Lipid nanodiscs for high-resolution NMR studies of membrane proteins

Journal:	*Chemical Reviews*
Manuscript ID	cr-2021-007023.R1
Manuscript Type:	Thematic Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Guensel, Umut; Technical University of Munich, Department of Chemistry Hagn, Franz; Technical University of Munich, Department of Chemistry

Lipid nanodiscs for high-resolution NMR studies of membrane proteins

Umut Günsel[1] & Franz Hagn[1,2,*]

[1] Bavarian NMR Center (BNMRZ) at the Department of Chemistry, Technical University of Munich, Ernst-Otto-Fischer-Str. 2, 85748 Garching, Germany

[2] Institute of Structural Biology, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

*Correspondence should be addressed to F.H. (e-mail: franz.hagn@tum.de, phone: +49-89-289-52624)

Abstract:

Membrane proteins (MPs) play essential roles in numerous cellular processes. Since 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 on the currently used lipid nanodisc systems as well as their application 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 a MP for subsequent NMR investigations. With this, we hope to provide a comprehensive background to enable an informed assessment on the applicability of lipid nanodiscs for NMR studies of a particular MP of interest.

Contents

1. Introduction

Membrane proteins (MPs) are essential players in a large number of cellular processes including intra- and inter-cellular signaling, transport of metabolites and proteins across the lipid bilayer membrane, enzymatic reactions, membrane fission-fusion, cell-cell interaction and energy production [1-5]. About 30 % of the human proteome are MPs[6, 7] and, 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 are in particular 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 non-covalent interactions, covalently-bound aliphatic membrane anchors or transmembrane helices[14-17]. Unlike soluble proteins, MPs require the presence of a lipid bilayer environment, often comprised 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 intra- molecular 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, since 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 can exist predominantly in the cell membrane, called lipid rafts [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, macro structural, collective behavior non-specifically or through specific direct chemical interactions between lipids and the 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 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 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, in order 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 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 head groups 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 shape. Instead, they tend to form micelles above the critical micellar concentration (CMC). In contrast, many lipid molecules have a roughly cylindrical overall shape since the cross sections of the polar head group 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 μM to mM range, this value is in 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 be screened individually[48, 87, 88]. Structurally,

detergents are classified as ionic (cationic or anionic), zwitterionic and non-ionic. Non-ionic 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 **Tab. 1** together with their CMC and micellar molecular weights.

Table 1. Commonly used detergents used for MP solubilization for nanodisc incorporation[68, 84, 89]. *CMC at 20-25 °C

Type	Detergent	CMC (mM)*	Micelle size (kDa)
Ionic	Sodium cholate[90]	9-15	0.9-1.3
	Sodium deoxycholate[91]	2-6	1.2-5
	CTAB	1	62
	N-lauroylsarcosine[92]	14.6	0.6
	SDS[93]	7-10	18
Zwitter-ionic	LysoPC	0.007	92
	CHAPS[94]	6	6.15
	Dodecyl-phosphocholine (DPC)[78]	1.5	19
	LDAO[95]	1-2	17
Non-ionic	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

Mild detergents are generally preferred for multi-pass integral MPs since intra- and inter-molecular 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 case of MP complexes, a harsh detergent might dissociate individual subunits. In line with this notion, it has been reported that individual subunits of a MP complex even require different detergent solubilization conditions[13, 101]. Furthermore, if a 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 of gram-negative bacteria but not the outer membrane can be solubilized in 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 as well as the compatibility with downstream applications for a 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 a 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, that are often highly sensitive to detergents, are investigated. Due to the various reasons mentioned above[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 are liposomes, providing 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. In order 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 chapter, we will introduce the most common nanodisc systems and will describe their suitability for solution NMR studies of MPs.

2. Lipid nanodiscs – a lipid-based and native-like membrane mimetic

Lipid nanodiscs have been 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] (**Fig. 1a**). This versatile membrane mimetic gained 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 (**Fig 1b**). In the next section, we will provide a compilation of all currently available lipid nanodisc systems and briefly summarize their use in NMR.

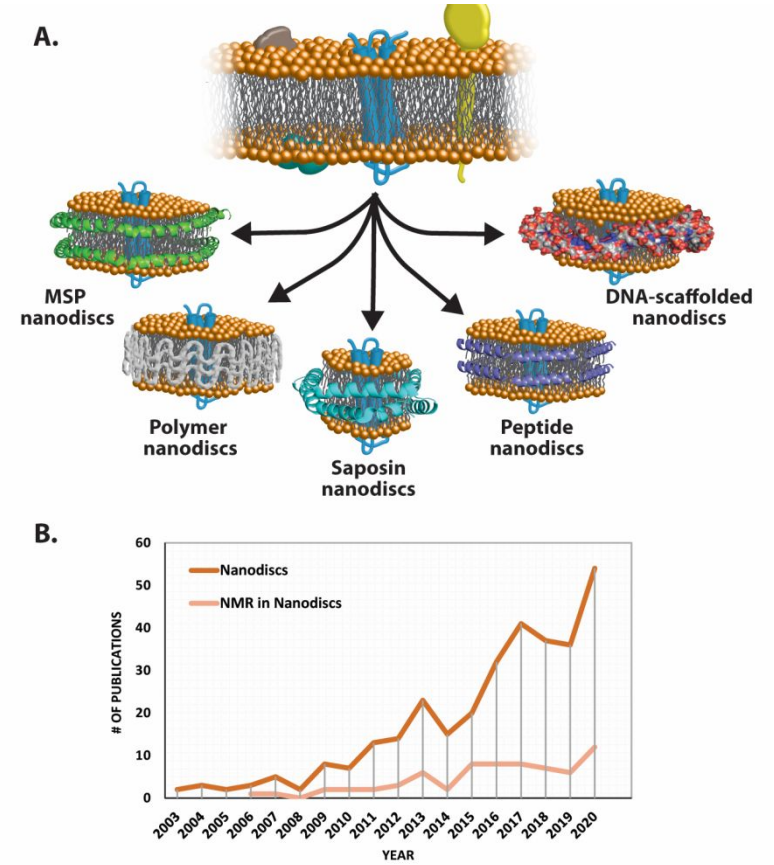


Fig. 1. Commonly used lipid nanodisc systems for NMR studies. (A) Lipid based nanodiscs systems can be used to mimic a native membrane environment for NMR structural studies. (B) Number of publications on the search terms "membrane protein" and "nanodiscs" with or without "NMR".

2.1. Membrane scaffold protein (MSP) 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 blood stream[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 anti-parallel orientation stabilizing the hydrophobic rim of the membrane (**Fig. 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 is comprised of 10 amphipathic α-helical segments generating nanodiscs of about 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 is very similar to 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 **Fig. 2B**.

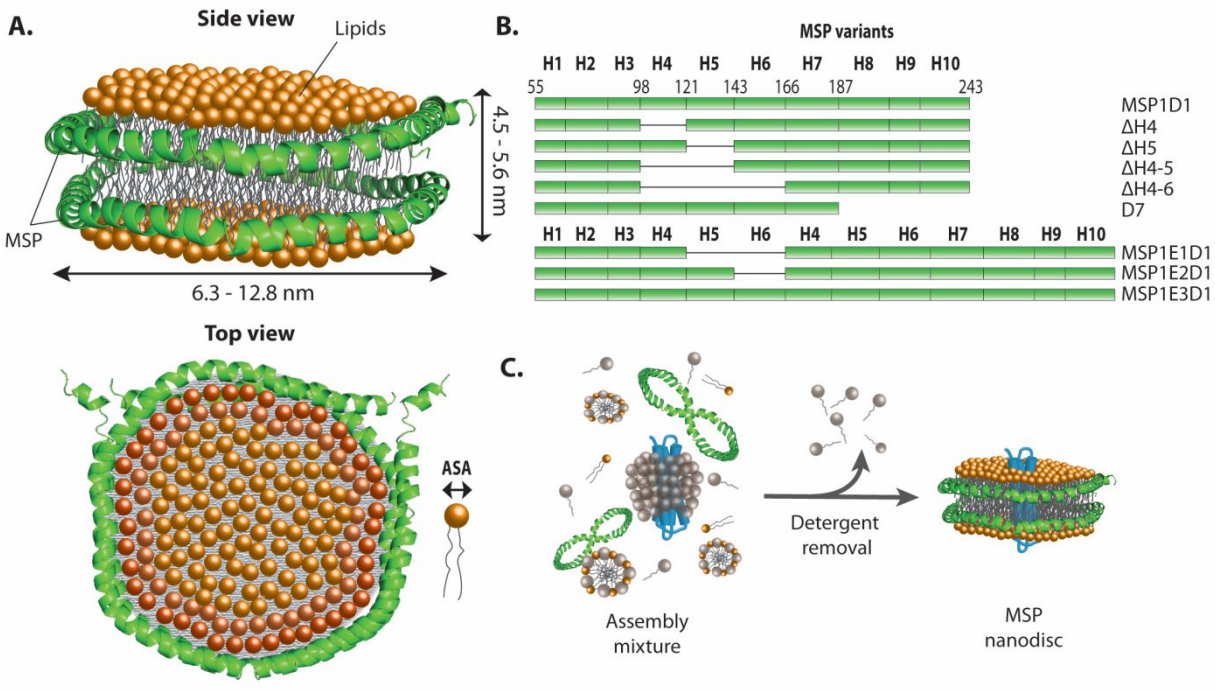


Fig. 2. MSP lipid nanodiscs. (A) Structure of an MSP lipid nanodiscs with two copies of the MSP (green) surrounding a patch of lipid bilayer membrane of tunable size (orange). (B) Available MSP constructs that have been used for solution NMR studies. In particular the truncated versions are attractive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

As mentioned above, MSP1D1 assembles into nanodiscs of 10 nm in diameter, corresponding to a molecular mass of about 150 kDa, representing a considerable challenge for NMR studies. Thus, in order 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, MSP1D1ΔH5) resulted in stable nanodiscs with a diameter of about 7.1 nm

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, D7 MSP1D1, a truncated MSP1D1 variant lacking the helices after helix 7, formed nanodiscs of 7 nm in diameter. However, further truncations resulted in larger particles, possibly due to formation of nanodiscs consisting of more than two MSP copies[128].

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]. Since 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 activity[136]. In a complimentary 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, 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 **Tab. 2**.

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]

MSP nanodiscs are formed via a self-assembly process by 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]. 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 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 co-solvents 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], 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, the amount of lipid molecules, which 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) head group, 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 surface area are shown in **Tab. 3**. In addition to the use of pure lipids and their defined mixtures, commercially available natural lipid blends extracted from different sources such as *E. coli*, chicken egg, yeast, soybean, 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].

Table 3. Commonly used lipids for nanodisc preparations, their phase transition temperatures (T_m) and surface area (SA).

Lipid	T_m (°C)[68, 90]	SA (Å²)
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]

The possibility to include lipids with various different head groups 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 head

groups, the thickness of the lipid bilayer can be adjusted by choosing lipids bearing longer or shorter acyl chain lengths. This aspect is highly relevant, since 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 non-bilayer 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]. In order 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 homogenous within a nanodisc particle but depends 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]. Due to 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 MSP belt and lipid head groups[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 a 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 thermodynamic 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 was 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]. Since detergent solubilization is not required for the formation of SMALPs, unwanted effects of detergents on MP structure can be avoided[181]. Hydrophobicity and solubilization properties of SMA copolymers can be adjusted by changing the ratio of styrene to maleic acid during 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 appears 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 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. Due to 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 (diisobuthylene/maleic acid), poly-styrene-co-maleimide (SMI), SMA-ethanolamine (SMA-EA), SMA-ethylenediamine (SMA-ED), and the covalently modifiable cysteamine derivative (SMA-SH) show better solubility 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 non-ionic, 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 nanodisc and also does 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 around 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 more than one order of magnitude[178]. In contrast to the well-understood size of 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 since no detergent is required for MP extraction[192]. Especially the increasing number 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 (saposin A-D). Saposins are small, lipid-binding proteins involved in lysosomal degradation pathways of sphingolipids[206]. They are comprised 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 can be also formed by 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 comprised 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 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

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-DWLKAFYDKVAEKLKEAF-NH_2), have been shown to form discoidal lipid particles[223] similar to MSP nanodiscs. While one side of the peptide is mainly comprised 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 towards the center[224]. Positively charged residues interact with negatively charged lipid head groups 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 hydrophobic match, as it is the case for MSP nanodiscs[224, 226, 227].

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, and its bendability for longer peptides, seems to be an important factor for the adjustability of the lipid-to-peptide ratio[230]. Since 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 use of chemically modified bifunctional peptides which 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].

Since 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 around 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 (RDC) (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]", is 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 is hybridized to non-circularized MSPs to generate larger discs up to ~70 nm diameter via lipid supplementation[236]. The DNA scaffold ensures positioning of protein-based MSP scaffolds rather than stabilizing lipid bilayer directly. However, since these scaffolds are designed to generate very large nanodiscs which are convenient to study larger assemblies such as MP oligomerization and early stages of viral entry, they are not compatible with solution-state NMR, and so, 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 about 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 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 head groups 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 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 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 homogenous 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 a 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 homogenous[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 relative 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 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 above, 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 homogenous 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 (**Tab. 2**) as well as the surface area of the used lipids (**Tab. 3**). This situation becomes a bit more complicated if a MP needs to be inserted, requiring a more systematic screening procedure, outlined in **Fig. 3a**.

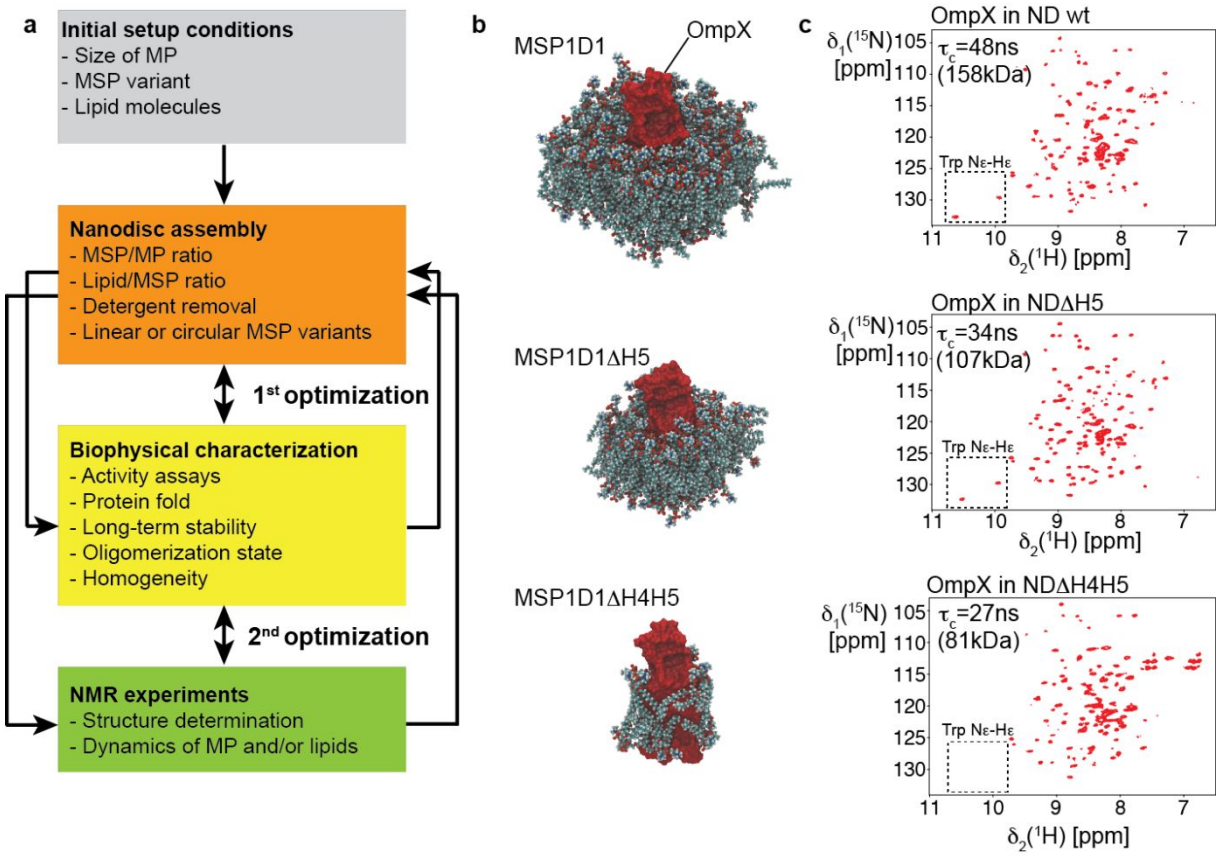


Fig. 3. Workflow for the optimization of nanodisc assembly and effect of nanodisc size on NMR spectral quality. Panels (b) and (c) are reproduced with permission from Ref. [78]. Copyright 2013 American Chemical Society.

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 approximately 6-10 Å, occupying a surface area of around 80 Å^2, whereas a 6-TMH protein would take up around 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 since 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. Since the both ratios affect each other, both values need to be considered simultaneously. For a 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 since 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 since 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 plays an important role for successful nanodisc formation. Typically, hydrophobic beads are used for this purpose since this is the most straightforward method. 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 slow, 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 homogenous oligomeric states can be selected, which could be analyzed by NMR [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 biophysical experiments on the folding state of the protein necessary, 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 homogenous oligomeric state. This can be done by multi angle light scattering (MALS), mass spectrometric or chemical crosslinking 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 homogenous nanodisc sample is at hand, the final validation step is to use isotope-labeled (typically *U*-^{2}H,^{15}N) MP for the assembly reaction and read out the spectral quality and the 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 (**Fig. 3b,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 MP and MSP leads to reduced spectral quality and in particular to the disappearance of tryptophane side-chain resonances (**Fig. 3c**). In this 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 on solution NMR studies of MPs in MSP nanodiscs and highlight the broad and versatile applications that became possible with this type of nanodiscs, 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 is an important factor 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, as well as 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 as well as 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 non-uniform sampling (NUS) strategies[268-273].

Since 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 decades, the most noticeable enhancement in NMR spectral quality of large protein systems can be 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. Since 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, Met) account for up to 45 % of the amino acids

in the TMHs of MPs [278], providing a multitude of distance information in multidimensional 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 non-specific[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 since the large molecular weight of MPs often prevents the establishment of connectivities between the side chains and the protein backbone using through-bond coherence transfer NMR experiments [296]. 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

Due to the inherent sparsity of NOE (nuclear Overhauser effect) 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 (RDC) [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], bicelles[323, 324] and 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 since 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 renders 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 applicability of nanodiscs for integral MPs. Shortly later, a 70 amino acid truncated and mutated version of CD4 (CD4mut), a core T cell receptor, comprised 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 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 MP 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 as well as 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 a 3D-[^{1}H,^{1}H]-NOESY-^{15}N-TROSY as well as a 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 could not be 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 since 2D-TROSY spectra of OmpA in MSP1D1 nanodiscs and in Fos-10 micelles were quite comparable[331]. In case of BamA, the β-barrel subunit of the bacterial β-barrel insertase complex, 2D-[^{15}N, ^{1}H]-TROSY spectra in MSP1D1 nanodiscs, in 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 a 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 multi-pass α-helical MP in nanodiscs was performed on ^{15}N-labeled KcsA, a tetrameric K^+ channel from *Streptomyces lividans*, which is comprised of an 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 find a detergent that resembles the nanodisc environment best 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 VSD of KvAP [339]. Furthermore, 2D-[^{15}N, ^{1}H]-TROSY spectra of VSD in DPC or DPC/LDAO micelles and in DMPC, DMPG or POPC/DOPG containing nanodiscs exhibited similar μs-ms inter-helical 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 case of the *E. coli* MP YgaP, comprised 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 about 80 % of the rhodanese domain with an additional ~40 peaks, possibly belonging to the TMH segment [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 on 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 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 GPCR 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 a 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

Due to the above-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 labs 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 (about 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 a MP in lipid nanodiscs [78]. This workflow was facilitated by the use of deuterated lipids and NMR non-uniform sampling (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* since 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 mimetics 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, D7 MSP1D1, which leads to nanodiscs of similar size as with ΔH5 nanodiscs, provided markedly improved 2D-[^{15}N, ^{1}H]-TROSY spectra for the integrin α_{IIb}

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 was 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 LMNG micelles [350]. The corresponding 2D-[^{13}C,^{1}H]-HSQC spectra of 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 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 above-mentioned examples, ΔH5 or its circular derivative seem to be the general choice for small nanodiscs. This intriguing preference is actually not surprising because ΔH5 construct is the most stable one among other internal deletion constructs [78], which even enabled the NMR structure determination of an empty nanodiscs containing DMPC lipids (PDB ID: 2N5E) [129]. The structure reveals that in the anti-parallel 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 about 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 since the C-terminal truncation mutants, D6 and D5 [128], had 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 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. Since Bcl2 proteins are inherently instable in a membrane environment [352], the use of detergents can lead to their unfolding, rendering structural studies difficult. It was recently shown that the anti-apoptotic 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 structural characterization by NMR[355].

Another well-studied membrane-associated protein system is the cytochrome P450 complex and its partner protein cytb_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 since the presence of detergents caused partial unfolding of the α-helices of P450 2B4 (CYP2B4) [357]. Recently, selective labeling of cytb_5 with 5-fluoro-tryptophan containing ^{19}F nuclei provided additional insights on 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 are 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 GDP or GTP using paramagnetic relaxation enhancement (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 homolog 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 binds 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 have 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 binding of peptides derived from the isoforms of the 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 above-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 compatibility of nanodiscs with most 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 since 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 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 revealed 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 lead 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 anti-apoptotic 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]. Since the soluble domain of BclxL is inherently instable in presence of detergent micelles, a different approach utilized the modular structure of BcxL to omit detergent contact of 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 demonstrate the versatility of the nanodisc system which not only allows for 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

Due to 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]. Based on this study, the shape heterogeneity of a MSP lipid nanodisc was later confirmed by a combined NMR, SAXS/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 phase is 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 above-mentioned solid-state NMR indicated restricted dynamics of a 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 ns-ps and ms-µs 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 ns-ps 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 a MP to adopt an active structural state was recently shown with the bacterial β-barrel assembly 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 on 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 at providing a detailed overview on 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 non-denaturing 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 as well as 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 ultra-high-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 heterogenic 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 what membrane mimetic would be the best choice for a particular application and sample situation. Initial NMR studies of a MP in detergent micelles are often a good starting point to get an impression on the behavior of the MP and to decide on the need for a more native nanodisc environment. As mentioned above, this is very often the case for structurally labile MPs, and 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 anyway 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 still possible with MSP circularization, leading to more homogenous and stable particles that are suitable for long-term NMR experiments at elevated temperatures, which can compensate for their larger size. Since 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.

5. References

(1) Cournia, Z.; Allen, T. W.; Andricioaei, I.; Antonny, B.; Baum, D.; Brannigan, G.; Buchete, N. V.; Deckman, J. T.; Delemotte, L.; Del Val, C.*, et al.* Membrane Protein Structure, Function, and Dynamics: A Perspective from Experiments and Theory. *J. Membr. Biol.* **2015,** *248*, 611-640.

(2) Engel, A.; Gaub, H. E. Structure and Mechanics of Membrane Proteins. *Annu. Rev. Biochem* **2008,** *77*, 127-148.

(3) Guo, L.; Wang, S. F.; Li, M. Y.; Cao, Z. C. Accurate Classification of Membrane Protein Types Based on Sequence and Evolutionary Information Using Deep Learning. *BMC Bioinformatics* **2019,** *20*.

(4) Von Heijne, G. The Membrane Protein Universe: What's out There and Why Bother? *J. Intern. Med.* **2007,** *261*, 543-557.

(5) Xian, H. X.; Liou, Y. C. Functions of Outer Mitochondrial Membrane Proteins: Mediating the Crosstalk between Mitochondrial Dynamics and Mitophagy. *Cell Death Differ.* **2021,** *28*, 827-842.

(6) Almen, M. S.; Nordstrom, K. J.; Fredriksson, R.; Schioth, H. B. Mapping the Human Membrane Proteome: A Majority of the Human Membrane Proteins Can Be Classified According to Function and Evolutionary Origin. *BMC Biol.* **2009,** *7*, 50.

(7) Krogh, A.; Larsson, B.; Von Heijne, G.; Sonnhammer, E. L. Predicting Transmembrane Protein Topology with a Hidden Markov Model: Application to Complete Genomes. *J. Mol. Biol.* **2001,** *305*, 567-580.

(8) Rask-Andersen, M.; Almen, M. S.; Schioth, H. B. Trends in the Exploitation of Novel Drug Targets. *Nat. Rev. Drug Discov.* **2011,** *10*, 579-590.

(9) Yin, H.; Flynn, A. D. Drugging Membrane Protein Interactions. *Annu. Rev. Biomed. Eng.* **2016,** *18*, 51-76.

(10) Lundstrom, K., An Overview on Gpcrs and Drug Discovery: Structure-Based Drug Design and Structural Biology on Gpcrs. In *G Protein-Coupled Receptors in Drug Discovery*, Leifert, W. R., Ed. Humana Press: Totowa, NJ, 2009; pp 51-66.

(11) Huggins, D. J.; Sherman, W.; Tidor, B. Rational Approaches to Improving Selectivity in Drug Design. *J. Med. Chem.* **2012,** *55*, 1424-1444.

(12) Jazayeri, A.; Dias, J. M.; Marshall, F. H. From G Protein-Coupled Receptor Structure Resolution to Rational Drug Design. *J. Biol. Chem.* **2015,** *290*, 19489-19495.

(13) Errasti-Murugarren, E.; Bartoccioni, P.; Palacín, M. Membrane Protein Stabilization Strategies for Structural and Functional Studies. *Membranes* **2021,** *11*, 155.

(14) Lodish, H.; Berk, A.; Zipursky, S. L.; Baltimore, D.; Darnell, J.; Matsudaira, P., Membrane Proteins. In *Molecular Cell Biology*, 4th ed.; W.H. Freeman: New York, 2000.

(15) Vinothkumar, K. R.; Henderson, R. Structures of Membrane Proteins. *Q. Rev. Biophys.* **2010,** *43*, 65-158.

(16) Boes, D. M.; Godoy-Hernandez, A.; Mcmillan, D. G. G. Peripheral Membrane Proteins: Promising Therapeutic Targets across Domains of Life. *Membranes* **2021,** *11*, 346.

(17) Sanders, C. R.; Sönnichsen, F. Solution NMR of Membrane Proteins: Practice and Challenges. *Magn. Reson. Chem.* **2006,** *44*, S24-S40.

(18) Sheetz, M. P.; Singer, S. J. Biological Membranes as Bilayer Couples. A Molecular Mechanism of Drug-Erythrocyte Interactions. *Proc. Natl. Acad. Sci. U. S. A.* **1974,** *71*, 4457-4461.

(19) Andersen, O. S.; Koeppe, R. E., 2nd Bilayer Thickness and Membrane Protein Function: An Energetic Perspective. *Annu. Rev. Biophys. Biomol. Struct.* **2007,** *36*, 107-130.

(20) Harayama, T.; Riezman, H. Understanding the Diversity of Membrane Lipid Composition. *Nat. Rev. Mol. Cell Biol.* **2018,** *19*, 281-296.
(21) Van Meer, G.; Voelker, D. R.; Feigenson, G. W. Membrane Lipids: Where They Are and How They Behave. *Nat. Rev. Mol. Cell Biol.* **2008,** *9*, 112-124.
(22) Galassi, V. V.; Wilke, N. On the Coupling between Mechanical Properties and Electrostatics in Biological Membranes. *Membranes* **2021,** *11*, 478.
(23) Simons, K.; Vaz, W. L. C. Model Systems, Lipid Rafts, and Cell Membranes. *Annu. Rev. Biophys. Biomol. Struct.* **2004,** *33*, 269-295.
(24) Lewis, R. N. a. H.; Mannock, D. A.; Mcelhaney, R. N., Chapter 2 Membrane Lipid Molecular Structure and Polymorphism. In *Curr. Top. Membr.*, Epand, R. M., Ed. Academic Press: 1997; Vol. 44, pp 25-102.
(25) Lee, A. G. Lipid-Protein Interactions in Biological Membranes: A Structural Perspective. *Biochim. Biophys. Acta* **2003,** *1612*, 1-40.
(26) Starling, A. P.; East, J. M.; Lee, A. G. Effects of Gel Phase Phospholipid on the Ca2+-Atpase. *Biochemistry* **1995,** *34*, 3084-3091.
(27) Lee, A. G. How Lipids Affect the Activities of Integral Membrane Proteins. *Biochim. Biophys. Acta* **2004,** *1666*, 62-87.
(28) Lewis, R. N. a. H.; Mcelhaney, R. N. Membrane Lipid Phase Transitions and Phase Organization Studied by Fourier Transform Infrared Spectroscopy. *Biochim. Biophys. Acta* **2013,** *1828*, 2347-2358.
(29) Mcelhaney, R. N. The Influence of Membrane Lipid Composition and Physical Properties of Membrane Structure and Function in Acholeplasma Laidlawii. *Crit. Rev. Microbiol.* **1989,** *17*, 1-32.
(30) Lee, A. Lipids and Their Effects on Membrane Proteins: Evidence against a Role for Fluidity. *Prog. Lipid Res.* **1991,** *30*, 323-348.
(31) Lingwood, D.; Simons, K. Lipid Rafts as a Membrane-Organizing Principle. *Science* **2010,** *327*, 46-50.
(32) Moore, D. J.; Snyder, R. G.; Rerek, M. E.; Mendelsohn, R. Kinetics of Membrane Raft Formation: Fatty Acid Domains in Stratum Corneum Lipid Models. *J. Phys. Chem. B* **2006,** *110*, 2378-2386.
(33) Regen, S. L. The Origin of Lipid Rafts. *Biochemistry* **2020,** *59*, 4617-4621.
(34) Sackmann, E. Physical Basis of Trigger Processes and Membrane Structures. *Biol. Membr.* **1984,** *5*, 105-143.
(35) Lundbæk, J. A.; Collingwood, S. A.; Ingólfsson, H. I.; Kapoor, R.; Andersen, O. S. Lipid Bilayer Regulation of Membrane Protein Function: Gramicidin Channels as Molecular Force Probes. *J. R. Soc. Interface* **2010,** *7*, 373-395.
(36) Sezgin, E.; Levental, I.; Mayor, S.; Eggeling, C. The Mystery of Membrane Organization: Composition, Regulation and Roles of Lipid Rafts. *Nat. Rev. Mol. Cell Bio.* **2017,** *18*, 361-374.
(37) Simunovic, M.; Prévost, C.; Callan-Jones, A.; Bassereau, P. Physical Basis of Some Membrane Shaping Mechanisms. *Phil. Trans. R. Soc. A* **2016,** *374*, 20160034.
(38) Huang, H. W. Deformation Free Energy of Bilayer Membrane and Its Effect on Gramicidin Channel Lifetime. *Biophys. J.* **1986,** *50*, 1061-1070.
(39) Nielsen, C.; Goulian, M.; Andersen, O. S. Energetics of Inclusion-Induced Bilayer Deformations. *Biophys. J.* **1998,** *74*, 1966-1983.
(40) Drew, D.; Froderberg, L.; Baars, L.; De Gier, J. W. Assembly and Overexpression of Membrane Proteins in Escherichia Coli. *Biochim. Biophys. Acta* **2003,** *1610*, 3-10.
(41) Kubicek, J.; Block, H.; Maertens, B.; Spriestersbach, A.; Labahn, J., Expression and Purification of Membrane Proteins. In *Methods Enzymol.*, Elsevier: 2014; pp 117-140.
(42) Pandey, A.; Shin, K.; Patterson, R. E.; Liu, X.-Q.; Rainey, J. K. Current Strategies for Protein Production and Purification Enabling Membrane Protein Structural Biology. *Biochem. Cell Biol.* **2016,** *94*, 507-527.
(43) Klöpfer, K.; Hagn, F. Beyond Detergent Micelles: The Advantages and Applications of Non-Micellar and Lipid-Based Membrane Mimetics for Solution-State NMR. *Prog. Nucl. Magn. Reson. Spectrosc.* **2019,** *114-115*, 271-283.
(44) Thoma, J.; Burmann, B. M. Fake It 'Till You Make It—the Pursuit of Suitable Membrane Mimetics for Membrane Protein Biophysics. *Int. J. Mol. Sci.* **2020,** *22*, 50.

(45) Warschawski, D. E.; Arnold, A. A.; Beaugrand, M.; Gravel, A.; Chartrand, É.; Marcotte, I. Choosing Membrane Mimetics for NMR Structural Studies of Transmembrane Proteins. *Biochim. Biophys. Acta* **2011,** *1808*, 1957-1974.
(46) Puthenveetil, R.; Vinogradova, O. Solution NMR: A Powerful Tool for Structural and Functional Studies of Membrane Proteins in Reconstituted Environments. *J. Biol. Chem.* **2019,** *294*, 15914-15931.
(47) Mineev, K. S.; Nadezhdin, K. D. Membrane Mimetics for Solution NMR Studies of Membrane Proteins. *Nanotechnology Reviews* **2017,** *6*, 15-32.
(48) Seddon, A. M.; Curnow, P.; Booth, P. J. Membrane Proteins, Lipids and Detergents: Not Just a Soap Opera. *Biochim. Biophys. Acta* **2004,** *1666*, 105-117.
(49) Linke, D. Detergents: An Overview. *Methods Enzymol.* **2009,** *463*, 603-617.
(50) Losonczi, J. A.; Olejniczak, E. T.; Betz, S. F.; Harlan, J. E.; Mack, J.; Fesik, S. W. NMR Studies of the Anti-Apoptotic Protein Bcl-Xl in Micelles. *Biochemistry* **2000,** *39*, 11024-11033.
(51) Malia, T. J.; Wagner, G. NMR Structural Investigation of the Mitochondrial Outer Membrane Protein VDAC and Its Interaction with Antiapoptotic Bcl-Xl. *Biochemistry* **2007,** *46*, 514-525.
(52) Denisov, I. G.; Sligar, S. G. Nanodiscs in Membrane Biochemistry and Biophysics. *Chem. Rev.* **2017,** *117*, 4669-4713.
(53) Chorev, D. S.; Robinson, C. V. The Importance of the Membrane for Biophysical Measurements. *Nat. Chem. Biol.* **2020,** *16*, 1285-1292.
(54) Danmaliki, G. I.; Hwang, P. M. Solution NMR Spectroscopy of Membrane Proteins. *Biochim. Biophys. Acta* **2020,** *1862*, 183356.
(55) Setyawati, I.; Stanek, W. K.; Majsnerowska, M.; Swier, L.; Pardon, E.; Steyaert, J.; Guskov, A.; Slotboom, D. J. In Vitro Reconstitution of Dynamically Interacting Integral Membrane Subunits of Energy-Coupling Factor Transporters. *Elife* **2020,** *9*.
(56) Jeter, V. L.; Escalante-Semerena, J. C. Insights into the Relationship between Cobamide Synthase and the Cell Membrane. *mBio* **2021,** *12*, e00215-00221.
(57) Zhao, H.; Lappalainen, P. A Simple Guide to Biochemical Approaches for Analyzing Protein-Lipid Interactions. *Mol. Biol. Cell* **2012,** *23*, 2823-2830.
(58) Yoneda, J. S.; Sebinelli, H. G.; Itri, R.; Ciancaglini, P. Overview on Solubilization and Lipid Reconstitution of Na,K-Atpase: Enzyme Kinetic and Biophysical Characterization. *Biophys. Rev.* **2020,** *12*, 49-64.
(59) Mclean, M. A.; Gregory, M. C.; Sligar, S. G. Nanodiscs: A Controlled Bilayer Surface for the Study of Membrane Proteins. *Annu. Rev. Biophys.* **2018,** *47*, 107-124.
(60) Lee, S.; Shin, J.; Jung, Y.; Son, H.; Shin, J.; Jeong, C.; Kweon, D. H.; Shin, Y. K. Munc18-1 Induces Conformational Changes of Syntaxin-1 in Multiple Intermediates for Snare Assembly. *Sci. Rep.* **2020,** *10*, 11623.
(61) Murphy, R. E.; Samal, A. B.; Vlach, J.; Mas, V.; Prevelige, P. E.; Saad, J. S. Structural and Biophysical Characterizations of Hiv-1 Matrix Trimer Binding to Lipid Nanodiscs Shed Light on Virus Assembly. *J. Biol. Chem.* **2019,** *294*, 18600-18612.
(62) Voleti, R.; Jaczynska, K.; Rizo, J. Ca(2+)-Dependent Release of Synaptotagmin-1 from the Snare Complex on Phosphatidylinositol 4,5-Bisphosphate-Containing Membranes. *Elife* **2020,** *9*.
(63) Hong, M.; Zhang, Y.; Hu, F. Membrane Protein Structure and Dynamics from NMR Spectroscopy. *Annu. Rev. Phys. Chem.* **2012,** *63*, 1-24.
(64) Andronesi, O. C.; Becker, S.; Seidel, K.; Heise, H.; Young, H. S.; Baldus, M. Determination of Membrane Protein Structure and Dynamics by Magic-Angle-Spinning Solid-State NMR Spectroscopy. *J. Am. Chem. Soc.* **2005,** *127*, 12965-12974.
(65) Ladizhansky, V. Applications of Solid-State NMR to Membrane Proteins. *Biochim. Biophys. Acta* **2017,** *1865*, 1577-1586.
(66) Murray, D. T.; Griffin, J.; Cross, T. A. Detergent Optimized Membrane Protein Reconstitution in Liposomes for Solid State NMR. *Biochemistry* **2014,** *53*, 2454-2463.
(67) Miehling, J.; Goricanec, D.; Hagn, F. A Split-Intein-Based Method for the Efficient Production of Circularized Nanodiscs for Structural Studies of Membrane Proteins. *ChemBioChem* **2018,** *19*, 1927-1933.
(68) Hagn, F.; Nasr, M. L.; Wagner, G. Assembly of Phospholipid Nanodiscs of Controlled Size for Structural Studies of Membrane Proteins by NMR. *Nat. Protoc.* **2018,** *13*, 79-98.

(69) Shah, N. R.; Voisin, T. B.; Parsons, E. S.; Boyd, C. M.; Hoogenboom, B. W.; Bubeck, D. Structural Basis for Tuning Activity and Membrane Specificity of Bacterial Cytolysins. *Nat. Commun.* **2020,** *11*, 5818.
(70) Gao, S.; Yan, N. Structural Basis of the Modulation of the Voltage-Gated Calcium Ion Channel Ca(V) 1.1 by Dihydropyridine Compounds. *Angew. Chem. Int. Ed. Engl.* **2021,** *60*, 3131-3137.
(71) Nosol, K.; Romane, K.; Irobalieva, R. N.; Alam, A.; Kowal, J.; Fujita, N.; Locher, K. P. Cryo-Em Structures Reveal Distinct Mechanisms of Inhibition of the Human Multidrug Transporter Abcb1. *Proc. Natl. Acad. Sci. U. S. A.* **2020,** *117*, 26245-26253.
(72) Shimada, H.; Kusakizako, T.; Dung Nguyen, T. H.; Nishizawa, T.; Hino, T.; Tominaga, M.; Nureki, O. The Structure of Lipid Nanodisc-Reconstituted Trpv3 Reveals the Gating Mechanism. *Nat. Struct. Mol. Biol.* **2020,** *27*, 645-652.
(73) Gao, S.; Valinsky, W. C.; On, N. C.; Houlihan, P. R.; Qu, Q.; Liu, L.; Pan, X.; Clapham, D. E.; Yan, N. Employing Nachbac for Cryo-EM Analysis of Toxin Action on Voltage-Gated Na(+) Channels in Nanodisc. *Proc. Natl. Acad. Sci. U. S. A.* **2020,** *117*, 14187-14193.
(74) Pleiner, T.; Tomaleri, G. P.; Januszyk, K.; Inglis, A. J.; Hazu, M.; Voorhees, R. M. Structural Basis for Membrane Insertion by the Human ER Membrane Protein Complex. *Science* **2020,** *369*, 433-436.
(75) Tan, Y. Z.; Zhang, L.; Rodrigues, J.; Zheng, R. B.; Giacometti, S. I.; Rosário, A. L.; Kloss, B.; Dandey, V. P.; Wei, H.; Brunton, R.*, et al.* Cryo-EM Structures and Regulation of Arabinofuranosyltransferase Aftd from Mycobacteria. *Mol. Cell* **2020,** *78*, 683-699.e611.
(76) Flores, J. A.; Haddad, B. G.; Dolan, K. A.; Myers, J. B.; Yoshioka, C. C.; Copperman, J.; Zuckerman, D. M.; Reichow, S. L. Connexin-46/50 in a Dynamic Lipid Environment Resolved by Cryoem at 1.9 Å. *Nat Commun* **2020,** *11*, 4331.
(77) Zhang, M.; Gui, M.; Wang, Z.-F.; Gorgulla, C.; Yu, J. J.; Wu, H.; Sun, Z.-Y. J.; Klenk, C.; Merklinger, L.; Morstein, L.*, et al.* Cryo-EM Structure of an Activated Gpcr–G Protein Complex in Lipid Nanodiscs. *Nat. Struct. Mol. Biol.* **2021,** *28*, 258-267.
(78) Hagn, F.; Etzkorn, M.; Raschle, T.; Wagner, G. Optimized Phospholipid Bilayer Nanodiscs Facilitate High-Resolution Structure Determination of Membrane Proteins. *J. Am. Chem. Soc.* **2013,** *135*, 1919-1925.
(79) Hagn, F.; Wagner, G. Structure Refinement and Membrane Positioning of Selectively Labeled OmpX in Phospholipid Nanodiscs. *J. Biomol. NMR* **2015,** *61*, 249-260.
(80) Kucharska, I.; Edrington, T. C.; Liang, B.; Tamm, L. K. Optimizing Nanodiscs and Bicelles for Solution NMR Studies of Two Beta-Barrel Membrane Proteins. *J. Biomol. NMR* **2015,** *61*, 261-274.
(81) Puthenveetil, R.; Nguyen, K.; Vinogradova, O. Nanodiscs and Solution NMR: Preparation, Application and Challenges. *Nanotechnol Rev* **2017,** *6*, 111-126.
(82) Kooijman, L.; Ansorge, P.; Schuster, M.; Baumann, C.; Löhr, F.; Jurt, S.; Güntert, P.; Zerbe, O. Backbone and Methyl Assignment of Bacteriorhodopsin Incorporated into Nanodiscs. *J. Biomol. NMR* **2020,** *74*, 45-60.
(83) Dutta, S. K.; Yao, Y.; Marassi, F. M. Structural Insights into Theyersinia Pestisouter Membrane Protein Ail in Lipid Bilayers. *The Journal of Physical Chemistry B* **2017,** *121*, 7561-7570.
(84) Yeagle, P. L., Chapter 4 - Detergents. In *The Membranes of Cells (Third Edition)*, Yeagle, P. L., Ed. Academic Press: Boston, 2016; pp 73-84.
(85) Van Den Brink-Van Der Laan, E.; Antoinette Killian, J.; De Kruijff, B. Nonbilayer Lipids Affect Peripheral and Integral Membrane Proteins Via Changes in the Lateral Pressure Profile. *Biochim. Biophys. Acta* **2004,** *1666*, 275-288.
(86) Chipot, C.; Dehez, F.; Schnell, J. R.; Zitzmann, N.; Pebay-Peyroula, E.; Catoire, L. J.; Miroux, B.; Kunji, E. R. S.; Veglia, G.; Cross, T. A.*, et al.* Perturbations of Native Membrane Protein Structure in Alkyl Phosphocholine Detergents: A Critical Assessment of NMR and Biophysical Studies. *Chem. Rev.* **2018,** *118*, 3559-3607.
(87) Gutmann, D. A.; Mizohata, E.; Newstead, S.; Ferrandon, S.; Postis, V.; Xia, X.; Henderson, P. J.; Van Veen, H. W.; Byrne, B. A High-Throughput Method for Membrane Protein Solubility Screening: The Ultracentrifugation Dispersity Sedimentation Assay. *Protein Sci.* **2007,** *16*, 1422-1428.

(88) Sjöstrand, D.; Diamanti, R.; Lundgren, C. a. K.; Wiseman, B.; Högbom, M. A Rapid Expression and Purification Condition Screening Protocol for Membrane Protein Structural Biology. *Prot. Sci.* **2017,** *26*, 1653-1666.
(89) Le Maire, M.; Champeil, P.; Møller, J. V. Interaction of Membrane Proteins and Lipids with Solubilizing Detergents. *Biochim. Biophys. Acta* **2000,** *1508*, 86-111.
(90) Denisov, I. G.; Grinkova, Y. V.; Lazarides, A. A.; Sligar, S. G. Directed Self-Assembly of Monodisperse Phospholipid Bilayer Nanodiscs with Controlled Size. *J. Am. Chem. Soc.* **2004,** *126*, 3477-3487.
(91) Morgan, C. R.; Hebling, C. M.; Rand, K. D.; Stafford, D. W.; Jorgenson, J. W.; Engen, J. R. Conformational Transitions in the Membrane Scaffold Protein of Phospholipid Bilayer Nanodiscs. *MCP* **2011,** *10*, M111.010876-M010111.010876.
(92) Ma, G.; Wei, M.; He, L.; Liu, C.; Wu, B.; Zhang, S. L.; Jing, J.; Liang, X.; Senes, A.; Tan, P.*, et al.* Inside-out Ca2+ Signalling Prompted by Stim1 Conformational Switch. *Nat. Commun.* **2015,** *6*, 7826.
(93) Shenkarev, Z. O.; Lyukmanova, E. N.; Butenko, I. O.; Petrovskaya, L. E.; Paramonov, A. S.; Shulepko, M. A.; Nekrasova, O. V.; Kirpichnikov, M. P.; Arseniev, A. S. Lipid-Protein Nanodiscs Promote in Vitro Folding of Transmembrane Domains of Multi-Helical and Multimeric Membrane Proteins. *Biochim. Biophys. Acta* **2013,** *1828*, 776-784.
(94) Mcdougle, D. R.; Palaria, A.; Magnetta, E.; Meling, D. D.; Das, A. Functional Studies of N-Terminally Modified Cyp2j2 Epoxygenase in Model Lipid Bilayers. *Protein Sci.* **2013,** *22*, 964-979.
(95) Raschle, T.; Hiller, S.; Yu, T.-Y.; Rice, A. J.; Walz, T.; Wagner, G. Structural and Functional Characterization of the Integral Membrane Protein VDAC-1 in Lipid Bilayer Nanodiscs. *J. Am. Chem. Soc.* **2009,** *131*, 17777-17779.
(96) Eggensperger, S.; Fisette, O.; Parcej, D.; Schäfer, L. V.; Tampé, R. An Annular Lipid Belt Is Essential for Allosteric Coupling and Viral Inhibition of the Antigen Translocation Complex Tap (Transporter Associated with Antigen Processing). *J. Biol. Chem.* **2014,** *289*, 33098-33108.
(97) Gawrisch, K.; Soubias, O.; Mihailescu, M. Insights from Biophysical Studies on the Role of Polyunsaturated Fatty Acids for Function of G-Protein Coupled Membrane Receptors. *Prostaglandins Leukot. Essent. Fatty Acids* **2008,** *79*, 131-134.
(98) Dalal, K.; Duong, F. Reconstitution of the Secy Translocon in Nanodiscs. *Methods Mol. Biol.* **2010,** *619*, 145-156.
(99) Goricanec, D.; Stehle, R.; Egloff, P.; Grigoriu, S.; Plückthun, A.; Wagner, G.; Hagn, F. Conformational Dynamics of a G-Protein A Subunit Is Tightly Regulated by Nucleotide Binding. *Proc. Natl. Acad. Sci. U. S. A.* **2016,** *113*, E3629-3638.
(100) Bayburt, T. H.; Leitz, A. J.; Xie, G.; Oprian, D. D.; Sligar, S. G. Transducin Activation by Nanoscale Lipid Bilayers Containing One and Two Rhodopsins. *J. Biol. Chem.* **2007,** *282*, 14875-14881.
(101) Kragh-Hansen, U.; Le Maire, M.; Møller, J. V. The Mechanism of Detergent Solubilization of Liposomes and Protein-Containing Membranes. *Biophys. J.* **1998,** *75*, 2932-2946.
(102) Schuck, S.; Honsho, M.; Ekroos, K.; Shevchenko, A.; Simons, K. Resistance of Cell Membranes to Different Detergents. *Proc. Natl. Acad. Sci. U. S. A.* **2003,** *100*, 5795-5800.
(103) London, E.; Brown, D. A. Insolubility of Lipids in Triton X-100: Physical Origin and Relationship to Sphingolipid/Cholesterol Membrane Domains (Rafts). *Biochim. Biophys. Acta* **2000,** *1508*, 182-195.
(104) Filip, C.; Fletcher, G.; Wulff, J. L.; Earhart, C. F. Solubilization of the Cytoplasmic Membrane of *Escherichia Coli* by the Ionic Detergent Sodium-Lauryl Sarcosinate. *J. Bacteriol.* **1973,** *115*, 717-722.
(105) Schnaitman, C. A. Solubilization of the Cytoplasmic Membrane of *Escherichia Coli* by Triton X-100. *J. Bacteriol.* **1971,** *108*, 545-552.
(106) Anandan, A.; Vrielink, A. Detergents in Membrane Protein Purification and Crystallisation. *Adv. Exp. Med. Biol.* **2016,** *922*, 13-28.
(107) Sonoda, Y.; Newstead, S.; Hu, N. J.; Alguel, Y.; Nji, E.; Beis, K.; Yashiro, S.; Lee, C.; Leung, J.; Cameron, A. D.*, et al.* Benchmarking Membrane Protein Detergent Stability for Improving Throughput of High-Resolution X-Ray Structures. *Structure* **2011,** *19*, 17-25.

(108) Zhou, H. X.; Cross, T. A. Influences of Membrane Mimetic Environments on Membrane Protein Structures. *Annu. Rev. Biophys.* **2013,** *42*, 361-392.
(109) Jones, M. B.; Garrison, J. C. Instability of the G-Protein β5 subunit in Detergent. *Anal. Biochem.* **1999,** *268*, 126-133.
(110) Infed, N.; Hanekop, N.; Driessen, A. J. M.; Smits, S. H. J.; Schmitt, L. Influence of Detergents on the Activity of the Abc Transporter Lmra. *Biochim. Biophys. Acta* **2011,** *1808*, 2313-2321.
(111) Lund, S.; Moller, J. V. Biphasic Kinetics of Sarcoplasmic Reticulum Ca2+-ATPase and the Detergent-Solubilized Monomer. *J. Biol. Chem.* **1988,** *263*, 1654-1664.
(112) Lavington, S.; Watts, A. Lipid Nanoparticle Technologies for the Study of G Protein-Coupled Receptors in Lipid Environments. *Biophys. Rev.* **2020,** *12*, 1287-1302.
(113) Sanders, C. R.; Landis, G. C. Reconstitution of Membrane Proteins into Lipid-Rich Bilayered Mixed Micelles for NMR Studies. *Biochemistry* **1995,** *34*, 4030-4040.
(114) Sanders, C. R.; Prosser, R. S. Bicelles: A Model Membrane System for All Seasons? *Structure* **1998,** *6*, 1227-1234.
(115) Dürr, U. H.; Gildenberg, M.; Ramamoorthy, A. The Magic of Bicelles Lights up Membrane Protein Structure. *Chem. Rev.* **2012,** *112*, 6054-6074.
(116) Piai, A.; Fu, Q.; Dev, J.; Chou, J. J. Optimal Bicelle Size q for Solution NMR Studies of the Protein Transmembrane Partition. *Chemistry* **2017,** *23*, 1361-1367.
(117) Bayburt, T. H.; Grinkova, Y. V.; Sligar, S. G. Self-Assembly of Discoidal Phospholipid Bilayer Nanoparticles with Membrane Scaffold Proteins. *Nano Lett.* **2002,** *2*, 853-856.
(118) Civjan, N. R.; Bayburt, T. H.; Schuler, M. A.; Sligar, S. G. Direct Solubilization of Heterologously Expressed Membrane Proteins by Incorporation into Nanoscale Lipid Bilayers. *BioTechniques* **2003,** *35*, 556-560, 562-553.
(119) Chen, A.; Majdinasab, E. J.; Fiori, M. C.; Liang, H.; Altenberg, G. A. Polymer-Encased Nanodiscs and Polymer Nanodiscs: New Platforms for Membrane Protein Research and Applications. *Front. Bioeng. Biotechnol.* **2020,** *8*, 598450.
(120) Sligar, S. G.; Denisov, I. G. Nanodiscs: A Toolkit for Membrane Protein Science. *Protein Sci.* **2021,** *30*, 297-315.
(121) Nasr, M. L. Large Nanodiscs Going Viral. *Curr. Opin. Struct. Biol.* **2020,** *60*, 150-156.
(122) Yokogawa, M.; Fukuda, M.; Osawa, M. Nanodiscs for Structural Biology in a Membranous Environment. *Chem. Pharm. Bull. (Tokyo)* **2019,** *67*, 321-326.
(123) Rouck, J. E.; Krapf, J. E.; Roy, J.; Huff, H. C.; Das, A. Recent Advances in Nanodisc Technology for Membrane Protein Studies (2012-2017). *FEBS Lett.* **2017,** *591*, 2057-2088.
(124) Ravula, T.; Hardin, N. Z.; Ramamoorthy, A. Polymer Nanodiscs: Advantages and Limitations. *Chem. Phys. Lipids* **2019,** *219*, 45-49.
(125) Brouillette, C. G.; Anantharamaiah, G. M.; Engler, J. A.; Borhani, D. W. Structural Models of Human Apolipoprotein a-I: A Critical Analysis and Review. *Biochim. Biophys. Acta* **2001,** *1531*, 4-46.
(126) Jonas, A. Reconstitution of High-Density Lipoproteins. *Methods Enzymol.* **1986,** *128*, 553-582.
(127) Nath, A.; Atkins, W. M.; Sligar, S. G. Applications of Phospholipid Bilayer Nanodiscs in the Study of Membranes and Membrane Proteins. *Biochemistry* **2007,** *46*, 2059-2069.
(128) Puthenveetil, R.; Vinogradova, O. Optimization of the Design and Preparation of Nanoscale Phospholipid Bilayers for Its Application to Solution NMR. *Proteins* **2013,** *81*, 1222-1231.
(129) Bibow, S.; Polyhach, Y.; Eichmann, C.; Chi, C. N.; Kowal, J.; Albiez, S.; Mcleod, R. A.; Stahlberg, H.; Jeschke, G.; Güntert, P.*, et al.* Solution Structure of Discoidal High-Density Lipoprotein Particles with a Shortened Apolipoprotein a-I. *Nat. Struct. Mol. Biol.* **2017,** *24*, 187-193.
(130) Ding, Y.; Fujimoto, L. M.; Yao, Y.; Plano, G. V.; Marassi, F. M. Influence of the Lipid Membrane Environment on Structure and Activity of the Outer Membrane Protein Ail from Yersinia Pestis. *Biochim. Biophys. Acta* **2015,** *1848*, 712-720.
(131) Singh, C.; Lee, H.; Tian, Y.; Schesser Bartra, S.; Hower, S.; Fujimoto, L. M.; Yao, Y.; Ivanov, S. A.; Shaikhutdinova, R. Z.; Anisimov, A. P.*, et al.* Mutually Constructive Roles of Ail and Lps in Yersinia Pestis Serum Survival. *Mol. Microbiol.* **2020,** *114*, 510-520.

(132) Nasr, M. L.; Baptista, D.; Strauss, M.; Sun, Z. J.; Grigoriu, S.; Huser, S.; Pluckthun, A.; Hagn, F.; Walz, T.; Hogle, J. M.*, et al.* Covalently Circularized Nanodiscs for Studying Membrane Proteins and Viral Entry. *Nat. Methods* **2017,** *14*, 49-52.
(133) Antos, J. M.; Popp, M. W.; Ernst, R.; Chew, G. L.; Spooner, E.; Ploegh, H. L. A Straight Path to Circular Proteins. *J. Biol. Chem.* **2009,** *284*, 16028-16036.
(134) Yusuf, Y.; Massiot, J.; Chang, Y.-T.; Wu, P.-H.; Yeh, V.; Kuo, P.-C.; Shiue, J.; Yu, T.-Y. Optimization of the Production of Covalently Circularized Nanodiscs and Their Characterization in Physiological Conditions. *Langmuir* **2018,** *34*, 3525-3532.
(135) Johansen, N. T.; Tidemand, F. G.; Nguyen, T. T. T. N.; Rand, K. D.; Pedersen, M. C.; Arleth, L. Circularized and Solubility-Enhanced Msps Facilitate Simple and High-Yield Production of Stable Nanodiscs for Studies of Membrane Proteins in Solution. *FEBS J.* **2019,** *286*, 1734-1751.
(136) Li, J.; Zhang, Y.; Soubias, O.; Khago, D.; Chao, F. A.; Li, Y.; Shaw, K.; Byrd, R. A. Optimization of Sortase a Ligation for Flexible Engineering of Complex Protein Systems. *J. Biol. Chem.* **2020,** *295*, 2664-2675.
(137) Ramirez, M.; Valdes, N.; Guan, D.; Chen, Z. Engineering Split Intein Dnae from Nostoc Punctiforme for Rapid Protein Purification. *Protein Eng. Des. Sel.* **2013,** *26*, 215-223.
(138) Bayburt, T. H.; Sligar, S. G. Membrane Protein Assembly into Nanodiscs. *FEBS Lett.* **2010,** *584*, 1721-1727.
(139) Camp, T.; Sligar, S. G. Nanodisc Self-Assembly Is Thermodynamically Reversible and Controllable. *Soft Matter* **2020,** *16*, 5615-5623.
(140) Catte, A.; Patterson, J. C.; Jones, M. K.; Gray Jerome, W.; Bashtovyy, D.; Su, Z.; Gu, F.; Chen, J.; Aliste, M. P.; Harvey, S. C.*, et al.* Novel Changes in Discoidal High Density Lipoprotein Morphology: A Molecular Dynamics Study. *Biophys. J.* **2006,** *90*, 4345-4360.
(141) Shih, A. Y.; Denisov, I. G.; Phillips, J. C.; Sligar, S. G.; Schulten, K. Molecular Dynamics Simulations of Discoidal Bilayers Assembled from Truncated Human Lipoproteins. *Biophys. J.* **2005,** *88*, 548-556.
(142) Miyazaki, M.; Nakano, M.; Fukuda, M.; Handa, T. Smaller Discoidal High-Density Lipoprotein Particles Form Saddle Surfaces, but Not Planar Bilayers. *Biochemistry* **2009,** *48*, 7756-7763.
(143) Li, M. J.; Atkins, W. M.; Mcclary, W. D. Preparation of Lipid Nanodiscs with Lipid Mixtures. *Curr. Protoc. Protein Sci.* **2019,** *98*, e100.
(144) Finkenwirth, F.; Sippach, M.; Landmesser, H.; Kirsch, F.; Ogienko, A.; Grunzel, M.; Kiesler, C.; Steinhoff, H. J.; Schneider, E.; Eitinger, T. Atp-Dependent Conformational Changes Trigger Substrate Capture and Release by an Ecf-Type Biotin Transporter. *J. Biol. Chem.* **2015,** *290*, 16929-16942.
(145) Hörnschemeyer, P.; Liss, V.; Heermann, R.; Jung, K.; Hunke, S. Interaction Analysis of a Two-Component System Using Nanodiscs. *PLoS One* **2016,** *11*, e0149187.
(146) Dominik, P. K.; Borowska, M. T.; Dalmas, O.; Kim, S. S.; Perozo, E.; Keenan, R. J.; Kossiakoff, A. A. Conformational Chaperones for Structural Studies of Membrane Proteins Using Antibody Phage Display with Nanodiscs. *Structure* **2016,** *24*, 300-309.
(147) Mi, L. Z.; Grey, M. J.; Nishida, N.; Walz, T.; Lu, C.; Springer, T. A. Functional and Structural Stability of the Epidermal Growth Factor Receptor in Detergent Micelles and Phospholipid Nanodiscs. *Biochemistry* **2008,** *47*, 10314-10323.
(148) Bajaj, R.; Bruce, K. E.; Davidson, A. L.; Rued, B. E.; Stauffacher, C. V.; Winkler, M. E. Biochemical Characterization of Essential Cell Division Proteins Ftsx and Ftse That Mediate Peptidoglycan Hydrolysis by Pcsb in Streptococcus Pneumoniae. *MicrobiologyOpen* **2016,** *5*, 738-752.
(149) Mitra, N.; Liu, Y.; Liu, J.; Serebryany, E.; Mooney, V.; Devree, B. T.; Sunahara, R. K.; Yan, E. C. Y. Calcium-Dependent Ligand Binding and G-Protein Signaling of Family B Gpcr Parathyroid Hormone 1 Receptor Purified in Nanodiscs. *ACS Chem. Biol.* **2013,** *8*, 617-625.
(150) Shirzad-Wasei, N.; Van Oostrum, J.; Bovee-Geurts, P. H.; Kusters, L. J.; Bosman, G. J.; Degrip, W. J. Rapid Transfer of Overexpressed Integral Membrane Protein from the Host Membrane into Soluble Lipid Nanodiscs without Previous Purification. *Biol. Chem.* **2015,** *396*, 903-915.
(151) Gregersen, J. L.; Fedosova, N. U.; Nissen, P.; Boesen, T. Reconstitution of Na(+),K(+)-Atpase in Nanodiscs. *Methods Mol. Biol.* **2016,** *1377*, 403-409.

(152) Marty, M. T.; Wilcox, K. C.; Klein, W. L.; Sligar, S. G. Nanodisc-Solubilized Membrane Protein Library Reflects the Membrane Proteome. *Anal. Bioanal. Chem.* **2013,** *405*, 4009-4016.
(153) Wilcox, K. C.; Marunde, M. R.; Das, A.; Velasco, P. T.; Kuhns, B. D.; Marty, M. T.; Jiang, H.; Luan, C.-H.; Sligar, S. G.; Klein, W. L. Nanoscale Synaptic Membrane Mimetic Allows Unbiased High Throughput Screen That Targets Binding Sites for Alzheimer's-Associated Aβ Oligomers. *PLoS One* **2015,** *10*, e0125263.
(154) Bocquet, N.; Kohler, J.; Hug, M. N.; Kusznir, E. A.; Rufer, A. C.; Dawson, R. J.; Hennig, M.; Ruf, A.; Huber, W.; Huber, S. Real-Time Monitoring of Binding Events on a Thermostabilized Human A2a Receptor Embedded in a Lipid Bilayer by Surface Plasmon Resonance. *Biochim. Biophys. Acta* **2015,** *1848*, 1224-1233.
(155) Kucerka, N.; Liu, Y.; Chu, N.; Petrache, H. I.; Tristram-Nagle, S.; Nagle, J. F. Structure of Fully Hydrated Fluid Phase Dmpc and Dlpc Lipid Bilayers Using X-Ray Scattering from Oriented Multilamellar Arrays and from Unilamellar Vesicles. *Biophys. J.* **2005,** *88*, 2626-2637.
(156) Ritchie, T. K.; Grinkova, Y. V.; Bayburt, T. H.; Denisov, I. G.; Zolnerciks, J. K.; Atkins, W. M.; Sligar, S. G. Chapter 11 - Reconstitution of Membrane Proteins in Phospholipid Bilayer Nanodiscs. *Methods Enzymol.* **2009,** *464*, 211-231.
(157) Kučerka, N.; Tristram-Nagle, S.; Nagle, J. F. Structure of Fully Hydrated Fluid Phase Lipid Bilayers with Monounsaturated Chains. *J. Membr. Biol.* **2006,** *208*, 193-202.
(158) Pan, J.; Heberle, F. A.; Tristram-Nagle, S.; Szymanski, M.; Koepfinger, M.; Katsaras, J.; Kučerka, N. Molecular Structures of Fluid Phase Phosphatidylglycerol Bilayers as Determined by Small Angle Neutron and X-Ray Scattering. *Biochim. Biophys. Acta* **2012,** *1818*, 2135-2148.
(159) Jämbeck, J. P. M.; Lyubartsev, A. P. An Extension and Further Validation of an All-Atomistic Force Field for Biological Membranes. *J. Chem. Theory Comput.* **2012,** *8*, 2938-2948.
(160) Petrache, H. I.; Tristram-Nagle, S.; Gawrisch, K.; Harries, D.; Parsegian, V. A.; Nagle, J. F. Structure and Fluctuations of Charged Phosphatidylserine Bilayers in the Absence of Salt. *Biophys. J.* **2004,** *86*, 1574-1586.
(161) Pan, J.; Cheng, X.; Monticelli, L.; Heberle, F. A.; Kučerka, N.; Tieleman, D. P.; Katsaras, J. The Molecular Structure of a Phosphatidylserine Bilayer Determined by Scattering and Molecular Dynamics Simulations. *Soft Matter* **2014,** *10*, 3716-3725.
(162) Kulig, W.; Korolainen, H.; Zatorska, M.; Kwolek, U.; Wydro, P.; Kepczynski, M.; Róg, T. Complex Behavior of Phosphatidylcholine–Phosphatidic Acid Bilayers and Monolayers: Effect of Acyl Chain Unsaturation. *Langmuir* **2019,** *35*, 5944-5956.
(163) Rues, R. B.; Dötsch, V.; Bernhard, F. Co-Translational Formation and Pharmacological Characterization of Beta1-Adrenergic Receptor/Nanodisc Complexes with Different Lipid Environments. *Biochim. Biophys. Acta* **2016,** *1858*, 1306-1316.
(164) Webb, R. J.; East, J. M.; Sharma, R. P.; Lee, A. G. Hydrophobic Mismatch and the Incorporation of Peptides into Lipid Bilayers: A Possible Mechanism for Retention in the Golgi. *Biochemistry* **1998,** *37*, 673-679.
(165) De Planque, M. R. R.; Killian, J. A. Protein–Lipid Interactions Studied with Designed Transmembrane Peptides: Role of Hydrophobic Matching and Interfacial Anchoring (Review). *Mol. Membr. Biol.* **2003,** *20*, 271-284.
(166) Killian, J. A. Hydrophobic Mismatch between Proteins and Lipids in Membranes. *Reviews on biomembranes, BBA* **1998,** *1376*, 401-416.
(167) Prabudiansyah, I.; Van Der Valk, R.; Aubin-Tam, M.-E. Reconstitution and Functional Characterization of the Ftsh Protease in Lipid Nanodiscs. *Biochim. Biophys. Acta* **2021,** *1863*, 183526.
(168) Denisov, I. G.; Mclean, M. A.; Shaw, A. W.; Grinkova, Y. V.; Sligar, S. G. Thermotropic Phase Transition in Soluble Nanoscale Lipid Bilayers. *J. Phys. Chem. B* **2005,** *109*, 15580-15588.
(169) Shaw, A. W.; Mclean, M. A.; Sligar, S. G. Phospholipid Phase Transitions in Homogeneous Nanometer Scale Bilayer Discs. *FEBS Lett.* **2004,** *556*, 260-264.
(170) Stepien, P.; Augustyn, B.; Poojari, C.; Galan, W.; Polit, A.; Vattulainen, I.; Wisnieska-Becker, A.; Rog, T. Complexity of Seemingly Simple Lipid Nanodiscs. *Biochim. Biophys. Acta* **2020,** *1862*, 183420.
(171) Schachter, I.; Allolio, C.; Khelashvili, G.; Harries, D. Confinement in Nanodiscs Anisotropically Modifies Lipid Bilayer Elastic Properties. *J. Phys. Chem. B* **2020,** *124*, 7166-7175.

(172) Bengtsen, T.; Holm, V. L.; Kjolbye, L. R.; Midtgaard, S. R.; Johansen, N. T.; Tesei, G.; Bottaro, S.; Schiott, B.; Arleth, L.; Lindorff-Larsen, K. Structure and Dynamics of a Nanodisc by Integrating NMR, Saxs and Sans Experiments with Molecular Dynamics Simulations. *Elife* **2020,** *9*.
(173) Johansen, N. T.; Luchini, A.; Tidemand, F. G.; Orioli, S.; Martel, A.; Porcar, L.; Arleth, L.; Pedersen, M. C. Structural and Biophysical Properties of Supercharged and Circularized Nanodiscs. *Langmuir* **2021**.
(174) Knowles, T. J.; Finka, R.; Smith, C.; Lin, Y.-P.; Dafforn, T.; Overduin, M. Membrane Proteins Solubilized Intact in Lipid Containing Nanoparticles Bounded by Styrene Maleic Acid Copolymer. *J. Am. Chem. Soc.* **2009,** *131*, 7484-7485.
(175) Orwick, M. C.; Judge, P. J.; Procek, J.; Lindholm, L.; Graziadei, A.; Engel, A.; Gröbner, G.; Watts, A. Detergent-Free Formation and Physicochemical Characterization of Nanosized Lipid–Polymer Complexes: Lipodisq. *Angew. Chem. Int. Ed.* **2012,** *51*, 4653-4657.
(176) Jamshad, M.; Charlton, J.; Lin, Y.-P.; Routledge, Sarah j.; Bawa, Z.; Knowles, Timothy j.; Overduin, M.; Dekker, N.; Dafforn, Tim r.; Bill, Roslyn m.*, et al.* G-Protein Coupled Receptor Solubilization and Purification for Biophysical Analysis and Functional Studies, in the Total Absence of Detergent. *Biosci. Rep.* **2015,** *35*.
(177) Lee, S. C.; Knowles, T. J.; Postis, V. L. G.; Jamshad, M.; Parslow, R. A.; Lin, Y.-P.; Goldman, A.; Sridhar, P.; Overduin, M.; Muench, S. P.*, et al.* A Method for Detergent-Free Isolation of Membrane Proteins in Their Local Lipid Environment. *Nat. Protoc.* **2016,** *11*, 1149-1162.
(178) Dörr, J. M.; Scheidelaar, S.; Koorengevel, M. C.; Dominguez, J. J.; Schäfer, M.; Van Walree, C. A.; Killian, J. A. The Styrene–Maleic Acid Copolymer: A Versatile Tool in Membrane Research. *Eur. Biophys. J.* **2016,** *45*, 3-21.
(179) Scheidelaar, S.; Koorengevel, M. C.; Pardo, J. D.; Meeldijk, J. D.; Breukink, E.; Killian, J. A. Molecular Model for the Solubilization of Membranes into Nanodisks by Styrene Maleic Acid Copolymers. *Biophys. J.* **2015,** *108*, 279-290.
(180) Jamshad, M.; Grimard, V.; Idini, I.; Knowles, T. J.; Dowle, M. R.; Schofield, N.; Sridhar, P.; Lin, Y.; Finka, R.; Wheatley, M.*, et al.* Structural Analysis of a Nanoparticle Containing a Lipid Bilayer Used for Detergent-Free Extraction of Membrane Proteins. *Nano Research* **2015,** *8*, 774-789.
(181) Stroud, Z.; Hall, S. C. L.; Dafforn, T. R. Purification of Membrane Proteins Free from Conventional Detergents: Sma, New Polymers, New Opportunities and New Insights. *Methods* **2018,** *147*, 106-117.
(182) Morrison, K. A.; Akram, A.; Mathews, A.; Khan, Z. A.; Patel, J. H.; Zhou, C.; Hardy, D. J.; Moore-Kelly, C.; Patel, R.; Odiba, V.*, et al.* Membrane Protein Extraction and Purification Using Styrene–Maleic Acid (Sma) Copolymer: Effect of Variations in Polymer Structure. *Biochem. J* **2016,** *473*, 4349-4360.
(183) Overduin, M.; Esmaili, M. Memtein: The Fundamental Unit of Membrane-Protein Structure and Function. *Chem. Phys. Lipids* **2019,** *218*, 73-84.
(184) Cunningham, R. D.; Kopf, A. H.; Elenbaas, B. O. W.; Staal, B. B. P.; Pfukwa, R.; Killian, J. A.; Klumperman, B. Iterative Raft-Mediated Copolymerization of Styrene and Maleic Anhydride toward Sequence- and Length-Controlled Copolymers and Their Applications for Solubilizing Lipid Membranes. *Biomacromolecules* **2020,** *21*, 3287-3300.
(185) Ravula, T.; Hardin, N. Z.; Ramadugu, S. K.; Cox, S. J.; Ramamoorthy, A. Formation of Ph-Resistant Monodispersed Polymer-Lipid Nanodiscs. *Angew. Chem. Int. Ed. Engl.* **2018,** *57*, 1342-1345.
(186) Ravula, T.; Hardin, N. Z.; Ramadugu, S. K.; Ramamoorthy, A. Ph Tunable and Divalent Metal Ion Tolerant Polymer Lipid Nanodiscs. *Langmuir* **2017,** *33*, 10655-10662.
(187) Hall, S. C. L.; Tognoloni, C.; Charlton, J.; Bragginton, É. C.; Rothnie, A. J.; Sridhar, P.; Wheatley, M.; Knowles, T. J.; Arnold, T.; Edler, K. J.*, et al.* An Acid-Compatible Co-Polymer for the Solubilization of Membranes and Proteins into Lipid Bilayer-Containing Nanoparticles. *Nanoscale* **2018,** *10*, 10609-10619.
(188) Oluwole, A. O.; Danielczak, B.; Meister, A.; Babalola, J. O.; Vargas, C.; Keller, S. Solubilization of Membrane Proteins into Functional Lipid-Bilayer Nanodiscs Using a Diisobutylene/Maleic Acid Copolymer. *Angew. Chem. Int. Ed.* **2017,** *56*, 1919-1924.

(189) Lindhoud, S.; Carvalho, V.; Pronk, J. W.; Aubin-Tam, M.-E. Sma-Sh: Modified Styrene–Maleic Acid Copolymer for Functionalization of Lipid Nanodiscs. *Biomacromolecules* **2016,** *17*, 1516-1522.
(190) Ravula, T.; Ramamoorthy, A. Synthesis, Characterization, and Nanodisc Formation of Non-Ionic Polymers. *Angew. Chem. Int. Ed.* **2021**.
(191) Ravula, T.; Ramadugu, S. K.; Di Mauro, G.; Ramamoorthy, A. Bioinspired, Size-Tunable Self-Assembly of Polymer-Lipid Bilayer Nanodiscs. *Angew. Chem. Int. Ed.* **2017,** *56*, 11466-11470.
(192) Brown, C. J.; Trieber, C.; Overduin, M. Structural Biology of Endogenous Membrane Protein Assemblies in Native Nanodiscs. *Curr. Opin. Struct. Biol.* **2021,** *69*, 70-77.
(193) Sun, C.; Benlekbir, S.; Venkatakrishnan, P.; Wang, Y.; Hong, S.; Hosler, J.; Tajkhorshid, E.; Rubinstein, J. L.; Gennis, R. B. Structure of the Alternative Complex Iii in a Supercomplex with Cytochrome Oxidase. *Nature* **2018,** *557*, 123-126.
(194) Qiu, W.; Fu, Z.; Xu, G. G.; Grassucci, R. A.; Zhang, Y.; Frank, J.; Hendrickson, W. A.; Guo, Y. Structure and Activity of Lipid Bilayer within a Membrane-Protein Transporter. *Proc. Natl. Acad. Sci. U. S. A.* **2018,** *115*, 12985.
(195) Yoder, N.; Gouaux, E. The His-Gly Motif of Acid-Sensing Ion Channels Resides in a Reentrant 'Loop' Implicated in Gating and Ion Selectivity. *eLife* **2020,** *9*, e56527.
(196) Yu, J.; Zhu, H.; Lape, R.; Greiner, T.; Du, J.; Lü, W.; Sivilotti, L.; Gouaux, E. Mechanism of Gating and Partial Agonist Action in the Glycine Receptor. *Cell* **2021,** *184*, 957-968.e921.
(197) Tascón, I.; Sousa, J. S.; Corey, R. A.; Mills, D. J.; Griwatz, D.; Aumüller, N.; Mikusevic, V.; Stansfeld, P. J.; Vonck, J.; Hänelt, I. Structural Basis of Proton-Coupled Potassium Transport in the Kup Family. *Nat. Commun.* **2020,** *11*, 626.
(198) Gulati, S.; Jamshad, M.; Knowles, T. J.; Morrison, K. A.; Downing, R.; Cant, N.; Collins, R.; Koenderink, J. B.; Ford, R. C.; Overduin, M.*, et al.* Detergent-Free Purification of Abc (Atp-Binding-Cassette) Transporters. *Biochem. J* **2014,** *461*, 269-278.
(199) Smith, A. a. A.; Autzen, H. E.; Faust, B.; Mann, J. L.; Muir, B. W.; Howard, S.; Postma, A.; Spakowitz, A. J.; Cheng, Y.; Appel, E. A. Lipid Nanodiscs Via Ordered Copolymers. *Chem* **2020,** *6*, 2782-2795.
(200) Voskoboynikova, N.; Karlova, M.; Kurre, R.; Mulkidjanian, A. Y.; Shaitan, K. V.; Sokolova, O. S.; Steinhoff, H.-J.; Heinisch, J. J. A Three-Dimensional Model of the Yeast Transmembrane Sensor Wsc1 Obtained by Sma-Based Detergent-Free Purification and Transmission Electron Microscopy. *J. Fungi* **2021,** *7*.
(201) Flegler, V. J.; Rasmussen, A.; Rao, S.; Wu, N.; Zenobi, R.; Sansom, M. S. P.; Hedrich, R.; Rasmussen, T.; Böttcher, B. The Mscs-Like Channel Ynai Has a Gating Mechanism Based on Flexible Pore Helices. *Proc. Natl. Acad. Sci. U.S.A.* **2020,** *117*, 28754.
(202) Lee, S. C.; Collins, R.; Lin, Y.-P.; Jamshad, M.; Broughton, C.; Harris, S. A.; Hanson, B. S.; Tognoloni, C.; Parslow, R. A.; Terry, A. E.*, et al.* Nano-Encapsulated Escherichia Coli Divisome Anchor Zipa, and in Complex with Ftsz. *Sci. Rep.* **2019,** *9*, 18712.
(203) Radoicic, J.; Park, S. H.; Opella, S. J. Macrodiscs Comprising Smalps for Oriented Sample Solid-State NMR Spectroscopy of Membrane Proteins. *Biophys. J.* **2018,** *115*, 22-25.
(204) Bersch, B.; Dörr, J. M.; Hessel, A.; Killian, J. A.; Schanda, P. Proton-Detected Solid-State NMR Spectroscopy of a Zinc Diffusion Facilitator Protein in Native Nanodiscs. *Angew. Chem. Int. Ed. Engl.* **2017,** *56*, 2508-2512.
(205) Broecker, J.; Eger, B. T.; Ernst, O. P. Crystallogenesis of Membrane Proteins Mediated by Polymer-Bounded Lipid Nanodiscs. *Structure* **2017,** *25*, 384-392.
(206) Kolter, T.; Sandhoff, K. Lysosomal Degradation of Membrane Lipids. *FEBS Lett.* **2010,** *584*, 1700-1712.
(207) Ahn, V. E.; Leyko, P.; Alattia, J.-R.; Chen, L.; Privé, G. G. Crystal Structures of Saposins a and C. *Protein Sci.* **2006,** *15*, 1849-1857.
(208) Ahn, V. E.; Faull, K. F.; Whitelegge, J. P.; Fluharty, A. L.; Privé, G. G. Crystal Structure of Saposin B Reveals a Dimeric Shell for Lipid Binding. *Proc. Natl. Acad. Sci. U. S. A.* **2003,** *100*, 38-43.
(209) Rossmann, M.; Schultz-Heienbrok, R.; Behlke, J.; Remmel, N.; Alings, C.; Sandhoff, K.; Saenger, W.; Maier, T. Crystal Structures of Human Saposins C and D: Implications for Lipid Recognition and Membrane Interactions. *Structure* **2008,** *16*, 809-817.

(210) Popovic, K.; Holyoake, J.; Pomes, R.; Prive, G. G. Structure of Saposin a Lipoprotein Discs. *Proc. Natl. Acad. Sci. U.S.A.* **2012,** *109*, 2908-2912.
(211) Flayhan, A.; Mertens, H. D. T.; Ural-Blimke, Y.; Martinez Molledo, M.; Svergun, D. I.; Löw, C. Saposin Lipid Nanoparticles: A Highly Versatile and Modular Tool for Membrane Protein Research. *Structure* **2018,** *26*, 345-355.e345.
(212) Chien, C.-T. H.; Helfinger, L. R.; Bostock, M. J.; Solt, A.; Tan, Y. L.; Nietlispach, D. An Adaptable Phospholipid Membrane Mimetic System for Solution NMR Studies of Membrane Proteins. *J. Am. Chem. Soc.* **2017,** *139*, 14829-14832.
(213) Frauenfeld, J.; Löving, R.; Armache, J.-P.; Sonnen, A. F.-P.; Guettou, F.; Moberg, P.; Zhu, L.; Jegerschöld, C.; Flayhan, A.; Briggs, J. a. G.*, et al.* A Saposin-Lipoprotein Nanoparticle System for Membrane Proteins. *Nat. Methods* **2016,** *13*, 345-351.
(214) Kurgan, K. W.; Chen, B.; Brown, K. A.; Falco Cobra, P.; Ye, X.; Ge, Y.; Gellman, S. H. Stable Picodisc Assemblies from Saposin Proteins and Branched Detergents. *Biochemistry* **2021,** *60*, 1108-1119.
(215) Li, J.; Richards, M. R.; Bagal, D.; Campuzano, I. D. G.; Kitova, E. N.; Xiong, Z. J.; Privé, G. G.; Klassen, J. S. Characterizing the Size and Composition of Saposin a Lipoprotein Picodiscs. *Anal. Chem.* **2016,** *88*, 9524-9531.
(216) Kintzer, A. F.; Green, E. M.; Dominik, P. K.; Bridges, M.; Armache, J.-P.; Deneka, D.; Kim, S. S.; Hubbell, W.; Kossiakoff, A. A.; Cheng, Y.*, et al.* Structural Basis for Activation of Voltage Sensor Domains in an Ion Channel Tpc1. *Proc. Natl. Acad. Sci. U.S.A.* **2018,** *115*, E9095.
(217) Nguyen, N. X.; Armache, J.-P.; Lee, C.; Yang, Y.; Zeng, W.; Mootha, V. K.; Cheng, Y.; Bai, X.-C.; Jiang, Y. Cryo-Em Structure of a Fungal Mitochondrial Calcium Uniporter. *Nature* **2018,** *559*, 570-574.
(218) Du, D.; Neuberger, A.; Orr, M. W.; Newman, C. E.; Hsu, P. C.; Samsudin, F.; Szewczak-Harris, A.; Ramos, L. M.; Debela, M.; Khalid, S.*, et al.* Interactions of a Bacterial Rnd Transporter with a Transmembrane Small Protein in a Lipid Environment. *Structure* **2020,** *28*, 625-634 e626.
(219) Lyons, J. A.; Bøggild, A.; Nissen, P.; Frauenfeld, J., Chapter Three - Saposin-Lipoprotein Scaffolds for Structure Determination of Membrane Transporters. In *Methods Enzymol.*, Ziegler, C., Ed. Academic Press: 2017; Vol. 594, pp 85-99.
(220) Lasitza-Male, T.; Bartels, K.; Jungwirth, J.; Wiggers, F.; Rosenblum, G.; Hofmann, H.; Löw, C. Membrane Chemistry Tunes the Structure of a Peptide Transporter. *Angew. Chem. Int. Ed.* **2020,** *59*, 19121-19128.
(221) Kanonenberg, K.; Smits, S. H. J.; Schmitt, L. Functional Reconstitution of Hlyb, a Type I Secretion Abc Transporter, in Saposin-a Nanoparticles. *Sci. Rep.* **2019,** *9*.
(222) Kehlenbeck, D.-M.; Josts, I.; Nitsche, J.; Busch, S.; Forsyth, V. T.; Tidow, H. Comparison of Lipidic Carrier Systems for Integral Membrane Proteins – Msba as Case Study. *Biol. Chem.* **2019,** *400*, 1509-1518.
(223) Miyazaki, M.; Tajima, Y.; Handa, T.; Nakano, M. Static and Dynamic Characterization of Nanodiscs with Apolipoprotein a-I and Its Model Peptide. *J. Phys. Chem. B* **2010,** *114*, 12376-12382.
(224) Anantharamaiah, G. M.; Jones, J. L.; Brouillette, C. G.; Schmidt, C. F.; Chung, B. H.; Hughes, T. A.; Bhown, A. S.; Segrest, J. P. Studies of Synthetic Peptide Analogs of the Amphipathic Helix. Structure of Complexes with Dimyristoyl Phosphatidylcholine. *J. Biol. Chem.* **1985,** *260*, 10248-10255.
(225) Mishra, V. K.; Palgunachari, M. N.; Segrest, J. P.; Anantharamaiah, G. M. Interactions of Synthetic Peptide Analogs of the Class a Amphipathic Helix with Lipids. Evidence for the Snorkel Hypothesis. *J. Biol. Chem.* **1994,** *269*, 7185-7191.
(226) Salnikov, E. S.; Anantharamaiah, G. M.; Bechinger, B. Supramolecular Organization of Apolipoprotein-a-I-Derived Peptides within Disc-Like Arrangements. *Biophys. J.* **2018,** *115*, 467-477.
(227) Mishra, V. K.; Anantharamaiah, G. M.; Segrest, J. P.; Palgunachari, M. N.; Chaddha, M.; Sham, S. W.; Krishna, N. R. Association of a Model Class a (Apolipoprotein) Amphipathic Alpha Helical Peptide with Lipid: High Resolution NMR Studies of Peptide.Lipid Discoidal Complexes. *J. Biol. Chem.* **2006,** *281*, 6511-6519.
(228) Park, S. H.; Berkamp, S.; Cook, G. A.; Chan, M. K.; Viadiu, H.; Opella, S. J. Nanodiscs Versus Macrodiscs for NMR of Membrane Proteins. *Biochemistry* **2011,** *50*, 8983-8985.

(229) Kondo, H.; Ikeda, K.; Nakano, M. Formation of Size-Controlled, Denaturation-Resistant Lipid Nanodiscs by an Amphiphilic Self-Polymerizing Peptide. *Colloids Surf. B. Biointerfaces* **2016,** *146*, 423-430.
(230) Larsen, A. N.; Sørensen, K. K.; Johansen, N. T.; Martel, A.; Kirkensgaard, J. J. K.; Jensen, K. J.; Arleth, L.; Midtgaard, S. R. Dimeric Peptides with Three Different Linkers Self-Assemble with Phospholipids to Form Peptide Nanodiscs That Stabilize Membrane Proteins. *Soft Matter* **2016,** *12*, 5937-5949.
(231) Midtgaard, S. R.; Pedersen, M. C.; Kirkensgaard, J. J.; Sørensen, K. K.; Mortensen, K.; Jensen, K. J.; Arleth, L. Self-Assembling Peptides Form Nanodiscs That Stabilize Membrane Proteins. *Soft Matter* **2014,** *10*, 738-752.
(232) Anada, C.; Ikeda, K.; Egawa, A.; Fujiwara, T.; Nakao, H.; Nakano, M. Temperature- and Composition-Dependent Conformational Transitions of Amphipathic Peptide-Phospholipid Nanodiscs. *J. Colloid Interface Sci.* **2021,** *588*, 522-530.
(233) Carlson, M. L.; Stacey, R. G.; Young, J. W.; Wason, I. S.; Zhao, Z.; Rattray, D. G.; Scott, N.; Kerr, C. H.; Babu, M.; Foster, L. J.*, et al.* Profiling the Escherichia Coli Membrane Protein Interactome Captured in Peptidisc Libraries. *eLife* **2019,** *8*, e46615.
(234) Rothemund, P. W. K. Folding DNA to Create Nanoscale Shapes and Patterns. *Nature* **2006,** *440*, 297-302.
(235) Wang, W.; Arias, D. S.; Deserno, M.; Ren, X.; Taylor, R. E. Emerging Applications at the Interface of DNA Nanotechnology and Cellular Membranes: Perspectives from Biology, Engineering, and Physics. *APL Bioeng.* **2020,** *4*, 041507.
(236) Zhao, Z.; Zhang, M.; Hogle, J. M.; Shih, W. M.; Wagner, G.; Nasr, M. L. DNA-Corralled Nanodiscs for the Structural and Functional Characterization of Membrane Proteins and Viral Entry. *J. Am. Chem. Soc.* **2018,** *140*, 10639-10643.
(237) Padmanabha Das, K. M.; Shih, W. M.; Wagner, G.; Nasr, M. L. Large Nanodiscs: A Potential Game Changer in Structural Biology of Membrane Protein Complexes and Virus Entry. *Front. Bioeng. Biotechnol.* **2020,** *8*, 539.
(238) Iric, K.; Subramanian, M.; Oertel, J.; Agarwal, N. P.; Matthies, M.; Periole, X.; Sakmar, T. P.; Huber, T.; Fahmy, K.; Schmidt, T. L. DNA-Encircled Lipid Bilayers. *Nanoscale* **2018,** *10*, 18463-18467.
(239) Maingi, V.; Rothemund, P. W. K. Properties of DNA- and Protein-Scaffolded Lipid Nanodiscs. *ACS Nano* **2021,** *15*, 751-764.
(240) Jones, S. F.; Joshi, H.; Terry, S. J.; Burns, J. R.; Aksimentiev, A.; Eggert, U. S.; Howorka, S. Hydrophobic Interactions between DNA Duplexes and Synthetic and Biological Membranes. *J. Am. Chem. Soc.* **2021**.
(241) Bibow, S. Opportunities and Challenges of Backbone, Sidechain, and Rdc Experiments to Study Membrane Protein Dynamics in a Detergent-Free Lipid Environment Using Solution State NMR. *Front. Mol. Biosci.* **2019,** *6*.
(242) Cuevas Arenas, R.; Danielczak, B.; Martel, A.; Porcar, L.; Breyton, C.; Ebel, C.; Keller, S. Fast Collisional Lipid Transfer among Polymer-Bounded Nanodiscs. *Sci. Rep.* **2017,** *7*, 45875.
(243) Gupta, K.; Donlan, J. a. C.; Hopper, J. T. S.; Uzdavinys, P.; Landreh, M.; Struwe, W. B.; Drew, D.; Baldwin, A. J.; Stansfeld, P. J.; Robinson, C. V. The Role of Interfacial Lipids in Stabilizing Membrane Protein Oligomers. *Nature* **2017,** *541*, 421-424.
(244) Henrich, E.; Peetz, O.; Hein, C.; Laguerre, A.; Hoffmann, B.; Hoffmann, J.; Dotsch, V.; Bernhard, F.; Morgner, N. Analyzing Native Membrane Protein Assembly in Nanodiscs by Combined Non-Covalent Mass Spectrometry and Synthetic Biology. *Elife* **2017,** *6*.
(245) Keener, J. E.; Zambrano, D. E.; Zhang, G.; Zak, C. K.; Reid, D. J.; Deodhar, B. S.; Pemberton, J. E.; Prell, J. S.; Marty, M. T. Chemical Additives Enable Native Mass Spectrometry Measurement of Membrane Protein Oligomeric State within Intact Nanodiscs. *J. Am. Chem. Soc.* **2019,** *141*, 1054-1061.
(246) Fu, Q.; Piai, A.; Chen, W.; Xia, K.; Chou, J. J. Structure Determination Protocol for Transmembrane Domain Oligomers. *Nat. Protoc.* **2019,** *14*, 2483-2520.
(247) Hausler, E.; Fredriksson, K.; Goba, I.; Peters, C.; Raltchev, K.; Sperl, L.; Steiner, A.; Weinkauf, S.; Hagn, F. Quantifying the Insertion of Membrane Proteins into Lipid Bilayer Nanodiscs Using a Fusion Protein Strategy. *Biochim. Biophys. Acta* **2020,** *1862*.

(248) Sperl, L. E.; Hagn, F. NMR Structural and Biophysical Analysis of the Disease-Linked Inner Mitochondrial Membrane Protein Mpv17. *J. Mol. Biol.* **2021,** *433*, 167098.
(249) Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. Attenuated T_2 Relaxation by Mutual Cancellation of Dipole–Dipole Coupling and Chemical Shift Anisotropy Indicates an Avenue to NMR Structures of Very Large Biological Macromolecules in Solution. *Proc. Natl. Acad. Sci. U.S.A.* **1997,** *94*, 12366-12371.
(250) Lee, D.; Hilty, C.; Wider, G.; Wuthrich, K. Effective Rotational Correlation Times of Proteins from NMR Relaxation Interference. *J. Magn. Reson.* **2006,** *178*, 72-76.
(251) Ernst, R. R.; Bodenhausen, G.; Wokaun, A., *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*. Oxford University Press: Oxford, UK, 1987.
(252) Kovermann, M.; Rogne, P.; Wolf-Watz, M. Protein Dynamics and Function from Solution State NMR Spectroscopy. *Q. Rev. Biophys.* **2016,** *49*, e6.
(253) Palmer, A. G. NMR Characterization of the Dynamics of Biomacromolecules. *Chem. Rev.* **2004,** *104*, 3623-3640.
(254) Bibow, S.; Hiller, S. A Guide to Quantifying Membrane Protein Dynamics in Lipids and Other Native-Like Environments by Solution-State NMR Spectroscopy. *FEBS J.* **2019,** *286*, 1610-1623.
(255) Gautier, A.; Mott, H. R.; Bostock, M. J.; Kirkpatrick, J. P.; Nietlispach, D. Structure Determination of the Seven-Helix Transmembrane Receptor Sensory Rhodopsin Ii by Solution NMR Spectroscopy. *Nat. Struct. Mol. Biol.* **2010,** *17*, 768-774.
(256) Hiller, S.; Garces, R. G.; Malia, T. J.; Orekhov, V. Y.; Colombini, M.; Wagner, G. Solution Structure of the Integral Human Membrane Protein VDAC-1 in Detergent Micelles. *Science* **2008,** *321*, 1206-1210.
(257) Kang, C.; Li, Q. Solution NMR Study of Integral Membrane Proteins. *Curr. Opin. Chem. Biol.* **2011,** *15*, 560-569.
(258) Liang, B.; Tamm, L. K. Structure of Outer Membrane Protein G by Solution NMR Spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* **2007,** *104*, 16140-16145.
(259) Oxenoid, K.; Chou, J. J. The Present and Future of Solution NMR in Investigating the Structure and Dynamics of Channels and Transporters. *Curr. Opin. Struct. Biol.* **2013,** *23*, 547-554.
(260) Rosenzweig, R.; Kay, L. E. Bringing Dynamic Molecular Machines into Focus by Methyl-TROSY NMR. *Annu. Rev. Biochem* **2014,** *83*, 291-315.
(261) Sprangers, R.; Kay, L. E. Quantitative Dynamics and Binding Studies of the 20s Proteasome by NMR. *Nature* **2007,** *445*, 618-622.
(262) Venters, R. A.; Farmer, B. T.; Fierke, C. A.; Spicer, L. D. Characterizing the Use of Perdeuteration in NMR Studies of Large Proteins C-13, N-15 and H-1 Assignments of Human Carbonic Anhydrase Ii. *J. Mol. Biol.* **1996,** *264*, 1101-1116.
(263) Yu, H. Extending the Size Limit of Protein Nuclear Magnetic Resonance. *Proc. Natl. Acad. Sci. U.S.A.* **1999,** *96*, 332.
(264) Tugarinov, V.; Kay, L. E. Ile, Leu, and Val Methyl Assignments of the 723-Residue Malate Synthase G Using a New Labeling Strategy and Novel NMR Methods. *J. Am. Chem. Soc.* **2003,** *125*, 13868-13878.
(265) Takeuchi, K.; Arthanari, H.; Shimada, I.; Wagner, G. Nitrogen Detected TROSY at High Field Yields High Resolution and Sensitivity for Protein NMR. *J. Biomol. NMR* **2015,** *63*, 323-331.
(266) Takeuchi, K.; Gal, M.; Shimada, I.; Wagner, G. Low-γ Nuclei Detection Experiments for Biomolecular NMR. *RSC Biomol. Sci.* **2012**, 25-52.
(267) Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. Transverse Relaxation-Optimized Spectroscopy (TROSY) for NMR Studies of Aromatic Spin Systems in 13C-Labeled Proteins. *J. Am. Chem. Soc.* **1998,** *120*, 6394-6400.
(268) Hyberts, S. G.; Arthanari, H.; Wagner, G. Applications of Non-Uniform Sampling and Processing. *Top. Curr. Chem.* **2012,** *316*, 125-148.
(269) Hoch, J. C.; Maciejewski, M. W.; Filipovic, B. Randomization Improves Sparse Sampling in Multidimensional NMR. *J. Magn. Reson.* **2008,** *193*, 317-320.
(270) Kazimierczuk, K.; Orekhov, V. Non-Uniform Sampling: Post-Fourier Era of NMR Data Collection and Processing. *Magn. Reson. Chem.* **2015,** *53*, 921-926.

(271) Palmer, M. R.; Suiter, C. L.; Henry, G. E.; Rovnyak, J.; Hoch, J. C.; Polenova, T.; Rovnyak, D. Sensitivity of Nonuniform Sampling NMR. *J. Phys. Chem. B* **2015,** *119*, 6502-6515.
(272) Robson, S.; Arthanari, H.; Hyberts, S. G.; Wagner, G. Nonuniform Sampling for NMR Spectroscopy. *Methods Enzymol.* **2019,** *614*, 263-291.
(273) Zambrello, M. A.; Schuyler, A. D.; Maciejewski, M. W.; Delaglio, F.; Bersonova, I.; Hoch, J. C. Nonuniform Sampling in Multidimensional NMR for Improving Spectral Sensitivity. *Methods* **2018,** *138*, 62-68.
(274) Varnay, I.; Truffault, V.; Djuranovic, S.; Ursinus, A.; Coles, M.; Kessler, H. Optimized Measurement Temperature Gives Access to the Solution Structure of a 49 kDa Homohexameric Beta-Propeller. *J. Am. Chem. Soc.* **2010,** *132*, 15692-15698.
(275) Rosen, M. K.; Gardner, K. H.; Willis, R. C.; Parris, W. E.; Pawson, T.; Kay, L. E. Selective Methyl Group Protonation of Perdeuterated Proteins. *J. Mol. Biol.* **1996,** *263*, 627-636.
(276) Gardner, K. H.; Rosen, M. K.; Kay, L. E. Global Folds of Highly Deuterated, Methyl-Protonated Proteins by Multidimensional NMR. *Biochemistry* **1997,** *36*, 1389-1401.
(277) Tugarinov, V.; Kay, L. E. Methyl Groups as Probes of Structure and Dynamics in NMR Studies of High-Molecular-Weight Proteins. *ChemBioChem* **2005,** *6*, 1567-1577.
(278) Ulmschneider, M. B.; Sansom, M. S. Amino Acid Distributions in Integral Membrane Protein Structures. *Biochim. Biophys. Acta* **2001,** *1512*, 1-14.
(279) Goto, N. K.; Gardner, K. H.; Mueller, G. A.; Willis, R. C.; Kay, L. E. A Robust and Cost-Effective Method for the Production of Val, Leu, Ile (δ1) Methyl-Protonated 15N-, 13C-, 2H-labeled Proteins. *J. Biomol. NMR* **1999,** *13*, 369-374.
(280) Tugarinov, V.; Kay, L. E. An Isotope Labeling Strategy for Methyl TROSY Spectroscopy. *J. Biomol. NMR* **2004,** *28*, 165-172.
(281) Hajduk, P. J.; Augeri, D. J.; Mack, J.; Mendoza, R.; Yang, J.; Betz, S. F.; Fesik, S. W. NMR-Based Screening of Proteins Containing13C-Labeled Methyl Groups. *J. Am. Chem. Soc.* **2000,** *122*, 7898-7904.
(282) Mas, G.; Crublet, E.; Hamelin, O.; Gans, P.; Boisbouvier, J. Specific Labeling and Assignment Strategies of Valine Methyl Groups for NMR Studies of High Molecular Weight Proteins. *J. Biomol. NMR* **2013,** *57*, 251-262.
(283) Miyanoiri, Y.; Takeda, M.; Okuma, K.; Ono, A. M.; Terauchi, T.; Kainosho, M. Differential Isotope-Labeling for Leu and Val Residues in a Protein by E. Coli Cellular Expression Using Stereo-Specifically Methyl Labeled Amino Acids. *J. Biomol. NMR* **2013,** *57*, 237-249.
(284) Tugarinov, V.; Kay, L. E. Stereospecific NMR Assignments of Prochiral Methyls, Rotameric States and Dynamics of Valine Residues in Malate Synthase G. *J. Am. Chem. Soc.* **2004,** *126*, 9827-9836.
(285) Gans, P.; Hamelin, O.; Sounier, R.; Ayala, I.; Durá, M. A.; Amero, C. D.; Noirclerc-Savoye, M.; Franzetti, B.; Plevin, M. J.; Boisbouvier, J. Stereospecific Isotopic Labeling of Methyl Groups for NMR Spectroscopic Studies of High-Molecular-Weight Proteins. *Angew. Chem. Int. Ed.* **2010,** *49*, 1958-1962.
(286) Kainosho, M.; Torizawa, T.; Iwashita, Y.; Terauchi, T.; Mei Ono, A.; Güntert, P. Optimal Isotope Labelling for NMR Protein Structure Determinations. *Nature* **2006,** *440*, 52-57.
(287) Miyanoiri, Y.; Takeda, M.; Kainosho, M., Stereo-Array Isotope Labeling Method for Studying Protein Structure and Dynamics. In *Isotope Labeling in Biomolecular NMR*, Atreya, H. S., Ed. Springer Netherlands: Dordrecht, 2012; pp 83-93.
(288) Isaacson, R. L.; Simpson, P. J.; Liu, M.; Cota, E.; Zhang, X.; Freemont, P.; Matthews, S. A New Labeling Method for Methyl Transverse Relaxation-Optimized Spectroscopy NMR Spectra of Alanine Residues. *J. Am. Chem. Soc.* **2007,** *129*, 15428-15429.
(289) Ayala, I.; Sounier, R.; Usé, N.; Gans, P.; Boisbouvier, J. An Efficient Protocol for the Complete Incorporation of Methyl-Protonated Alanine in Perdeuterated Protein. *J. Biomol. NMR* **2009,** *43*, 111-119.
(290) Velyvis, A.; Ruschak, A. M.; Kay, L. E. An Economical Method for Production of 2h,13ch3-Threonine for Solution NMR Studies of Large Protein Complexes: Application to the 670 kDa Proteasome. *PLoS One* **2012,** *7*, e43725.

(291) Ayala, I.; Chiari, L.; Kerfah, R.; Boisbouvier, J.; Gans, P.; Hamelin, O. Asymmetric Synthesis of Methyl Specifically Labelled L-Threonine and Application to the NMR Studies of High Molecular Weight Proteins. *ChemistrySelect* **2020,** *5*, 5092-5098.
(292) Gelis, I.; Bonvin, A. M. J. J.; Keramisanou, D.; Koukaki, M.; Gouridis, G.; Karamanou, S.; Economou, A.; Kalodimos, C. G. Structural Basis for Signal-Sequence Recognition by the Translocase Motor Seca as Determined by NMR. *Cell* **2007,** *131*, 756-769.
(293) Stoffregen, M.; Schwer, M.; Renschler, F.; Wiesner, S. Methionine Scanning as an NMR Tool for Detecting and Analyzing Biomolecular Interaction Surfaces. *Structure* **2012,** *20*, 573-581.
(294) Fischer, M.; Kloiber, K.; Häusler, J.; Ledolter, K.; Konrat, R.; Schmid, W. Synthesis of a 13C-Methyl-Group-Labeled Methionine Precursor as a Useful Tool for Simplifying Protein Structural Analysis by NMR Spectroscopy. *ChemBioChem* **2007,** *8*, 610-612.
(295) Linser, R.; Gelev, V.; Hagn, F.; Arthanari, H.; Hyberts, S. G.; Wagner, G. Selective Methyl Labeling of Eukaryotic Membrane Proteins Using Cell-Free Expression. *J. Am. Chem. Soc.* **2014,** *136*, 11308-11310.
(296) Gorman, S. D.; Sahu, D.; O'rourke, K. F.; Boehr, D. D. Assigning Methyl Resonances for Protein Solution-State NMR Studies. *Methods* **2018,** *148*, 88-99.
(297) Gross, J. D.; Gelev, V. M.; Wagner, G. A Sensitive and Robust Method for Obtaining Intermolecular NOEs between Side Chains in Large Protein Complexes. *J. Biomol. NMR* **2003,** *25*, 235-242.
(298) Kerfah, R.; Plevin, M. J.; Pessey, O.; Hamelin, O.; Gans, P.; Boisbouvier, J. Scrambling Free Combinatorial Labeling of Alanine-B, Isoleucine-δ1, Leucine-ProS and Valine-ProS Methyl Groups for the Detection of Long Range NOEs. *J. Biomol. NMR* **2015,** *61*, 73-82.
(299) Nerli, S.; De Paula, V. S.; Mcshan, A. C.; Sgourakis, N. G. Backbone-Independent NMR Resonance Assignments of Methyl Probes in Large Proteins. *Nat Commun* **2021,** *12*, 691.
(300) Amero, C.; Asunción Durá, M.; Noirclerc-Savoye, M.; Perollier, A.; Gallet, B.; Plevin, M. J.; Vernet, T.; Franzetti, B.; Boisbouvier, J. A Systematic Mutagenesis-Driven Strategy for Site-Resolved NMR Studies of Supramolecular Assemblies. *J. Biomol. NMR* **2011,** *50*, 229-236.
(301) Schütz, S.; Sprangers, R. Methyl TROSY Spectroscopy: A Versatile NMR Approach to Study Challenging Biological Systems. *Prog. Nucl. Magn. Reson. Spectrosc.* **2020,** *116*, 56-84.
(302) Eddy, M. T.; Lee, M. Y.; Gao, Z. G.; White, K. L.; Didenko, T.; Horst, R.; Audet, M.; Stanczak, P.; Mcclary, K. M.; Han, G. W.*, et al.* Allosteric Coupling of Drug Binding and Intracellular Signaling in the A2a Adenosine Receptor. *Cell* **2018,** *172*, 68-80 e12.
(303) Nygaard, R.; Zou, Y.; Dror, R. O.; Mildorf, T. J.; Arlow, D. H.; Manglik, A.; Pan, A. C.; Liu, C. W.; Fung, J. J.; Bokoch, M. P.*, et al.* The Dynamic Process of Beta(2)-Adrenergic Receptor Activation. *Cell* **2013,** *152*, 532-542.
(304) Kitevski-Leblanc, J. L.; Prosser, R. S. Current Applications of F-19 NMR to Studies of Protein Structure and Dynamics. *Prog. Nucl. Magn. Reson. Spectrosc.* **2012,** *62*, 1-33.
(305) Rose-Sperling, D.; Tran, M. A.; Lauth, L. M.; Goretzki, B.; Hellmich, U. A. F-19 NMR as a Versatile Tool to Study Membrane Protein Structure and Dynamics. *Biol. Chem.* **2019,** *400*, 1277-1288.
(306) Frieden, C.; Hoeltzli, S. D.; Bann, J. G. The Preparation of 19F-Labeled Proteins for NMR Studies. *Methods Enzymol.* **2004,** *380*, 400-415.
(307) Goba, I.; Goricanec, D.; Schum, D.; Hillenbrand, M.; Plückthun, A.; Hagn, F. Probing the Conformation States of Neurotensin Receptor 1 Variants by NMR Site-Directed Methyl Labeling. *ChemBioChem* **2021,** *22*, 139-146.
(308) Religa, T. L.; Ruschak, A. M.; Rosenzweig, R.; Kay, L. E. Site-Directed Methyl Group Labeling as an NMR Probe of Structure and Dynamics in Supramolecular Protein Systems: Applications to the Proteasome and to the Clpp Protease. *J. Am. Chem. Soc.* **2011,** *133*, 9063-9068.
(309) Clore, G. M.; Iwahara, J. Theory, Practice, and Applications of Paramagnetic Relaxation Enhancement for the Characterization of Transient Low-Population States of Biological Macromolecules and Their Complexes. *Chem. Rev.* **2009,** *109*, 4108-4139.
(310) Gottstein, D.; Reckel, S.; Dötsch, V.; Güntert, P. Requirements on Paramagnetic Relaxation Enhancement Data for Membrane Protein Structure Determination by NMR. *Structure* **2012,** *20*, 1019-1027.

(311) Prestegard, J. H.; Bougault, C. M.; Kishore, A. I. Residual Dipolar Couplings in Structure Determination of Biomolecules. *Chem. Rev.* **2004,** *104*, 3519-3540.
(312) Tolman, J. R.; Ruan, K. NMR Residual Dipolar Couplings as Probes of Biomolecular Dynamics. *Chem. Rev.* **2006,** *106*, 1720-1736.
(313) Battiste, J. L.; Wagner, G. Utilization of Site-Directed Spin Labeling and High-Resolution Heteronuclear Nuclear Magnetic Resonance for Global Fold Determination of Large Proteins with Limited Nuclear Overhauser Effect Data. *Biochemistry* **2000,** *39*, 5355-5365.
(314) Liang, B. Y.; Bushweller, J. H.; Tamm, L. K. Site-Directed Parallel Spin-Labeling and Paramagnetic Relaxation Enhancement in Structure Determination of Membrane Proteins by Solution NMR Spectroscopy. *J. Am. Chem. Soc.* **2006,** *128*, 4389-4397.
(315) Hilty, C.; Wider, G.; Fernández, C.; Wüthrich, K. Membrane Protein-Lipid Interactions in Mixed Micelles Studied by NMR Spectroscopy with the Use of Paramagnetic Reagents. *ChemBioChem* **2004,** *5*, 467-473.
(316) Butterwick, J. A.; Mackinnon, R. Solution Structure and Phospholipid Interactions of the Isolated Voltage-Sensor Domain from Kvap. *J. Mol. Biol.* **2010,** *403*, 591-606.
(317) Mazhab-Jafari, M. T.; Marshall, C. B.; Stathopulos, P. B.; Kobashigawa, Y.; Stambolic, V.; Kay, L. E.; Inagaki, F.; Ikura, M. Membrane-Dependent Modulation of the Mtor Activator Rheb: NMR Observations of a GTPase Tethered to a Lipid-Bilayer Nanodisc. *J. Am. Chem. Soc.* **2013,** *135*, 3367-3370.
(318) Jarvet, J.; Danielsson, J.; Damberg, P.; Oleszczuk, M.; Gräslund, A. Positioning of the Alzheimer Abeta(1-40) Peptide in SDS Micelles Using NMR and Paramagnetic Probes. *J. Biomol. NMR* **2007,** *39*, 63-72.
(319) Chen, K.; Tjandra, N., The Use of Residual Dipolar Coupling in Studying Proteins by NMR. In *NMR of Proteins and Small Biomolecules. Topics in Current Chemistry*, Springer: 2012; Vol. 326, pp 47-67.
(320) Bibow, S.; Carneiro, M. G.; Sabo, T. M.; Schwiegk, C.; Becker, S.; Riek, R.; Lee, D. Measuring Membrane Protein Bond Orientations in Nanodiscs Via Residual Dipolar Couplings. *Protein Sci.* **2014,** *23*, 851-856.
(321) Douglas, S. M.; Chou, J. J.; Shih, W. M. DNA-Nanotube-Induced Alignment of Membrane Proteins for NMR Structure Determination. *Proc. Natl. Acad. Sci. U. S. A.* **2007,** *104*, 6644-6648.
(322) Bellot, G.; Mcclintock, M. A.; Chou, J. J.; Shih, W. M. DNA Nanotubes for NMR Structure Determination of Membrane Proteins. *Nat. Protoc.* **2013,** *8*, 755-770.
(323) Canlas, C. G.; Ma, D.; Tang, P.; Xu, Y. Residual Dipolar Coupling Measurements of Transmembrane Proteins Using Aligned Low-q Bicelles and High-Resolution Magic Angle Spinning NMR Spectroscopy. *J. Am. Chem. Soc.* **2008,** *130*, 13294-13300.
(324) Ottiger, M.; Bax, A. Characterization of Magnetically Oriented Phospholipid Micelles for Measurement of Dipolar Couplings in Macromolecules. *J. Biomol. NMR* **1998,** *12*, 361-372.
(325) Kamen, D. E.; Cahill, S. M.; Girvin, M. E. Multiple Alignment of Membrane Proteins for Measuring Residual Dipolar Couplings Using Lanthanide Ions Bound to a Small Metal Chelator. *J. Am. Chem. Soc.* **2007,** *129*, 1846-1847.
(326) Strickland, M.; Tjandra, N., Residual Dipolar Coupling for Conformational and Dynamic Studies. In *Modern Magnetic Resonance*, Webb, G. A., Ed. Springer International Publishing: Cham, 2017; pp 1-16.
(327) Ma, C.; Opella, S. J. Lanthanide Ions Bind Specifically to an Added "Ef-Hand" and Orient a Membrane Protein in Micelles for Solution NMR Spectroscopy. *J. Magn. Reson.* **2000,** *146*, 381-384.
(328) Lyukmanova, E. N.; Shenkarev, Z. O.; Paramonov, A. S.; Sobol, A. G.; Ovchinnikova, T. V.; Chupin, V. V.; Kirpichnikov, M. P.; Blommers, M. J. J.; Arseniev, A. S. Lipid−Protein Nanoscale Bilayers: A Versatile Medium for NMR Investigations of Membrane Proteins and Membrane-Active Peptides. *J. Am. Chem. Soc.* **2008,** *130*, 2140-2141.
(329) Glück, J. M.; Wittlich, M.; Feuerstein, S.; Hoffmann, S.; Willbold, D.; Koenig, B. W. Integral Membrane Proteins in Nanodiscs Can Be Studied by Solution NMR Spectroscopy. *J. Am. Chem. Soc.* **2009,** *131*, 12060-12061.
(330) Yu, T.-Y.; Raschle, T.; Hiller, S.; Wagner, G. Solution NMR Spectroscopic Characterization of Human VDAC-2 in Detergent Micelles and Lipid Bilayer Nanodiscs. *Biochim. Biophys. Acta* **2012,** *1818*, 1562-1569.

(331) Sušac, L.; Horst, R.; Wüthrich, K. Solution-NMR Characterization of Outer-Membrane Protein a Frome. Coliin Lipid Bilayer Nanodiscs and Detergent Micelles. *ChemBioChem* **2014,** *15*, 995-1000.
(332) Morgado, L.; Zeth, K.; Burmann, B. M.; Maier, T.; Hiller, S. Characterization of the Insertase BamA in Three Different Membrane Mimetics by Solution NMR Spectroscopy. *J. Biomol. NMR* **2015,** *61*, 333-345.
(333) Bibow, S.; Bohm, R.; Modaresi, S. M.; Hiller, S. Detergent Titration as an Efficient Method for NMR Resonance Assignments of Membrane Proteins in Lipid-Bilayer Nanodiscs. *Anal. Chem.* **2020,** *92*, 7786-7793.
(334) Laguerre, A.; Lohr, F.; Henrich, E.; Hoffmann, B.; Abdul-Manan, N.; Connolly, P. J.; Perozo, E.; Moore, J. M.; Bernhard, F.; Dotsch, V. From Nanodiscs to Isotropic Bicelles: A Procedure for Solution Nuclear Magnetic Resonance Studies of Detergent-Sensitive Integral Membrane Proteins. *Structure* **2016,** *24*, 1830-1841.
(335) Shenkarev, Z. O.; Lyukmanova, E. N.; Solozhenkin, O. I.; Gagnidze, I. E.; Nekrasova, O. V.; Chupin, V. V.; Tagaev, A. A.; Yakimenko, Z. A.; Ovchinnikova, T. V.; Kirpichnikov, M. P.*, et al.* Lipid-Protein Nanodiscs: Possible Application in High-Resolution NMR Investigations of Membrane Proteins and Membrane-Active Peptides. *Biochemistry (Moscow)* **2009,** *74*, 756-765.
(336) Qasim, A.; Sher, I.; Hirschhorn, O.; Shaked, H.; Qasem, Z.; Ruthstein, S.; Chill, J. H. Investigation of a KcsA Cytoplasmic Ph Gate in Lipoprotein Nanodiscs. *ChemBioChem* **2019,** *20*, 813-821.
(337) Imai, S.; Osawa, M.; Mita, K.; Toyonaga, S.; Machiyama, A.; Ueda, T.; Takeuchi, K.; Oiki, S.; Shimada, I. Functional Equilibrium of the KcsA Structure Revealed by NMR. *J. Biol. Chem.* **2012,** *287*, 39634-39641.
(338) Olerinyova, A.; Sonn-Segev, A.; Gault, J.; Eichmann, C.; Schimpf, J.; Kopf, A. H.; Rudden, L. S. P.; Ashkinadze, D.; Bomba, R.; Frey, L.*, et al.* Mass Photometry of Membrane Proteins. *Chem* **2021,** *7*, 224-236.
(339) Shenkarev, Z. O.; Lyukmanova, E. N.; Paramonov, A. S.; Shingarova, L. N.; Chupin, V. V.; Kirpichnikov, M. P.; Blommers, M. J. J.; Arseniev, A. S. Lipid−Protein Nanodiscs as Reference Medium in Detergent Screening for High-Resolution NMR Studies of Integral Membrane Proteins. *J. Am. Chem. Soc.* **2010,** *132*, 5628-5629.
(340) Shenkarev, Z. O.; Paramonov, A. S.; Lyukmanova, E. N.; Shingarova, L. N.; Yakimov, S. A.; Dubinnyi, M. A.; Chupin, V. V.; Kirpichnikov, M. P.; Blommers, M. J. J.; Arseniev, A. S. NMR Structural and Dynamical Investigation of the Isolated Voltage-Sensing Domain of the Potassium Channel Kvap: Implications for Voltage Gating. *J. Am. Chem. Soc.* **2010,** *132*, 5630-5637.
(341) Etzkorn, M.; Raschle, T.; Hagn, F.; Gelev, V.; Rice, J., Amanda; Walz, T.; Wagner, G. Cell-Free Expressed Bacteriorhodopsin in Different Soluble Membrane Mimetics: Biophysical Properties and NMR Accessibility. *Structure* **2013,** *21*, 394-401.
(342) Viennet, T.; Bungert-Plümke, S.; Elter, S.; Viegas, A.; Fahlke, C.; Etzkorn, M. Reconstitution and NMR Characterization of the Ion-Channel Accessory Subunit Barttin in Detergents and Lipid-Bilayer Nanodiscs. *Front. Mol. Biosci.* **2019,** *6*.
(343) Tzitzilonis, C.; Eichmann, C.; Maslennikov, I.; Choe, S.; Riek, R. Detergent/Nanodisc Screening for High-Resolution NMR Studies of an Integral Membrane Protein Containing a Cytoplasmic Domain. *PLoS One* **2013,** *8*, e54378.
(344) Kofuku, Y.; Ueda, T.; Okude, J.; Shiraishi, Y.; Kondo, K.; Mizumura, T.; Suzuki, S.; Shimada, I. Functional Dynamics of Deuterated β2-Adrenergic Receptor in Lipid Bilayers Revealed by NMR Spectroscopy. *Angew. Chem. Int. Ed.* **2014,** *53*, 13376-13379.
(345) Mizumura, T.; Kondo, K.; Kurita, M.; Kofuku, Y.; Natsume, M.; Imai, S.; Shiraishi, Y.; Ueda, T.; Shimada, I. Activation of Adenosine A2a Receptor by Lipids from Docosahexaenoic Acid Revealed by NMR. *Sci. Adv.* **2020,** *6*, eaay8544.
(346) Casiraghi, M.; Damian, M.; Lescop, E.; Point, E.; Moncoq, K.; Morellet, N.; Levy, D.; Marie, J.; Guittet, E.; Banères, J.-L.*, et al.* Functional Modulation of a G Protein-Coupled Receptor Conformational Landscape in a Lipid Bilayer. *J. Am. Chem. Soc.* **2016,** *138*, 11170-11175.
(347) Shiraishi, Y.; Natsume, M.; Kofuku, Y.; Imai, S.; Nakata, K.; Mizukoshi, T.; Ueda, T.; Iwaï, H.; Shimada, I. Phosphorylation-Induced Conformation of B(2)-Adrenoceptor Related to Arrestin Recruitment Revealed by NMR. *Nat. Commun.* **2018,** *9*, 194.

(348) Salzmann, M.; Pervushin, K.; Wider, G.; Senn, H.; Wuthrich, K. TROSY in Triple-Resonance Experiments: New Perspectives for Sequential NMR Assignment of Large Proteins. *Proc. Natl. Acad. Sci. U. S. A.* **1998,** *95*, 13585-13590.
(349) Deshmukh, L.; Meller, N.; Alder, N.; Byzova, T.; Vinogradova, O. Tyrosine Phosphorylation as a Conformational Switch: A Case Study of Integrin β3 Cytoplasmic Tail. *J. Biol. Chem.* **2011,** *286*, 40943-40953.
(350) Schuster, M.; Deluigi, M.; Pantić, M.; Vacca, S.; Baumann, C.; Scott, D. J.; Plückthun, A.; Zerbe, O. Optimizing the A1b-Adrenergic Receptor for Solution NMR Studies. *Biochim. Biophys. Acta* **2020,** *1862*, 183354.
(351) Mineev, K. S.; Goncharuk, S. A.; Kuzmichev, P. K.; Vilar, M.; Arseniev, A. S. NMR Dynamics of Transmembrane and Intracellular Domains of P75ntr in Lipid-Protein Nanodiscs. *Biophys. J.* **2015,** *109*, 772-782.
(352) Raltchev, K.; Pipercevic, J.; Hagn, F. Production and Structural Analysis of Membrane-Anchored Proteins in Phospholipid Nanodiscs. *Chem. Eur. J.* **2018,** *24*, 5493-5499.
(353) Yao, Y.; Fujimoto, L. M.; Hirshman, N.; Bobkov, A. A.; Antignani, A.; Youle, R. J.; Marassi, F. M. Conformation of Bcl-Xl Upon Membrane Integration. *J. Mol. Biol.* **2015,** *427*, 2262-2270.
(354) Yao, Y.; Nisan, D.; Fujimoto, L. M.; Antignani, A.; Barnes, A.; Tjandra, N.; Youle, R. J.; Marassi, F. M. Characterization of the Membrane-Inserted C-Terminus of Cytoprotective Bcl-Xl. *Protein Expression Purif.* **2016,** *122*, 56-63.
(355) Sperl, L. E.; Ruhrnossl, F.; Schiller, A.; Haslbeck, M.; Hagn, F. High-Resolution Analysis of the Conformational Transition of Pro-Apoptotic Bak at the Lipid Membrane. *EMBO J.* **2021**, e107159.
(356) Zhang, M.; Huang, R.; Ackermann, R.; Im, S.-C.; Waskell, L.; Schwendeman, A.; Ramamoorthy, A. Reconstitution of the Cytb5-Cytp450 Complex in Nanodiscs for Structural Studies Using NMR Spectroscopy. *Angew. Chem. Int. Ed.* **2016,** *55*, 4497-4499.
(357) Zhang, M.; Huang, R.; Im, S.-C.; Waskell, L.; Ramamoorthy, A. Effects of Membrane Mimetics on Cytochrome P450-Cytochrome β5 Interactions Characterized by NMR Spectroscopy. *J. Biol. Chem.* **2015,** *290*, 12705-12718.
(358) Bai, J.; Wang, J.; Ravula, T.; Im, S. C.; Anantharamaiah, G. M.; Waskell, L.; Ramamoorthy, A. Expression, Purification, and Functional Reconstitution of (19)F-Labeled Cytochrome β5 in Peptide Nanodiscs for NMR Studies. *Biochim. Biophys. Acta* **2020,** *1862*, 183194.
(359) Prade, E.; Mahajan, M.; Im, S.-C.; Zhang, M.; Gentry, K. A.; Anantharamaiah, G. M.; Waskell, L.; Ramamoorthy, A. A Minimal Functional Complex of Cytochrome P450 and Fbd of Cytochrome P450 Reductase in Nanodiscs. *Angew. Chem. Int. Ed.* **2018,** *57*, 8458-8462.
(360) Galiakhmetov, A. R.; Kovrigina, E. A.; Xia, C.; Kim, J. P.; Kovrigin, E. L. Application of Methyl-TROSY to a Large Paramagnetic Membrane Protein without Perdeuteration: (13)C-MMTS-Labeled NADPH-Cytochrome P450 Oxidoreductase. *J. Biomol. NMR* **2018,** *70*, 21-31.
(361) Gentry, K. A.; Anantharamaiah, G. M.; Ramamoorthy, A. Probing Protein–Protein and Protein–Substrate Interactions in the Dynamic Membrane-Associated Ternary Complex of Cytochromes P450, β5, and Reductase. *Chem. Commun.* **2019,** *55*, 13422-13425.
(362) Reiner, D. J.; Lundquist, E. A. Small Gtpases. *WormBook* **2018,** *2018*, 1-65.
(363) Mazhab-Jafari, M. T.; Marshall, C. B.; Smith, M. J.; Gasmi-Seabrook, G. M. C.; Stathopulos, P. B.; Inagaki, F.; Kay, L. E.; Neel, B. G.; Ikura, M. Oncogenic and Rasopathy-Associated K-Ras Mutations Relieve Membrane-Dependent Occlusion of the Effector-Binding Site. *Proc. Natl. Acad. Sci. U.S.A.* **2015,** *112*, 6625-6630.
(364) Lee, K. Y.; Fang, Z.; Enomoto, M.; Gasmi-Seabrook, G.; Zheng, L.; Koide, S.; Ikura, M.; Marshall, C. B. Two Distinct Structures of Membrane-Associated Homodimers of GTP- and GDP-Bound Kras4b Revealed by Paramagnetic Relaxation Enhancement. *Angew. Chem. Int. Ed.* **2020,** *59*, 11037-11045.
(365) Fang, Z.; Marshall, C. B.; Nishikawa, T.; Gossert, A. D.; Jansen, J. M.; Jahnke, W.; Ikura, M. Inhibition of K-Ras4b by a Unique Mechanism of Action: Stabilizing Membrane-Dependent Occlusion of the Effector-Binding Site. *Cell Chem. Biol.* **2018,** *25*, 1327-1336.e1324.

(366) Marcink, T. C.; Simoncic, J. A.; An, B.; Knapinska, A. M.; Fulcher, Y. G.; Akkaladevi, N.; Fields, G. B.; Van Doren, S. R. Mt1-Mmp Binds Membranes by Opposite Tips of Its β Propeller to Position It for Pericellular Proteolysis. *Structure* **2019,** *27*, 281-292.e286.
(367) Viennet, T.; Wördehoff, M. M.; Uluca, B.; Poojari, C.; Shaykhalishahi, H.; Willbold, D.; Strodel, B.; Heise, H.; Buell, A. K.; Hoyer, W.*, et al.* Structural Insights from Lipid-Bilayer Nanodiscs Link A-Synuclein Membrane-Binding Modes to Amyloid Fibril Formation. *Commun. Biol.* **2018,** *1*.
(368) Jakubec, M.; Bariås, E.; Furse, S.; Govasli, M. L.; George, V.; Turcu, D.; Iashchishyn, I. A.; Morozova-Roche, L. A.; Halskau, Ø. Cholesterol-Containing Lipid Nanodiscs Promote an A-Synuclein Binding Mode That Accelerates Oligomerization. *FEBS J.* **2020**.
(369) Sahoo, B. R.; Genjo, T.; Cox, S. J.; Stoddard, A. K.; Anantharamaiah, G. M.; Fierke, C.; Ramamoorthy, A. Nanodisc-Forming Scaffold Protein Promoted Retardation of Amyloid-Beta Aggregation. *J. Mol. Biol.* **2018,** *430*, 4230-4244.
(370) Rodriguez Camargo, D. C.; Korshavn, K. J.; Jussupow, A.; Raltchev, K.; Goricanec, D.; Fleisch, M.; Sarkar, R.; Xue, K.; Aichler, M.; Mettenleiter, G.*, et al.* Stabilization and Structural Analysis of a Membrane-Associated Hiapp Aggregation Intermediate. *eLife* **2017,** *6*, e31226.
(371) Reif, M. M.; Fischer, M.; Fredriksson, K.; Hagn, F.; Zacharias, M. The N-Terminal Segment of the Voltage-Dependent Anion Channel: A Possible Membrane-Bound Intermediate in Pore Unbinding. *J. Mol. Biol.* **2019,** *431*, 223-243.
(372) Gaussmann, S.; Gopalswamy, M.; Eberhardt, M.; Reuter, M.; Zou, P.; Schliebs, W.; Erdmann, R.; Sattler, M. Membrane Interactions of the Peroxisomal Proteins Pex5 and Pex14. *Front. Cell Dev. Biol.* **2021,** *9*.
(373) Zhang, A. H.; Edwards, I. A.; Mishra, B. P.; Sharma, G.; Healy, M. D.; Elliott, A. G.; Blaskovich, M. a. T.; Cooper, M. A.; Collins, B. M.; Jia, X.*, et al.* Elucidating the Lipid Binding Properties of Membrane-Active Peptides Using Cyclised Nanodiscs. *Front. Chem.* **2019,** *7*.
(374) Sharma, G.; Deuis, J. R.; Jia, X.; Mueller, A.; Vetter, I.; Mobli, M. Recombinant Production, Bioconjugation and Membrane Binding Studies Ofpn3a, a Selective Na(V)1.7 Inhibitor. *Biochem. Pharmacol.* **2020,** *181*, 114148.
(375) Yahalom, A.; Davidov, G.; Kolusheva, S.; Shaked, H.; Barber-Zucker, S.; Zarivach, R.; Chill, J. H. Structure and Membrane-Targeting of a Bordetella Pertussis Effector N-Terminal Domain. *Biochim. Biophys. Acta* **2019,** *1861*, 183054.
(376) Aleshin, A. E.; Yao, Y.; Iftikhar, A.; Bobkov, A. A.; Yu, J.; Cadwell, G.; Klein, M. G.; Dong, C.; Bankston, L. A.; Liddington, R. C.*, et al.* Structural Basis for the Association of Plekha7 with Membrane-Embedded Phosphatidylinositol Lipids. *Structure* **2021**.
(377) Ghazarian, H.; Hu, W.; Mao, A.; Nguyen, T.; Vaidehi, N.; Sligar, S.; Shively, J. E. NMR Analysis of Free and Lipid Nanodisc Anchored Ceacam1 Membrane Proximal Peptides with Ca(2+)/Cam. *Biochim. Biophys. Acta* **2019,** *1861*, 787-797.
(378) Pahl, M. C.; Askinazi, O. L.; Hamilton, C.; Cheng, I.; Cichewicz, K.; Kuhn, J.; Manohar, S.; Deppmann, C. D., Signalling Via Single-Pass Transmembrane Proteins. In *Els*, John Wiley & Sons, Ltd: 2013.
(379) Bugge, K.; Lindorff-Larsen, K.; Kragelund, B. B. Understanding Single-Pass Transmembrane Receptor Signaling from a Structural Viewpoint-What Are We Missing? *FEBS J.* **2016,** *283*, 4424-4451.
(380) Li, E.; Wimley, W. C.; Hristova, K. Transmembrane Helix Dimerization: Beyond the Search for Sequence Motifs. *Biochim. Biophys. Acta* **2012,** *1818*, 183-193.
(381) Steiner, A.; Schlepckow, K.; Brunner, B.; Steiner, H.; Haass, C.; Hagn, F. γ-Secretase Cleavage of the Alzheimer Risk Factor TREM2 Is Determined by Its Intrinsic Structural Dynamics. *EMBO J.* **2020,** *39*, e104247.
(382) Wunderlich, P.; Glebov, K.; Kemmerling, N.; Tien, N. T.; Neumann, H.; Walter, J. Sequential Proteolytic Processing of the Triggering Receptor Expressed on Myeloid Cells-2 (Trem2) Protein by Ectodomain Shedding and γ-Secretase-Dependent Intramembranous Cleavage. *J. Biol. Chem.* **2013,** *288*, 33027-33036.

(383) Jonsson, T.; Stefansson, H.; Steinberg, S.; Jonsdottir, I.; Jonsson, P. V.; Snaedal, J.; Bjornsson, S.; Huttenlocher, J.; Levey, A. I.; Lah, J. J.*, et al.* Variant of TREM2 Associated with the Risk of Alzheimer's Disease. *New Engl. J. Med.* **2012,** *368*, 107-116.
(384) Kale, J.; Osterlund, E. J.; Andrews, D. W. Bcl-2 Family Proteins: Changing Partners in the Dance Towards Death. *Cell Death Differ.* **2018,** *25*, 65-80.
(385) Mors, K.; Roos, C.; Scholz, F.; Wachtveitl, J.; Dotsch, V.; Bernhard, F.; Glaubitz, C. Modified Lipid and Protein Dynamics in Nanodiscs. *Biochim. Biophys. Acta* **2013,** *1828*, 1222-1229.
(386) Martinez, D.; Decossas, M.; Kowal, J.; Frey, L.; Stahlberg, H.; Dufourc, E. J.; Riek, R.; Habenstein, B.; Bibow, S.; Loquet, A. Lipid Internal Dynamics Probed in Nanodiscs. *ChemPhysChem* **2017,** *18*, 2651-2657.
(387) Frey, L.; Lakomek, N. A.; Riek, R.; Bibow, S. Micelles, Bicelles, and Nanodiscs: Comparing the Impact of Membrane Mimetics on Membrane Protein Backbone Dynamics. *Angew. Chem. Int. Ed.* **2017,** *56*, 380-383.
(388) Frey, L.; Hiller, S.; Riek, R.; Bibow, S. Lipid- and Cholesterol-Mediated Time-Scale-Specific Modulation of the Outer Membrane Protein X Dynamics in Lipid Bilayers. *J. Am. Chem. Soc.* **2018,** *140*, 15402-15411.
(389) Iadanza, M. G.; Schiffrin, B.; White, P.; Watson, M. A.; Horne, J. E.; Higgins, A. J.; Calabrese, A. N.; Brockwell, D. J.; Tuma, R.; Kalli, A. C.*, et al.* Distortion of the Bilayer and Dynamics of the Bam Complex in Lipid Nanodiscs. *Commun. Biol.* **2020,** *3*.
(390) Yin, J.; Chen, K. M.; Clark, M. J.; Hijazi, M.; Kumari, P.; Bai, X. C.; Sunahara, R. K.; Barth, P.; Rosenbaum, D. M. Structure of a D2 Dopamine Receptor-G-Protein Complex in a Lipid Membrane. *Nature* **2020,** *584*, 125-129.
(391) Ebersberger, L.; Schindler, T.; Kirsch, S. A.; Pluhackova, K.; Schambony, A.; Seydel, T.; Bockmann, R. A.; Unruh, T. Lipid Dynamics in Membranes Slowed Down by Transmembrane Proteins. *Front. Cell Dev. Biol.* **2020,** *8*, 579388.
(392) Engelman, D. M. Membranes Are More Mosaic Than Fluid. *Nature* **2005,** *438*, 578-580.
(393) Pourmousa, M.; Pastor, R. W. Molecular Dynamics Simulations of Lipid Nanodiscs. *Biochim. Biophys. Acta* **2018,** *1860*, 2094-2107.
(394) Voskoboynikova, N.; Orekhov, P.; Bozdaganyan, M.; Kodde, F.; Rademacher, M.; Schowe, M.; Budke-Gieseking, A.; Brickwedde, B.; Psathaki, O. E.; Mulkidjanian, A. Y.*, et al.* Lipid Dynamics in Diisobutylene-Maleic Acid (DIBMA) Lipid Particles in Presence of Sensory Rhodopsin Ii. *Int. J. Mol. Sci.* **2021,** *22*.
(395) Duncan, A. L.; Corey, R. A.; Sansom, M. S. P. Defining How Multiple Lipid Species Interact with Inward Rectifier Potassium (Kir2) Channels. *Proc. Natl. Acad. Sci. U. S. A.* **2020,** *117*, 7803-7813.
(396) Duncan, A. L.; Song, W.; Sansom, M. S. P. Lipid-Dependent Regulation of Ion Channels and G Protein-Coupled Receptors: Insights from Structures and Simulations. *Annu. Rev. Pharmacol. Toxicol.* **2020,** *60*, 31-50.
(397) Moradi, S.; Nowroozi, A.; Shahlaei, M. Correction: Shedding Light on the Structural Properties of Lipid Bilayers Using Molecular Dynamics Simulation: A Review Study (Vol 9, Pg 4644, 2019). *RSC Adv.* **2019,** *9*, 7687-7687.

Author information

Corresponding author

*E-mail: franz.hagn@tum.de. Phone: +49 89 289 52624

Biographies

Umut Günsel

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 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 starts with chaperone proteins during his M.Sc. and continues with protein translocation studies into mitochondria during his Ph.D. Currently, as the new 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

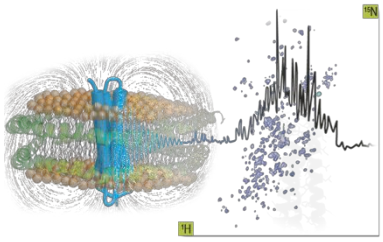
Franz Hagn studied biochemistry from 1998 to 2003 at the Universities of Bayreuth, Germany and Stockholm, Sweden. He worked in the lab of Astrid Gräslund and Lena Mäler in Stockholm on the dynamics of a membrane-bound peptide in different membrane-mimicking media and with Jochen Balbach on using real-time NMR to follow protein folding processes. In 2004, funded by the Elite Network of Bavaria, he moved to the Technical University of Munich and conducted his doctoral research with Horst Kessler where he used NMR to determine the structure and dynamics of molecular chaperones, tumorsupressor and spider silk proteins. After graduation in 2009 with "*summa cum laude*" pursued postdoctoral research with Gerhard Wagner at Harvard Medical School in

Boston, USA, 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 was promoted to associate professor at TUM in 2020.

Acknowledgements

The authors acknowledge funding by the Helmholtz Society (VH-NG-1039), the Technical University of Munich – Institute for Advanced Study, funded by the German Excellence Initiative and the European Union Seventh Framework Program under grant agreement n° 291763, and the Center for Integrated Protein Science Munich (CIPSM). All current and former members of the Hagn Lab are gratefully acknowledged for their enthusiasm and dedication in the development and application of lipid nanodiscs for structural studies of membrane proteins. Furthermore, we want to thank Dr. Gerhard Wagner (Harvard Medical School) for his support and collaboration in the field of lipid nanodiscs and their application to challenging membrane protein systems.

TOC Figure



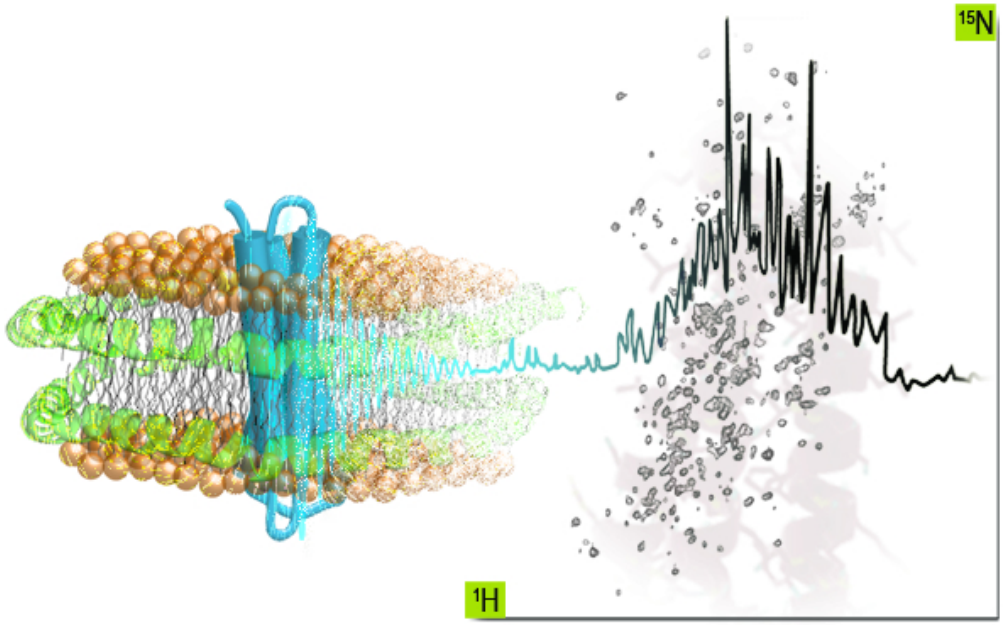


Table of Content Figure

50x50mm (300 x 300 DPI)

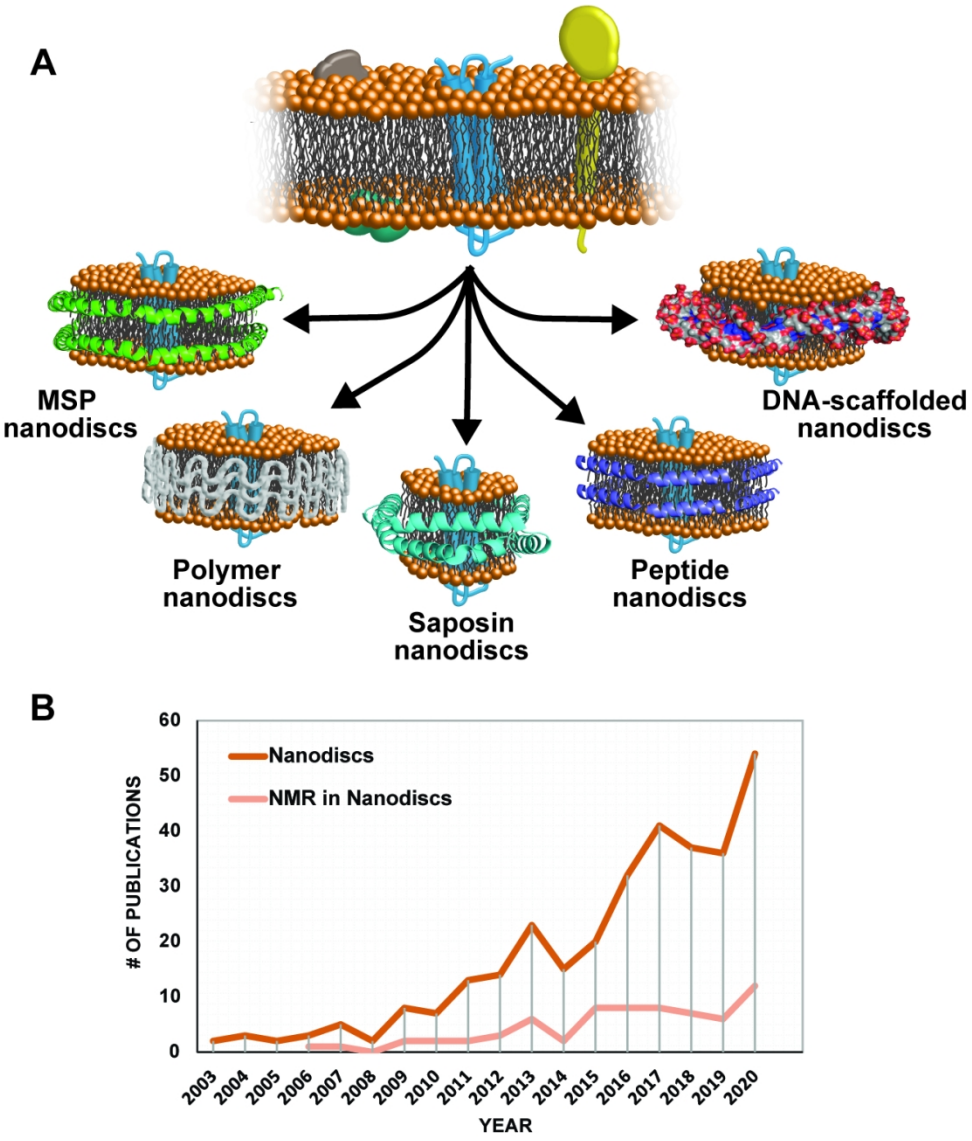

A
MSP nanodiscs
Polymer nanodiscs
Saposin nanodiscs
Peptide nanodiscs
DNA-scaffolded nanodiscs
B
Nanodiscs
NMR in Nanodiscs
OF PUBLICATIONS
YEAR
0
10
20
30
40
50
60
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020

Figure 1

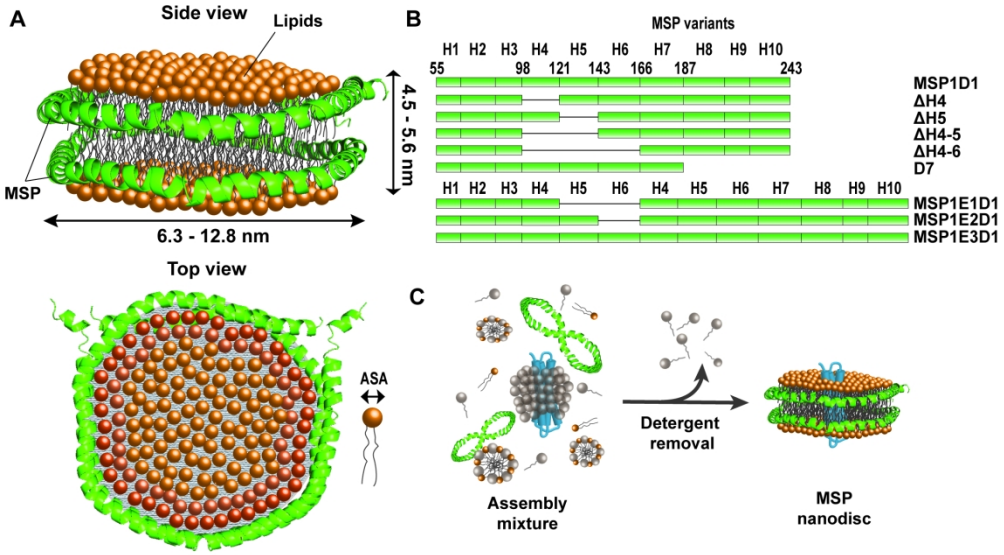


Figure 2

187x106mm (300 x 300 DPI)

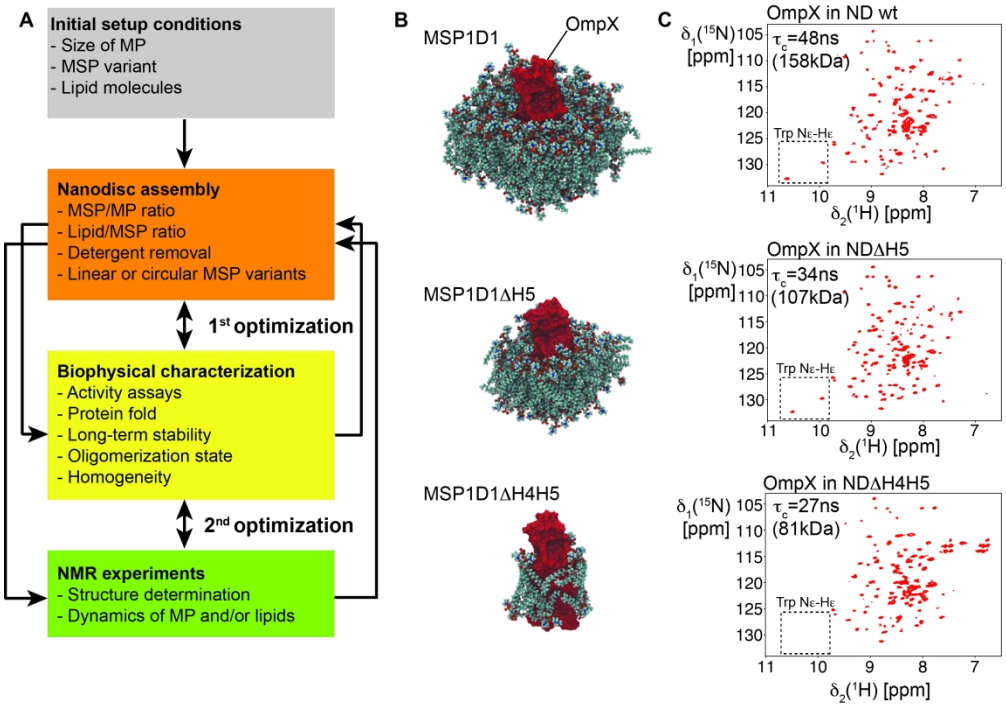

A
Initial setup conditions
- Size of MP
- MSP variant
- Lipid molecules
Nanodisc assembly
- MSP/MP ratio
- Lipid/MSP ratio
- Detergent removal
- Linear or circular MSP variants
1st optimization
Biophysical characterization
- Activity assays
- Protein fold
- Long-term stability
- Oligomerization state
- Homogeneity
2nd optimization
NMR experiments
- Structure determination
- Dynamics of MP and/or lipids
B
MSP1D1
OmpX
MSP1D1ΔH5
MSP1D1ΔH4H5
C
OmpX in ND wt
τc=48ns
(158kDa)
OmpX in NDΔH5
τc=34ns
(107kDa)
OmpX in NDΔH4H5
τc=27ns
(81kDa)
Trp Nε-Hε
δ1(15N) [ppm]
δ2(1H) [ppm]

Figure 3