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Assembly of phospholipid nanodiscs of controlled size for structural studies of membrane proteins by NMR

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Abstract

Suitable membrane mimetics are crucial for performing structural and functional studies of membrane proteins. Phospholipid nanodiscs (formed when a membrane scaffold protein encircles a small portion of a lipid bilayer) have native-like membrane properties. These have been used for a variety of functional studies, but structural studies by high-resolution solution-state NMR spectroscopy of membrane proteins in commonly-used nanodiscs of 10 nm diameter were limited by the high molecular weight of these particles causing unfavorably large NMR line widths. We have recently constructed truncated versions of the membrane scaffold protein allowing the preparation of a range of stepwise smaller nanodiscs (6–8 nm diameter) to overcome this limitation. Here, we present a protocol on the assembly of phospholipid nanodiscs of various sizes for structural studies of membrane proteins with solution-state NMR spectroscopy. We describe specific isotope labeling schemes required for working with large membrane protein systems in nanodiscs and provide guidelines on the setup of NMR non-uniform sampling (NUS) data acquisition and high-resolution NMR spectra reconstruction. We discuss critical points and pitfalls relating to optimizing nanodiscs for NMR spectroscopy and outline a strategy for the high-resolution structure determination and positioning of isotope-labeled membrane proteins in nanodiscs using NOESY spectroscopy, residual dipolar couplings (RDCs) and paramagnetic relaxation enhancements (PREs). Depending on the target protein of interest, nanodisc assembly and purification can be achieved within 12 – 24h. While the focus of this protocol is on protein NMR, these nanodiscs can also be used for (cryo-) EM and small-angle X-ray and neutron scattering studies.

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Author contributions

F.H. performed experiments and analyzed data; F.H. wrote the paper and G.W. and M.L.N. commented on and edited the paper.

Competing financial interests

The authors declare no conflict of interest.

INTRODUCTION

Despite the high relevance of membrane proteins in health and disease, as well as drug design, little is known about the structures of this protein class. Membrane proteins only represent about 2% of all structures deposited in the protein data bank. This is primarily due to difficulties of producing integral membrane proteins in folded and active forms as well as in sufficient yields. A major part of this problem is the selection of an appropriate membrane-mimicking environment supporting both function and stability of a particular membrane protein. Usually detergents are employed for membrane protein preparation and detergent micelles are the most common media for structural investigations¹. This is because these systems are easy to prepare and are often of small size, which is favorable for solution NMR spectroscopy. However, crystal structures of membrane proteins solubilized in detergents often contain bound lipids emphasizing the beneficial effect of a lipid environment for their structure and stability².

Detergents may also lower membrane protein stability, alter their structure and interfere with their biological function³. This can result in loss of enzymatic activity or the inhibition of ligand binding properties that induce allosteric conformational changes required for signal transduction across a biological membrane. In addition, detergents can hamper interaction studies between membrane proteins and soluble factors, as they tend to destabilize or even denature soluble interaction partners^{4,5}. Bicelles⁶, a mixture of long-chain and short-chain lipids or detergents, provide a more realistic membrane environment and have been extensively used for solution NMR studies^{7–9}. However, due to the presence of a short-chain lipid or detergent, similar problems arise as encountered with detergent micelles.

In contrast, the use of phospholipid bilayers can overcome these problems, as they resemble a considerably more native membrane environment. Phospholipid bilayers can be prepared to match any desired lipid composition, required to stabilize the respective membrane protein. Until recently, phospholipid liposomes and supported bilayers were the only means to harbor membrane proteins in a detergent-free lipid environment. Unfortunately, these systems are not compatible with crystallization and these particles are too big for solution-state NMR as well. However, crystallization in the lipid cubic phase has had an important impact for structural studies of membrane proteins^{10,11}. Among NMR methods, solid-state NMR has made progress in studying membrane proteins in large phospholipid bilayer systems¹² but signal overlap and low sensitivity are preventing structural work at high-resolution in most cases.

The recent introduction of phospholipid bilayer nanodiscs as membrane mimetics^{13–15} appears promising for studying membrane proteins in a native like environment by solution NMR. The formation of nanodiscs is based on the observation that apolipoprotein A-I (ApoA-I) can wrap around small patches of bilayer creating small membrane-like particles of defined size^{16,17}. Different versions of Apo A-I have been engineered for biophysical studies and are called membrane-scaffolding proteins (MSPs)¹³. Ideally, two copies of MSP wrap around a patch of a lipid bilayer to form a disc-like particle or nanodisc^{13,18} of a well-defined diameter, which is solely dependent on the length of the MSP. For assembly of these particles, any kind of bilayer-forming lipid can be used, depending on the requirements of

the particular membrane protein of interest. Nanodiscs with a diameter of around 10 nm and higher have been widely used for 2D heteronuclear NMR spectroscopy^{19–21}. However, the use of nanodiscs for multidimensional NMR experiments in solution as required for chemical shift assignments and structure determination remained elusive due to the still large molecular weight of these particles. To resolve these problems, we recently introduced a set of truncated MSP variants that produce smaller nanodiscs of diameters ranging from 6–8 nm²² (Fig. 1). Excitingly, the solution structure of nanodiscs assembled from one of our truncated variants, MSP1D1DH5, has been solved recently by solution NMR spectroscopy²³. The structure shows a protein double belt surrounding a lipid bilayer in an antiparallel manner and provides important insights on how the integrity of nanodisc structure is maintained. Furthermore, the authors used chemical shift comparison and paramagnetic relaxation enhancement experiments to show that the nanodiscs with and without helix 5 are structurally similar. In addition to the wide use in solution^{24–27} and solid-state NMR^{28–30}, these smaller nanodiscs have the potentials to be useful in (cryo-) electron microscopy^{31,32}, small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) studies of membrane proteins as the smaller diameters of these nanodiscs help decrease diffusion of the membrane proteins embedded inside. Moreover, these smaller nanodiscs have been used to interrogate the structure and compositions of exocytotic fusion pores as they prevent the dilation of the fusion pores³³.

Overview of the Procedure

The presented protocol covers all stages of nanodisc assembly. General protocols for MSP production and nanodisc assembly can also be found in previously published papers^{14,15,28} or online (<http://sligarlab.life.uiuc.edu/nanodisc/protocols.html>).

A basic overview of our nanodisc assembly procedure for NMR structural studies is shown in Fig. 2. In order to get started with nanodisc assembly, MSP proteins of various lengths have to be produced by heterologous expression (Steps 1–36 of the Procedure), lipid stocks need to be prepared (REAGENT SETUP), and properly folded membrane protein of interest (MPI) in detergent micelles needs to be available for the assembly of loaded nanodiscs (Steps 37–46 of the Procedure).

We usually screen for optimal nanodisc assembly conditions in a small volume. Parameters that need to be adjusted in each case are lipid composition, nanodisc size and MSP-to-MPI ratio. The way of detergent removal is another point to consider. We use either Biobeads (Biorad) or dialysis in a 10–20kD MWCO device. If the MPI contains an affinity purification tag (e.g. His₆ or His₁₀-tag), the loaded discs can be separated from the empty ones right after assembly. Such samples can then be further analyzed by size exclusion chromatography.

Once good conditions for nanodisc assembly and MPI incorporation are found, we usually scale-up the reaction to around 1–5 mL in volume to obtain at least 200 μM of MPI-containing nanodiscs for further biophysical and structural analyses. The quality of the obtained nanodisc preparation is further assessed by CD-spectroscopy (CD far-UV spectra and thermal melting experiments) and 2D-TROSY NMR experiments. Conditions that

provide highest possible thermal stability, as well as good NMR spectral quality will subsequently be used for more sophisticated NMR experiments.

We routinely employ sparse NMR data acquisition techniques (non-uniform sampling or NUS, BOX 1) to increase spectral resolution and minimize the required experimental time. For structure determination of an MPI, we prepare the selectively methyl-group-isotope-labeled protein (BOX 2) and use deuterated lipids for nanodisc assembly to ensure optimal NMR spectral quality. To determine the position of the MPI relative to the phospholipid bilayer membrane, Gd^{3+} -bound-lipids can be incorporated into nanodiscs and measure the paramagnetic relaxation enhancement (PRE) effect³⁴ of the Gd^{3+} on the signals of the MPI.

Taken together, this protocol will cover the assembly of smaller nanodiscs, the optimization of spectral quality for solution-state NMR as well as the procedure to obtain high-resolution structures of membrane proteins and their membrane position in a native-like phospholipid bilayer environment.

Experimental Design

Choice of MSP variants—In order to enable solution NMR structural studies of membrane proteins in phospholipid nanodiscs, we previously designed a series of truncated MSP variants²² (Fig. 1a) that form nanodiscs of subsequently smaller sizes, as probed by size exclusion chromatography and negative-stain EM (Fig. 1b). Production and purification of these MSP variants are described in detail in Steps 1–36. The long-term stability of the assembled discs strongly depends on their size, where the smallest discs (6–7 nm in diameter) are only stable for 1–2 weeks whereas the medium-sized discs (with MSP1D1DH4 and DH5, around 8 nm diameter) are stable for at least 50 days and thus are well-suited for NMR structural studies (Fig. 1c). This medium size still permits high-resolution work with solution state NMR spectroscopy and provides long-term stability of the incorporated membrane protein.

Any combination of bilayer-forming lipids as well as mixtures with cholesterol can be used for nanodisc assembly.

In any of these cases, the optimal MSP to lipid ratio needs to be empirically determined by a series of test assembly reactions and subsequent screening for sample homogeneity by size exclusion chromatography (See Steps 47–49 of the Procedure, and Table 1 for advice on MSP-to-lipid ratios). The required surface area of a membrane protein can be estimated based on the secondary structure, i.e. a single transmembrane (TM) helix has a diameter of approximately 6–10 Å, taking up around 80 Å². A six TM-helical protein would then require around 500 Å². The mean surface area of commonly used lipids is known, e.g. 57 Å² for DMPC, 52 Å² for DPPC, and 69 Å² for POPC¹⁵. Also, the lipid surface areas available in the various nanodiscs can easily be calculated using the known diameter of particular nanodiscs (Fig. 3). For the 8 nm diameter (6 nm inner diameter) nanodisc formed with MSP1D1DH5 (Table 1 and Ref.²²), the overall lipid surface area is around 2800 Å², providing, in theory, enough space for medium sized MPIs. However, in order to form a stable nanodisc complex, at least one layer of lipids between the MPI and the MSP is required. One DMPC lipid layer has a diameter of ~8.5 Å, thus reducing the available

diameter for a membrane protein from 6 nm to 4.3 nm, corresponding to a surface area occupied by 25 DMPC molecules. A second layer of lipids turns out to be empirically required for obtaining decent NMR spectra. In this case, this would reduce the diameter of the nanodisc that can be replaced by a membrane protein by another 1.7 nm, resulting in a diameter of 2.6 nm, or 10 DMPC molecules or around 600 Å². These straightforward calculations usually save valuable time during nanodisc screening at a later stage and provide a fair estimate of the spatial requirements of a given membrane protein.

The screening process—The overall task during the screening process is to find optimal values for a number of variables in the nanodisc assembly setup (Fig. 4). This includes nanodisc size, lipid composition, ratios between MSP and the MPI, as well as between MSP and lipids. This multidimensional search space can be reduced by the geometric considerations addressed in the previous paragraph. In addition, it is often known from previous studies whether the MPI is a monomer or an oligomer, which is crucial for finding the correct nanodisc size.

To screen for a suitable nanodisc size, we usually use a standard lipid (DMPC or POPC) and perform small-scale nanodisc assemblies with different MSP variants, as shown in Fig. 1b. The corresponding lipid-to-MSP ratios for empty nanodiscs are listed in Table 1. Depending on required surface area of a membrane protein that needs to be inserted, we usually subtract the corresponding number of lipid molecules from the initial lipid-to-MSP ratio, taking into account the mean surface area of the used lipid. We then set up assembly reactions using different MSP variants and the assumed MSP-to-lipid ratio. The ratio between MPI and MSP is kept constant at 1:4, ensuring that empty nanodiscs are present in excess. This situation prevents artificial oligomer formation, but in many cases still needs to be optimized at a later stage. The resulting nanodiscs are purified by Ni-NTA affinity chromatography, utilizing a His-tag (or any other affinity tag) on the target membrane protein to selectively pull out occupied nanodiscs. These preparations are then further analyzed by size exclusion chromatography and thermal melting experiments. A typical size exclusion profile during optimization of the lipid-to-MSP ratio is shown in Fig. 3c.

A similar screening procedure can be applied to identify optimal lipids or lipid blends required for a particular MPI. Changes in this parameter usually lead to an increase in the yield of MPI insertion and in the thermal stability of the resulting particle.

For solution state NMR, the resulting particle needs to be small enough to obtain reasonably narrow linewidths, but big enough to accommodate for the membrane protein and the 1–2 layers of lipids. Proper insertion of MPI and nanodisc assembly also leads to high thermal stability of the MSP and the MPI. This can be assayed using thermal melting experiments detected with circular dichroism (CD) or fluorescence spectroscopy, and finally 2D-NMR spectroscopy (Fig. 4).

As prototype membrane proteins, we used the VDAC1 ion channel and the bacterial outer membrane protein OmpX, as shown in Fig. 5. Optimization of the lipid composition has been done with the VDAC1 anion channel (Fig. 5, left). It turned out that 25 % of negatively charged lipids (i.e. 75 % DMPC / 25 % DMPG) is beneficial for NMR spectral quality in

this case. Considering the fact that most biological membranes contain a negative net charge, the use of such a lipid blend reflects a more physiological situation. Another parameter is the nanodisc size. As mentioned in the above paragraphs, it can be estimated fairly well what MSP protein might be suitable for a certain membrane protein. Screening by size exclusion chromatography and thermal melting experiments provides initial hints for subsequent NMR experiments. Due to its medium size that permits insertion into a variety of nanodiscs, we used the bacterial outer membrane protein OmpX (Fig. 5, right) for NMR screening. A reduction in nanodisc size can increase the spectral quality whenever the size of the membrane protein of interest permits this procedure. A simple readout of NMR spectral quality are 1D- ^{15}N ; ^1H -TRACT experiments³⁵, where the R_2 relaxation rates of the TROSY (Rb rate) and anti-TROSY (Ra rate) components are used to calculate an apparent rotational correlation time that is directly related to the hydrodynamic size of the nanodisc particle (Fig. 5, right). In Fig. 5b, the particle size of OmpX nanodiscs decreases with subsequently smaller nanodiscs, as expected. However, in the smallest tested nanodisc (composed of MSP1D1DH4H5) a pronounced number of signals originating from isotope-labeled OmpX are missing, especially tryptophan side chain resonances (yellow box). This is indicative of the presence of direct contacts between OmpX and the MSP protein, i.e. a lack of lipid molecules around the target protein. Therefore, the medium sized nanodiscs (composed of MSP1D1 H5) have been selected for follow up NMR experiments in this case.

In general, the introduced procedure can be adapted to any kind of membrane protein of interest. However, prior knowledge of the overall size of the membrane protein or oligomers thereof is required. In terms of lipid requirements, it is helpful to check the original membrane location of the MPI in a cellular environment and investigate the lipid composition of the particular membrane, which can be found in the literature^{36,37} or online provided by the University of Michigan (<http://opm.phar.umich.edu/atlas.php>). With this *ab initio* information, it is possible to optimize nanodisc assembly conditions for any given membrane protein. Another important factor is the thermodynamic stability of the MPI. In general, we observe that transfer of a MPI from detergent to a lipid bilayer environment depends on the stability of the protein, caused by precipitation occurring during the transfer from a detergent to a lipid environment. Furthermore, the transfer step from detergent micelles into a lipid environment can serve as quality control. If the MPI is not properly folded in detergent micelles, the yield of nanodisc incorporation will be low as well. In such a case, it is recommended to revisit the purification or refolding protocol of the particular MPI.

So far, nanodisc assembly has been reported for a large variety of integral membrane proteins, including α -helical 7-TM helical proteins^{19,22,38,39} or β -barrel proteins^{20,22,24,40–42}.

For successful NMR work, besides optimizing nanodisc assembly, sophisticated isotope labeling is an important factor. For initial sample optimization as shown in Fig. 5, U - ^{2}H ; ^{15}N -labeled protein is required as well as the use of TROSY NMR spectroscopy⁴³, simply due to the large size of any nanodisc system. For TROSY-based triple resonance backbone assignment experiments⁴⁴, the labeling pattern has to be expanded to U - ^{2}H ; ^{13}C ; ^{15}N .

Finally, for structure determination, selective methyl-group labeling⁴⁵ in a highly deuterated background is crucial to obtain high quality spectra and reduce spectral overlap but still keep a sufficient number of signals for high-resolution structure determination (BOX 1 and Fig. 6). At this step, the use of deuterated lipids is also recommended to further enhance spectral quality and reduce the signals arising from lipid molecules to a minimum.

Due to the fact that any lipid of interest can be inserted into phospholipid nanodiscs, the use of paramagnetically labeled lipids (like Gd³⁺-DTPA-DMPE), is a very convenient way of mapping the position of a membrane protein in nanodiscs. This has been applied to membrane anchored soluble proteins⁴⁶ and the integral membrane protein OmpX⁴⁰. Such paramagnetic lipid molecules lead to distance-dependent NMR signal attenuation and consequently provide information on the location of an amino acid residue relative to the lipid bilayer surface (Fig. 6).

For NMR backbone resonance assignment of a given membrane protein, we usually perform standard TROSY-based 3-dimensional triple resonance experiments, as summarized in Ref. ⁴⁴. For obtaining distance restraints for structure calculation, we run 3D or 4D ¹⁵N- or ¹³C-edited [¹H, ¹H]-NOESY experiments. Usually, all of these experiments are run in a non-uniformly sampled (NUS) manner⁴⁷, i.e. not all points are being recorded in the indirect dimensions and the missing points are reconstructed during data processing. The general setup of NUS experiments is described in BOX 2. This procedure leads to enhanced NMR spectral resolution and sensitivity^{48,49}.

Taken together, the presented protocol provides essential information on the practical aspects of nanodisc formation for NMR applications and the use of NMR to obtain high-resolution structures in a phospholipid nanodisc membrane environment. More recently, our group applied a SortaseA mediated ligation approach to construct even more robust nanodiscs⁵⁰. The presented protocols (the assembly of nanodiscs and the optimization of NMR spectral quality) still apply to these newly designed nanodiscs.

Limitations

In comparison to micellar systems, nanodiscs require controlled assembly conditions. Especially, for large (oligomeric) membrane proteins prior knowledge of the final size is needed for the selection of a suitable MSP. Furthermore, a defined composition of phospholipids is required to favor incorporation of membrane proteins into nanodiscs. These factors require a certain level of experience and additional time for optimization. More “open” membrane mimetics, like detergent micelles, phospholipid liposomes or bicelles, are somewhat easier to handle by a less experienced user.

MATERIALS

REAGENTS

For all items marked with a **! CAUTION** marker, please use proper personal protective equipment (gloves, eye protection and proper attire). For more information, please refer to each item's Material Safety Data Sheet (MSDS).

- *E. coli* expression plasmids, e.g. the pET28a-MSP plasmid, for the production of truncated MSP proteins can be obtained through Addgene Inc. (https://www.addgene.org/Gerhard_Wagner/)
- *E. coli* BL21 (DE3) cells can be purchased from e.g. EMD Millipore (cat. no. 69450)
- Kanamycin sulfate (Sigma-Aldrich, cat. no. K4000)
- LB powder (AMRESCO, cat. no. J106-2KG)
- Isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma, cat. no. I6758) ! **CAUTION** Do not breathe the dust. Avoid contact with skin and eyes.
- Sodium cholate hydrate (Sigma-Aldrich, cat. no. C6445)
- Triton X-100 (Sigma-Aldrich, cat. no. X100) ! **CAUTION** It is harmful; avoid contact with eyes.
- Lysozyme (Sigma-Aldrich, cat. no. L6876)
- 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids, cat. no. 850345)
- 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG) (Avanti Polar Lipids, cat. no. 840445)
- Fatty-acid deuterated lipids DMPC-*d*₅₄ and DMPG-*d*₅₄ (FB-reagents, Cambridge, MA or Avanti Polar Lipids)
- 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (gadolinium salt) (Gd-DTPE-DMPE) (Avanti Polar Lipids, cat. no. 790981P)
- Chloroform (American Bioanalytical, cat. no. AB00350) ! **CAUTION** Chloroform liquid and vapors are toxic. Wear a mask, gloves and chemical safety goggles while handling it.
- DNase I (Roche, cat. no. 10104159001)
- Ni-NTA agarose resin (Qiagen, cat. no. 30210)
- Imidazole (Alfa Aesar, cat. No. A10221) ! **CAUTION** imidazole is harmful.
- Biobeads SM-2 (Biorad, cat. no. 1528920)
- Methanol (EMD, cat. No MX0488) ! **CAUTION** methanol is toxic and flammable.
- Sodium chloride (NaCl; American Bioanalytical, cat. no. AB01915)
- Hydrochloric acid (HCl; EMD Millipore, cat. no. HX0603) ! **CAUTION** HCl is toxic and corrosive.
- Tris-HCl (American Bioanalytical, cat. no. AB02005)
- Sodium phosphate dibasic anhydrous (Fisher Scientific, cat. no. S375-500)

- Sodium phosphate monobasic anhydrous (Fisher Scientific, cat. no. S397-500)
- DTT (Roche, cat. no. 10708992001)
- Magnesium chloride (MgCl_2) (Fisher Scientific, cat. no. BP214-500)
- EDTA (Sigma-Aldrich, cat. no. EDS)
- Guanidine hydrochloride (GuHCl) (Sigma-Aldrich, cat. no. G4505)
- Tobacco etch virus (TEV) protease produced in house according to published protocols⁵¹. Also commercially available (Sigma-Aldrich, cat. no. T4455)
- Deuterium oxide (D_2O) (Sigma-Aldrich, cat. no. 151882 or Cambridge Isotope Labs, cat. no. DLM-4-99 or Cortecnet, cat. no. CD5250L)
- $^{15}\text{NH}_4\text{Cl}$ (Sigma-Aldrich cat.no. 299251 or Cambridge Isotope Labs, cat. no. NLM-467 or Cortecnet, cat. no. CN80P)
- D-glucose- $^{13}\text{C}_6$, D_7 (Sigma-Aldrich, cat. no. 552151, Cambridge Isotope Labs, cat. no. CDLM-3813 or Cortecnet, cat. no. CCD860P)
- D-glucose- $^{12}\text{C}_6$, D_7 (Sigma-Aldrich, cat. no. 552003, Cambridge Isotope Labs, cat. no. DLM-2062 or Cortecnet, cat. no. CD860P)
- ^1H , ^{15}N -labeled L-Phenylalanine and Tyrosine (Sigma-Aldrich, cat. no. 490105 and 332151)
- L-Alanine 3- ^{13}C ; 2-D (Sigma-Aldrich, cat. no. 740055 or Cambridge Isotope Labs, cat. no. CDLM-8649)
- L-Succinic acid- d_6 (Sigma-Aldrich cat. no. 488356 or Cambridge Isotope Labs, cat. no. DLM-584)
- Precursors for stereospecific methyl-group labeling: Ile- d_1 : 4- ^{13}C -3,3- d_2 ketobutyrate (Cambridge Isotope Labs, cat. no. CDLM-7318, NMR-Bio, cat. no. SLAM-I81, Sigma-Aldrich, cat. no. 594903) and Val- γ^2 / Leu-82 (pro-S): ethyl 2-hydroxy-2-(^{13}C)-methyl-3-oxobutanoate (Sigma-Aldrich, cat. no. 729531)
- Pf1-phage for partial alignment of nanodiscs (Asla Biotech, cat. no. P-50-P)

EQUIPMENT

- Cell-shaker (e.g. New Brunswick Innova 44, cat. no. M1282-0002)
- Thermomixer C (Eppendorf, cat. no. 5382000015)
- Sonicator (e.g. Branson Sonifier W-250 Digital)
- Swinging bucket centrifuge (e.g. Eppendorf type 5810 R)
- Centrifuge (e.g. Thermo Lynx-6000)
- Protein purification instrument, e.g. Äkta Pure (GE Healthcare, cat. no. 29-0182-24)

- Size exclusion columns (S200 Hi Load 16/600 and S200 Increase 10/300 GL) (GE Healthcare, cat. no. 28989335 and 28990944)
- Rocking platform shaker (e.g. VWR, cat. no. 10127-872)
- Amicon Ultra-15 centrifugal devices 10kD and 30kD MWCO (Millipore, cat no. UFC901096 and UFC903096)
- Glass syringe (Hamilton, cat. no. 1710 RN, 1750 RN)
- Argon or Nitrogen tank and gauge
- Glass tube, 12 × 75 mm (Fisher, cat. no. 14-961-26)
- NMR spectrometer (600 MHz or higher) equipped with a triple resonance cryogenic probe (Bruker BioSpin)
- Circular dichroism spectrometer (Jasco or others)
- Shigemi low-volume NMR tubes (Sigma-Aldrich cat. no. Z529451-1EA)
- NMRPipe, nmrDraw (<http://spin.niddk.nih.gov/NMRPipe/>), Topspin (Bruker BioSpin) and Sparky (<https://www.cgl.ucsf.edu/home/sparky/>), Cara (<http://cara.nmr.ch/doku.php>) or CCPNMR (<http://www.ccpn.ac.uk/v2-software/software/analysis>) software for processing and analysis of NMR data
- Structure calculation software (e.g. XPLOR-NIH, <https://nmr.cit.nih.gov/xplor-nih/>)
- Poisson-gap non-uniform sampling schedule generator and iterative soft thresholding (IST) reconstruction software (<http://gwagner.med.harvard.edu/intranet/istHMS/>)

REAGENT SETUP

Lipid preparation—Add the required lipid – we routinely use DMPC, DMPG, POPC, POPG DOPC or DPPC – (typically 10 – 25 mg/ml solution in chloroform) into a glass culture tube. Dry up organic solvent using nitrogen or argon gas stream in a fume hood. To remove residual solvent, place the tube in a vacuum dessicator overnight. Add buffer containing sodium cholate or other detergent (that is well-suited with membrane protein of interest) to the dried lipid film to obtain a 50 mM lipid, 100 mM cholate/other detergent solution. Typically, the final detergent concentration is at least twice the final lipid concentration in order to efficiently solubilize lipids. Vortex, sonicate and/or perform several freeze-thaw cycles by repeatedly placing the tube in liquid nitrogen and under hot tap water until the solution becomes clear. Quantification of lipid stocks can be done with ¹H NMR spectroscopy by peak integration of the methyl group signal at ~0.95 ppm proton chemical shift and comparison with an internal standard like DSS of known concentration, or phosphorus analysis according to established protocols (<https://avantilipids.com/tech-support/analytical-procedures/determination-of-total-phosphorus>)^{52,53}. These should be optimally prepared freshly but can also be stored for up to 30 days at –20 °C.

Bio-Beads—Before use, wash the Bio-beads in a gravity flow column with 2 CV methanol then extensively with ddH₂O to remove organic solvent. The beads can be stored at 4 °C in ddH₂O and 0.01% NaN₃ until further use (for at least 1–3 months). The amount of beads used for nanodisc assembly is ~ 0.5 – 1 g of the wet beads per every ml of the reconstitution mixture.

PROCEDURE

<CRITICAL> This protocol is focused on the insertion of membrane proteins into nanodiscs for subsequent structural studies using NMR spectroscopy. As mentioned in the section ‘experimental design’, the most critical step is the optimization of nanodisc assembly conditions taking into account various parameters like nanodisc size, lipid composition, relative ratios of lipids vs. MSP or MSP vs. membrane protein.

Expression of truncated MSP variants <TIMING> 1 d

<CRITICAL> Membrane scaffold protein (MSP) production is done according to established protocols. We generally observe that some MSP proteins are more soluble than others. For the MSP1D1 protein, roughly 50% of the protein is in the soluble fraction when produced at 37°C, for MSP1D1 H5 and MSP1D1 H4 more than 75% of the protein is in the soluble fraction. The shorter MSP variants (MSP1D1 H4H5, MSP1D1 H4-H6) are less soluble with a similar behavior as MSP1D1, i.e. 50/50 (soluble/insoluble). The MSP1D1 H4/2 construct behaves least favorably in terms of solubility and cannot be concentrated higher than ~ 150 µM.

In order to optimize the amount of soluble protein, expression can optionally be done at lower temperatures (30 °C or below). However, as MSP can be refolded from the guanidine hydrochloride (GuHCl) denatured state, we usually solubilize the insoluble inclusion bodies (IBs) in GuHCl. This procedure maximizes the overall protein yield as the MSP efficiently accumulates in the cells over time protected from degradation. The expression yield per liter culture is also dependent on the MSP construct and the use of a fermenter *versus* standard shaker flasks.

After NiNTA-purification of the MSP, we recommend cleaving off the His-tag. If the only protein containing a His-tag is the protein of interest, it is possible to isolate nanodiscs that contain the membrane protein of interest after assembly.

- 1 Freshly transform *E. coli* BL21 (DE3) cells with 50 ng of pET28a-MSP plasmid
- 2 Shake 50 mL LB culture supplemented with 35 mg/L Kanamycin in a baffled flask over night at 37 °C
- 3 Inoculate 4L LB medium supplemented with 35 mg/L Kanamycin (2x 2L in 6 L baffled flasks) and shake at 180 rpm at 37 °C until OD_{600nm} reaches 0.6–0.8
- 4 Induce protein production by addition of 1 mM sterile filtered IPTG (1 M stock in ddH₂O)
- 5 Let shake for further 3–4 h at 180 rpm and 37 °C

- 6 Harvest cells by centrifugation at 5000 g in 1 L bottles for 20 minutes at 4°C. The expected yield should be around 5g wet cell paste per liter culture.
 - 7 Re-suspend cell paste in ~50 mL MSP buffer (20 mM Tris/HCl pH7.5, 100 mM NaCl, 0.5 mM EDTA) using a serological pipette and transfer cells into 50 mL Falcon tubes
 - 8 Spin down cells at 3000 g for 20 min and at 4 °C in a swinging bucket table centrifuge
 - 9 Remove supernatant and store cell pellet at –80 °C.
- <PAUSE POINT> The cell pellet can be stored for up to 12 months.

Purification of truncated MSP variants <TIMING> 3 d

- 10 Thaw cells at RT for 30 minutes and resuspend cell pellet in ~4 mL 50 mM Tris/HCl pH 8.0 500 mM NaCl, 1 % TritonX-100, 1 mM EDTA per gram of cell paste and add one protease inhibitor cocktail tablet (Roche)
- 11 Add 1 mg lysozyme per mL of cell suspension (10 mg/mL stock solution in ddH₂O) and stir on ice for 30 minutes
- 12 Sonicate cells on ice (30 % duty cycle, 40 % power, 10 minutes net sonication time)
- 13 Add 5 mM MgCl₂ and tip of DNase I and stir on ice for 30 minutes
- 14 Spin down cell debris and insoluble material in SS34 rotor at 30000 g and 4°C for 45 min
- 15 Apply supernatant to Ni-NTA column (~1 mL resin/1 L cell culture) equilibrated with 50 mM Tris/HCl pH 8.0, 500 mM NaCl (= buffer A) + 1 % TritonX-100
- 16 Wash with 5–10 column volumes (CV) buffer A+ 1% TritonX-100 to wash off bound proteins from the MSP.
- 17 Wash with 5–10 CV buffer A + 50 mM Cholate to wash off bound lipids and residual TritonX-100 from the MSP
- 18 Wash with 5–10 CV buffer A
- 19 Wash with 5–10 CV buffer A + 20 mM imidazole
- 20 Elute protein with 3–5 CV buffer A+ 500 mM imidazole. Collect 2 mL fractions during elution
- 21 Monitor protein content in final wash fractions using a Bradford assay in a 96 well plate (90 µL (1x) Bradford reagent + 10 µL sample) and check protein purity with SDS-PAGE.
- 22 Combine protein-containing fractions and dialyze against 50 mM Tris/HCl pH 8.0, 20 mM NaCl, 0.5 mM EDTA using a 6–8 kD MWCO membrane

- 23 **His-tag cleavage:** To cleave off His-tag, add TEV protease (1 A₂₈₀ TEV for 100 A₂₈₀ MSP protein) and 1 mM DTT, incubate 4–12 h at 4 °C
- 24 Dialyze against 2 L 20 mM Tris/HCl pH 8.0, 500 mM NaCl using a 6–8 kD MWCO membrane
- 25 Apply protein solution to Ni-NTA column, wash with 10 mM imidazole, collect flow-through and wash fractions.
- 26 Change buffer to 20 mM Tris pH7.5, 100 mM NaCl, 0.5 mM EDTA by dialysis
- 27 Concentrate up to 500–600 µM, flash freeze in liquid N₂, store at –80 °C.
- 28 As a lot of MSP protein can be found in the insoluble fraction after cell lysis, we dissolve the pellet in 6 M GuHCl, 50 mM Tris/HCl pH 8.0, 500 mM NaCl
- 29 Centrifuge in SS34 rotor (30000 g, 45 min, 4 °C) to remove insoluble debris
- 30 Apply onto NiNTA equilibrated with 6 M GuHCl buffer
- 31 Wash with 5–10 CV 6 M GuHCl buffer
- 32 Wash with 50 mM Tris/HCl pH 8.0 500 mM NaCl (buffer A) to refold the MSP on the NiNTA column. This procedure gives quantitative refolding yields and the resulting refolded MSP preparations are behaving identical to the soluble MSP fraction during nanodisc assembly.
- 33 Wash with buffer A + TritonX-100, buffer A + cholate, buffer A, and buffer A + 20 mM Imidazole as mentioned above
- 34 Elute with buffer A + 500 mM Imidazole.
- 35 Perform buffer exchange, TEV cleavage, reverse Ni-NTA as described in steps 23–26.
- 36 Determine the yield of MSP protein. It should be around 20–50 mg per liter LB culture.

<PAUSE POINT> The MSP protein can be stored at –80 °C for at least 1 year.

Nanodisc assembly <TIMING> 1 – 3 d

<CRITICAL> Steps 37–46 describe nanodisc assembly. The conditions will need to be optimized for each new protein of interest. Nanodisc assembly and purification is identical for empty nanodiscs as for nanodiscs containing proteins. However, an affinity tag (e.g. His₆ or His₁₀) on the MPI can be used to selectively purify the loaded discs before SEC. This ensures that the sample homogeneity is monitored only for loaded discs during SEC.

- 37 Prepare MSP protein stock of ~500 µM in MSP buffer (20 mM Tris/HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA)
- 38 Prepare Cholate buffer: 100 mM sodium cholate in MSP buffer
- 39 Prepare Lipid stocks: 50 mM lipid in cholate buffer. See below for additional considerations on what lipids can be used for nanodisc assembly.

To do this, place an appropriate amount of lipid powder in cholate buffer and slightly heat the solution in hot tap water and vortex until dissolved

<CRITICAL STEP> If lipid in chloroform: add required amount of lipid to glass tube and dry under N₂ or Argon steam, place in vacuum dessicator overnight and dissolve white or clear pellet in cholate buffer.

<CRITICAL STEP> Depending on which MSP is used, the final lipid and cholate concentrations vary considerably. It is therefore critical to add additional cholate to a final concentration of above its critical micelle concentration (cmc), which is ~13 mM. We usually adjust the final cholate concentration to 20 mM. For cmc values of other detergents, see Table 2.

- 40** Add the components in the following order: MSP buffer, then Cholate buffer, then Lipids, then MSP, and lastly the membrane protein of interest.

e.g. For empty nanodiscs composed of MSP1D1 H5 (MSP:lipid = 1:50), to prepare a 3 ml reaction volume, combine the following reagents:

	Volume	Final concentration
MSP buffer	1.2 mL	
DMPC (50 mM stock in 100 mM cholate)	600 µL	10 mM lipid 20 mM cholate
MSP1D1 H5 (500 µM stock)	1.2 mL	0.2 mM

No extra cholate buffer is required, because the final cholate concentration is already at 20 mM.

For nanodisc assembly with a membrane protein of interest (MPI), e.g. OmpX, with an MSP-to-MPI ratio of 2:1 and a DMPC-to-MSP ratio of 40 (accounting for 10 lipid molecules to be subtracted per bilayer leaflet for each inserted OmpX molecule), a 3 ml volume might have the following composition:

	Volume	Final concentration
MSP buffer	600 µL	
100 mM cholate buffer	120 µL	up to 20 mM
DMPC (50 mM stock in 100 mM cholate)	480 µL	8 mM
MSP1D1 H5 (500 µM stock)	1.2 mL	0.2 mM
OmpX (500 µM stock)	600 µL	0.1 mM

CRITICAL STEP: The ratio of lipid to MSP needs to be adjusted in each case, see Table 1. The mean surface area per lipid in nanodiscs is 52 Å² for DPPC, 57 Å² for DMPC, and 69 Å² for POPC¹⁵. The presence of a to be inserted membrane protein also needs to be considered. If the structure of the membrane protein is known, the cross section of the trans-membrane area can be calculated

and divided by the surface area of the used lipids. With a membrane protein of unknown structure, the exact ratio needs to be determined experimentally, which can in the case of an α -helical membrane protein be guided by the estimation that the area of one transmembrane helix is around $140 \text{ \AA}^2$ (see 'experimental design' section for details)

- 41 Incubate the assembly mixture for 1–2 hours at the right temperature; this should be near the phase transition temperature of the lipids being used (see Table 3). The lipid mixtures we use the most are DMPC/DMPG (T_m are 24 and 23 °C, respectively) and POPC/POPG (T_m is -2 °C for each). Therefore, we perform assembly at room temperature and over ice, respectively. If we use other lipid mixtures where lipid components have significantly different T_m values, we perform the assembly at a temperature close to the T_m of the major lipid component. **[AUTHOR: I have moved this from the table legend to here, because it seems more appropriate to include it as part of the step.]** In the example of empty nanodiscs composed of MSP1D1 H5 (MSP:lipid = 1:50), incubate for 30 min-1 h at RT (for DMPC, on ice for e.g. POPC) **!! CRITICAL STEP:** Solution needs to be clear after incubation
- 42 To remove detergents, add 0.5 to 1 g Biobeads (Biorad) / mL assembly mixture. We add half the amount first, shake for 1 hour, then add second half and shake for another 1–3 hours. We usually perform this step at the same temperature as the nanodisc assembly. In the example of empty nanodiscs composed of MSP1D1 H5 (MSP:lipid = 1:50), let the mixture shake on orbital shaker for 2 h at room temperature.

<CRITICAL STEP> If a membrane protein is inserted into nanodiscs the temperature at this step depends on its thermal stability. In the case of a membrane protein with significantly lower stability than OmpX, we recommend performing the reaction at 4°C and using lipids with a phase transition temperature below 4°C, like POPC.
- 43 Remove Biobeads by punching a hole into the bottom of the reaction tube using a 20-gauge needle. The reaction mixture is then collected in a fresh tube of appropriate size. The remaining Biobeads should be washed once with MSP buffer and both fractions should be pooled afterwards.
- 44 Check whether the membrane protein of interest binds to Biobeads. If this is the case, detergent needs to be removed by dialysis against an at least 1000-fold higher volume of MSP buffer, which needs to be changed 3-4 times over 1–2 days. This step can be done e.g. with 20 kDa MWCO dialysis devices (Thermo Scientific). <TROUBLESHOOTING>
- 45 **OPTIONAL:** Remove empty nanodiscs from those containing the membrane protein of interest (MPI) by affinity chromatography (e.g. NiNTA for a His-tag containing MPI). We usually remove the His₆-tag from the MSP before nanodisc assembly and keep a His₆-tag on the inserted membrane protein.

- 46 Run size exclusion chromatography using a S200 column (see Equipment section) to check size of the assembled nanodiscs and their uniformity.

<PAUSE POINT> Empty nanodiscs can be stored for up to 2 months at 4 °C. For protein containing nanodiscs, this storage time needs to be determined experimentally. For the OmpX example, they can be stored for more than 12 months at 4 °C.

Optimization of nanodisc preparations for NMR spectroscopy <TIMING> 2–4 weeks

- 47 Referring to general protocols for MSP production (Steps 1–36) and nanodisc assembly (Steps 37–46), Table 1 and Table 2 (also previously published papers^{14,15} or online protocols (<http://sligarlab.life.uiuc.edu/nanodisc/protocols.html>)), screen for the optimal MSP-to-lipid ratio. As a starting point, the approximate values in table 1 should be used. Consider that the inserted membrane protein of interest (MPI) takes up space and competes with the lipids (see also **Step 40** and section “**Experimental design / The screening process**”).
- 48 Screen for optimal MSP-to-MPI ratio. This is important to obtain a homogenous nanodiscs preparation and/or shift the equilibrium to the monomeric form. Usually, we use a 4-10-fold excess of MSP. We usually have a His₆ or His₁₀ tag fused to the membrane protein which allows to pull out the occupied discs for further analysis.
- 49 Run S200 size exclusion column to check the homogeneity of the assembled nanodiscs and use conditions that give symmetric and narrow peaks at the smallest possible nanodiscs size.
- 50 Pool SEC peak and analyze the stability of the nanodiscs preparation using circular dichroism-detected thermal melting experiments. The melting point of the inserted membrane protein should be as high as possible. Nanodiscs usually melt at temperatures above 80°C, which does not interfere with the unfolding transition of the MPI. **! CRITICAL STEP:** Screen for high nanodisc and MPI stability at a smallest possible nanodisc size that allows for high spectral quality.
- 51 To determine which nanodisc size will result in the best NMR spectral quality, perform 1D-[¹⁵N;¹H]-TRACT experiments, where the T2 relaxation rate of the TROSY and anti-TROSY components are used to calculate an apparent rotational correlation time³⁵ (Fig. 5b).

! CRITICAL STEP: If nanodiscs size is too small for the inserted membrane protein, its spectral quality will be lower, with broader lines in the ¹H dimension and disappearing resonances, especially side-chain tryptophan signals (see Fig. 3b). **CRITICAL STEP:** Another important factor is the temperature during the NMR experiment. As a rule of thumb, NMR spectral quality is relatively low if the temperature is close to or below the phase transition temperature of the used lipids. Above that transition, the NMR spectral quality improves more linearly with temperature, due to faster rotational motion.

High-resolution non-uniformly sampled 3D-triple resonance NMR experiments

Structure determination and refinement <TIMING> > 1 month (strongly depending on inserted membrane protein)

- 52 For backbone assignment, record a conventional suite of TROSY-based triple resonance experiments⁴⁴ using a *U*-[²H,¹³C,¹⁵N]-labeled sample of a membrane protein in phospholipid nanodiscs of suitable size.

<CRITICAL STEP> The use of non-uniform sampling during data acquisition leads to high-resolution spectra and optimized sensitivity⁴⁸ (see BOX 1).

<CRITICAL STEP> After optimization of nanodisc assembly for NMR spectroscopy, the additional use of deuterated lipids is beneficial for NMR spectral quality, especially for less sensitive triple resonance experiments involving side-chain atoms, e.g. HNCACB. Furthermore, the use of deuterated lipids is highly recommended for NOESY experiments used for subsequent structure calculation as shown in Ref⁴⁰.
- 53 Perform data analysis and sequence specific resonance assignment. These can be done with various software packages, e.g. Sparky (T.D. Goddard and D.G. Kneller, UCSF), CCPNMR⁶² or CARA (Rochus Keller, Wüthrich lab, ETH Zurich), See Materials section for further information.
- 54 (optional) Perform 3D-¹⁵N-edited-[¹H,¹H]-NOESY to determine initial protein structural models. This is especially helpful for analysis of β -sheet proteins, because the protein fold can be easily determined by using backbone amide-amide NOE contacts. In other cases, this experiment is still helpful for backbone resonance assignment and the mapping of helical secondary structure (strong NOE contacts between neighboring amide HN spin pairs, see also Ref.⁶³)
- 55 For structure refinement, we use specifically methyl- and aromatics-labeled protein samples (Fig. 6a and BOX 2) in order to detect contacts in 3D or 4D ¹⁵N/¹³C-edited NOESY experiments between side-chain methyl groups, between methyl groups and aromatics, as well as between backbone amides and methyl groups or aromatics in an otherwise highly deuterated background in nanodiscs containing deuterated lipids (Fig. 4b,c). To perform structure refinement, the membrane protein of interest needs to be isotope-labeled as outlined in BOX 2 and inserted into suitable nanodiscs containing deuterated lipids. To identify NOE contacts for structure determination, run a suite of 3D NOESY experiments that are ¹⁵N and/or ¹³C edited in one or two dimensions. The NOE cross-peaks in these experiments can be used to extract distance restraints for subsequent structure calculation. The inclusion of distances involving side-chain atoms is particularly helpful to refine the overall side-chain packing and the overall shape of initial structures (Fig. 6d)
- 56 To further validate and refine the resulting structural model, we⁴⁰ and others²⁵ established the use of conventional Pf1 phage alignment medium⁶⁴ for the measurement of residual dipolar couplings (RDCs)⁶⁵ of membrane proteins in phospholipid nanodiscs. Due to their detergent-free nature, nanodiscs do not

interfere with the Pf1 phage medium and permit stable partial alignment (Fig. 7d–f). To do this, add 5–10 mg/mL Pf1 phage to the U -[^2H , ^{15}N]-labeled membrane protein of interest in nanodiscs and record two sets of 2D NMR experiments consisting of a 2D-[^1H , ^{15}N]-TROSY and 2D-[^1H , ^{15}N]-HSQC or 2D-[^1H , ^{15}N]-semi-TROSY experiment. These experiments need to be recorded in the presence and absence of Pf1 phage. The distance (in Hz) between the signals of a particular HN-amide pair in the TROSY and semi-TROSY spectra corresponds to the one-bond scalar coupling constant (1J) in the absence of an alignment medium (isotropic) and to the sum of the scalar and residual dipolar coupling ($^1J + ^1D$) in the aligned case. The residual dipolar coupling (RDC) can be easily extracted by subtracting the apparent coupling in the isotropic case from the value obtained in the aligned case. The value of the RDCs is dependent on the orientation of the HN bond vector and therefore suitable for structure refinement, independently from the NOE data.

(optional) Membrane positioning in nanodiscs using paramagnetic lipids

- 57 To probe the orientation of a membrane protein in respect to the phospholipid bilayer membrane, spin-labeled or paramagnetic lipids can be used like Gd-DTPE-DMPE⁶⁶ (Fig. 5a), which carries a chelated Gd^{3+} ion in its head-group. Prepare modified nanodisc assemblies by following Steps 37–46, using the paramagnetic lipid in a final concentration corresponding to one molecule per bilayer leaflet (1:50 for MSP1D1 H5 nanodiscs).
- 58 Compare the intensity in 2D-[^1H , ^{15}N]-TROSY NMR spectra performed with and without Gd-DTPE-DMPE. In NMR experiments, the presence of a paramagnetic agent leads to the distance-dependent signal decrease of resonances corresponding to amino acids that are close to the bilayer head-group region or even outside the membrane. Any residue in the hydrophobic bilayer region is protected from the effect of the paramagnetic label and consequently does not experience a reduction in signal intensity (Fig. 7a,b).
- 59 Use the signal intensity pattern to identify whether the protein of interest is an integral membrane protein and how the protein is oriented in the membrane, i.e. determination of the approximate tilt angle relative to the bilayer normal (Fig. 7c).

TIMING

These numbers are rough estimates and in particular the NMR structure determination time frame can differ substantially, depending on the complexity and size of the investigated membrane protein.

- Preparation of MSP protein: 4 d
- Preparation of lipids from chloroform: 12 h
- Incubation of the assembly ingredients to ensure proper mixing: 2 h

- Detergent removal and nanodisc assembly: 2–4 h using Biobeads; 1–2 d with dialysis
- OPTIONAL: Affinity purification of membrane-protein-containing nanodiscs: 3 h
- Final size exclusion chromatography: 2–4 h
- NMR backbone assignment experiments: ~ 7 d
- NMR NOESY experiments: ~ 7 d
- Resonance assignment: ~ 1–3 months
- Structure determination: > 1 month

TROUBLESHOOTING

General Comments

- Never add more than 5% glycerol (used as co-solvent for stabilization of a membrane protein before insertion into nanodiscs) to the assembly reaction as this interferes with nanodisc assembly
- If the MPI tends to precipitate during assembly, one possible reason might be sensitivity against cholate that is used for lipid solubilization. This can be resolved by preparing lipid stocks in the same detergent that is used for MPI purification or other suitable detergent.
- In case the nanodisc assembly gives poor yields and results in larger particles than anticipated for a certain MPI and MSP combination, the user might consider possible oligomerization of the membrane protein. This can be solved by either using a longer MSP variant or a larger excess of MSP to target protein. For mostly monomeric MPIs we use a 4:1 (MSP:MPI) ratio (i.e. 2 nanodiscs per target protein molecule), but this can be adjusted to up to 10–20 in sensitive cases.

Specific problems

See Table 4 for further troubleshooting advice.

ANTICIPATED RESULTS

The presented protocol will provide the necessary skills to successfully produce MSP proteins of various lengths (BOX 1) and assemble nanodiscs of different sizes and lipid compositions, as outlined in Figs. 1 and 2 and BOX 2. In Fig. 1c, we give an estimation of the nanodisc stability, which goes down with decreasing size. This aspect should also be considered for the selection of an appropriate MSP variant. The main purpose of this protocol is the optimization of nanodisc assembly for NMR. This is outlined in the **procedure** section and is schematically shown in Fig. 2. The protocol discusses the parameters that can be altered to obtain high-quality nanodisc preparations, such as considerations on the correct nanodisc size and lipid-to-MSP ratios (Fig. 3). Our preferred

nanodisc quality check is a combination of size exclusion chromatography, circular dichroism and ultimately NMR spectroscopy (Fig. 4).

This optimization process for NMR is shown in Fig. 5 for the VDAC1 anion channel and the outer membrane protein X (OmpX). The user can apply NMR to monitor the effect of lipid composition, temperature and nanodisc size on the spectral quality of the membrane protein spectrum and relaxation properties (indicative of the apparent molecular weight). Another important piece of information in these spectra is the possibility to detect tight packing of the MPI into nanodiscs. In these cases, line broadening and disappearing signals, especially of tryptophan side chain resonances (Fig. 5b) are indicative of direct contacts between the MPI and the MSP possibly due to lacking of sufficient layers of lipids between them (see also Fig. 3). The charge of the lipids used during assembly might also play a role in the interaction between the MSP and the MPI, as shown for the VDAC1 anion channel in DMPC²⁰ vs. DMPC/PG nanodiscs⁵⁰. There, a net negative lipid charge resulted in the disruption of the MSP-VDAC1 interaction.

Due to the large size of nanodisc-MPI complexes, sophisticated NMR experiments require specific isotope labeling as published earlier⁴⁵ and outlined in Box 2. The overall goal is to obtain highest possible spectral quality using protein per-deuteration and, at the same time, introduce a specific set of protonated and isotope-labeled sites to facilitate the extraction of distance information for NMR structure determination (Fig. 6). For NMR data acquisition, the user should be aware of the possibility to apply non-uniform sampling (NUS) methods (see Box 1) that will speed up the data acquisition process and lead to high-resolution spectra. Furthermore, paramagnetically labeled lipids, like Gd³⁺-DTPA-DMPE can easily be incorporated into nanodiscs to obtain information on the membrane protein position relative to the lipid bilayer surface (Fig. 7a–c).

Finally, we emphasized the use of a conventional alignment medium, such as filamentous Pf1 phages, for the measurement of RDCs of membrane proteins in nanodiscs. Membrane proteins in detergents are not compatible with Pf1 phages as detergents tend to destabilize their liquid crystalline properties (Fig. 7d–f).

Overall, this protocol should provide sufficient information to perform the most important steps of nanodisc assembly and structure determination of membrane proteins by NMR. The cited references should provide additional information on the individual steps. However, the details and the overview provided in this protocol paper can only serve as a starting point for further optimization, which will heavily depend on the particular target membrane protein of interest.

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BOX 1**NMR assignment experiment setup with NUS <TIMING> 1 h**

In non-uniform sampling methods for NMR data acquisition only a subset of data points (sampling schedule) is recorded. The missing points are reconstructed by computational methods. This procedure offers the advantage of high-resolution spectra acquisition and increased sensitivity. It is crucial to use a sampling schedule that is adapted to the size and relaxation properties of the particular NMR sample. Reconstruction of the final NMR data can be done by a variety of computational methods^{54–59}. In our setup, we use Poisson-gap sampling^{49,60} and perform spectral reconstruction using the iterative soft thresholding algorithm⁶⁰.

Sampling schedule generation

- 1.) We use our in-house non-uniform sampling (NUS) schedule generator that employs Poisson-gap sampling^{49,60}. A web-based generator can be found at: http://gwagner.med.harvard.edu/intranet/istHMS/gensched_new.html. This step is required to determine the to-be-recorded data points for a particular protein and needs to be calculated for each case.
- 2.) Required input is the number of dimensions (3D in most cases), the sampling density (10–30% for 3Ds, 1–5% for 4Ds), the number of complex points in the two indirect dimensions and the spectral widths in ppm. As a rough estimate, the number of complex points should be expected number of signals in the respective NMR experiment. The first indirect “slow” dimension is ¹³C in triple resonance experiments, the second indirect “fast” dimension is ¹⁵N. An estimation of the relaxation properties of each nucleus can be performed based on temperature and molecular weight of the protein system.
- 3.) Click on “Generate Schedule” and save it as a sorted or random list. This schedule can then be used for NUS setup in Topspin. Alternatively, the user can employ the Topspin NUS module for setup, according to the manufacturer’s instruction and based on published methods⁵⁴.

NUS NMR experiment setup in Topspin

- 4.) In the Bruker Topspin (version 3.0 or higher) acquisition parameter menu adjust spectral widths and number of increments (which is double the value as the number of complex indirect points that has been set in the schedule generator) in the same manner as done for NUS schedule generation. The parameter “FnTYPE” should be “non-uniform_sampling”. Scroll down the parameter window to “NUS parameters” and adjust the values for NusPOINTS to the number of points in the NUS schedule. Choose a name for the Bruker topspin-generated NUS list “NUSLIST”, i.e. nuslist_{expno} and press calculate. This will generate a nuslist in the variable delay (vd) directory in the topspin list directory. This schedule is already suitable for

- running the NUS experiment. However, we generally use our own schedules that have been generated with Poisson-gap sampling.
- 5.) Copy the Poisson-gap sampling schedule to the “vd” directory. These files have a two-column format for a 3D experiment, or 3 columns for a 4D experiment, respectively. In order to use the sampling schedule in Topspin, the two columns need to be switched, i.e. ^{15}N dimension is the 1st column, ^{13}C is the 2nd. Replace the Topspin-generated schedule file by this file. Keep the same file name.
 - 6.) The 3D NMR experiment can now be started just as in the uniformly-sampled case. The progress of the experiment and the sampled points can be monitored by typing ‘eda’ in the command line and clicking on the “Show” bottom under “NUS parameters”.

Spectra reconstruction using iterative soft thresholding (IST)

For processing of NUS data we use NMRpipe software⁶¹ and in-house scripts as described in an online tutorial (<http://gwagner.med.harvard.edu/intranet/istHMS/234Proc.html>). In brief, a Fourier transformation of the direct detected ^1H dimension is performed first. Then we use the hmsIST software⁶⁰ (available from the authors upon request for Linux/Mac OS) to reconstruct the missing data points in the indirect 2D matrix. Afterwards, the remaining two dimensions are processed in a standard manner using NMRpipe, as covered in the online tutorial.

BOX 2**Specific labeling of methyl groups and aromatic amino acids**

1. Protein production is done in *E. coli* strain BL21 (DE3), however, any other suitable *E. coli* strain that is required for efficient protein production in each particular case works as well. Use established protocols for protein expression and bacterial cell adaptation in M9 D₂O medium⁶⁷.
2. We prefer methyl-group labeling⁶⁸ of proteins in a stereospecific manner^{69–71}. We routinely produce protein samples with the following labeling pattern: U-[²H,¹⁵N],[¹H,¹³C]-Ala-β, Val-γ2, Leu-δ2, Ile-δ1-U-[¹H,¹⁵N]-Phe, Tyr (Fig. 6a)⁴⁰. In order to achieve isotope labeling, add the following components 1 hour prior to induction of protein production: 2.5 g L-succinic acid-d₆ ! **CRITICAL STEP:** check pH of medium after succinate addition ! and 0.8 g L-alanine 3-¹³C; 2-D (for alanine β labeling)⁷⁰, 300 mg ethyl 2-hydroxy-2-(¹³C)-methyl-3-oxobutanoate (for Val-γ2 and Leu-d2 labeling)^{69,71} and 80 mg 4-¹³C-3,3-d₂ ketobutyrate (for Ile-d1 labeling)⁴⁵ (see REAGENTS section), as well as 80 mg of each U-[¹H,¹⁵N]-Phe and Tyr per liter of deuterated M9 medium prepared with 99.8%-[D]-D₂O and supplemented with 1 g ¹⁵NH₄Cl and 2 g U-[²H,¹²C]-D-glucose according to established protocols^{45,68}. In general, the spectral quality of such samples as monitored with 2D-[¹⁵N;¹H]-TROSY or 2D-[¹³C;¹H]-HMQC experiments is good enough for multidimensional NOESY experiments for structure determination (Fig. 6b).

Fig. 1. Design of smaller nanodiscs for solution NMR spectroscopy

(a) Structural model of a phospholipid nanodisc where two copies of membrane scaffold protein (MSP) wrap around a patch of phospholipid bilayer, the length of the MSP determines the diameter of the disc. For empty nanodiscs, an empirical formula can be used to calculate the diameter of the resulting nanodisc assembled with a particular truncated MSP variant²²: $D = (n(\text{aa}) \cdot 0.15 \text{ nm}/\pi) + 1 \text{ nm}$, where $n(\text{aa})$ is the number of amino acids in contact with lipids, which is 180 for MSP1D1 (b) Schematic overview of available truncated MSP constructs. (c) Size exclusion chromatography (SEC) profiles using a S200 increase 10/300 GL column (x-axis: Elution volume (mL), y-axis: A280nm (a.u.)). Middle: negative-stain EM showing single nanodisc particles for the MSP variants. Right: Average diameter of nanodisc preparations showing a decrease in nanodisc diameter with shorter MSP variants. The number of particles used for the size analysis are indicated. Error bars represent standard deviation. (d) Long-term stability of these nanodiscs. SEC runs of freshly assembled nanodiscs (blue) and after 50 days at 4°C (red) are shown. The smallest nanodiscs (ΔH4H5 and ΔH4-H6) disassemble after some weeks, whereas the medium-size discs (ΔH4, ΔH5) are stable for at least 50 days. Figure adapted from Ref²².

Fig. 2.
Overview of the general procedure of nanodisc assembly for NMR structural studies.

Fig. 3. Geometrical considerations on the available lipid bilayer surface area in a nanodisc that can be replaced by a target membrane protein

(a) Values are calculated for DMPC, which occupies a surface area of 57 Å², leading to an occupied surface diameter of 8.5 Å, taking into account a circular shape. Other lipids differ in their mean surface area occupation, as indicated in the Procedure, step 40 and the Experimental design section. (b) Required surface area of a GPCR (left) and the β-barrel protein OmpX. The 8 nm nanodiscs, having an available inner diameter of 2.6 nm will most likely be too small for the GPCR, but perfectly suited for OmpX. (c) Experimental screen for an optimal lipid-to-MSP ratio using two truncated MSP variants and DMPC lipid

without target membrane protein. In cases where a too low amount of lipid is used, free MSP is visible in the SEC profile, whereas in the opposite case, lipid-MSP aggregates (A) build up with increasing lipid:MSP ratio. A: Aggregates, ND: nanodiscs, MSP: free membrane scaffold protein

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Fig. 4. Flow scheme for the optimization of nanodisc preparations for NMR spectroscopy

The first step is the screening for a suitable lipid composition using initial nanodiscs assembled with MSP1D1. We often screen with an increasing amount of negatively charged lipids, but this can also be done with other functionally relevant lipids and cholesterol. The first readout is the homogeneity of the preparation by SEC and the NMR spectral quality. The second step is to vary the size of the nanodiscs in order to optimize NMR spectral quality. We routinely used MSP1D1, MSP1D1 H4 or H5 and MSP1D1 H4H5 nanodiscs. The resulting nanodiscs are analyzed with SEC, circular dichroism (CD) thermal melting experiments and NMR spectroscopy. For NMR, a series of 2D- $[^{15}\text{N};^1\text{H}]$ -TROSY experiments of an isotope-labeled inserted membrane protein are recorded at increasing temperatures, ranging from 30 to 50 °C. There, in order to obtain reasonable spectral quality, the temperature of the NMR experiment should be kept above the phase transition temperature of the used lipid (see Table 3).

Fig. 5. Optimization of NMR spectra

(a) 2D- ^1H ; ^{15}N -TROSY spectra of VDAC1 in DMPC (upper panel) and DMPC:DMPG (3:1) (lower panel) nanodiscs assembled with MSP1D1. The latter spectrum was recoded at higher temperature. Projections of TROSY spectra at various temperatures along the ^{15}N -dimension are shown to emphasize the effect of temperature on spectral quality. 1D NMR spectra in subpanel (a) represent projections in the ^1H dimension of the shown 2D-spectra at the indicated experimental conditions (b) 2D- ^1H ; ^{15}N -TROSY spectra and ^{15}N -TRACT experiments³⁵ of U- ^2H , ^{15}N -labeled OmpX in nanodiscs of subsequently smaller size; τ_c : correlation time. The apparent relaxation rates indicate particles of 158, 107 and 81 kDa molecular weight for nanodiscs assembled with MSP1D1, MSP1D1 H5 and MSP1D1 H4H5, respectively. However, the spectral quality of OmpX in the smallest nanodisc is worse than in the intermediate-sized discs, possibly due to direct contact between the protein side chains and the MSP protein, leading to the disappearance of tryptophan side-chain Ne-He resonances.

Fig. 6. High-resolution structure determination of membrane proteins in smaller nanodiscs using multidimensional NMR spectroscopy and selective isotope labeling

(a) $U\text{-}^2\text{H}$, ^{12}C , ^{15}N -OmpX was selectively isotope labeled with stereospecific [^{13}C , ^1H] methyl groups and [^1H , ^{15}N]-labeled phenylalanine and tyrosine. (b) 2D- ^1H ; ^{15}N -TROSY and 2D- ^1H ; ^{13}C -HMQC spectra of OmpX incorporated into MSP1D1 H5 nanodiscs assembled with d_{54} DMPC: d_{54} DMPG (3:1) deuterated lipids. (c) This labeling pattern gives abundant NOE cross-peaks between amides, methyl groups and aromatics that can be used for further high-resolution structure determination. (d) Structure of OmpX determined with the outlined approach. Due to side-chain – side-chain contacts, the geometry of the β -barrel could be accurately defined. Figure adapted from Ref⁴⁰.

Fig. 7. Measurement of residual dipolar couplings and paramagnetic relaxation enhancements of membrane proteins in phospholipid nanodiscs

(a) Relative signal intensities in 2D- ^{15}N , ^1H -TROSY experiments of $U\text{-}^2\text{H}$, ^{15}N -OmpX in nanodiscs assembled with the Gd^{3+} -chelating lipid Gd-DTPA-DMPE and DMPG/DMPG (1:50) or DMPC/DMPG only are plotted against residue number. (b) Signal intensity decrease is mapped onto the OmpX structure (from grey to blue) monitoring proximity to the lipid head-group region. (c) These PRE data can be used to determine the position of OmpX in the nanodiscs. The orange spiral round the outside is the scaffold protein. (d) Partial alignment of 8mg/mL Pf1-phage containing solution as monitored by the magnitude of the HDO splitting in a ^2H spectrum. Splitting of the water resonance indicates partial alignment, which is possible with nanodiscs but not in detergent solution. (e) Example spectra indicating the magnitude of the residual dipolar coupling (= aligned minus isotropic peak splitting in the ^1H dimension) and correlation between the experimental RDCs (D_{obs}) and couplings back-calculated from the OmpX structure (D_{calc}). (f) OmpX in nanodiscs oriented relative to the alignment tensor determined with the experimental RDCs. The orange helix round the outside is the membrane scaffold protein. Figure adapted from Ref⁴⁰.

Table 1

Properties and MSP to lipid ratios for truncated MSP variants (without His₆ tag).

MSP	MW (kDa)	Length (aa)	Lipid-to-MSP ratio ¹	MPI-to-MSP-Lipids ²
MSP1D1	22.00	189	70	1:2:120
DH4/2	20.57	178	60	1:2:100
H4	19.12	167	45	1:2:70
H5	19.45	167	50	1:2:80
H4/2H5	18.01	156	30	1:2:40
H4H5	16.57	145	15	1:2:10
H4-H6	14.07	123	10	n/a

¹ empirically determined for DMPC, will vary for other lipids according to their surface area relative to DMPC (Box 2).

² Assuming a 1:2 ratio of MPI to MSP (i.e. only loaded nanodiscs are present) and depending on the size of the inserted membrane protein (numbers reported for insertion of OmpX, which is replacing ~10 lipid molecules per bilayer leaflet). The indicated values are intended to serve as starting points for further optimization.

Table 2CMC and micellar molecular weight of common detergents used in nanodisc assembly⁷²

Detergent	CMC* (mM)	Micellar Molecular Weight
Sodium cholate ¹³	9–15	900–1,300
Sodium deoxycholate ⁷³	2–6	1,200–5,000
CTAB	1	62,000
LysoPC	0.007	92,000
CHAPS ⁷⁴	6	6,150
Octyl- β -D-glucopyranoside ⁷⁵	23–25	25,000
<i>n</i> -Dodecyl- β -D-Maltoside (DDM) ⁷⁶	0.15	50,000
Digitonin ⁷⁷	< 0.5	70,000
Triton X-100 ⁷⁸	0.2–0.9	80,000
Decyl- β -D-Maltoside (DM) ³⁸	1.6	–
LDAO ²⁰	1–2	17,000
Cymal-5	2.4–5	32,600
Cymal-6	0.56	32,000
Dodecyl-phosphocholine (DPC) ²²	1.5	19,000
N-lauroylsarcosine ⁷⁹	14.6	600
SDS ⁸⁰	7–10	18,000

* CMC in water at 20–25 °C

Table 3

Phase transition temperatures of common lipids used in nanodisc assembly (from the membrane protein data bank: <http://www.lipidat.tcd.ie> and Ref.⁸¹)

Lipid	$T_m(^{\circ}\text{C})$
POPC	-2°C
POPS	14°C
POPG	-2°C
DPPC	41°C
DMPC	24°C
DMPG	23°C
DOPG	-18°C
DPPC	41°C
DLPC	-2°C
DOPC	-17°C
DLPG	-3°C
DMPS	35°C
DOPS	-11°C
DOPA	-4°C
DOPE	-16°C
POPE	25°C

Table 4**Troubleshooting**

Problem	Possible reason	Solution
Expression and purification of MSP		
MSP expression yield is low	-Extended post-induction growth results in significant decrease of MSP yield as MSPs are considerably prone to proteolysis - Occurrence of inclusion bodies	-Make sure you do not exceed 4 hours post-induction time at 37°C. If MSP proteolytic degradation still is an issue, then reduce the post-induction growth time and/or lower the temperature to 30°C in comparison with the standard protocol above. - Check for the presence of inclusion bodies and follow the above mentioned protocol for the purification of the insoluble fraction. -Use rich medium such as terrific broth (TB) to improve the yield.
Removal of His tag by TEV is not complete	-Not enough TEV -TEV activity is low -Incubation time and temperature not ideal -Autolysis of TEV -Buffer is not ideal -MSP is aggregated and the cleavage site not accessible to TEV	-Use more TEV and/or longer incubation. -Perform TEV cleavage at higher temperature. We usually perform TEV cleavage at 4°C as TEV retains significant activity at this temperature. However, optimum activity of TEV is between 30- 34°C. If one wants to perform the reaction at 30-34 °C they must be very careful as not to exceed 37°C as TEV activity drops off abruptly above this temperature. -Autolysis of TEV protease can be avoided by using (S219V) mutant. This mutant is approximately 100-fold more resistant to autoinactivation than the wild-type. -Sometimes MSP precipitate during dialysis against the TEV buffer (50 mM Tris/HCl pH 8.0, 20 mM NaCl, 0.5 mM EDTA). In this case adjust NaCl concentration to 100 -200 mM instead of 20 mM and perform dialysis for only 4 hours. TEV activity is highest in the absence of monovalent salt, but decreases only moderately at NaCl concentrations up to 200 mM.
MSP purity is not good enough after Ni column.	-Nonspecific binding can occur on Ni column during long incubation times	-Apply extensive washes and/or increase the concentration of imidazole to 20 or 30 mM during the last washing step. -Add 20 mM of imidazole to the incubation buffer. -Run anion exchange chromatography (size exclusion chromatography does not yield higher MSP purity due to structural inhomogeneity of the protein)
Preparation of nanodisc reconstitution mixture		
Difficulty in solubilizing the dried lipids in cholate or detergent buffer.	-Presence of residual chloroform -Not enough detergent	-Spread lipid over large surface area inside the glass tube while drying the chloroform under nitrogen gas. -Place the glass tube in a vacuum dessicator under high vacuum overnight to remove residual chloroform. -Vortex the tube, heat it under hot tap water, and sonicate in an ultrasonic bath until the solution is clear. Freeze-thaw cycles are also recommended. -Add more detergent.
Nanodisc assembly		
Protein degradation during nanodisc assembly	-Contamination with proteases can degrade proteins during assembly	Protease inhibitors should be included in the assembly mixture if protein degradation is a concern.
Nanodisc yield is low	-Detergent removal is not complete -MSP to lipid ratio is not optimal -Assembly temperature is not ideal -Contamination with proteases	-Detergent should be removed from the mixture by extensive incubation with Biobeads or dialysis. -Add fresh batch of Biobeads (remove the old batch first before adding fresh one) -Use the right lipid to MSP ratio. The optimal MSP to lipid ratio needs to be tested by a series of test assembly reactions and subsequent screening with an analytical S200 size exclusion column. -The temperature of the self-assembly should be near the T _m of the lipid being used. T _m of commonly used phospholipids are listed in table 3.
Yield of loaded nanodiscs (i.e nanodiscs containing the protein of interest) is low	- Membrane protein of interest sticks to the Biobeads - Membrane protein:MSP:lipid ratio not ideal - Oligomerization of the membrane protein	- In cases where the membrane protein of interest sticks to the Biobeads (often helical membrane proteins or single spanners), dialysis should be used. Volume of the dialysis buffer should be at least 1000x the assembly reaction volume, change buffer 2-4 times within 24h. - Test for the optimal protein: lipid: MSP ratio by a series of assembly reactions and subsequent screening using size exclusion chromatography. -If oligomerization of the membrane protein is an issue, use a larger excess of MSP to target protein.