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Tidemand, Frederik Grønbæk; Blemmer, Sara; Johansen, Nicolai Tidemand; Arleth, Lise; Pedersen, Martin Cramer

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Non-ionic detergent assists formation of supercharged nanodiscs and insertion of membrane proteins

Frederik G. Tidemand a,1, Sara Blemmer a,1, Nicolai T. Johansen a,b,1, Lise Arleth a, Martin Cramer Pedersen a,*

a Niels Bohr Institute, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen, Denmark
b Section for Transport Biology, Department of Plant and Environmental Sciences, University of Copenhagen, 1871 Frederiksberg C, Denmark

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ABSTRACT

Nanodiscs are used to stabilize membrane proteins in a lipid environment and enable investigations of the function and structure of these. Membrane proteins are often only available in small amounts, and thus the stability and ease of use of the nanodiscs are essential. We have recently explored circularizing and supercharging membrane scaffolding proteins (MSPs) for nanodisc formation and found increased temporal stability at elevated temperatures. In the present study, we investigate six different supercharged MSPs and their ability to form nanodiscs: three covalently circularized and the three non-circularized, linear versions. Using standard reconstitution protocols using cholate as the reconstitution detergent, we found that two of the linear constructs formed multiple lipid-protein species, whereas adding n-Dodecyl-B-D-maltoside (DDM) with the cholate in the reconstitution gave rise to single-species nanodisc formation for these MSPs. For all MSPs, the formed nanodiscs were analyzed by small-angle X-ray scattering (SAXS), which showed similar structures for each MSP, respectively, suggesting that the structures of the formed nanodiscs are independent of the initial DDM content, as long as cholate is present. Lastly, we incorporated the membrane protein proteorhodopsin into the supercharged nanodiscs and observed a considerable increase in incorporation yield with the addition of DDM. For the three circularized MSPs, a single major species appeared in the size exclusion chromatography (SEC) chromatogram, suggesting monodisperse nanodiscs with proteorhodopsin incorporated, which is in strong contrast to the samples without DDM showing almost no incorporation and high polydispersity.

1. Introduction

Nanodiscs are discoidal bilayer patches surrounded by membrane scaffold protein (MSP) belts [1,2] that can be used to incorporate membrane proteins [3,4]. Since the first reports on the structure and function of nanodiscs [1], the field has undergone tremendous development, and nanodiscs are now available with a variety of diameters ranging from 7 nm [5] to 50 nm [6], making them compatible with many different biophysical techniques and membrane proteins, whereas the development of circularized MSPs have improved nanodisc stability and provided better control of membrane protein reconstitution [6–10]. Furthermore, we have recently explored supercharging the MSP in combination with circularization to enhance repulsion between nanodiscs, which also resulted in improved nanodisc stability and in high production yields of circularized MSP [7]. Following the same design principles, we recently presented supercharged and circularized MSPs of different sizes and investigated the structural and biophysical properties of nanodiscs formed with these and the phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) [11].

Nanodiscs are formed, or reconstituted, by a self-assembly process that is initiated by detergent removal from the so-called pre-aggregates, which is a mix of lipids, MSP, and detergent [12]. Given appropriate concentrations and mixing ratios of lipids, MSP and detergent during the reconstitution, the choice of detergent appears to be the single most important factor for shape and homogeneity of the finally formed nanodiscs [13]. Here, sodium cholate is the most common choice as reconstitution detergent and generally gives rise to highly loaded and homogeneous nanodiscs [14]. Cholate is a steroid-based anionic...
detergent with a relatively high CMC of ~13 mM that forms small mixed micelles with phospholipids, e.g. 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), above its critical solubilization ratio [15]. As recently reported, cholate-assisted nanodisc self-assembly is a reversible process with respect to the cholate concentration [16]. However, while cholate works well for reconstituting nanodiscs, it is seldom used for handling membrane proteins, possibly due to issues with membrane protein stability and aggregation propensity. More often, non-ionic detergents are used for this purpose, including n-Dodecyl-B-D-maltoside (DDM), which is considered mild [17]. However, we recently showed that using DDM for reconstituting nanodiscs resulted in poor lipid loading efficiency [13], likely because of the large size of mixed micelles formed by DDM and lipids [18] compared to the small size of those formed using cholate in combination with the lipids [13]. When we instead used a mixture of cholate and DDM, in a ratio close to what would be the case for reconstitution of a DDM-solubilized membrane protein in nanodiscs formed with cholate, we obtained well-formed nanodiscs [13]. This suggested that the poor reconstitution properties of DDM alone were compensated by the addition of cholate in the mixed system which would hold promise for nanodisc reconstitution of membrane proteins. However, despite their importance for optimizing membrane protein reconstitution, systematic studies on the influence of detergent composition and concentration on nanodisc reconstitution are scarce.

In this study, we investigated the formation of nanodiscs as a function of the size of the MSP, circularization of the MSP, and concentration of DDM. While we have previously shown that the ~270 amino acid circularized and/or supercharged MSP1E3D1 (csMSP1E3D1) form nanodiscs with POPC lipids using the cholate-assisted method [7], we find that when we reconstitute shorter, supercharged MSPs with the cholate-assisted method, we obtain inhomogeneous samples containing a mix of nanodiscs and larger MSP-lipid particles. However, despite their importance for optimizing membrane protein reconstitution, systematic studies on the influence of detergent composition and concentration on nanodisc reconstitution are scarce.

2. Materials and methods

2.1. MSPs used for the present study

Six different MSPs were used in this study: three covalently circularized and supercharged (cs) and three non-circularized (linear) and supercharged (ls) (Fig. 1). The proteins were expressed in *E. coli* as pre-proteins called His-IsMSPs. After purification, Tobacco Etch Virus (TEV) protease was used to remove the N-terminal black region, resulting in the IsMSPs. To obtain circularized MSPs, the evolved Sortase (eSt) enzyme [19] (Sortase motif in blue) was used to create csMSPs (the reaction removes the C-terminal red region and the C-terminal G of the sortase motif and links the termini of the remaining sequence). The sequences of the three pre-proteins (His-IsMSP) used to prepare both ls- and csMSPs for this study are as follows:

**His-IsMSP1D1ΔH5:**

**His-IsMSP1D1:**

**His-IsMSP1E3D1E3 (as also reported in [7]):**

2.2. Protein expression and purification

The genes encoding the His-IsMSPs were synthesized and cloned into the pET28a vector by Genscript (His-IsMSP1D1ΔH5 and His-IsMSP1E3D1) and Twist Bioscience (His-IsMSP1D1). The vectors were transformed into heat competent BL21(DE3) (Merck) cells using standard protocols. IsMSP1D1, IsMSP1E3D1, csMSP1D1 and csMSP1E3D1
were all produced using the same protocol as described for IsMSP1E3D1 and csMSP1E3D1 previously [7]. IsMSP1D1H5 and csMSP1D1H5 were prepared using a similar protocol, with the exceptions that protein expression was carried out at 20 °C overnight using 0.1 mM IPTG instead of at 3 h at 37 °C using 1 mM IPTG and that eSrt mediated circularization was achieved at a MSP:eSrt ratio of 50:1. The ratio of 50:1 was used to avoid oligomers and thus maximize formation of monomeric csMSP1D1H5.

Proteorhodopsin was expressed and purified as described previously [20,21].

2.3. Nanodisc preparation

1-Palmitoyl-2-oleoyl-glycerol-3-phosphocholine (POPC, Merck) lipids were dissolved in detergent solutions: For samples containing cholate, POPC was dissolved to 50 mM in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 100 mM cholate, whereas for samples without cholate, POPC was dissolved to 25 mM in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 75 mM DDM (50 mM POPC, 150 mM DDM was too viscous to handle). The lipid:MSP ratios of the three different sized MSPs were as follows: MSP1D1H5: 55:1, MSP1D1: 70:1, and MSP1E3D1: 130:1. The detergent-solubilized lipids were mixed with relevant MSP (corresponding to the lipid:MSP ratio) and additional detergent in buffer to a final POPC concentration of 10 mM. In total, 12 samples were prepared for each MSP. 11 samples contained 20 mM cholate and increasing concentrations of DDM (0 mM to 50 mM), where DDM was added from a 185 mM stock solution in buffer. The final sample was produced from the DDM solubilized POPC solution and contained 10 mM POPC and 30 mM DDM (no cholate). This mixture of detergent, POPC and MSP is hereafter denoted pre-aggregates. The pre-aggregates were incubated at 15 °C for 10 min with 500 rpm agitation before Amberlite XAD-2 beads (Merck) were added to remove detergent and facilitate the self-assembly of nanodiscs. To ensure complete removal of the detergent, the mass of wetted beads added to the sample was calculated using the equation [22]:

\[
\frac{m_{\text{beads}}}{V_{\text{sample}}} = 150 \frac{m_{\text{beads}}}{m_{\text{sample}}} + 1.5c_{\text{DDM}} \frac{m_{\text{beads}}}{m_{\text{sample}}} m_{\text{MSP}},
\]

Here, \(c_{\text{DDM}}\) is the DDM concentration in mM. For example, in a 400 μl sample prepared with 10 mM DDM, 66 mg beads were added. The samples were incubated while shaking overnight at 15 °C. Next day, Amberlite XAD-2 beads were removed, and the samples were centrifuged and loaded onto a Superdex 200 Increase 10/300 column (GE Healthcare) equilibrated in 20 mM Tris-HCl pH 7.5, 150 mM NaCl. Fractions were collected, and their concentrations were determined by UV absorption at 280 nm using a Nanodrop1000 (Thermofisher). Molar concentrations were calculated using extinction coefficients calculated using the Expasy ProtParam tool [23].

For nanodiscs with PR incorporated, slightly lower MSP:POPC ratios were used to account for the bilayer patch displaced by PR: MSP1D1H5: 45:1, MSP1D1: 60:1 and MSP1E3D1: 115:1. Aiming for monomeric insertion, the MSP:PR ratio was kept at 6:1 for all samples [24]. The DDM concentration in the pre-aggregates was either 0 mM or 30 mM.

2.4. SAXS measurements

All samples were prepared at room temperature, then stored and shipped on ice until measured at the B21 BioSAXS beamline at Diamond, Oxfordshire [25]. All measurements were performed at 10 °C and the data were automatically reduced using the pipeline available on the GenApp.rocks server (https://somo.chem.utk.edu/bayesapp/) [29]. The error bars on the SAXS data were rescaled using a new implementation in BayesApp [30].

2.5. SAXS analysis

From our SAXS data, we refine the structural properties of our samples using the nanodisc model [31,32] available in the WillItFit analysis software [33]. The model has been utilized in a number of scattering studies of nanodiscs [7,13,21]. In this model, the nanodisc is represented by simple geometric shapes, the scattering properties of which are readily calculated. Chemical compositions and scattering lengths of all sample components can be found in Table S1. Specifically, the nanodisc model consists of a set of cylinders with elliptical cross-section, each of which represents a specific part of the nanodisc such as the MSP belt, the headgroups of the phospholipids in the upper bilayer leaflet or the methyl groups in the center of the bilayer. The model has been used in a number of studies of nanodiscs and their structural properties [7,13,31,32] and is sketched in Fig. 4E+F; we parametrize the model using the following quantities:

- The number of phospholipid molecules per nanodisc, \(N_{\text{lip}}\)
- The axis ratio of the phospholipid bilayers, \(\kappa\)
- The area per phospholipid headgroup in the bilayer, \(A_{\text{head}}\)
- The height of the MSP belt, which we fix at 25.78 Å in line with previous studies [2,7]
- The partial specific molecular volume of a phospholipid, \(V_{\text{lip}}\)
- The partial specific molecular volume of a single MSP protein, \(V_{\text{MSP}}\).

Additionally, we refine the following two parameters that do not directly impact the geometry of the model:

- A term accounting for any roughness between the interfaces in the model, \(R\), as outlined in [34].
- A constant background added to the model, \(b\).

These parameters allow us to derive the full set of dimensions describing a particular nanodisc. From these, we calculate form factor scattering amplitudes [31,32,35], thus accounting for the scattering contribution from each of the geometric components of the nanodiscs. We assign the cylinders with scattering properties corresponding to the respective chemical composition. These are based on the scattering lengths in Table S1; as an example, the total scattering length of the cylinder representing the methyl layer is \(N_{\text{lip}}\) times the scattering length for a methyl group.

2.6. Phosphate analysis

The lipid content of the ND samples was determined using phosphate analysis [36]. This analysis relies on the release of the phosphate group from the POPC lipids and subsequent binding of molybdate which gives rise to absorption at 812 nm. Initially, the samples were mixed with perchloric acid in a long-necked glass tube loosely capped with a glass bead and boiled at 180 °C for 2 h to release the phosphate group. Subsequently, molybdate and ascorbic acid were added, and the samples were incubated at 80 °C for 10 min in a water bath. After cooling to room temperature, absorption at 812 nm was measured. Reference samples with known phosphate contents were treated in the same way and used to generate a standard curve, enabling determination of the lipid content of the ND samples.

2.7. Fitting SEC curves

To quantify areas under the different peaks in our SEC data, we fitted exponentially modified Gaussian functions to the datasets. To quantify the effect of DDM on ND reconstitution we found inspiration in the
Michaels-Menten equation used in enzyme kinetics [37]. The modified equation is as follows:

Modified Michaelis-Menten equation used to describe the impact of DDM concentration on the formation of nanodiscs.

\[
\frac{A_{\text{Main}}}{A_{\text{Tot}}} = \frac{(A_{\text{Max}} - A_0)c_{\text{DDM}}}{K_{\text{DDM}} + c_{\text{DDM}}} + A_0
\]  

(1)

where \(A_{\text{Tot}}\) is the total area under the SEC curve, \(A_{\text{Main}}\) is the area fitted using EMG under the nanodisc peak, \(A_{\text{Max}}\) and \(A_0\) are the highest and the lowest fraction of \(A_{\text{Main}}\) divided by \(A_{\text{Tot}}\), respectively, and \(K_{\text{DDM}}\) is the DDM concentration that gives rise to the value halfway between \(A_{\text{Max}}\) and \(A_0\).

3. Results and discussion

3.1. Large POPC-MSP particle form when using standard protocols

Recently, we developed supercharged versions of MSPs that can be circularized and produce nanodiscs with diameters of roughly 9, 10, and 13 nm using 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipids [7,11]. With DMPC, monodisperse nanodiscs were formed using the standard cholate-assisted protocol, i.e. single SEC peaks at the expected retention volumes were obtained in all cases. In the present study, we use the more physiologically relevant POPC [38], which in comparison to DMPC has longer carbon tails, one of which is monounsaturated. Fig. 2 shows the SEC purification of nanodiscs formed using the standard cholate-assisted protocol, which reveals that populations of different larger particles are formed in most cases. As mentioned, this is very different from the reconstitution with DMPC. This variance likely arises from the difference in hydrophobic height of DMPC and POPC in relation to the hydrophobic height of the MSP. In the present case, for each MSP, the rightmost peak is at the expected retention volume for a nanodisc (Fig. 2). While small side peaks are commonly encountered in SEC purification of nanodiscs [1,24], as is the case for lsMSP1E3D1 (Fig. 2C), the effect is extremely pronounced for the smaller lsMSP1D1H5 (Fig. 2A) and lsMSP1D1 nanodiscs (Fig. 2B), where the majority of the SEC area corresponds to unwanted larger particles. To investigate if these larger particles arise due to a suboptimal POPC:MSM ratio, lipid titrations were performed for all six MSPs (Fig. S 1). These titrations revealed that changing the POPC:MSM ratio did not result in optimal reconstitution conditions, as larger particles were still formed. These inhomogeneous samples are contrary to the original MSP1D1H5 and MSP1D1 which self-assemble into a single major nanodisc species under these reconstitution conditions [1,39] and imply that the additional charges in the supercharged MSPs affect the reconstitution of these smaller nanodiscs. Notably, the SEC chromatograms for the circularized nanodiscs in all cases contain only a single major peak, corresponding to well-formed nanodiscs. Our results here suggest that the confining structure of the circularized MSPs pushes the self-assembly towards the local energy minimum that is given by the nanodisc structure, whereas the less restricting linear MSPs allow formation of larger lipid-MSP species that, probably due to the high charge density of the closed disc shapes, are thermodynamically more favorable. We note that this effect is only seen for the two smaller MSPs, which suggests that the larger lsMSP1E3D1 allow for accommodating the higher charge density of the nanodiscs. The ratio between the number of negatively charged residues in the MSPs (two per nanodisc) and the number of lipids per nanodisc in the reconstitution is 0.84, 0.76 and 0.56 for MSP1D1, MSP1D1 and MSP1E3D1-based nanodiscs, respectively. The number of lipids per nanodisc can be taken as a proxy for the bilayer surface area in the nanodiscs and we speculate that the lower charge to lipid ratio and hence the lower charge density in lsMSP1E3D1 based nanodiscs is the enabling factor for these structures.

To further investigate the nature of the particles formed with lsMSP1D1H5 and lsMSP1D1, we collected SAXS data on relevant SEC fractions (Fig. S 2). The longest distance in the particle, \(D_{\text{max}}\) from the SAXS data on the rightmost peak (labeled ‘ND’, Fig. S 2) exhibits the expected size for the nanodiscs, whereas the two other peaks in both cases are considerably bigger. However, all the datasets are reminiscent of a multi-contrast system, suggesting the presence of both MSP and lipids. Independent phosphate quantification validated the presence of lipids in all species, allowing determination of lipid:MSP ratios (Fig. S 2). In line with expectations, these ratios are higher for all the larger species compared to the nanodisc peak. With the lack of baseline separation in SEC, monodisperse samples could not be extracted, and consequently, these lipid:MSP ratios as well as the estimated sizes from SAXS only serve as preliminary data to understanding the nature of these larger particles.

3.2. DDM has a positive effect on nanodisc self-assembly with supercharged MSPs

While the cholate-assisted reconstitution protocol [1] yields a fraction of well-formed nanodiscs with the lsMSPs, it is desirable to maximize the yield of the self-assembly reaction. To optimize the reconstitution protocol, we added the non-ionic detergent DDM to the components of the standard cholate-assisted protocol, as DDM in combination with cholate has previously been shown to be compatible with nanodisc formation [12]. Fig. 3A shows the SEC purification of lsMSP1D1H5 nanodiscs that were reconstituted from pre-aggregates with cholate and different concentrations of DDM as well as a sample produced with DDM-solubilized POPC and no cholate. The nanodisc formation shows a DDM dependence where the undesired side peaks at low retention volume gradually diminish relative to the expected

Fig. 2. SEC analysis of nanodisc formation with POPC lipids using the standard cholate-assisted reconstitution. Nanodiscs were formed from a mixture containing 20 mM cholate, 10 mM POPC, and MSP in concentrations specified in the method section.
nanodisc peak. In the DDM-only sample, a single peak is observed, but it should be noted that this sample was opaque prior to loading on the SEC column, suggesting formation of large lipid particles that had to be removed by centrifugation prior to SEC analysis. As such, the overall yield is relatively lower when using DDM alone. Furthermore, the peak for the DDM-only sample is located at a higher retention volume than corresponding nanodisc peaks in samples formed using cholate. This implies the formation of smaller nanodiscs, likely due to a lower lipid loading efficiency.

By fitting the SEC curves with exponentially modified Gaussians, we estimate the fraction of well-formed nanodiscs, which we define as the ratio between the area of the nanodisc peak (shaded in Fig. 3A) and the total area under the SEC curve. Fig. 3B–D shows these ratios as a function of DDM concentration included in the pre-aggregates. For all lsMSPs as well as csMSP1D1, these plots show a saturating dependence upon increased DDM concentration. The most striking improvements are seen for the small lsMSP1D1ΔH5 and lsMSP1D1 nanodiscs, while for the larger MSP1E3D1 nanodiscs, there is only little room for improvement by including DDM.

We note that fitting of Eq. (1) to the data in Fig. 3B–D yields good fits capturing the effect of DDM. From these fits, we obtain the saturation value, $A_{\text{Max}}$, the initial ratio (at 0 mM DDM), $A_0$, as well as the DDM concentration, $K_{\text{DDM}}$, providing the value halfway between $A_0$ and $A_{\text{Max}}$ (Table 1). These values summarize the data in Fig. 3B–D and allow a quantitative comparison between all MSPs. Here, $A_{\text{Max}}$ values are generally high (≥0.89), showing that well-formed nanodiscs can be achieved for all MSPs at sufficiently high DDM concentrations in the pre-aggregates. On the other hand, $A_0$ values indicate the efficiency of

| MSP Type             | $A_{\text{Max}}$ | $A_0$  | $K_{\text{DDM}}$, mM |
|----------------------|------------------|-------|----------------------|
| IsMSP1D1ΔH5          | 0.95             | 0.28  | 9.11                 |
| csMSP1D1ΔH5          | 0.91             | 0.86  | 0.08                 |
| IsMSP1D1             | 0.99             | 0.28  | 40.64                |
| csMSP1D1             | 0.96             | 0.70  | 2.45                 |
| lsMSP1E3D1           | 0.89             | 0.81  | 5.00                 |
| csMSP1E3D1           | 0.92             | 0.87  | 1.04                 |
detergent removal is known to affect vesicle formation [41] but a recent study using an increasing amount of detergent absorbing beads found no apparent effect on nanodisc reconstitution using only cholate [13]. Secondly, DDM is known to form larger micelles compared to those formed by cholate [17,40]. This means that the size of the pre-aggregates will change as DDM concentration is increased. However, as the original MSP1D1 and MSP1D1ΔH5 are compatible with reconstitution without DDM, this does not seem to be the issue either. Lastly, DDM is non-ionic whereas cholate is anionic, and the lack of charge could mean that DDM is less affected by the high charge density of the negatively supercharged MSPs. In contrast, the anionic cholate will have a repulsive interaction with the supercharged MSPs, which could make

![Figure 4](https://example.com/fig4.png)

Fig. 4. SAXS on lsMSP1D1ΔH5 nanodisc samples. A: SAXS data measured on the fractions shown in Fig. S 3. DDM concentration in the pre-aggregates as indicated in the figure. Refined fits of the nanodisc model shown in black. The data for x = 0 is shown in correct units and the remaining datasets are scaled by a factor of 10^{−1.2}. B: p(r)-function of corresponding to the data in A. A nanodisc model was fitted to the SAXS data providing different parameters of which some are shown in C and D. C: Number of lipids per nanodisc. D: Area per headgroup of the lipids. E and F: Graphical representation of the geometrical nanodisc model used to fit the SAXS data. A detailed description of the model is presented in the method section.
nanodisc formation less favorable. Using a zwitterionic steroid detergent such as CHAPS, instead of or in combination with cholate, might also favor the reconstitution, but this is out of the scope of the present study. However, we observe that circularizing the supercharged MSPs completely mitigates this problem, implying that it is a fine balance between nanodisc formation and formation of larger species.

3.3. Structural properties of the nanodiscs

So far, we have evaluated nanodisc reconstitution by the presence of a SEC peak at the expected retention volume. To further characterize the formed lsMSP1D1ΔH5 nanodiscs, we collected fractions from the peaks in all SEC runs (Fig. S 3) and measured SAXS on these. An example of this is shown in Fig. 4A, while all SAXS data are shown in Fig. S 4. All of the collected SAXS curves are indicative of nanodiscs with a minimum around \( q = 0.07 \, \text{Å}^{-1} \) and a double bump feature in the \( q \)-range 0.08 Å\(^{-1} \) to 0.25 Å\(^{-1} \) [31]. Likewise, the \( p(r) \)-distributions (Fig. 4B) are characteristic for nanodiscs with two peaks separated by a negative minimum due to the negative contrast of the POPC tails. Comparing the different samples, we find that the SAXS curves and \( p(r) \)-distributions are quite similar throughout the series, suggesting that the particles collected in the expected nanodisc peak in SEC are indeed nanodiscs, independent of the DDM content in the pre-aggregates. Two samples contained slightly larger particles (1 mM DDM and 3 mM DDM, red asterisks). As seen from the SEC data in Fig. S 3, these samples do not deviate from the overall trend, and we conclude that the fractions collected from exactly these samples were contaminated by a minor fraction of the adjacent peaks containing larger particles.

To characterize the nanodisc structures in more detail, we refined the nanodisc model (graphical representation in Fig. 4E and F) introduced earlier to each data set using WillItFit [33]. The model generally captures all features in the SAXS data and provides good fits for all DDM concentrations. The refined parameters can be found in Table S 2. Fig. 4C shows the number of lipids per nanodisc as a function of DDM concentration in the pre-aggregates. This number is steadily refined to a value around 130, which surprisingly is higher than the 110 POPC used for forming nanodiscs with lsMSP1D1ΔH5. A notable exception in the number of lipids is seen for the sample prepared with DDM-solubilized POPC, which is only loaded with roughly 100 POPC per nanodisc. This is in line with our previous findings that using only DDM as reconstitution detergent yields poorly loaded nanodiscs [13]. Another important parameter, the area per POPC headgroup, is reported in Fig. 4D. For the areas per headgroup, the two samples marked with a red asterisk are located slightly above the remaining values which trend around 58 Å\(^2\). This value is slightly lower than the 61.5 Å\(^2\) which is reported for POPC in planar bilayers at 10 °C [42,43].

Subsequently, we performed similar model fitting and phosphate analysis on the remaining five MSPs (data overview in Fig. S 4). Again, the nanodisc model captured all features in the data, and provided good fits for all datasets. The number of lipids refined from the fits can be seen in Fig. 5 (overview of all refined parameters from model fits in Table S 2) together with values obtained by phosphate analysis (labeled ‘Pi’). Nanodiscs based on five of the six MSPs exhibit almost constant number of lipids versus DDM concentration as determined by SAXS, with only csMSP1D1 showing an increase around 10 mM to 30 mM DDM. We note that these values are also the only ones that are systematically different from those determined by phosphate analysis, in which they exhibit a trend of constant values. This suggests that these samples are affected by a small population of larger species, causing the SAXS fits to show high lipid contents. For all six MSPs, the nanodiscs reconstituted with DDM...
solubilized lipids contain fewer lipids compared to the samples reconstituted with cholate solubilized lipids. The area per head group and the axis ratios of the nanodiscs (Table S2) are generally stable and independent of initial DDM concentration. This underlines the importance of optimizing the detergent composition in the pre-aggregate, as this can increase the yield of the self-assembly process, i.e. single SEC peaks, without affecting the structural parameters of the formed nanodiscs.

3.4. DDM increases proteorhodopsin incorporation into nanodiscs

After having optimized the reconstitution of the empty nanodiscs, we investigated the impact of DDM on membrane protein incorporation into nanodiscs. We used proteorhodopsin (PR) as a model membrane protein as the protein’s inherent absorption at 530 nm allows for specific visualization of PR loaded nanodiscs during a SEC run. Samples with PR (stored in 1% octyl glucoside) were prepared with either 0 mM or 30 mM DDM in the pre-aggregates. The samples were centrifuged before the SEC runs to eliminate large aggregates, resulting in lower absorption in the chromatograms in cases with unsuccessful reconstitution. As PR consists mainly of transmembrane helices, the success criterion is a monodisperse peak at the same retention volume as that of an empty nanodisc, i.e. around 11 ml to 14 ml depending on the length of the MSP.

Fig. 6 shows the obtained SEC chromatograms with normalized areas to facilitate comparison of reconstitution efficiency. For all pairs of samples, it is apparent that DDM facilitates the incorporation of PR: With DDM, there are no peaks at the void volume and the areas are larger. Finally, the samples prepared with circularized MSPs and DDM included show the best-defined peaks at the expected retention volumes. PR is known to form high order oligomers [44], but the spatial restriction of the covalently linked MSP likely counteracts the insertion of PR.
oligomers that cannot fit inside the final nanodisc. Indeed, for the samples prepared with non-circularized MSPs, several side-peaks are observed, which likely contains large lipid-protein particles with PR oligomers, in addition to a minor peak at the expected retention volume for our nanodiscs.

4. Conclusions

We have utilized novel supercharged MSPs with increased stability and optimized the nanodisc reconstitution by tuning the composition of the pre-aggregates used for the self-assembly. A standard nanodisc reconstitution is mediated by the anionic detergent cholate, but we find that this protocol does not yield satisfactory amounts of nanodiscs with the linear versions of the two smaller supercharged MSPs: lsMSP1D1LSH and lsMSP1D1. However, adding increasing amounts of the non-charged detergent, DDM, to the pre-aggregate mitigated this effect, leading to high yields for all MSPs investigated. Contrary to the nanodiscs based on linear MSPs, the samples reconstituted using circularized MSPs provided high yields only using cholate. The observation that the extra charges on the MSP and the presence of DDM have such a major impact on the outcome of the self-assembly process emphasizes the fine balance between different energy minima for the system and that it is essential to optimize the composition of the pre-aggregate.

We investigated the resulting nanodiscs by SAXS and phosphate analysis, and generally found them to be independent of DDM content in the pre-aggregate. The only exception was the sample without cholate, which for all six MSPs showed a lower lipid loading compared to all other samples, underlining that DDM alone is not optimal for nanodisc reconstitution is mediated by the anionic detergent cholate, but we find that this protocol does not yield satisfactory amounts of nanodiscs with the linear versions of the two smaller supercharged MSPs: lsMSP1D1LSH and lsMSP1D1. However, adding increasing amounts of the non-charged detergent, DDM, to the pre-aggregate mitigated this effect, leading to high yields for all MSPs investigated. Contrary to the nanodiscs based on linear MSPs, the samples reconstituted using circularized MSPs provided high yields only using cholate. The observation that the extra charges on the MSP and the presence of DDM have such a major impact on the outcome of the self-assembly process emphasizes the fine balance between different energy minima for the system and that it is essential to optimize the composition of the pre-aggregate.

We investigated the resulting nanodiscs by SAXS and phosphate analysis, and generally found them to be independent of DDM content in the pre-aggregate. The only exception was the sample without cholate, which for all six MSPs showed a lower lipid loading compared to all other samples, underlining that DDM alone is not optimal for nanodisc formation. Lastly, we found that DDM also facilitated incorporation of the membrane protein PR into nanodiscs based on six MSPs under investigation. Here, the circularized variants all exhibited a major peak at the expected retention volume, indicating monodisperse nanodiscs, whereas the linear MSPs showed the presence of different species. However, in all cases, major improvements were seen in comparison to the samples without DDM added, again showing the benefits of including DDM in the pre-aggregate. Other detergents are likely to show the same positive effect on the nanodisc reconstitution efficiency when used in combination with a detergent efficient for lipid solubilization, such as cholate.

CRediT authorship contribution statement

NTJ, FGT, LA and MCP conceived the project. NTJ and FGT produced all proteins used for this study. SB made all nanodisc samples and fitted SEC and SAXS data. FGT and NTJ co-wrote the manuscript with inputs from all authors.

Declaration of competing interest

FGT, NTJ and LA have founded TideDisc Biotech Aps involved in commercialization of supercharged MSPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbbamem.2022.183884.
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