

# Integral Membrane Proteins Can Be Crystallized Directly from Nanodiscs

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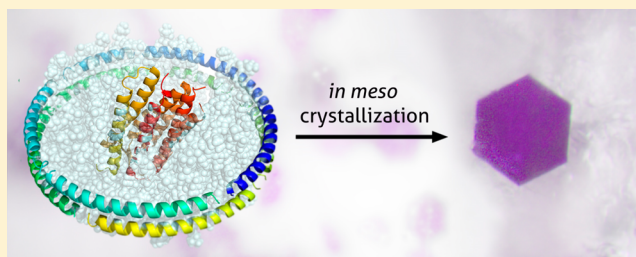
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## S Supporting Information

**ABSTRACT:** Membrane-like nanodiscs (ND) have become an important tool for the cell-free expression, solubilization, folding, and in vitro structural and functional studies of membrane proteins (MPs). Direct crystallization of MPs embedded in NDs would be of high importance for structural biology. However, despite considerable efforts we have been as yet unable to obtain crystals suitable for X-ray crystallography. In the present work, we show that an ND-trapped MP can be transferred into the cubic phase and crystallized in meso. Bacteriorhodopsin (BR) reconstituted into nanodiscs was mixed with a lipidic mesophase and crystallization was induced by adding a precipitant. The resulting crystals diffract beyond 1.8 Å. The structure of BR was solved at 1.9 Å and found to be indistinguishable from previous structures obtained with the protein solubilized in detergent. We suggest the proposed protocol of in meso crystallization to be generally applicable to ND-trapped MPs.



Membrane proteins (MPs) are the main functional units of biological membranes, which are responsible for many diverse processes in a living cell. They are highly important drug targets and their structures are of great interest to pharmacology.<sup>1,2</sup> Unfortunately, elucidation of MPs structures is a major challenge for membrane protein research; out of 7000 predicted human MPs, structures are only known for several dozens of them.<sup>3,4</sup> Due to their amphipathic nature, membrane proteins must be solubilized for biophysical and biochemical studies and crystallization. Previously, mainly detergents were used for solubilization, but they tend to destabilize MPs structure and function. Several alternative membrane mimicking systems have therefore been developed to maintain membrane proteins extracted from their native environment in a soluble and functional state.<sup>5,6</sup>

A new promising system for the membrane protein solubilization and stabilization is the nanodisc system (or nanodiscs, NDs).<sup>7,8</sup> NDs consist of discoidal patches of lipid bilayer, surrounded by two molecules of truncated human apolipoprotein A1, designated as membrane scaffold protein

(MSP).<sup>9–11</sup> The MSP molecules cover the hydrophobic perimeter of the lipidic bilayer. NDs technology greatly simplifies the process of MP stabilization. The lipid composition and diameter of NDs can be varied in accordance with needs of the target integral membrane protein.<sup>12</sup> Thus, NDs are considered an excellent system for solubilization and stabilization of different kinds of MPs and their complexes. At present, the list of MPs which were reconstructed into nanodiscs comprises a large variety of bacterial and eukaryotic membrane proteins, including bacterial rhodopsins,<sup>13–17</sup> receptor tyrosine kinases,<sup>18</sup> cytochrome C450,<sup>7,19–21</sup> G-protein coupled receptors,<sup>22–25</sup> translocon complexes,<sup>26,27</sup> and many others.

Furthermore, NDs as a membrane mimicking medium are used not only to solubilize MPs, but to harbor nascent membrane proteins in cell-free expression system.<sup>18,28,29</sup>

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Moreover, there are successful examples of ND applications in a variety of experimental techniques, including NMR,<sup>30,31</sup> single-molecule fluorescence,<sup>32</sup> atomic force microscopy,<sup>10</sup> Cryo-EM,<sup>33</sup> microscale thermophoresis,<sup>25</sup> surface plasmon resonance,<sup>24</sup> laser flash photolysis,<sup>14,34</sup> small-angle X-ray and neutron scattering,<sup>35–37</sup> and others. Direct usage of MPs embedded in NDs for crystallization would be of high importance for structural biology. However, so far our considerable attempts to obtain crystals suitable for X-ray diffraction have been unsuccessful. In the present work, we used well-known model MP bacteriorhodopsin (BR) to show the possibility of crystallizing ND-trapped MPs by their direct transfer to a lipid based crystallization matrix (a mesophase).

BR was purified and solubilized from purple membranes of *Halobacterium salinarum* S9<sup>38</sup> as described in detail.<sup>39,40</sup> MSPs were expressed and purified according to the protocols<sup>41</sup> and experimental details can be found in the [Supporting Information](#).

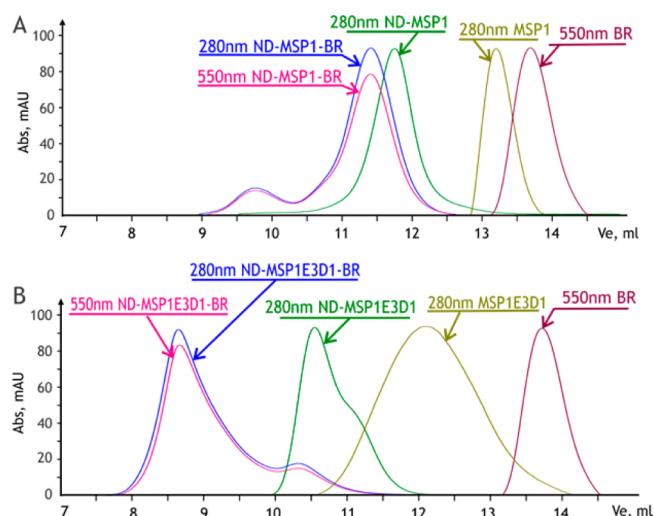
The bilayer area of the existing NDs varies from 4500 Å<sup>2</sup> for the smallest NDs, surrounded by scaffold protein MSP1 (ND-MSP1) to 8900 Å<sup>2</sup> for the largest NDs, surrounded by elongated scaffold protein construct MSP1E3D1 (ND-MSP1E3D1).<sup>41</sup> Both of them, ND-MSP1 and ND-MSP1E3D1, were used in this work to examine the influence of the NDs size on the MP crystallization. For ND assembly with and without reconstituted BR we have adapted protocols from refs 10, 41, and 42. In brief, NDs reconstitution mixture was prepared by extrusion DMPC/sodium cholate mixture. Then MSP and BR presolubilized in 0.05% DDM were added using following molar ratios: for empty ND assembly, MSP1:DMPC as 1:90 and MSP1E3D1:DMPC as 1:160; for NDs with BR, MSP1:DMPC:BR as 2:100:0.5 and MSP1E3D1:DMPC:BR as 2:160:3. These ratios between membrane protein, lipids, and MSP play an important role in correct and monodisperse nanodisc assembly. A good estimation can be obtained from simple geometric considerations, counting the cross-sectional area of the membrane protein, which displaces a certain number of lipids from inside of the ND.<sup>41</sup> Nevertheless, the lipid:MSP:MP ratio was experimentally adjusted to optimize the formation of NDs.

Theoretically, BR trimer could fit into MSP1 nanodiscs, but experimentally we observed mostly assembly NDs with BR monomers what was concluded from the results of size exclusion chromatography (SEC) ([Figure 1A](#)), polyacrylamide gel electrophoresis (SDS-PAGE) ([Figure 2](#)), and small-angle X-ray scattering (SAXS) (see [SI Figure 1](#)). In the larger NDs-MSP1E3D1, BR was prone to be incorporated in the form of trimers ([Figure 1](#) and [Figure 2](#)).

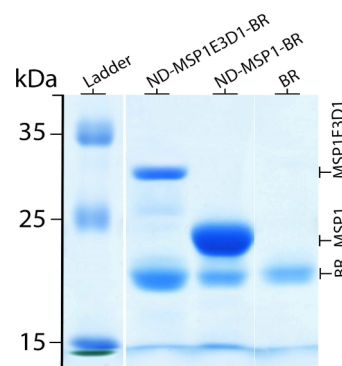
Self-assembly of NDs was initiated in a process of detergent removal using either dialysis or detergent-adsorbing beads.

The analysis of the reconstituted samples using a calibrated gel filtration column allowed us to determine size and homogeneity of the NDs. Typical elution profiles after assembly of NDs-MSP1-BR is shown in [Figure 1A](#) in comparison with empty NDs of the same size ND-MSP1. The majority of the BR-nanodiscs eluted as a single peak, while a minimal amount did as larger aggregates also containing BR. One possible explanation for the presence of aggregates is that multiple BR interactions promoted an aggregation pathway as opposed to formation of NDs of fixed size.

Despite using the optimal MSP:DMPC:BR ratio for BR reconstitution into the larger NDs-MSP1E3D1, the major NDs peak with BR trimers was still accompanied by a smaller one



**Figure 1.** Overlay of the SEC elution profiles for solubilized BR, MSP, and NDs with and without reconstituted BR. (A) Smaller NDs assembled with MSP1 and (B) larger NDs assembled with MSP1E3D1.



**Figure 2.** SDS-PAGE with NDs main peak fractions after SEC and BR (as a reference), Ladder - PageRuler Plus (Thermo) Prestained Protein Ladder (Thermo Scientific).

with BR monomers ([Figure 1B](#)). The main peak fractions of ND-trapped BRs were pooled and assessed by SDS-PAGE ([Figure 2](#)) and SAXS.

The technique of SAXS allowed us to determine shape and structural parameters of NDs. The data collection process and treatment are presented in detail in the [Supporting Information](#).

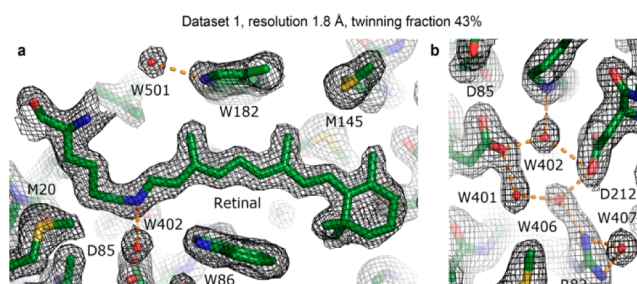
The in meso technique with modifications was used for crystallization both samples of ND-trapped BR.<sup>39,43</sup> Samples were concentrated to 18 mg/mL of BR and then were mixed with monoolein (MO, 1-oleoyl-*rac*-glycerol)-based mesophase by two gastight syringes.<sup>44</sup> Well-homogenized mesophase was automatically dispensed by an NT8 Formulatrix crystallization robot in 96-well LCP Sandwich Set glass plates (Marienfeld, Germany). Each well consisted of 150 nL of mesophase covered with 800 nL of the precipitant solution. Na/K-Pi, pH 5.6, solutions at different concentrations were used as precipitant.

First crystals in both cases were obtained in a month after addition of the precipitant solution and incubation at 22 °C. Crystals reached maturity within 2 months (defined by colorless phase surrounding the crystals). The crystals were well-shaped hexagonal plates, about 100 μm × 100 μm × 20

$\mu\text{m}$  (SI Figure 2). It should be noted that crystals of BR trapped in ND-MSP1 preferably appeared at low concentrations of salt (lower than 1.2 M). In contrast, the crystals of BR inserted into ND-MSP1E3D1 did not grow at low concentrations of the salt and were observed only at high concentrations of the salt (2.2–2.8 M). The probability to obtain crystals with BR-trapped in ND-MSP1E3D1 was about 2 times higher, as crystals were observed with a larger number of different precipitants, than for BR inserted into ND-MSP1.

To test their quality, the crystals were mounted in a loop and flash-cooled in liquid nitrogen without further cryo-protection. X-ray diffraction data were collected at the beamline ID23–1 at the ESRF, using a PILATUS 6 M detector, at the wavelength of 0.973 Å. The data statistics is presented in the Supporting Information in Table 1.

The structures of BR crystallized from ND-MSP1 and ND-MSP1E3D1 were solved at the resolutions of 1.8 and 2.0 Å, correspondingly (Supporting Information Table 1, and Figure 3). Overall, the packing of BR in crystals and its structure are



**Figure 3.** Example of the electron density map obtained with the BR crystals grown from nanodiscs. The  $2F_o - F_c$  electron density maps are drawn at the level of  $1.5 \sigma$ . (a,b) Data from Data set 1. Panel a shows the electron density map around the retinal, and panel b shows the electron density map in the retinal's Schiff base region. The configurations are identical to those observed in the crystals grown by traditional lipidic cubic phase approach.

identical to those observed in the crystals grown by traditional in meso protocols<sup>45</sup> (r.m.s.d. of backbone atom positions are 0.3 and 0.4 Å, correspondingly) or in the crystals obtained with BR transferred from Amphipol<sup>46</sup> (r.m.s.d. of the backbone atom positions are 0.3 and 0.3 Å, correspondingly). Similarly to the latter case, there are electron densities corresponding to ordered lipid fragments, but no unexpected electron densities that could be ascribed either to the MSP1 or MSP1E3D1.

We suggest also that this approach to crystallization of a particular membrane protein trapped in NDs explored in the present work can be applied for MP crystallization in general. The plausible mechanism of crystallization is as follows. NDs harboring MP “dissolve” in the bilayer of the in meso crystallization matrix after which the properties of lipidic bilayer phase as whole rule crystallization. MPs separate from former NDs surrounding and scaffold proteins do not interfere with MP crystallization.

NDs have proven to be very useful both to stabilize MPs in aqueous solution<sup>41,47</sup> and to obtain them, either by assisting their folding from a denatured state, such as that obtained by solubilization of inclusion bodies, or by acting as the host medium during cell-free synthesis.<sup>18</sup> Combining ND-assisted production and/or stabilization of MPs with the in meso crystallization offers a new promising route to MP crystallization.

## ■ ASSOCIATED CONTENT

### § Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.cgd.6b01631.

Protocols of MSP expression, purification, and NDs assembly. SAXS curves and pair distance distribution functions of NDs. Photos of BR crystals in mesophase and table with their parameters. (PDF)

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○M.N. and E.R. contributed equally to this work.

### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

- (1) Overington, J. P.; Al-Lazikani, B.; Hopkins, A. L. *Nat. Rev. Drug Discovery* **2006**, 5 (12), 993.
- (2) Bakheet, T. M.; Doig, A. J. *Bioinformatics* **2009**, 25 (4), 451.
- (3) Fagerberg, L.; Jonasson, K.; von Heijne, G.; Uhlén, M.; Berglund, L. *Proteomics* **2010**, 10 (6), 1141.
- (4) Kozma, D.; Simon, I.; Tusnády, G. E. *Nucleic Acids Res.* **2013**, 41, D524.
- (5) Popot, J.-L. *Annu. Rev. Biochem.* **2010**, 79 (1), 737.
- (6) Serebryany, E.; Zhu, G. A.; Yan, E. C. Y. *Biochim. Biophys. Acta, Biomembr.* **2012**, 1818 (2), 225.
- (7) Civjan, N. R.; Bayburt, T. H.; Schuler, M. A.; Sligar, S. G. *Biotechnology* **2003**, 35 (3), 556.
- (8) Nath, A.; Atkins, W. M.; Sligar, S. G. *Biochemistry* **2007**, 46 (8), 2059.
- (9) Shaw, A. W.; McLean, M. A.; Sligar, S. G. *FEBS Lett.* **2004**, 556 (1–3), 260.
- (10) Bayburt, T. H.; Sligar, S. G. *Protein Sci.* **2003**, 12 (11), 2476.
- (11) Sligar, S. G.; Bayburt, T. H.; Grinkova, Y. V. *Nano Lett.* **2002**, 2, 853.
- (12) Grinkova, Y. V.; Denisov, I. G.; Sligar, S. G. *Protein Eng., Des. Sel.* **2010**, 23 (11), 843.



- (13) Bayburt, T. H.; Grinkova, Y. V.; Sligar, S. G. *Arch. Biochem. Biophys.* **2006**, 450 (2), 215.
- (14) Johnson, P. J. M.; Halpin, A.; Morizumi, T.; Brown, L. S.; Prokhorenko, V. I.; Ernst, O. P.; Miller, R. J. D. *Phys. Chem. Chem. Phys.* **2014**, 16, 21310.
- (15) Bayburt, T. H.; Leitz, A. J.; Xie, G.; Oprian, D. D.; Sligar, S. G. *J. Biol. Chem.* **2007**, 282 (20), 14875.
- (16) Wang, J.; Sasaki, J.; Tsai, A.-L.; Spudich, J. L. *J. Biol. Chem.* **2012**, 287 (25), 21316.
- (17) Ranaghan, M. J.; Schwall, C. T.; Alder, N. N.; Birge, R. R. *J. Am. Chem. Soc.* **2011**, 133 (45), 18318.
- (18) Lyukmanova, E. N.; Shenkarev, Z. O.; Khabibullina, N. F.; Kopeina, G. S.; Shulepko, M. A.; Paramonov, A. S.; Mineev, K. S.; Tikhonov, R. V.; Shingarova, L. N.; Petrovskaya, L. E.; Dolgikh, D. A.; Arseniev, A. S.; Kirpichnikov, M. P. *Biochim. Biophys. Acta, Biomembr.* **2012**, 1818 (3), 349.
- (19) Baas, B. J.; Denisov, I. G.; Sligar, S. G. *Arch. Biochem. Biophys.* **2004**, 430 (2), 218.
- (20) Das, A.; Grinkova, Y. V.; Sligar, S. G. *J. Am. Chem. Soc.* **2007**, 129 (45), 13778.
- (21) Das, A.; Sligar, S. G. *Biochemistry* **2009**, 48 (51), 12104.
- (22) Leitz, A.; Bayburt, T.; Barnakov, A.; Springer, B.; Sligar, S. G. *BioTechniques* **2006**, 40 (5), 601.
- (23) Marin, V. L.; Bayburt, T. H.; Sligar, S. G.; Mrksich, M. *Angew. Chem., Int. Ed.* **2007**, 46 (46), 8796.
- (24) Bocquet, N.; Kohler, J.; Hug, M. N.; Kuszniir, E. A.; Rufer, A. C.; Dawson, R. J.; Hennig, M.; Ruf, A.; Huber, W.; Huber, S. *Biochim. Biophys. Acta, Biomembr.* **2015**, 1848 (5), 1224.
- (25) Dijkman, P. M.; Watts, A. *Biochim. Biophys. Acta, Biomembr.* **2015**, 1848, 2889.
- (26) Alami, M.; Dalal, K.; Lelj-Garolla, B.; Sligar, S. G.; Duong, F. *EMBO J.* **2007**, 26 (8), 1995.
- (27) Dalal, K.; Nguyen, N.; Alami, M.; Tan, J.; Moraes, T. F.; Lee, W. C.; Maurus, R.; Sligar, S. S.; Brayer, G. D.; Duong, F. *J. Biol. Chem.* **2009**, 284 (12), 7897.
- (28) Roos, C.; Kai, L.; Proverbio, D.; Ghoshdastider, U.; Filipek, S.; Dötsch, V.; Bernhard, F. *Mol. Membr. Biol.* **2013**, 30 (1), 75.
- (29) Katzen, F.; Fletcher, J. E.; Yang, J.-P.; Kang, D.; Peterson, T. C.; Cappuccio, J. A.; Blanchette, C. D.; Sulchek, T.; Chromy, B. A.; Hoeprich, P. D.; Coleman, M. A.; Kudlicki, W. *J. Proteome Res.* **2008**, 7 (8), 3535.
- (30) Kijac, A. Z.; Li, Y.; Sligar, S. G.; Rienstra, C. M. *Biochemistry* **2007**, 46 (48), 13696.
- (31) Li, Y.; Kijac, A. Z.; Sligar, S. G.; Rienstra, C. M. *Biophys. J.* **2006**, 91 (10), 3819.
- (32) Nath, A.; Koo, P. K.; Rhoades, E.; Atkins, W. M. *J. Am. Chem. Soc.* **2008**, 130 (47), 15746.
- (33) Efremov, R. G.; Leitner, A.; Aebersold, R.; Raunser, S. *Nature* **2014**, 517 (7532), 39.
- (34) Mörs, K.; Roos, C.; Scholz, F.; Wachtveitl, J.; Dötsch, V.; Bernhard, F.; Glaubitz, C. *Biochim. Biophys. Acta, Biomembr.* **2013**, 1828 (4), 1222.
- (35) Kynde, S. A. R.; Skar-Gislinge, N.; Pedersen, M. C.; Midtgaard, S. R.; Simonsen, J. B.; Schweins, R.; Mortensen, K.; Arleth, L. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2014**, 70, 371.
- (36) Maric, S.; Skar-Gislinge, N.; Midtgaard, S.; Thygesen, M. B.; Schiller, J.; Frielinghaus, H.; Moulin, M.; Haertlein, M.; Forsyth, V. T.; Pomorski, T. G.; Arleth, L. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2014**, 70, 317.
- (37) Skar-Gislinge, N.; Simonsen, J. B.; Mortensen, K.; Feidenhans'l, R.; Sligar, S. G.; Lindberg Møller, B.; Bjørnholm, T.; Arleth, L. *J. Am. Chem. Soc.* **2010**, 132 (39), 13713.
- (38) Oosterhelt, D.; Stoekenius, W. *Proc. Natl. Acad. Sci. U. S. A.* **1973**, 70 (10), 2853.
- (39) Gordeliy, V. I.; Schlesinger, R.; Efremov, R.; Büldt, G.; Heberle, J. *Methods Mol. Biol.* **2003**, 228, 305.
- (40) Borshchevskiy, V. I.; Round, E. S.; Popov, A. N.; Büldt, G.; Gordeliy, V. I. *J. Mol. Biol.* **2011**, 409 (5), 813.
- (41) Ritchie, T. K.; Bayburt, T. H.; Denisov, I. G.; Zolnerciks, J. K.; Atkins, W. M.; Sligar, S. G.; Grinkova, Y. V. In *Methods in Enzymology*; Elsevier Inc., 2009; Vol. 464, pp 211–231.
- (42) Bayburt, T. H.; Grinkova, Y. V.; Sligar, S. G. *Arch. Biochem. Biophys.* **2006**, 450 (2), 215.
- (43) Caffrey, M.; Cherezov, V. *Nat. Protoc.* **2009**, 4 (5), 706.
- (44) Cheng, A.; Hummel, B.; Qiu, H.; Caffrey, M. *Chem. Phys. Lipids* **1998**, 95 (1), 11.
- (45) Luecke, H.; Schobert, B.; Richter, H. T.; Cartailler, J. P.; Lanyi, J. K. *J. Mol. Biol.* **1999**, 291 (4), 899.
- (46) Polovinkin, V.; Gushchin, I.; Sintsov, M.; Round, E.; Balandin, T.; Chervakov, P.; Schevchenko, V.; Utrobin, P.; Popov, A.; Borshchevskiy, V.; Mishin, A.; Kuklin, A.; Willbold, D.; Chupin, V.; Popot, J.-L.; Gordeliy, V. *J. Membr. Biol.* **2014**, 247 (9–10), 997.
- (47) Shirzad-Wasei, N.; van Oostrum, J.; Bovee-Geurts, P. H. M.; Kusters, L. J. A.; Bosman, G. J. C. G. M.; DeGrip, W. J. *Biol. Chem.* **2015**, 396 (8), 903.