CHAPTER FOURTEEN

Studying integral membrane protein by SANS using stealth reconstitution systems

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Contents

Abstract

Structural studies of integral membrane proteins (IMPs) are challenging as many of them require a lipid environment for full activity and stability. Reconstitution of IMPs into carrier systems such as nanodiscs or Salipro that mimic the native lipidic environment allow structural studies of membrane proteins in solution. The difficulty with this approach when applied to scattering techniques is the contribution of the carrier system to the scattering intensity and the subsequent challenging data analysis. Recently, so-called stealth carrier systems have been developed and applied to small-angle neutron scattering (SANS) studies of integral membrane proteins that become invisible to neutrons due to specific deuteration and solvent contrast-variation.

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In this chapter, we describe in detail how the well-studied ATP-binding cassette (ABC) transporter protein MsbA can be reconstituted into stealth nanodiscs and subsequently be studied by SANS. This approach allows for a direct observation of the scattering signal from MsbA without the contribution of the surrounding carrier system and enables detection of different conformational states. The protocols can also be adapted to other stealth carrier systems (such as stealth Salipro).

Abbreviations

ABC	ATP-binding cassette
DDM	dodecylmaltoside
DIBMA	diisobutylene-maleic acid
dMSP1D1	deuterated membrane scaffold protein variant D1
dPC	deuterated mixed acyl phosphatidylcholine
IMP	integral membrane protein
LMNG	lauryl maltose neopentyl glycol
LPS	lipopolysaccharide
MSP	membrane scaffold protein
NBD	nucleotide-binding domain
ND	nanodisc
PC	phosphatidylcholine
SANS	small-angle neutron scattering
SAXS	small-angle X-ray scattering
SLD	scattering length density
sND	stealth nanodiscs
TMD	transmembrane domain

1. Introduction

Integral membrane proteins (IMPs) are essential for life as they transport matter and signals across cell membranes and generate electrochemical gradients. They play key roles in signal transduction, nutrient uptake and energy generation and are key targets for drug development (Li et al., 2021; Wacker, Stevens, & Roth, 2017). Structural and functional characterization of integral membrane proteins is still very challenging due to their low abundance and difficulties in purifying functional IMPs from their native membrane environment. Many IMPs require specifically-bound lipids or a lipid environment for full stability and activity (Bechara et al., 2015; Clay & Sharom, 2013; Doerrler & Raetz, 2002; Eggensperger, Fisette, Parcej, Schafer, & Tampe, 2014).

Several reconstitution systems have been developed in the last decade that allow membrane proteins to be incorporated in a native-like lipidic

environment while still being amendable to structural studies (in solution). These scaffold/carrier systems include nanodiscs, Salipro and peptidiscs (Angiulli et al., 2020; Denisov & Sligar, 2016; Frauenfeld et al., 2016) that all have successfully been used for structural studies of IMPs.

Nanodiscs are discoidal protein-lipid complexes that are composed of two amphipathic membrane scaffold proteins (MSPs) encircling a lipid bilayer as a helical belt, yielding a lipidic assembly that is soluble in aqueous solution (Bayburt & Sligar, 2010; Denisov & Sligar, 2016). Nanodisc reconstitutions have been successfully used for structural studies of different IMP classes by scattering techniques (Bayburt, Grinkova, & Sligar, 2006; Choi, Rice, Stokes, & Coller, 2013; Skar-Gislinge et al., 2015). However, solution scattering studies of nanodisc-incorporated IMPs are challenging due to the complex multi-contrast scattering contribution of the scaffold disc.

To bypass this problem, so-called stealth carrier systems have been developed that become invisible to neutrons due to specific deuteration and solvent contrast-variation (Maric et al., 2014). We recently applied this stealth nanodisc (sND) technology to the ABC transporter MsbA and succeeded in observing different conformational states of MsbA in a lipid environment by SANS (Josts et al., 2018). We also used stealth nanodiscs to generate a structural model for the full-length Ca^{2+} pump ACA8 in its calmodulin-activated state (Nitsche et al., 2018).

Neutron sources such as ILL, SNS, MLZ, ISIS and ANSTO are scarce and access to SANS beamlines is limited. For this reason, an optimal sample preparation is of utmost importance for efficient use of resources and to optimize the scientific knowledge gain.

In this chapter, we describe the detailed experimental procedure to reconstitute integral membrane proteins into stealth nanodiscs using MsbA as example. MsbA is a prokaryotic ABC transporter that acts as lipid flippase, translocating lipid A, glycolipids and lipopolysaccharides (LPS) from the inner to the outer leaflet of the bacterial inner membrane (Raetz, Reynolds, Trent, & Bishop, 2007). Our method described here yields monodisperse IMP samples incorporated in stealth carrier systems, that are invisible to low-resolution neutron diffraction yet provide a favorable lipidic environment for the embedded membrane protein of interest, and thus paves the way for low-resolution structure determination of IMPs in solution using both *ab initio* and rigid body analysis approaches.

2. Contrast variation in small-angle neutron scattering (SANS) by selective deuteration of lipids and proteins for use as stealth carrier systems

A major advantage of SANS compared to SAXS is the possibility to make use of the different scattering length densities (SLD) of biological macromolecules and exploit contrast variation to distinguish between different components in the sample. This can be achieved by exploiting the natural contrast of biomolecules containing different hydrogen composition (e.g., protein/DNA or protein/lipids), by exchanging H_2O against D_2O in appropriate amounts to achieve the contrast-match point, or by controlled deuterium labeling of biomolecules leading to artificial contrast (Mahieu & Gabel, 2018).

With regard to integral membrane proteins, SANS is a particularly suitable technique due to the natural contrast between membrane protein and surrounding detergent or lipid molecules (Breyton et al., 2013). This feature has been exploited in several studies where different membrane mimetics were made "invisible" (i.e., turning them into "stealth" carriers). The detergent LMNG becomes experimentally undetectable in SANS in a solvent with 21.4% (v/v) D_2O and can be used to solubilize IMPs (Vermot et al., 2020). Mixed micelles containing a deuterated detergent analog have been described that eliminate neutron contrast between core and shell and allow the micelle scattering to be fully contrast-matched (Oliver, Pingali, & Urban, 2017). The diisobutylene maleic acid copolymer DIBMA has a contrast match point of 12% (v/v) D_2O (Guo, Sumner, & Qian, 2021). A present limitation of these contrast-matching experiments using detergents or amphipols at low D_2O content (<25%) is the incoherent scattering of water that limits acquisition of high-quality data essential for reliable structural studies (Gabel, 2017). In addition, detergent based membrane mimetics are lacking the lipid component that is often essential for the activity and structural integrity of the integral membrane protein.

An elegant alternative for the study of IMPs by SANS is the stealth nanodisc system, which combines deuterated MSP belt proteins with selectively deuterated phosphatidylcholine (PC) lipids resulting in a negligible SANS signal in 100% D_2O, thus effectively rendering the bilayer scaffold invisible (Maric et al., 2014) (Figs. 1 and 2). As the high D_2O concentration used with stealth nanodiscs minimize background from incoherent neutron

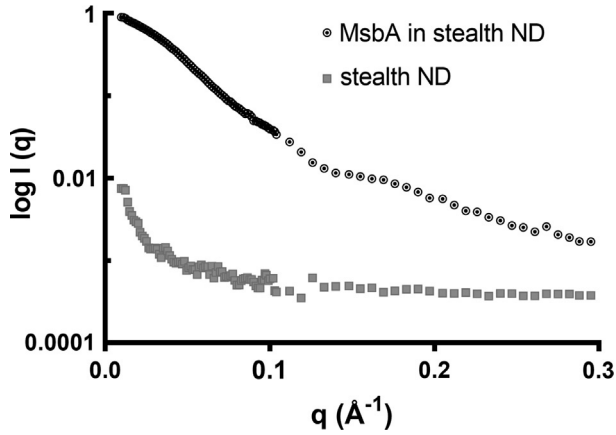

Fig. 1 Comparison of SANS data obtained from empty stealth nanodiscs (sND) (containing deuterated lipids and membrane scaffold protein only) (gray squares) with those recorded from nanodiscs that contain incorporated MsbA (black dots). The scattering signal from MsbA-sND is more than two orders of magnitude stronger than that from empty sNDs, indicating "an invisible nanodisc", i.e., that the contribution from lipids and belt protein would not influence the data analysis and structural modeling (Josts et al., 2018).

scattering and thus improves signal-to-noise ratio (Gabel et al., 2002), this method has the potential to yield high-quality data that allow to distinguish between different conformational states of incorporated membrane proteins as shown for the ABC transporter MsbA (Josts et al., 2018).

Further details regarding SANS contrast variation can be found in chapters "Contrast variation SAXS: Sample preparation protocols, experimental procedures, and data analysis" by Emeterio et al. as well as "Planning, executing, and assessing the validity of SANS contrast variation experiments" by Krueger of this edition of *Methods in Enzymology*.

For the preparation of the aforementioned stealth nanodiscs the theoretical contrast match points in 100% D_2O were calculated as 75% deuteration for the MSP1D1 belt protein (taking into account the exchangeable H atoms bound to O, N and S atoms) (Engelman & Moore, 1975) as well as 78% and 92% deuteration, respectively, for the head group atoms and acyl chain atoms of phospatidylcholine (Maric et al., 2014). These components can be produced by *Escherichia coli*-based biosynthesis. For the deuterated MSP belt protein the *E. coli* strain BL21 (DE3) was adapted and grown in

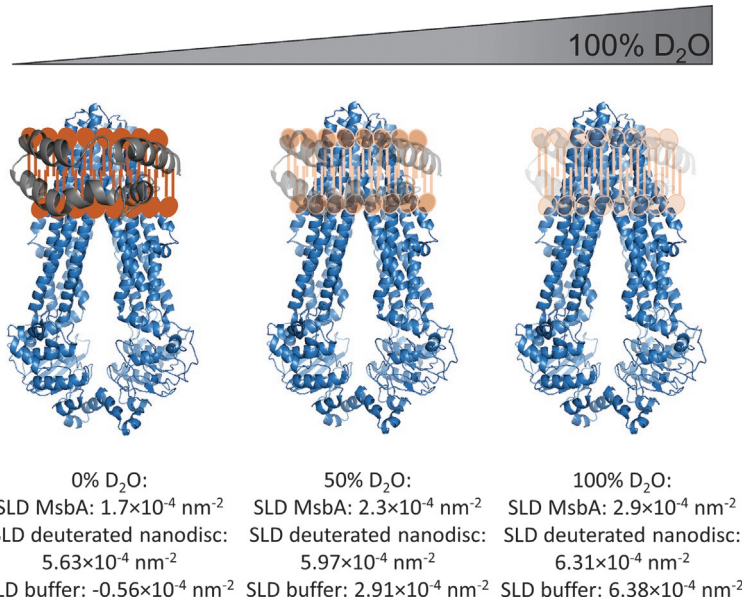

Fig. 2 Schematic representation of SANS scattering contribution from stealth nanodisc-reconstituted MsbA when measured in gradually increasing level of D_2O. At 100% H_2O based buffer (0% D_2O), all components (i.e., MsbA, lipids and belt protein) contribute to the SANS signal. With increasing D_2O concentrations, the stealth nanodiscs components (d-MSP1D1 and d-POPC, respectively) become increasingly invisible. Thus, the measured scattering data can be attributed entirely to the integrated membrane protein (MsbA). The relevant scattering length densities (SLDs) are indicated.

85% deuterated minimal medium with glycerol as carbon source (Artero, Hartlein, McSweeney, & Timmins, 2005; Rochel et al., 2011) and subsequently purified using standard procedures (Ritchie et al., 2009).

The selectively deuterated PC lipids can be produced using the genetically modified *E. coli* strain AL95 (Bogdanov, Heacock, Guan, & Dowhan, 2010) in close to 100% deuterated minimal medium supplemented with deuterated d_8-glycerol and partially deuterated choline chloride (Maric et al., 2015). Deuterated PC lipids (dPC) were then extracted and purified using silica-gel chromatography (Bligh & Dyer, 1959; Maric et al., 2015).

Further general information regarding deuteration for SANS experiments can be found in the chapter "Deuteration for biological SANS: Case studies, success and challenges in chemistry and biology" by Duff et al. of this edition of *Methods in Enzymology* as well as in several recent reviews (Abelson, Simon, Pyle, & Christianson, 2015; Haertlein et al., 2016; Pyle & Christianson, 2016).

3. Reconstitution of integral membrane proteins (IMP) into stealth carrier systems/example: Reconstitution of MsbA into stealth nanodiscs

This section describes reconstitution of the ABC transporter MsbA into stealth nanodiscs using the previously described deuterated components. The assembly process starts from detergent solubilized MsbA and dried lipids and consists of a biobead-driven assembly followed by size-exclusion chromatography (Fig. 3). The reconstitution efficiency, stability and activity are monitored by SDS-PAGE, nDSF and (Baginski) activity assays, respectively. After successful reconstitution, the samples can be directly used for SANS experiments or stored at −80 °C.

3.1 Equipment

- Glass vials (Carl Roth)
- Roller table (Cole-Parmer)
- ÄKTA pure chromatography system (Cytiva)
- Superdex200 10/300 GL size-exclusion chromatography column (Cytiva)
- Electrophoresis chamber (Bio-Rad)
- SDS-PAGE gels (Bio-Rad)
- Concentrators (Millipore)
- Centrifuge 5810 R (Eppendorf)
- Ultracentrifuge Optima XE-90 (Beckman Coulter)
- Microtiter plates (ThermoFisher)
- Tecan Infinite200 microplate reader (Tecan)
- Prometheus NT.48 nanoDSF differential scanning fluorimeter (NanoTemper Technologies)
- Prometheus NT.48 Capillaries nanoDSF Grade (NanoTemper Technologies)

3.2 Chemicals

- Tris (Carl Roth)
- NaCl (Carl Roth)
- β-mercaptoethanol (Carl Roth)
- sodium cholate (Carl Roth)
- biobeads (Bio-Rad)
- $MgCl_2$ (Carl Roth)
- ATP (Jena Biosciences)

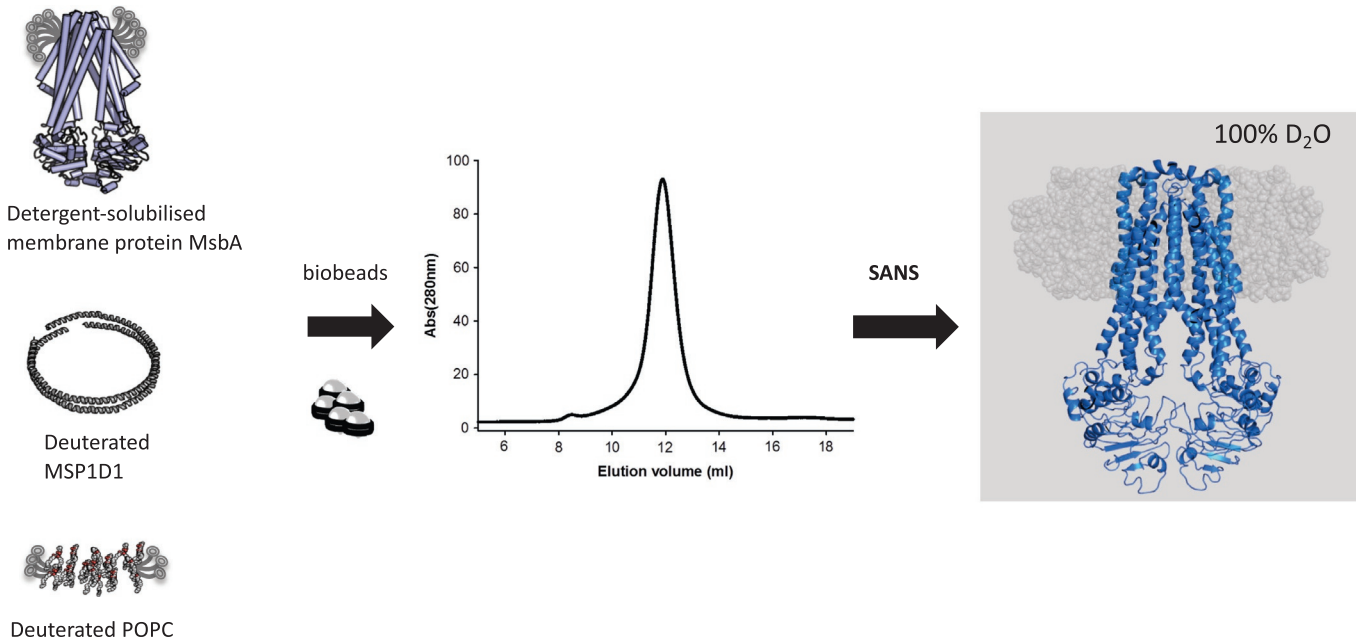

Fig. 3 Overview of stealth nanodisc assembly. The assembly process starts from detergent solubilized MsbA, dried deuterated lipids and deuterated MSP1 belt protein and consists of a biobead-driven assembly followed by size-exclusion chromatography. The resulting stealth nanodisc is invisible to neutron radiation in 100% D_2O and allows the structural investigation of the incorporated integral membrane protein by SANS.

- Ascorbic acid (Carl Roth)
- SDS (Carl Roth)
- HCl (Carl Roth)
- ammonium heptamolybdate (Sigma-Aldrich)
- sodium citrate (Carl Roth)
- sodium metaarsenite (Sigma-Aldrich)
- acetic acid (Carl Roth)
- D_2O (Sigma-Aldrich)

3.3 Protocol

Reconstitution of MsbA into stealth nanodiscs

- Resuspend selectively deuterated PC lipid (see above) from a dry lipid film in buffer containing 30 mM Tris (pH7.5), 150 mM NaCl, 1 mM β-mercaptoethanol and 100 mM cholate to yield a 50 mM stock solution. Several mild sonication steps are necessary to fully dissolve the lipids.
- Mix detergent solubilized MsbA (in Tris/NaCl buffer with 0.03% DDM), deuterated MSP1D1 and deuterated PC (see above) in a molar ratio of 1:1:25 (i.e., two MSP1D1 belt proteins per MsbA homodimer) in buffer containing 20 mM cholate and incubate at 4 °C for 1 h.
- Initiate the nanodisc formation by adding 0.5–0.8 g/mL biobeads to the reaction and incubate overnight at 4 °C under constant agitation.
- Purify the stealth nanodisc-reconstituted MsbA by size-exclusion chromatography (SEC) on a Superdex200 10/300 GL column (GE Healthcare) in a buffer containing 30 mM Tris (pH7.5), 150 mM NaCl and 1 mM β-mercaptoethanol (Fig. 4B).
- Analyze the elution fractions by SDS-PAGE (Fig. 4A)
- Concentrate fractions containing stealth nanodisc-embedded MsbA up to 5–8 mg/mL using a 100 kDa cut-off concentrator.
- Samples can be snap-frozen in liquid nitrogen and stored at −80 °C or directly used for stealth SANS experiments.
- On the day before to the SANS experiments, MsbA-nanodisc assemblies are dialyzed extensively against buffer prepared with 100% D_2O.

Evaluate activity of stealth nanodisc-reconstituted MsbA using activity assay

- Prepare solutions A and B of Baginski assays (Baginski, Epstein, & Zak, 1975; Chifflet, Torriglia, Chiesa, & Tolosa, 1988)
  - solution A: 140 mM ascorbic acid, 0.5 M HCl, 0.1% SDS, 5 mM ammonium heptamolybdate
  - solution B: 75 mM sodium citrate, 2% (w/v) sodium metaarsenite and 2% (v/v) acetic acid

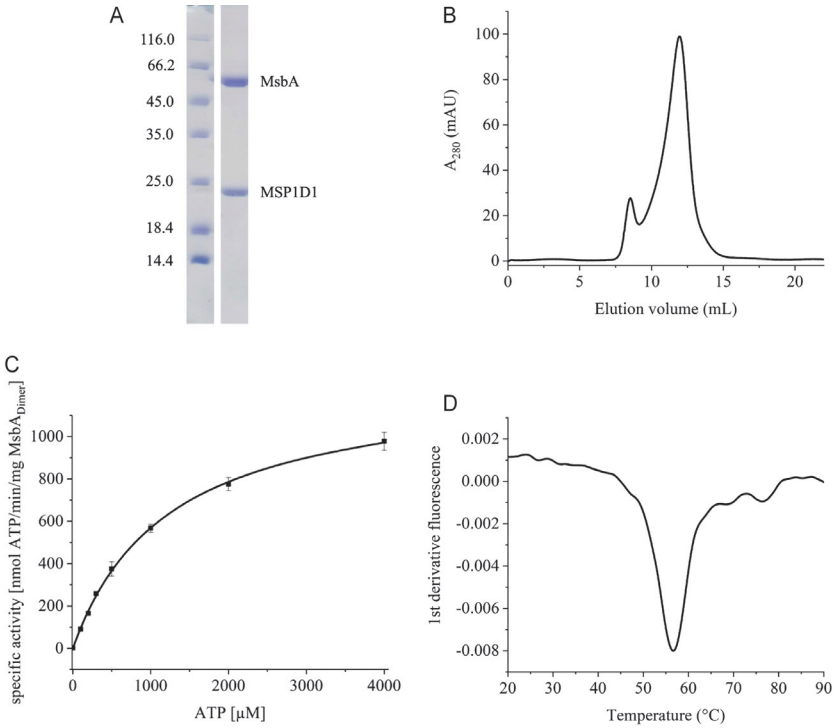

Fig. 4 Recommended quality control experiments during stealth nanodisc reconstitution. (A) SDS-PAGE sowing bands for belt protein (MSP1D1) and incorporated membrane protein (MsbA). (B) Size-exclusion chromatography (SEC) profile showing a minor void peak and a main peak corresponding to stealth nanodisc-reconstituted MsbA. (C) Activity assay based on detection of inorganic phosphate from ATP hydrolysis showing classical enzyme kinetics. (D) nDSF thermal melt profiles of stealth nanodisc-reconstituted MsbA.

- Incubate 5 μg MsbA in stealth nanodiscs in a buffer containing 30 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$ in 50 μL volume with different ATP concentrations (0/100/200/300/500/1000/2000/4000 μM) for 12 min at 25 °C.
- Stop the reaction by adding 50 μL ascorbic acid solution A (140 mM ascorbic acid, 0.5 M HCl, 0.1% SDS, 5 mM ammonium heptamolybdate).
- Stop the colorimetric reaction after a defined time by addition of 75 μL solution B (75 mM sodium citrate, 2% (w/v) sodium metaarsenite and 2% (v/v) acetic acid).
- Measure the absorbance at 860 nm using a Tecan Infinite200 microplate reader after 20 min.
- Calculate MsbA activity using standard curve obtained from defined phosphate concentrations (Fig. 4C).

Evaluate stability of stealth nanodisc-reconstituted MsbA using differential scanning fluorimetry (nDSF)

- Load stealth nanodisc-reconstituted MsbA (c = 0.5 mg/mL, V = 10 μL) in a buffer containing 20 mM Tris (pH 7.5) and 150 mM NaCl in a capillary (Prometheus NT.48 capillaries nanoDSF Grade) and measure the unfolding at a heating rate of 1 °C/min.
- Use the intrinsic fluorescence of tryptophan residues at 330 and 350 nm after excitation at 280 nm to monitor the fluorescence change upon heat unfolding.
- Use the first derivative of the unfolding curves (F350/F330) to determine the transition midpoint (Fig. 4D).

3.4 Notes

- It is recommended to evaluate the long-term stability of reconstituted samples before long-lasting SANS studies. For this purpose, the samples should be analyzed by analytical size-exclusion chromatography after several days using a Superdex S200 10/300 GL column (GE Healthcare):
  - Centrifuge identical volumes of the samples and load them onto the column to allow peak area comparison
  - Quantify the amount of intact MsbA/stealthND by peak integration
- Empty stealth nanodiscs should be prepared in a similar way as described above (using 1:40 molar ratio of dMSP1D1:dPC) in order confirm that they are really "invisible" to neutron radiation. They should show neglectable scattering when measured in 100% D_2O.
- A non-optimal ratio of IMP/MSP1D1/lipid can result in the formation of empty nanodiscs in addition to the correctly reconstituted complex. This can be detected by an additional peak in the SEC-profile as the empty nanodiscs elute significantly later (if the incorporated IMP has a reasonable size). If the IMP still contains an affinity tag (e.g., His-tag), reconstituted complexes could be re-bound to an affinity column (e.g., Ni-NTA) and empty discs could be washed off.
- For several detergents with low cmc (such as LMNG), detergent removal using biobeads is not very efficient and often results in the assembly of empty nanodiscs. For these cases, the use of cyclodextrin is recommended. Cyclodextrin is composed of a sugar ring with a hydrophobic core inside which has a higher affinity for detergents and is applicable to a wide range of detergents (Degrip, Vanoostrum, & Bovee-Geurts, 1998; Vargas, Schonbeck, Heimann, & Keller, 2018).

- It is recommended to acquire SAXS data of the reconstituted IMP samples in stealth nanodiscs before SANS measurements (using different samples from the same batch). These SAXS data contains the signal from the IMP as well as from the stealth disc and should therefore results in larger R_g and D_{max} values compared to the SANS data.
- As an additional quality control, we further recommend to measure SAXS data on the identical samples as used for SANS (after samples are released from radiation safety procedures) as soon as possible after the SANS measurements to confirm sample stability and integrity.
- Some IMPs co-purify with endogenous lipids and in the case of MsbA we introduced a 1% DDM wash (5–10 column volumes) while the protein was still bound to the Ni-NTA resin to remove the hydrogenated endogenous lipids from the sample prior to incorporation into the stealth nanodiscs.
- This methodology could also be applied (with minor adjustments) to other scaffold/carrier systems such as Salipro.

4. SANS data acquisition and analysis of IMP/stealth nanodisc samples

SANS data were measured in 100% D_2O buffer (30 mM Tris (pH 7.5), 150 mM NaCl) at 1–6 mg/mL protein concentration at 10 °C. Data were measured at the D11 beamline at ILL in two different configurations of 1.5 m/1.5 m and 8 m/8 m (collimation length/sample-detector distances) to cover a sufficiently wide q-range ($0.03 < q < 0.58\,Å^{-1}$), with a fixed neutron wavelength (λ) of 4.6 Å (Josts et al., 2018). Data reduction was performed using GRASP after measuring water reference samples, buffers, empty cell, direct beam, and the total absorber boron-cadmium.

As the previously described fractional deuteration of the components make the stealth nanodiscs invisible to neutron diffraction, the measured data only contain the signal from the incorporated membrane protein without contribution from the surrounding nanodisc. Thus, the reduced one-dimensional scattering intensities (I(q)) can be treated as homogeneous IMP scattering data and analyzed using standard small-angle scattering procedures. In the examples case of MsbA in stealth nanodiscs, the pipeline included:

- Buffer subtraction using PRIMUS (Konarev, Volkov, Sokolova, Koch, & Svergun, 2003)
- Calculation of radii of gyration (R_g) using the Guinier approximation

- Comparison with back-calculated data from atomic models using CRYSON (Svergun et al., 1998)
- Use of the MULCH server (http://smb-research.smb.usyd.edu.au/NCVWeb/) (Whitten, Cai, & Trewhella, 2008) to determine contrast and partial specific volume at 100% D_2O from the sample components and protein sequence
- Calculation of low-resolution *ab initio* models and their alignment using DAMMIF and SUPCOMP (Franke & Svergun, 2009; Petoukhov & Svergun, 2015)
- Model averaging and filtering using DAMAVER and DAMFILT (Kozin & Svergun, 2001; Petoukhov & Svergun, 2015)
- Rigid-body modeling using SASREF (Petoukhov & Svergun, 2005)

5. Summary and conclusions

The stealth nanodiscs methodology could not only be adapted to other carrier systems such as Salipro but it can also be used to study complexes between integral membrane proteins and soluble protein binding partners (Nitsche et al., 2018). In this case, contrast variation via selective deuteration of one of the proteins in the complex will make this component invisible when measured at 100% D_2O. Combining several datasets with different contrasts (deuteration/hydrogenation) will then allow more accurate modeling of the IMP complex in the stealth environment. Additionally, combining X-ray and neutron scattering of IMPs with the stealth technology allows for modeling multiple protein conformations and characterizing highly flexible regions of IMPs systems such as disordered regions that are often not visible using high-resolution techniques such as X-ray crystallography and cryo-EM. These models could then finally be combined with AlphaFold2 predictions of full-length proteins.

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