detergent that best resembles the nanodisc environment and continue with more detailed NMR studies in the identified detergent system. According to the findings of this report, zwitterionic detergents provided the most "nanodisc-like" environment for the VSD of KvAP.[339] Furthermore, 2D-[^{15}N,^{1}H]-TROSY spectra of the VSD in DPC or DPC/LDAO micelles and in DMPC-, dimyristoyl phosphatidylglycerol (DMPG)-, or POPC/1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG)-containing nanodiscs exhibited similar microsecond to millisecond interhelical motions, suggesting that these motions are an inherent property of the VSD independent of the membrane-mimetic environment.[340] The comparison of TROSY-HSQC spectra of the light-activated proton pump bacteriorhodopsin (bR) in different membrane environments revealed that MSP1D1 nanodiscs are suitable for 7-TMH MPs.[341] NMR spectra of bR displayed larger chemical-shift perturbations for the regions that are exposed to the different environments, while the residues in the hydrophobic core of the protein showed smaller shifts, indicating that the membrane-mimetic environment does not affect the overall protein fold in that case. Notably, the nanodisc environment provided the highest stability for a much longer time, allowing NMR data acquisition at 50 °C, resulting in 209 resolved peaks.[341] Likewise, MSP nanodiscs improved the stability of Barttin, an accessory subunit of CIC-K chloride channels containing two α-helical TMH segments, although LDAO micelles resulted in a better-resolved NMR spectrum, presumably due to the smaller size of the detergent micelle. Nonetheless, a final assessment of the functionally most suitable membrane environment for Barttin requires functional studies.[342] In the case of the *E. coli* MP YgaP, composed of two TMH segments and a cytoplasmic rhodanese domain, a 2D-[^{15}N,^{1}H]-TROSY spectrum of the protein in deuterated d_{54}-DMPC MSP1D1 nanodiscs resolved ∼80% of the rhodanese domain with an additional ∼40 peaks possibly belonging to the TMH segments.[343]

Of particular interest, nanodiscs have been used for the investigation of the conformational landscape of various G-protein-coupled receptors (GPCRs), a pharmacologically highly important class of MPs. Notably, the conformational equilibrium of β_2-adrenoceptor (β_2AR) in nanodiscs was found to be significantly different from the equilibrium in detergent micelles utilizing methyl-labeled methionine residues as probes in 2D methyl-TROSY NMR experiments.[344] A similar strategy was used by the same group for adenosine A_{2A} receptor (A_{2A}AR) to gain insight into the conformational dynamics of the protein in the presence of ligand molecules in MSP1E1 nanodiscs containing POPC/POPG lipids. It was found that the presence of acyl chains derived from docosahexaenoic (C_{22}) acid in a lipid bilayer affected the conformational states of the receptor, leading to enhanced G-protein binding.[345] Similarly, the effect of the cholesterol analogue cholesteryl

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hemisuccinate (CHS) on the conformational dynamics of the leukotriene B4 receptor, BLT2, was demonstrated in MSP1D1 nanodiscs.[346] More recently, the intrinsically disordered C-terminal region of β_2AR upon phosphorylation and β-arrestin binding was studied in MSP1D1 nanodiscs using NMR.[347] In this report, an isotope-labeled C-terminal peptide was produced in *E. coli* cells and then ligated to the C-terminally truncated GPCRs expressed in insect cells via intein-mediated protein trans splicing[347] instead of labeling the entire protein.

Despite the impressive advances demonstrated by these early studies of MPs in lipid nanodiscs, a high-resolution structure determination of an MP appeared out of reach for some time. The main reason for this situation was the still large particle size of the available nanodiscs that prevented the successful recording of high-quality multidimensional NMR experiments that are commonly used for backbone resonance assignment, even if cutting-edge isotope labeling and TROSY-based NMR experiments[348] were used. Consequently, this issue led to the idea of designing smaller nanodiscs for NMR.

3.3.2. Smaller Lipid Nanodiscs for High-Resolution NMR Studies of MPs. Because of the earlier-mentioned size-related limitations in solution-NMR studies of MPs, a logical next step in the optimization process of nanodiscs for solution NMR was the design of smaller nanodiscs. Efforts from different laboratories in engineering the MSP1D1 construct resulted in the generation of smaller nanodiscs either by internal deletions[78] or N- and C-terminal truncations.[90,128]

Notably, the Wagner group obtained NMR spectra of the *E. coli* outer-membrane β-barrel protein OmpX with exceptional spectral quality in MSP1D1ΔH5 (ΔH5) nanodiscs containing d_{54}-DMPC/DMPG (3:1 ratio) lipids. The deletion of helix 4 or 5 led to a stable MSP construct that assembled into nanodiscs of markedly smaller size (~50 kDa reduction in molecular weight). Furthermore, in this system, 3D-TROSY-based triple-resonance experiments were possible, which enabled an almost-complete backbone resonance assignment of OmpX. In addition, an NOE-based structure determination was possible, leading to the first high-resolution structure of an MP in lipid nanodiscs.[78] This workflow was facilitated by the use of deuterated lipids and NMR NUS setup. Later, refinement of the structure was performed for OmpX in ΔH5 nanodiscs by the use of N−H RDCs in Pf1 phage medium,[320] as well as utilizing side-chain NOE information and PRE restraints for positioning OmpX in the lipid-bilayer membrane.[79] Likewise, in another study by a different group, ΔH5 nanodiscs allowed for the acquisition of improved NMR spectra of the *Pseudomonas aeruginosa* outer-membrane proteins OprG and OprH, as compared to spectra obtained in the larger MSP1D1 nanodiscs.[80] Eventually, the authors of this study chose to use lipid bicelles (q = 0.3 DHPC/DMPC/DHPS/DMPS) for subsequent NMR studies of these two proteins. However, shortening the long and flexible loops of the β-barrel protein OprH (OprHΔL1ΔL4) led to a strong improvement of the spectral quality in lipid nanodiscs.[80] ΔH5 nanodiscs were also used for the β-barrel outer-membrane protein Ail from *Y. pestis* because the presence of detergents markedly impaired its ligand-binding activity.[130] The NMR spectrum of Ail in nanodiscs was comparable to the one in detergent micelles, but, due to the larger particle size, the peaks were broader, indicating a similar overall fold in both membrane-mimetic systems. Moreover, a backbone structure determination of Ail was possible with solution NMR in addition to binding studies with ΔH5 nanodiscs containing lipopolysaccharide (LPS),[131] in agreement with prior solid-state NMR experiments with LPS in liposomes.[83] Another smaller nanodisc, D7MSP1D1, which leads to nanodiscs of similar size as with ΔH5 nanodiscs, provided markedly improved 2D-[^{15}N,^{1}H]-TROSY spectra for the integrin αIIb fragment containing a single TMH and the cytoplasmic domain compared to the larger D13 (MSP1E3D1) nanodiscs.[128] In addition, conformational changes of the C-terminal tail of integrin β_3 in D7 nanodiscs upon phosphorylation by the kinase domain of Src kinase were studied by the same group,[81] following up previous studies in detergent micelles.[349] Lastly, 2D-[^{15}N,^{1}H]-TROSY spectra of a thermally stabilized version of human α_{1B}-adrenoceptor (α_{1B}-AR) in circularized ΔH5 nanodiscs (cNW9) containing DMPG/DMPC was of almost comparable quality as in lauryl maltose neopentyl glycol (LMNG) micelles.[350] The corresponding 2D-[^{13}C,^{1}H]-HSQC spectra of a specifically ILV methyl-labeled receptor showed quite identical quality due to the advantageous relaxation properties of methyl groups.

On the other hand, smaller nanodiscs have been shown to impact the conformational landscape of the inserted MPs, as demonstrated recently with the isotope-labeled neurotensin receptor subtype-1 (NTR1) in circularized cNW9 nanodiscs, where a higher G-protein binding affinity was observed, presumably due to a higher population of active receptor. In addition, 2D-[^{15}N,^{1}H]-TROSY NMR spectra of ^{2}H,^{15}N-labeled NTR1 in these nanodiscs were well-dispersed, and the protein was stable for 15 days at 45 °C.[77] In the case of the p75NTR, a member of the tumor necrosis factor receptor family and a single TMH MP harboring large soluble domains, a comparison of nanodiscs assembled with different MSP constructs showed that smaller discs may not result in better NMR spectra if the soluble domains artificially interact with the MSP in smaller nanodiscs.[351] Therefore, it is important to make use of the broad selection of available MSP variants and screen for the best lipid nanodisc system using NMR guided by the size properties of the MP of interest.

Evidently, size reduction in nanodisc particles containing MPs by generation of smaller nanodiscs allows for the acquisition of better NMR spectra, enabling backbone resonance assignments as well as the structure determination of MPs. As it becomes quickly evident from the earlier-mentioned examples, ΔH5 or its circular derivative seem to be the general choice for small nanodiscs. This intriguing preference is actually not surprising because the ΔH5 construct is the most stable one among other internal deletion constructs,[78] which even enabled the NMR structure determination of empty nanodiscs containing DMPC lipids (PDB ID: 2N5E).[129] The structure reveals that, in the antiparallel double ring formed by two MSP1D1ΔH5 copies, helix 5 (H5) of one monomer of the original MSP1D1 construct would interact with H5 of the second monomer. Therefore, the deletion of H5 does not perturb the overall packing of the MSP belt, which is stabilized by ~30 salt bridges and cation-π interactions in H4 and H6.[129] In addition, the interaction between the N- and C-termini of MSP proteins seems to be important for ring closure because the C-terminal truncation mutants, D6 and D5,[128] have a stronger tendency for ring fusion.

3.3.3. NMR Investigations of Membrane-Attached Proteins. Nanodiscs present a particularly suitable environment to study interactions of membrane-attached proteins with other proteins or small-molecule compounds by NMR. A

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prominent example of such a protein class is the Bcl2 protein family. Most Bcl2 proteins are composed of a compactly folded α-helical domain and a C-terminal TMH that serves as a membrane anchor. Upon apoptotic simulation, certain pro-apoptotic Bcl2 family members (so-called effectors) can insert into the outer mitochondrial membrane and form pores that lead to the release of pro-apoptotic proteins. Because Bcl2 proteins are inherently unstable in a membrane environment,[352] the use of detergents can lead to their unfolding, rendering structural studies difficult. It was recently shown that the antiapoptotic Bcl2 protein BclxL can be transferred from detergent micelles into ΔH5 nanodiscs containing DMPC and DMPG lipids, leading to good spectral quality as probed by 2D-[^{15}N,^{1}H]-TROSY NMR[353] experiments. Moreover, the transmembrane helix was found to be quite flexible within the membrane, suggesting a potential functional role in apoptosis.[354] In a later NMR study on full-length BclxL,[352] an alternative strategy based on SortaseA-mediated protein ligation was applied to obtain the full-length protein to prevent the adverse effects of detergents on the soluble domain of BclxL. Therein, the TMH and soluble domain of BclxL were produced and purified separately and, after nanodisc insertion of the TMH, ligated in a detergent-free environment. This approach might be useful for the production of membrane-anchored proteins that cannot otherwise be produced or refolded in a full-length context[352] and where segmental isotope labeling for NMR is desirable. In a recent study, lipid nanodiscs were used for the structural characterization of the pro-apoptotic Bcl2 protein Bak in a membrane environment by NMR.[355] Therein, the use of nanodiscs of a defined size (MSP1D1ΔH5) prevented the premature membrane incorporation and oligomerization of the Bak soluble domain, which would lead to membrane pore formation. This setup enabled the defined activation of Bak membrane incorporation by BH3-only proteins for a subsequent structural characterization by NMR and hydrogen-deuterium exchange (HDX) mass spectrometry.[355]

Another well-studied membrane-associated protein system is the cytochrome P450 complex and its partner protein cyt-b_5. The interaction between these proteins was studied in 22A peptide nanodiscs containing DMPC lipids.[356] The use of lipid nanodiscs was critical for this study because the presence of detergents caused partial unfolding of the α-helices of P450 2B4 (CYP2B4).[357] Recently, selective labeling of cyt-b_5 with 5-fluorotryptophan-containing ^{19}F nuclei provided additional insights into the interaction between cytb_5 and full-length CYP2B4 within the lipid bilayer formed by 4F peptide nanodiscs.[358] The same group also reported well-resolved 2D-[^{15}N,^{1}H]-TROSY spectra of the FMN (flavin mononucleotide)-binding domain (FBD) of CYP450-reductase alone and in complex with CYP2B4 attached to 4F peptide nanodiscs containing DMPC lipids.[359] Moreover, ^{13}C-methyl methanethiosulfonate (^{13}C-MMTS) labeling of surface-exposed cysteines on the full-length CYP450 reductase in MSP1D1 nanodiscs containing DOPG/DOPC lipids allowed methyl-TROSY experiments to be performed in the oxidized and reduced states of the protein to detect conformational transitions without the need for perdeuteration.[360] By this approach, the interaction of the components in the ternary complex in the absence or presence of substrates was possible, emphasizing the power of the nanodisc technology in combination with NMR spectroscopy.[361]

Another prominent membrane-associated protein class is small GTPases.[362] Using MSP1D1 nanodiscs, the membrane-binding properties of Rheb, a GTPase belonging to the Ras superfamily of proteins, was studied by NMR.[317] NMR chemical-shift perturbation experiments suggested that the protein is not tightly associated with the lipid bilayer in the absence of a membrane anchor. Thus, the authors established a stable membrane location by covalently linking Rheb, harboring a single cysteine residue, to a cysteine-selective maleimide-headgroup-modified lipid incorporated into nanodiscs. In that setup, the authors could elegantly probe the orientation of Rheb on the membrane surface in complex with guanosine diphosphate (GDP) or guanosine triphosphate (GTP) using PRE experiments in the presence of Gd^{3+}-chelated lipids.[317] In a similar workflow, the effector-dependent membrane interaction of K-RAS4B (Kirsten rat sarcoma viral oncogene homologue 4B) GTPase was observed by PRE experiments using MSP1D1 nanodiscs.[363] Later, this approach was extended to an analysis of the K-RAS4B dimerization mechanism on the membrane surface,[364] as well as its interaction with the small-molecule compound Cmpd2, which stabilizes its membrane interaction and leads to impaired RAF kinase activation.[365] In addition to GTPases, a similar strategy was used for the NMR investigation of the membrane-bound metalloprotease MT1-MMP.[366] A stabilized neurotensin receptor in MSP1D1 nanodiscs was used to study its interaction with an isotope-labeled inhibitory Gα subunit by NMR and probe the impact of the GPCR on the conformational equilibrium of the G-protein.[99]

The membrane-binding properties of α-synuclein (α-syn), a protein linked to Parkinson's disease, was investigated by solution NMR using nanodiscs containing a suitable lipid composition that was required for binding. 2D-[^{15}N,^{1}H]-TROSY spectroscopy showed that α-syn preferentially bound to nanodiscs containing negatively charged lipids, which stimulated the nucleation of amyloid fibril formation.[367] More recently, the effect of cholesterol on fibrillation was investigated using polymer nanodiscs.[368] In contrast, 4F peptide nanodiscs were shown to have an inhibitory effect on the formation of Aβ40 fibers, a major player in Alzheimer's disease, suggesting a possible therapeutic potential of these nanodiscs in amyloidosis.[369] MSP1D1ΔH5 nanodiscs of different lipid compositions were also used to capture a human islet amyloid polypeptide (IAPP) aggregation intermediate, enabling its initial structural characterization by NMR,[370] and the same type of nanodiscs has been used to probe the binding of the VDAC1 N-terminal helix to the membrane surface.[371] Recently, 2D-[^{15}N,^{1}H]-HSQC spectra of different Pex14 constructs, a component of the peroxisomal translocon complex, in the presence of ΔH5 nanodiscs and bicelles, were recorded to investigate its membrane-binding properties.[372]

Similarly, nanodiscs allowed for the NMR characterization of the membrane-binding properties of membrane-active peptides, such as the ion-channel inhibitor VSTx1, the antimicrobial β-hairpin peptide AA193,[373] the spider-venom inhibitor Pn3a,[374] the PI(4,5)P_2-mediated membrane-targeting of the cytotoxin BteA from *Bordetella pertussis*,[375] and the binding of the major intracellular component of the epithelial adherent junctions pleckstrin homology domain containing family A member 7 (PLEKHA7) to phosphatidylinositol lipids (PIPs).[376] Moreover, involvement of a membrane environment in the binding of peptides derived from the isoforms of the

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cytosolic domain of the homotypic cell-adhesion receptor CEACAM1 with calcium-calmodulin (Ca^{2+}/CaM) was investigated using MSP1D1 nanodiscs containing DMPC lipids, revealing its stepwise interaction with Ca^{2+}/CaM and actin.[377]

The earlier-mentioned examples demonstrate the benefits of nanodiscs for the NMR investigation of membrane-associated proteins and peptides without the adverse effects of detergents that often lead to structural alterations or even unfolding of its soluble parts. Thus, conformational changes of these proteins in response to membrane/lipid interaction can be monitored in a realistic environment. Moreover, effects of specific lipids can be analyzed due to the compatibility of nanodiscs with most of the relevant lipid types.

3.3.4. Studying Single-Pass Helical MPs in Nanodiscs by NMR. Single-pass transmembrane-helical MPs are a large class of MPs with essential biological functions.[378] The members of this highly relevant MP class are often too small and flexible to be studied by X-ray crystallography or cryo-EM. This results in an excellent niche for the application of solution-state NMR to investigate the structure and dynamics of TMHs in membrane environments. Most solution-NMR structural studies of TMHs have been conducted in detergent micelles, as recently summarized in ref 379. A rather harsh detergent environment is commonly considered to be compatible with TMH structure formation due to the lack of helix−helix interactions if single TMHs are investigated. However, the use of lipid nanodiscs to study not only the structure but also the functional dynamics of TMHs provides the opportunity to capture its functional properties. This topic appears to be important because the conformational landscape of TMHs is most likely affected by the surrounding membrane environment.[108,380]

So far, only a few high-resolution NMR structural studies of TMHs in lipid nanodiscs are available. One of them is the TMH of the Alzheimer risk factor TREM2 (triggering receptor expressed on myeloid cells 2).[381] The TMH of TREM2 is subjected to sequential intramembrane proteolysis by γ-secretase following the initial cleavage at a C-terminal location within the TMH.[382,383] Therefore, the structural and dynamical properties of the TREM2 TMH can be used as a model system to better understand the substrate-recognition features of γ-secretase. NMR spectra of the TMH in both DPC micelles and DMPC/DMPG MSP1D1ΔH5 nanodiscs reveal an intrinsic regulation mechanism that modulates the stability of the TMH upon exposure of a positively charged lysine residue in the transmembrane region. This residue is normally protected from the hydrophobic membrane environment by binding to its partner protein DAP12, which most likely dissociates from TREM2 once proteolytic processing by other proteases is initiated. This study revealed that a dynamic region within the TREM2 TMH is recognized and cleaved by γ-secretase. Charge removal of the TREM2 TMH leads to structure formation in that region and to an altered processing pattern by γ-secretase. Thus, γ-secretase appears to preferentially bind to flexible regions of a TMH, leading to proper proteolysis of the TREM2 fragment.[381]

Another prominent example is the C-terminal TMH of antiapoptotic BclxL, which serves as a membrane anchor and is essential for its functionality.[384] The BclxL-TMH was initially investigated in a full-length context and in isolation using detergent micelles and lipid nanodiscs.[353,354] Because the soluble domain of BclxL is inherently unstable in the presence of detergent micelles, a different approach utilized the modular structure of BcxL to omit detergent contact with this domain. The isotope-labeled TMH was first incorporated into lipid nanodiscs using SDS or DPC detergent for initial solubilization.[352] After assembly, the detergent-free TMH in nanodiscs was ligated to the soluble domain of BclxL, as mentioned in section 3.3.3. In this study, the high-resolution structure of lipid-incorporated BclxL-TMH was determined, as well as the membrane location of the soluble domain, enabling the construction of a structural model of the full-length protein.[352] These initial examples further demonstrated the versatility of the nanodisc system, which allows for not only the high-resolution structural studies of TMHs but also the study of the corresponding full-length proteins containing detergent-sensitive soluble domains.

3.3.5. Investigation of Lipid and MP Dynamics in Nanodiscs. Because of the presence of a rather fluid lipid-bilayer membrane, lipid nanodiscs are highly dynamic particles, which could be seen by the quite large alterations in the nanodisc shape in the NMR structural bundle of MSP1D1ΔH5 nanodiscs.[129] On the basis of this study, the shape heterogeneity of a MSP lipid nanodisc was later confirmed by a combined NMR, small-angle X-ray scattering (SAXS)/small-angle neutron scattering (SANS), and molecular dynamics simulation study, where various elliptical shapes were detected as well as different degrees of lipid ordering within the nanodisc.[172]

Due to the presence of the lipid-binding MSP, the lipid molecules in nanodiscs are somewhat more restricted than in a pure lipid-membrane mimetic, such as liposomes. Thus, it is important to probe the dynamics of the lipids in nanodiscs. In a solid-state NMR study, it was shown that the dynamics of lipids in both the gel- and liquid-crystalline phases are restricted as compared to lamellar-liposome preparations. In turn, reduced dynamics in nanodiscs was also shown for the incorporated MP proteorhodopsin.[385] This finding was refined in another solid-state NMR study, reporting an almost abolished gel-to-fluid phase transition in nanodiscs caused by a higher degree of lipid fluidity, whereas the addition of cholesterol led to chain ordering.[386] Nonetheless, the reduction in nanodisc size or covalent circularization of the MSP did not have a marked effect on lipid dynamics in empty nanodiscs.[78,132]

These changes in lipid dynamics have implications on the dynamics of an incorporated MP. Ideally, the best membrane-mimetic system is liposomes. However, due to the size limitations in solution-state NMR, smaller lipid-based membrane mimetics are required. Even though the earlier-mentioned solid-state NMR indicated restricted dynamics of an MP in nanodiscs as compared to liposomes,[385] it has been shown by a comparative solution-state NMR study with OmpX in different membrane mimetics[387] that nanodiscs still permit nanosecond to picosecond and millisecond to microsecond motions within membrane-embedded areas that are absent in detergent micelles. Loop regions of OmpX outside the membrane were shown to exhibit similar dynamical features in the nanosecond to picosecond time scale.[78] Due to the ability to incorporate basically any lipid into nanodiscs, it could be shown that a modulation of lipid dynamics by the addition of cholesterol had an impact on the dynamics of the incorporated model protein OmpX.[388] Despite the minor restrictions in lipid dynamics, the native-like properties of lipid nanodiscs required for an MP to adopt an active structural state were recently shown with the bacterial β-barrel assembly

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machinery (BAM), where the lipid surface of the nanodisc could be deformed by the disruptase activity of BAM.[389] However, due to limitations in the membrane surface area, the MP insertase activity of the BAM complex was higher in larger nanodiscs or liposomes. Furthermore, the active G-protein-binding-competent state of a GPCR can still be adopted in smaller nanodiscs, as evident from recent complex structures.[77,390]

Taken together, as compared to liposomes, the MSP-encircled structure of a nanodisc leads to a restriction in the lipid dynamics, in particular at the lipid−MSP interface. However, compared to other membrane mimetics, nanodiscs can be considered the most native-like system. Due to the abundance of various MPs[391] as well as cholesterol in biological membranes,[20,392] it appears likely that lipid motion is already heavily restricted in living cells, but the modulation of MP activity by the surrounding lipids might represent an important level of regulation in cells. Apart from experimental approaches, state-of-the-art molecular dynamics (MD) simulations on lipid nanodiscs or other bilayer systems[171,393−397] have the potential to provide valuable insights into this topic. The possibility to vary the nanodisc size and lipid composition will be beneficial to mimic the native situation for structural, dynamical, and functional investigations of MPs.

4. SUMMARY AND FUTURE DIRECTIONS

In this Review, we aimed to provide a detailed overview of the currently used membrane-mimetic systems for the structural and functional investigation of MPs, with a strong focus on lipid nanodiscs. We strongly believe that lipid nanodiscs have superior properties as compared to other membrane-mimicking media. Lipid nanodiscs provide a native-like lipid-bilayer environment containing any desired lipid composition, which generally facilitates MP stability and functionality. Furthermore, due to their realistic bilayer and nondenaturing properties, lipid nanodiscs are a perfect tool to investigate the (lipid-dependent) interaction of peripheral MPs with the lipid-bilayer surface, as well as to study the structure and function of membrane-anchored proteins or complexes between membrane-associated soluble proteins and integral MPs. Especially the introduction of small lipid nanodiscs almost a decade ago was essential for making this membrane mimetic popular in the NMR community, enabling structural studies of integral and peripheral MPs as well as investigations on the dynamical features of the entrapped lipids and the incorporated MP.

However, these clear advantages of lipid nanodiscs do not come without limitations. For solution NMR, the still quite large size of the available lipid nanodiscs is challenging, requiring cutting-edge sample-preparation methods as well as NMR methodology, including ultrahigh-field NMR magnets and cryogenic probes. Further truncation of the MSP to produce even smaller nanodiscs is very difficult due to stability issues and the requirement for a certain amount of incorporated lipids. By comparison, the use of detergent micelles often leads to a better NMR spectral quality, simply due to the smaller size and possibly the lower tendency of MPs to form heterogeneous mixtures of monomers, dimers, or oligomers in this harsher environment. In addition, detergent micelles are very easy to use without the need for the production of additional components, such as the MSP. Thus, in each particular case, it has to be carefully considered which membrane mimetic would be the best choice for a particular application and sample situation. Initial NMR studies of an MP in detergent micelles are often a good starting point to get an impression of the behavior of the MP and to decide on the need for a more native nanodisc environment. As mentioned earlier, this is very often the case for structurally labile MPs, as well as for any MP system where detergents are unfavorable.

With nanodiscs at hand that have a diameter of ∼6 nm, the size limitations of the MSP lipid nanodisc system are most likely reached. However, most integral MPs would require a larger membrane surface; thus, the use of 8−10 nm nanodiscs or larger is required in many cases. The improvement of the NMR spectral quality of MPs in nanodiscs has been shown to be possible with MSP circularization, leading to more homogeneous and stable particles that are suitable for long-term NMR experiments at elevated temperatures, which can compensate for their larger size. Because the MSP is a protein that can be mutated, truncated, circularized, or otherwise modified, we are very optimistic that the MSP nanodisc technology can be further developed and refined for specific applications in structural biology as well as functional investigations of MPs in the future.

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The authors declare no competing financial interest.

Biographies

Umut Günsel completed his B.Sc. in 2011 and M.Sc. in 2013 in the Department of Molecular Biology and Genetics in Istanbul Technical University, Turkey. He received his Ph.D. from the laboratory of Dr. Dejana Mokranjac in the Department of Physiological Chemistry, Ludwig Maximilian University of Munich, Germany, in 2020. Since January 2021 he has been working as a postdoctoral scientist in the laboratory of Prof. Dr. Franz Hagn on the structural biology of membrane proteins in Bavarian NMR Center (BNMRZ) at the Technical University of Munich. His journey in the field of protein structure and function relationship as his main area of interest started with chaperone proteins during his M.Sc. and continued with protein translocation studies into mitochondria during his Ph.D. Currently, as the newest member of the group of Prof. Hagn, he works on structure and dynamics of mitochondrial membrane proteins and GPCRs associated with metabolic diseases, neurological disorders, and cancer.

Franz Hagn studied biochemistry from 1998 to 2003 at the Universities of Bayreuth, Germany, and Stockholm, Sweden. He worked in the lab of Astrid Gräslund and Lena Mäler in Stockholm on the dynamics of a membrane-bound peptide in different membrane-mimicking media and with Jochen Balbach on using real-time NMR to follow protein-folding processes. In 2004, funded by the Elite

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Network of Bavaria, he moved to the Technical University of Munich and conducted his doctoral research with Horst Kessler, in which he used NMR to determine the structure and dynamics of molecular chaperones, tumor suppressors, and spider silk proteins. After graduation in 2009 with summa cum laude, he pursued postdoctoral research with Gerhard Wagner at Harvard Medical School in Boston, U.S.A., funded by EMBO and HFSP long-term fellowships. In the Wagner lab, he developed and applied size-optimized lipid nanodiscs for structural studies of membrane proteins by solution-state NMR and determined the first high-resolution structure of a membrane protein in lipid nanodiscs. In 2014, he moved back to Munich and accepted a faculty position at the TUM as well as a junior group leader position at the Helmholtz Center Munich, funded by the Helmholtz Young Investigator's group program of the Helmholtz Society. In Munich he is still interested in the development of lipid nanodiscs for NMR spectroscopy as well as in the structural investigation of membrane proteins involved in signal transduction, metabolite transport, and apoptosis. He has been awarded the Friedrich-Weygand Award of the Max-Bergmann Kreis for peptide chemistry in 2011, the Hans-Fischer Award of the Department of Chemistry of the TUM in 2011, the Arnold-Sommerfeld Award of the Bavarian Academy of Sciences in 2012, and the Felix-Bloch-Lecture of the Magnetic Resonance Division of the German Chemical Society in 2019, and he was promoted to associate professor at TUM in 2020.

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