Supplemental Figures

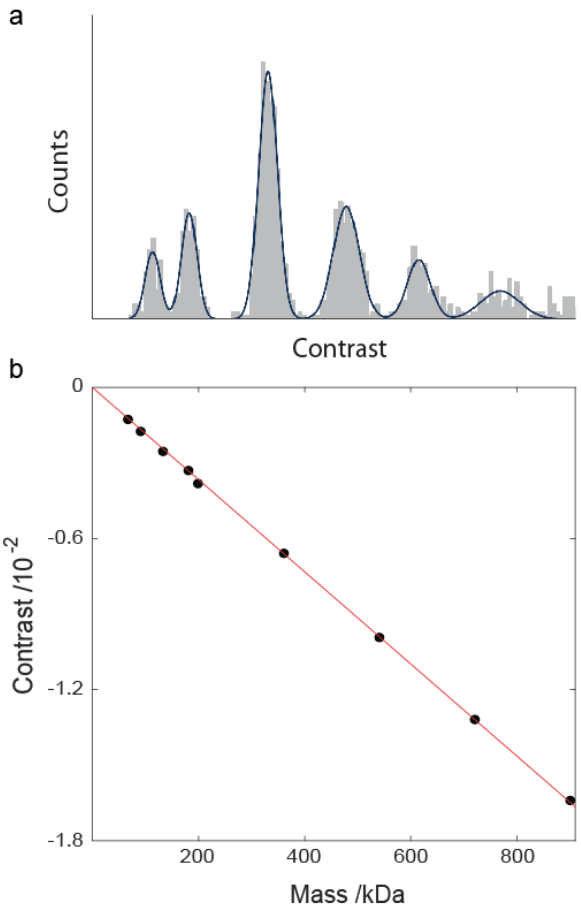


Figure S1. Contrast-to-mass (C2M) calibration (see also Figure 1A). (a) Example histogram of a mass calibrant as measured by MP. (b) The contrast of two proteins of known mass with different oligomeric states were plotted against their mass (66, 90, 132, 180, 198, 360, 540, 720, 900 kDa). The red line is the linear fit to the data according to $y = b.x$, with $b = -1.83 \cdot 10^{-5}$ being the C2M calibration factor.

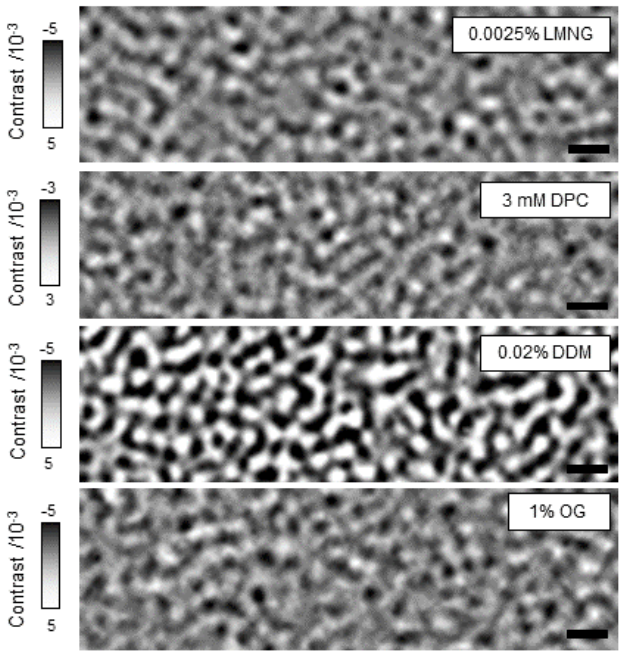


Figure S2. MP of detergent micelles (see also Figure 1C). Images of detergents LMNG, DPC, DDM and OG at or above the critical micelle concentration (CMC). Accompanying movies are: Supplemental Video 2 (LMNG), Supplemental Video 3 (DPC), Supplemental Video 4 (DDM) and Supplemental Video 5 (OG). Scale bar: 1 µm.

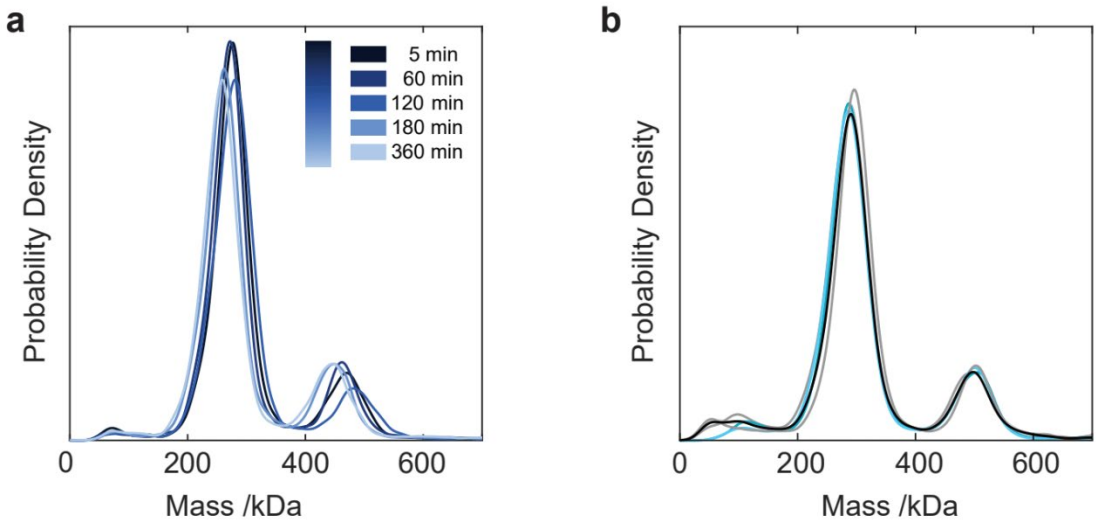


Figure S3: *E. coli bo₃* oxidase reproducibility and stability upon drop dilution (see also Figure 2B). (**a**) MP measurements of 20 nM *bo₃* oxidase at different time point after drop-diluting in detergent-free buffer. Final LMNG concentration is ~0.001x CMC. Colors correspond to different waiting times after dilution. (**b**) Repeats of MP measurements of 10 nM (turquoise lines) and 20 nM (grey lines) *bo₃* oxidase showing the reproducibility of the measurement. Black line corresponds to the average overall repeats.

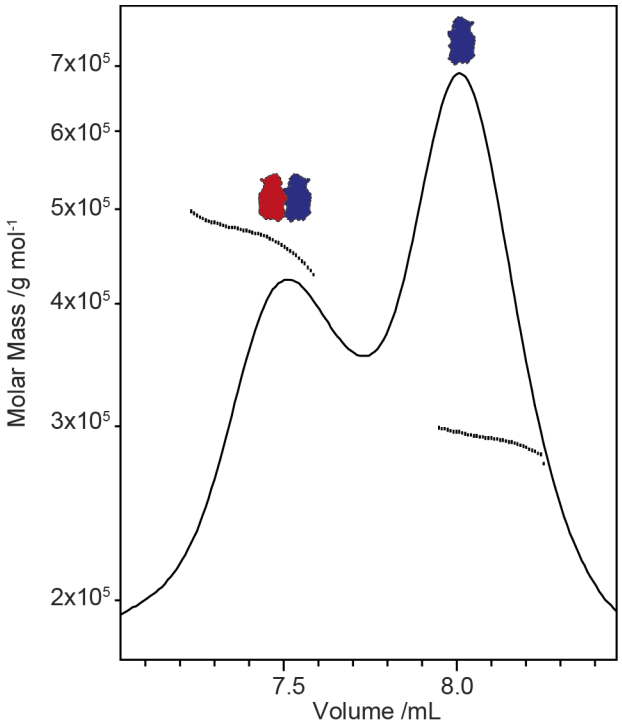


Figure S4: SEC-MALS of *E. coli bo₃* oxidase (see also Figure 2B). UV absorbance chromatogram and MALS traces (black dotted lines) of *bo₃*-LMNG micelle complexes in 0.003% LMNG containing running buffer. The conjugate protein-detergent molar mass was 293 kDa (monomer) and 464 kDa (dimer). The molar mass of individual components were 140 kDa *bo₃* plus 153 kDa LMNG (monomer) and 259 kDa *bo₃* plus 205 kDa LMNG (dimer). The red and blue molecules both represent *bo₃* oxidase monomers.

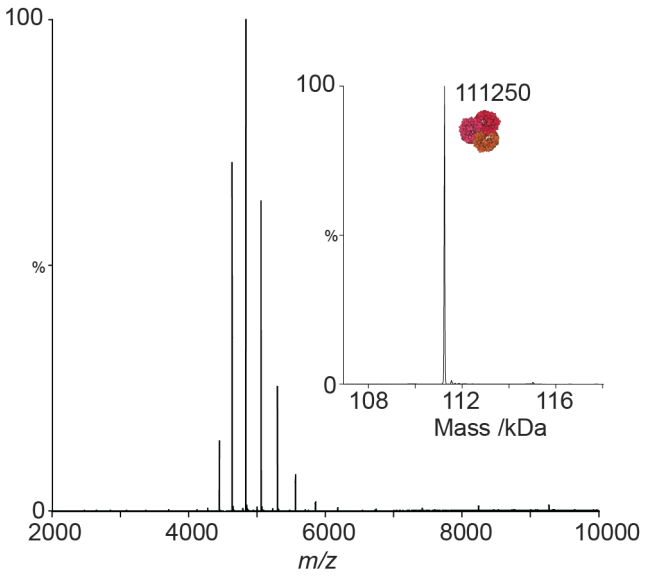


Figure S5. Native mass spectrometry of OmpF (see also Figure 2D). OmpF at 2 µM measured in 100 mM ammonium acetate with 2 x CMC n-octyl-β-D-glucopyranoside (OG).

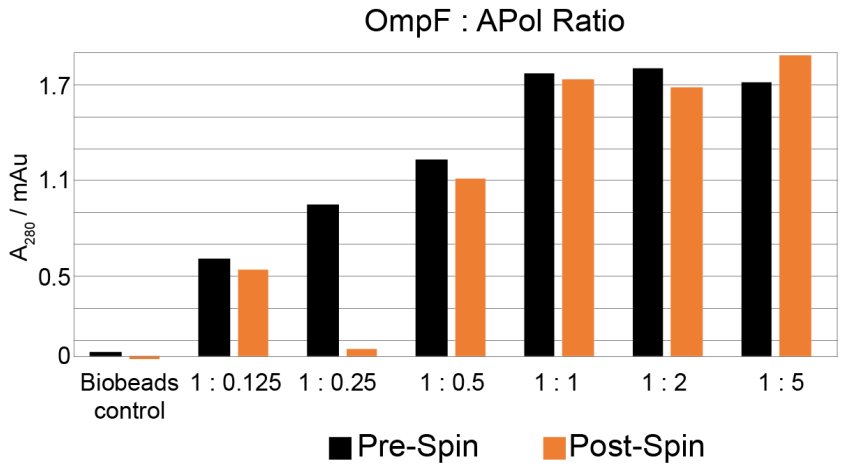


Figure S6. Analysis of OmpF detergent-amphipol exchange (see also Figure 2D). UV-VIS A_{280} measurement of OmpF prior to ultra-centrifugation step in the amphipol insertion procedure (black) compared to the absorbance of the supernatant post-ultracentrifugation (orange).

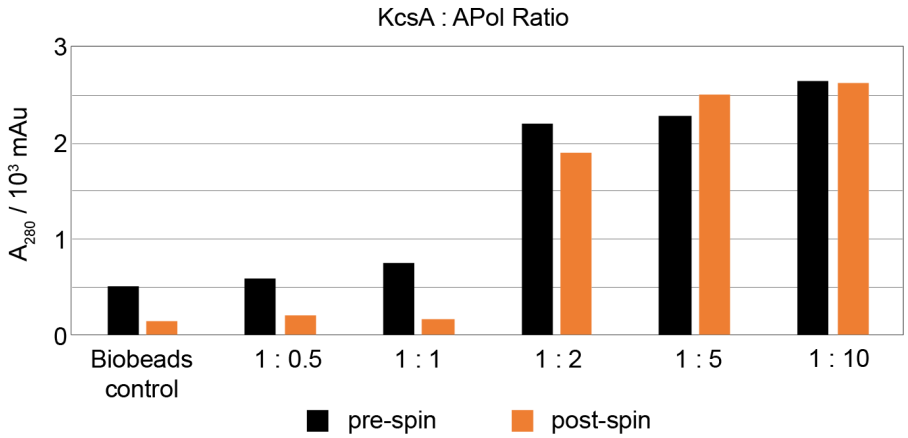


Figure S7. Analysis of KcsA detergent-amphipol exchange (see also Figure 2E). UV-VIS A_{280} measurement of KcsA prior to ultra-centrifugation step in the amphipol insertion procedure (black) compared to the absorbance of the supernatant post-ultracentrifugation (orange).

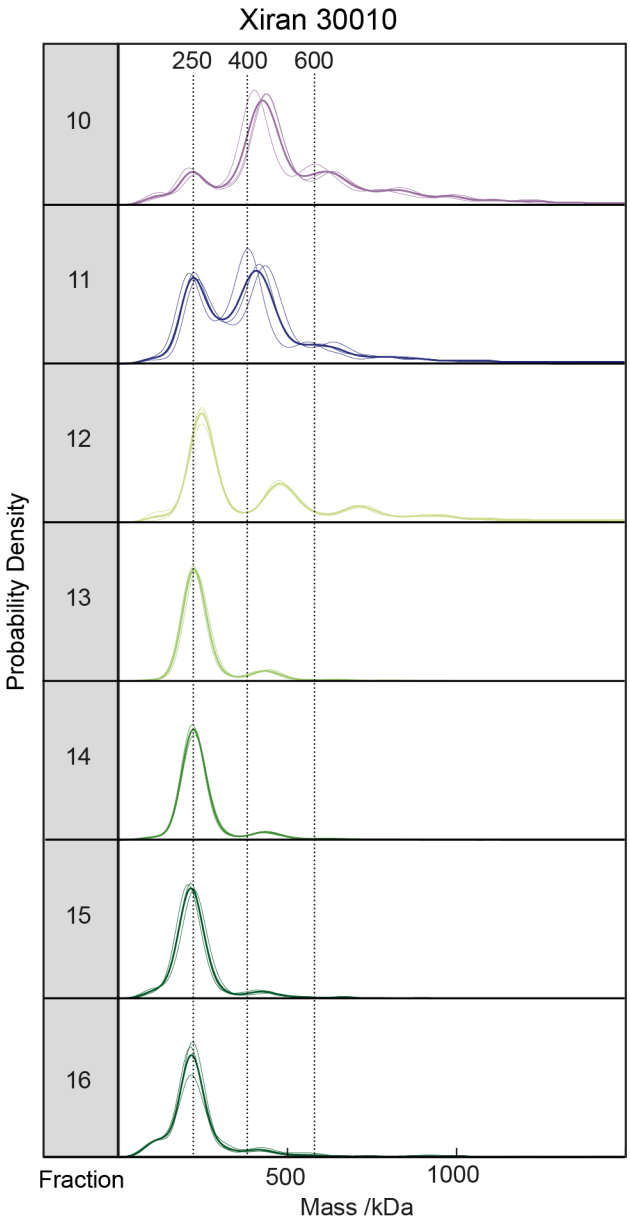


Figure S8. MP replicates of *E. coli* KcsA native ND SEC fractions (see also Figure 3D). Each fraction was diluted 5-10x and measured immediately three times on separate coverslips. The thick outline for each fraction represents the average data from the three separate experiments.

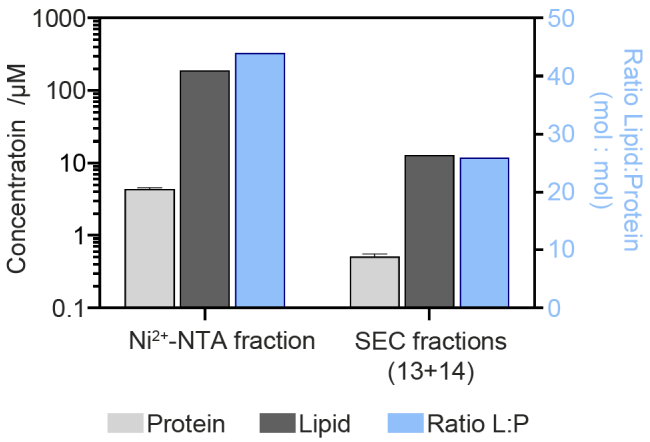


Figure S9. Lipid/protein analysis of *E. coli* KcsA native NDs (see also Figure 3D). Experimentally derived protein and lipid concentrations and their respective molar ratios for the Ni^{2+}-NTA fraction compared to combined fractions 13 and 14 from size-exclusion chromatography. Protein concentration is plotted with ± SEM.

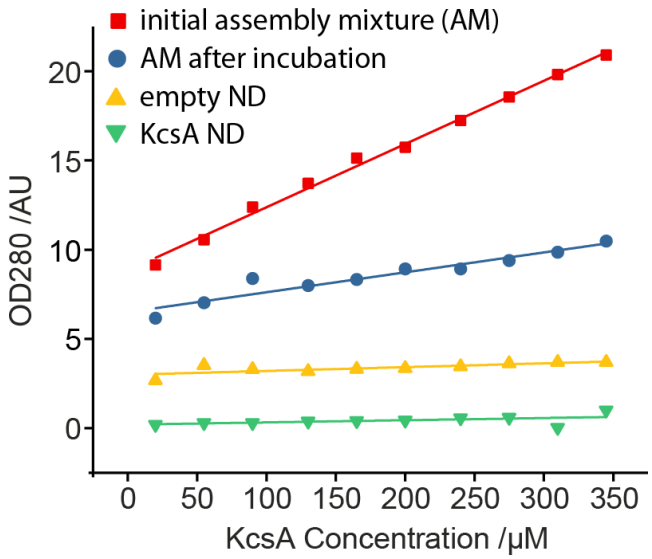


Figure S10. KcsA ND assembly screen *via* OD_{280} (see also Figure 4). A series of small-scale assemblies (50 µL each) of potassium channel KcsA into MSP nanodisc was carried out to screen for optimal membrane protein to scaffold ratio. Sample absorbance at 280 nm was monitored for the initial assembly mixture, assembly mixture after overnight incubation, washing fraction from batch Ni^{2+}-NTA chromatography and elution fraction or purified KcsA nanodiscs. Recorded absorbances are depicted as a function of the input KcsA concentration.

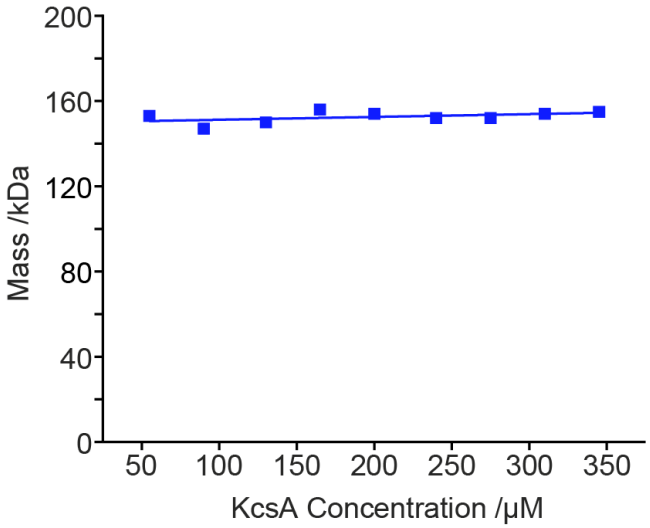


Figure S11. KcsA ND assembly mass distributions (see also Figure 4). The average mass of the final nanodisc particle distributions was measured with MP. All of the test assemblies resulted in a homogeneous sample with a consistent mass. This shows that membrane protein to scaffold ratio of up to 1:4.5 does not compromise the sample fitness and therefore is optimal for maximum ND yield per scaffold.

Supplemental Tables

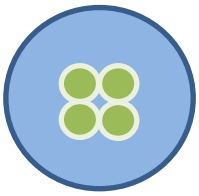
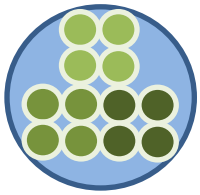
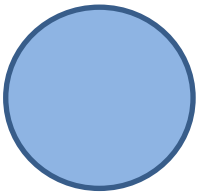
Theoretical assemblies				
~Ratio of lipid-to-protein (mol/mol)	200	42	0	300 [lipids]
Supramolecular organisation	Single tetramer	Dimer of tetramers	Trimer of tetramers	Empty SMALP
Protein (*)	80 kDa (1 mol)	160 kDa (2 mol)	240 kDa (3 mol)	0 kDa (0 mol)
Lipid (**)	140 kDa (195 mol)	60 kDa (84 mol)	0 kDa (0 mol)	220 kDa (306 mol)
Polymer (***)	30 kDa (10 mol)	30 kDa (10 mol)	30 kDa (10 mol)	30 kDa (10 mol)
Total mass (†)	250 kDa	250 kDa	270 kDa	250 kDa
*Protein: KcsA tetramer = 80 kDa. **Lipid: POPE = 0.718 kDa. ***Polymer: M_n = 3 kDa. †Total in one nanodisc.				

Table S1. Theoretical lipid/protein/SMA stoichiometry of KcsA native NDs (see also Figure 3).

	KcsA tetramer : MSP1D1 : DMPC (molar ratio)	KcsA tetramer / µM	MSP1D1 / µM	DMPC lipids / mM
Assembly A	1:20:800	20	400	16
Assembly B	1:4:100	50	200	5

Table S2. KcsA:MSP1D1:DMPC ND assembly (see also Figure 4). Two assembly ratios screened for the reconstitution of the potassium channel KcsA into the lipid nanodiscs.

Protein	Dilution / Concentration
KcsA in MSP NDs	20 nM
KcsA in SMALP	5-10 fold dilution
KcsA in APol	~ 50 nM
bo$_3$ oxidase	10 or 20 nM
bo$_3$ oxidase – time stability assay	20 nM
complex I	15 nM
OmpF in Apol	30 nM
Empty MSP NDs	~10-50 nM
Empty SMALP	20-40 nM
SMA polymer	2 or 6.7 µg/ml

Table S3. Protein/carrier concentrations in MP measurements (see also Materials and Methods). Detailed information on the concentration of samples. In cases where the concentrations were not precisely known, a dilution factor was given (*e.g.* KcsA in native nanodiscs).

Protein	Number of binned frames, n	Threshold 1
KcsA in MSP NDs	5	1.5
KcsA in native NDs	5	1.5
KcsA in APol	10	1
bo$_3$ oxidase	10	0.5
bo$_3$ oxidase – time stability assay	5	0.9
Complex I	3	1.5
OmpF in Apol	5	1
Empty MSP NDs	5	1.5
Empty SMALP	3	1.5
SMA polymer	3	1.5

Table S4. DiscoverMP analysis parameters (see also Materials and Methods). The values for threshold 2 (= 0.25) and median filter kernel (= 15) remained constant for all protein samples.

Supplemental Materials and Methods

Mass photometry. Mass photometry data was acquired in microscope flow chambers. All microscope coverslips (No. 1.5, 24x50 and 24x24 mm^2, VMR) were cleaned by sonication with 50% isopropanol (HPLC grade)/Milli-Q H_2O, followed by sonication in Milli-Q H_2O (5 minutes each). Microscope coverslips were dried by either a clean nitrogen stream or in the oven at 110 ˚C for 1 hour. A small proportion of coverslips was cleaned by applying a layer of First Contact Polymer Optics Cleaner onto the surface, letting it dry for 15 minutes, peeling off the solidified layer, followed by rinsing in ethanol (HPLC grade)/Milli-Q H_2O and drying with a clean nitrogen stream. Flow chambers were assembled from the clean coverslip immediately after the cleaning process, using double-sided-sticky tape (3M),[1] and stored prior to use for up to three weeks. MP measurements were performed at a range of concentrations ~10-50 nM, with the exact concentration specified in *Supplemental Information,* Table S3. Where the protein stock concentration was higher, diluting to nM was done immediately prior to the measurement (unless stated otherwise). For each MP measurement, a buffer solution was added to the flow chamber and the focus position identified and secured for the entire measurement using a focus feedback loop based on total internal reflection of a reference laser beam.[2] Each measurement was taken for either 60 or 90 seconds after ~15 µL of the diluted sample was introduced into the flow-chamber.

All measurements were performed on three similar mass photometers. Most of the data was acquired using a home-built mass photometer as described previously.[1] Briefly, the output of a 520 nm laser diode (Lasertack) was collimated and sent through a pair of acousto-optic deflectors (AODs, AA Optoelectronic DTSXY-400). A 4f telecentric lens system images the deflection by the AODs into the back focal plane of the microscope objective (Olympus UApo N, 100x, 1.49 NA). The objective collects light reflected at the interface between a glass coverslip and some of the light scattered by the sample, with efficient separation of illumination and detection achieved through the combination of a polarizing beamsplitter and quarter-wave plate (Thorlabs). The same telecentric lens system images the back focal plane of the objective onto a partial reflector made from a thin layer of silver of 2.5 mm diameter deposited onto a window, which selectively attenuates the reflected light compared to light from point scatterers at the surface by a factor of about 1000. A final lens images the sample onto a CMOS camera (Ximea, MC023MG-SY) with 277.8x magnification, resulting in a final pixel size of 21.1 nm/pixel. Before saving each movie file, areas of 4x4 pixels were binned for an effective pixel size of 84.4 nm/pixel, and frames were averaged 5-fold in time. The entire setup was constructed on a thick (50 mm) metal aluminum plate, and fully enclosed to minimize the influence of air currents.

The bo_3 oxidase and respiratory complex I data was acquired with a similar, but commercial mass photometer, One^{MP} (Refeyn LTD, Oxford, UK), with effective pixel size and frame rate as described above. Empty SMALP and OmpF-APol data were acquired on a home-built mass photometer which uses 445 nm Laser diode. Here, the pixel size is 23.4 nm and frame rate is 1 kHz. Prior to saving the images, a 5-fold time average and 3x3 pixel binning were applied, resulting in effective frame rate of 200 Hz and effective pixel size of 70.2 nm. Data acquisition was performed using either custom software written in Labview (for the home-built mass photometers) or AcquireMP (Refeyn LTD, v1.2.1) for the commercial instrument.

Image processing. *Mass photometry landing assays:* The videos of proteins binding to the glass surface were analyzed with DiscoverMP (Refeyn Ltd). The software detects binding events and determines the respective interferometric scattering contrasts. The user can choose how many frames are averaged for continuous background removal (n_{avg}) and can set the thresholds T_1 and T_2 for the two image filters, which are used to detect the binding events.

Filter 1 is based on T-tests of the pixel intensity fluctuations. As a particle (*i.e.* IMP) binds to the glass, the pixel intensity changes suddenly. This change is associated with an increase of the filter 1 score calculated as $-\ln(p)$, where p is the p-value of the T-test comparing pixel values at n_{avg} frames before and n_{avg} frames after the event. The smallest intensity jump amplitude that exceeds random noise fluctuations and is associated with a binding event is controlled by the value of threshold T_1.

The signatures of the binding events in interferometric images are radially symmetric. Filter 2 measures the radial symmetry of all pixel neighborhoods of the interferometric images.[3] The lowest symmetry score expected at the center of a peak is defined as threshold T_2.

Pixel clusters that exceed both thresholds T_1 and T_2 are used for peak fitting. The amplitude of the peak fit provides an estimate for the interferometric peak contrast. The peak signature (point spread function) is modelled as a superposition of two Sombrero functions multiplied by two Gaussians.[1]

Calibration Procedure. Contrast-to-mass (C2M) calibration protocol included measurement of several different protein oligomer solutions, with known masses. Each MP calibration was analyzed using DiscoverMP, where mean contrast values of all peaks were determined in the software using Gaussian fitting. The mean contrast values were then plotted against the known mass of the proteins (*Supplemental Information,* Fig. S1) and fitted to a line, $y = bx$, with y – contrast, x – mass and b – C2M calibration factor. For the data shown in Figure *1f, 4b-c*, and *Supplemental Information* Figure S11, calibration was performed using a mixture of protein standards of known molecular weight, namely bovine serum albumin, alcohol dehydrogenase, apo-ferritin and thyroglobulin.

Integral membrane protein preparation: MSP Nanodiscs. *MSPΔH5 nanodiscs.* Empty nanodiscs were prepared as described previously.[4] Briefly, MSPΔH5 was overexpressed in BL21 Star (DE3) cells (Thermo Fisher) at 28 °C for 5 h and was purified after harvest by His-tag affinity chromatography involving a number of washing steps. Then, 200 µM MSPΔH5 were assembled with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) 14:0 lipids (Avanti Polar Lipids) in a molar ratio of 1:50 by shaking them in a glass vial for 8 h at 27 °C. Subsequently, 1 g/mL Bio-Beads SM-2 (Biorad) were added for 4 h and the MSPΔH5 nanodiscs were purified by size-exclusion chromatography using a Superdex 200 Increase 10/300GL column (GE Healthcare).
Potassium channel KcsA: KcsA from *Streptomyces lividans* was produced using a pET-28a vector containing the coding sequence of the wild-type protein fused to a thrombin-cleavable N-terminal His-tag (monomer 19.9 kDa).[5] KcsA was expressed in the *E. coli* strain BL21 Star (DE3) (Invitrogen) in Luria-Bertani media. Cells were grown at 37 °C until OD_{600}=0.8 and protein production was initiated by addition of 0.5 mM IPTG (Invitrogen). The culture was incubated for additional 5 hours at 37 °C. After protein expression, the cells were lysed by two microfluidizer (Microfluidics) cycles. KcsA was extracted from the membrane with 20 mM dodecylphosphocholine (DPC) detergent by gentle stirring at 4 °C overnight. The cleared extraction mixture was loaded on a Ni^{2+} Sepharose 6 Fast Flow resin (GE Healthcare). After washing the resin with 3.3 mM DPC, KcsA was eluted with 300 mM imidazole and 3.3 mM DPC. Fractions containing the protein were pooled together and the buffer was exchanged to 20 mM Tris-HCl pH 7.4 and 3 mM DPC using a PD-10 (GE Healthcare) desalting column.
Expression of membrane scaffold protein (MSP1D1): MSP1D1 fused to a TEV (Tobacco Etch Virus) protease cleavable N-terminal His-tag was encoded in a pET-28a vector. The MSP1D1 variant of MSP1 deletes the first 11 amino acids of the original MSP1 sequence.[6] Expression and purification of the protein was carried out as described previously.[7] The His-tag on MSP1D1 was cleaved by addition of TEV protease for 16 hours at room temperature. MSP1D1 without His-tag has a molecular weight of 22.0 kDa.

Reconstitution of KcsA into DMPC Nanodiscs and empty nanodisc formation: **1**) Following the protocol of Shenkarev *et al*. [8] KcsA was incorporated into saturated DMPC 14:0 MSP1D1 nanodiscs. DMPC lipids were first solubilized in sodium cholate (cholate/DMPC 2:1 molar ratio). The purified KcsA sample was mixed with scaffolding protein MSP1D1, DMPC, and sodium cholate at a molar ratio of 1:20:800:1600 in a buffer containing 20 mM Tris-HCl pH 7.4 and 150 mM NaCl. Our choice of the KcsA tetramer:MSP:DMPC ratio was based on published reports from membrane protein groups that have extensively studied KcsA in lipid nanodiscs.[8,9] The final concentration of KcsA was 15 µM. The mixture was incubated overnight at 27 °C while shaking at 150 rpm. Incorporation of KcsA into nanodiscs was initiated by addition of 80% w/v Bio-Beads for 2 hours at 27 °C while shaking at 150 rpm. Empty nanodiscs were separated from nanodiscs containing N-terminal His-tagged KcsA by Ni^{2+} affinity chromatography. The fraction of nanodiscs containing KcsA was eluted with 300 mM imidazole and used for mass photometry measurements. **2**) DMPC lipids were purchased from Avanti and solubilized in 200 mM sodium cholate at a concentration of 100 mM. Lipid nanodisc assembly mixtures containing membrane protein KcsA (tetramer),

scaffolding protein MSPdH5 and DMPC lipids were premixed at two different ratios (*Supplemental Information,* Table S2) to a volume of 500 µL and incubated at room temperature overnight. Lipid nanodisc reconstitution was triggered by the addition of 50% (m/v) Bio-Beads to the assembly mixtures over 4 hours. Assembled nanodiscs were purified with size exclusion chromatography using Superdex 200 chromatographic column (GE Healthcare).

Small scale reconstitution of KcsA into lipid nanodiscs: Lipid nanodisc assembly mixtures containing membrane protein KcsA (tetramer), scaffolding protein MSP1D1 and DMPC lipids were premixed at varying ratios to a volume of 50 µL. Concentration of DMPC lipids was kept at 16 mM, concentration of MSP was kept at 400 µM and concentration of KcsA (monomer) was varied in range from 20 µM up to 350 µM. After the overnight incubation at room temperature lipid nanodisc reconstitution was triggered by the addition of 50% (m/v) Bio-Beads to the assembly mixtures over 4 hours. Assembled nanodiscs were purified with batch Ni-NTA chromatography.

Integral membrane protein preparation: Native Nanodiscs / SMALPs. Commercially available Styrene-Maleic Anhydride (SMAnh) copolymer, Xiran30010 (number-average molecular weight (M_n) ~2.5 kDa, polydispersity index (PDI) ~2.6), was a kind gift from Polyscope Polysciences (Geleen, NL). Conversion of the SMAnh polymers into the acid form (SMA) was achieved by hydrolysis under base-catalyzed conditions as detailed previously.[10] SMA stock solutions were prepared at final concentrations of 5% (w/v).

Preparation of E. coli membranes overexpressing KcsA: Total membrane fractions of *E. coli* cells (strain BL21(λDE3)) producing KcsA were obtained as described previously.[11] Briefly, cells were transformed with an N-terminal His-tagged pT7-KcsA vector, containing the *KcsA* gene. Membrane preparations were obtained by differential centrifugation after cell wall lysis and mechanical disruption through a French press. Membrane pellets were resuspended in buffer (Tris-HCl 5 mM, NaCl 300 mM, KCl 15 mM, pH 8) to an OD_{600} of ~4. After lipid extraction according to the method of Bligh and Dyer,[12] the total phosphate content was determined to be 10 mM using the method of Rouser *et al*.[13] Membrane suspensions were stored at −20 °C until further use.

SMA-mediated solubilization of KcsA from E. coli membranes: Membrane stocks (4.8 mL) were thawed on ice and diluted with solubilization buffer (Tris-HCl 50 mM, NaCl 300 mM, KCl 15 mM, imidazole 10mM, pH 8) to total volume of 33 mL, resulting in phosphate concentration of ~1.5 mM and protein concentration of ~1 mg/mL. Polymer was added to the suspensions at a final concentration 1% (w/v). The mixture was incubated on a rotary disc at 4°C overnight, during which the suspension cleared up significantly. To remove any unsolubilized material the mixture was centrifuged at ~40,000 g for 1 h at 4 °C to pellet the non-soluble fraction. The solubilized fraction (supernatant) was carefully removed and used for His-tag purification of the nanodiscs.

Purification of KcsA native nanodiscs: The solubilized fraction containing the nanodiscs was added to 6 mL of HisPure Ni-NTA agarose beads (Thermo Scientific) and incubated on a rotary disc at 4 °C overnight. The beads were then loaded on a gravity-flow column for the affinity purification. First, the flow-through was collected, followed by washing with increasing amounts of imidazole (10 mM, then 50 mM) and finally elution with a high concentration of imidazole (300 mM). Elution fractions containing pure KcsA were combined and the pooled fractions were concentrated using centrifuge spin filters (Amicon, 15 mL, 10 kDa MWCO). The imidazole was removed by washing the concentrate three times with buffer (Tris-HCl 50 mM, NaCl 300 mM, KCl 15 mM, pH 8).

The isolated KcsA native nanodiscs were further purified and analyzed by size exclusion chromatography (SEC) on an ÄKTA pure system (GE Healthcare), using a Superdex 200 10/300 GL size exclusion column (GE Healthcare). Protein was eluted using buffer (Tris-HCl 50 mM, NaCl 150 mM, KCl 15 mM, NaN_3 1 mM, pH 8). Fractions were collected on an autosampler and UV detection was performed at λ= 280 nm. Collected fractions were frozen and stored at -80 °C until further use.

Determination of sample purity using SDS-PAGE: Samples were incubated with Laemilli buffer without any reducing agent, loaded onto 13% SDS-PAGE gel, and run at 175 V for 1 hour. The gels were stained with coomasie blue and scanned for further analysis.

Determination of protein-to-lipid ratios in KcsA native nanodiscs: Protein concentration was determined using Pierce micro BCA protein assay kit (Thermo Scientific). The standard protocol was modified by using 2.5-fold the recommended amount of reagent C, as the SMA copolymers chelate copper and thus an excess is required. Furthermore, 1% SDS was added in order to ensure that the membrane proteins remained in solution. Weight concentration was converted to mole concentration of protein on the basis of a KcsA tetramer of ~80 kDa.

Lipids were extracted according to a modified version of the method of Bligh and Dyer,[12] namely without the use of hydrochloric acid but rather under alkaline conditions to prevent possible aggregation and precipitation of SMA bound to nanodiscs or lipids. The lipid extraction was performed to separate lipids from proteins and copolymers present, in order to prevent potential interference with analysis. The lipid concentration was determined based on total phosphate content according to the method of Rouser *et al*.[13] For the SEC fractions, two samples had to be combined (fraction 13 and 14) in order to give sufficient material to be above the lower limit of detection.

The contribution of polymers in terms of mass to a single nanodisc is difficult to determine. If we assume two belts of polymers per disc, the total amount of polymer per nanodisc is approximately 30 kDa, or about ten polymer molecules with M_n = 3 kDa.[14]

Integral membrane protein preparation: Detergent Micelles. *bo_3 oxidase: E. coli bo_3* oxidase was expressed in *E. coli* strain BL21(DE3) star/pET*cyo $_{his}$cyoC*. Plasmid pET*cyo $_{his}$cyoC* was a kind gift from Prof. Christoph von Ballmoos (Bern, Switzerland). Cells were grown at 37 °C in LB medium supplemented with 0.5% glycerol, 2 mM $MgSO_4$, 0.03 mM $FeSO_4$ and 0.01 mM $CuSO_4$. At an optical density of 1.5, gene expression was induced by an addition of 0.5 mM IPTG and cells were grown for further 2 hours. Cytoplasmic membranes were suspended in buffer A (50 mM MOPS and 20 mM NaCl, pH 7.5) and membrane proteins were solubilized by adding LMNG dropwise to a final concentration of 2% (w/v). Solubilized proteins were loaded onto a Probond Ni^{2+}-IDA column (25 mL) equilibrated in buffer A containing 20 mM imidazole and 0.005% LMNG. After washing with 92 mM imidazole, bound proteins were eluted in a single step to 284 mM imidazole. The eluate was concentrated by ultrafiltration (Amicon Ultra-15, 100 kDa molecular weight cut-off) and subjected to size exclusion chromatography on Superose 6 (300 mL) in buffer A containing 0.005% LMNG. The main peak eluting after 177 mL was concentrated to 100 µM, aliquots were flash frozen in liquid nitrogen and stored at -80 °C until further use.

Respiratory complex I: Respiratory complex I from *E. coli* was prepared as described previously[15] with slight modifications. After affinity chromatography on a Probond Ni^{2+}-IDA column (35 mL), the complex was subjected to a Superose 6 (24 mL) size exclusion column. Peak fractions were concentrated to 30 µM (Amicon Ultra-15, 100 kDa MWCO) and stored in 50 mM MES/NaOH, 50 mM NaCl, 5 mM $MgCl_2$, pH 6.0 with 0.005% LMNG at -80 °C. MP measurements of complex I were performed at 15 nM. Dilution to measured concentrations was performed with buffer A either without any detergents or with 0.001% LMNG.

Integral membrane protein preparation: Amphipols. *E. coli outer membrane protein F (OmpF) in APol:* OmpF was expressed and purified as described previously[16] in 20 mM phosphate buffer (pH 6.5, 1% OG) at a concentration of 11 µM. Bio-Beads (Bio-Rad) were used for exchange from detergent to amphipols and detergent adsorption according to Zoonens, *et al*. [17] Approximately 1 g of Bio-Beads was washed for 25 min with methanol (HPLC grade), 20 min with Milli-Q H_2O, and 2x 20 min with OmpF buffer (20 mM phosphate buffer, pH 6.5). Each wash was done at room temperature with gentle agitation. The beads were rinsed twice with Milli-Q in between steps. Fresh A8-35 amphipol (Anatrace) stock solution (10%) was prepared and diluted 10x in OmpF buffer. A range of amphipol to integral membrane protein ratios was calculated, from 0:1 (control) to 5:1, in order to determine the lowest ratio for effective protein extraction. Amphipol solution, integral membrane protein and buffer were added to a final exchange volume of 200 µL. An excess of prepared Bio-Beads was added to each combination, which was then incubated at 4 °C for a total of 16 hours, with a change of BioBeads after 2 hours.

Once detergent removal was complete, the protein – amphipol solution was carefully removed and OmpF – amphipol was buffer-exchanged into OmpF Tris buffer (20 mM Tris, 100 mM NaCl, pH 8.0) using 0.5 mL molecular concentrators with molecular weight cut-off of 30 kDa (Amicon). The sample was spun at 10,000 x g at 4 °C for 2 minutes, after which the flow through was discarded and 300 µL of fresh buffer was added. This was repeated 5 times, checking for protein precipitation after each spin cycle. After OmpF buffer exchange, light absorbance at 280nm was measured for each sample using Nanodrop device in order to determine protein concentration. The samples were subsequently ultra-centrifuged at 150,000 x g for 20 minutes. After centrifugation, absorbance at 280 nm of the supernatant of each sample was measured again using Nanodrop for comparison of the amount of protein retained in the supernatant (*Supplemental Information,* Fig. S6). This was used to help determine the minimal amount of amphipol needed to solubilize the membrane protein. Once the insertion was complete, the samples were stored at 4 °C for up to a few weeks.

Potassium channel – KcsA in APol: KcsA solubilized in DPC micelles (preparation described above) was exchanged to amphipols using the same protocol described above for OmpF, with a few minor differences; KcsA buffer (20 mM Bis-Tris, 150 mM NaCl, pH 7) was used throughout the whole protocol and a second exchange of Bio-Beads was added after 4 hours of incubation in the amphipol preparation. *Supplemental Information,* Fig. S7 shows the absorbance values for the different ratio for KcsA-amphipol.

SEC-MALS. SEC-MALS analysis was performed on an Agilent 1200 HPLC system with an autosampler, diode array detector and differential refractive index detector connected in-line with a 3-angle light scattering detector (Treos, Wyatt Technology). Light scattering data were collected from an injection of 30 µL bo_3 oxidase at 87 µM onto a SHODEX KW-803 300 mm x 8 mm column equilibrated in 50 mM MOPS pH 7.0, 300 mM NaCl and 0.003% LMNG at 0.5 mL/min and analyzed as a protein conjugate using the Astra V software (v 5.3.4.20). The extinction coefficient of bo_3 was predicted from its sequence to be 299 $M^{-1}cm^{-1}$ at 280 nm and the detergent assumed to have no absorbance at 280 nm (an extinction coefficient = 0). We measured the refractive index increment, d*n*/d*c,* for LMNG by injecting a series of dilutions of LMNG in water onto our differential refractive index detector and obtained a value of 0.151 mL/g. In addition, the average d*n*/d*c* for proteins of 0.185 mL/g was used for the analysis.

Negative staining electron microscopy. For negative staining, grids (300 mesh Cu carbon film) were glow discharged for 20 seconds at 15 mA (Leica EM ACE 200). 10 µL of sample was applied to the grid for 2 minutes, blotted, stained with 2% uranyl acetate for 20 seconds, blotted and allowed to air dry. Images were acquired on a 120kV Tecnai 12 (Thermofisher) TEM equipped with an OneView digital camera (Gatan).

NMR Spectroscopy. 2D [^{15}N, ^{1}H]-HMQC experiments with $H_2O/^{15}NH_4^+$ defocusing and selective excitation burp pulses were all collected at 305 K on a Bruker 700 MHz Avance III spectrometer equipped with a triple resonance cryoprobe.

Molecular dynamics simulations. We carried out Molecular Dynamics (MD) simulations of *E. coli* bo_3 ubiquin oxidase (PDB: 1FFT)[18] in a lipid bilayer using GROMACS 2016.4 CUDA.[19] The Amber14sb all-atom force field [20] was used to describe the protein and the lipid all-atom force field to describe the palmitoyloleoyl-phosphocholine (POPC) lipids. All simulations were run in the NPT ensemble. We first assembled a POPC membrane using packmol [21] and subsequently equilibrated it in TIP3P water for 20 ns. bo_3 was then inserted into the equilibrated bilayer according to the arrangement predicted by the OPM server.[22] The resulting protein-lipid system was then filled with TIP3P water, charge neutralized with chloride ions and energy minimized *via* steepest descent, with the maximum force tolerance set to 200 kJ mol^{-1} nm^{-1}. Finally, the system was simulated for 60 ns, where the first 50 ns were excluded as part of the equilibration, and the last 10 ns used for production.

In all simulations long-range interactions were calculated using the particle mesh Ewald (PME) method and a cut-off of 12 Å was used for van der Waals and Columbic interactions. The LINCS constraint was used to restrain bonds involving hydrogen atoms. Simulations utilized a 2 fs integration time step, updating the neighbor lists every 10 steps. An atmospheric pressure of 1 bar was maintained *via* anisotropic pressure coupling using a compressibility $k_x = k_y = k_z = 4.5 \times 10^{-5}$

bar^{-1}, with off-diagonal terms k_{xy} = k_{xz} = k_{yz} = 0 bar^{-1} and time constant τ_p = 1.0 ps. The protein, lipids and solvent (water and ions) were individually coupled to a heat bath at 310.15 K with time constant τ_t = 0.1 ps.

Protein-protein docking. The prediction of a possible *bo*$_3$ dimeric arrangement was carried out using the protein-protein docking software JabberDock 1.0.[23] JabberDock generates protein surface representations (STID maps) simultaneously describing atomic charge, distribution and dynamics, and then produces docking candidates of maximal surface complementarity by exploiting the POW^{er} optimization engine.[24] To this end, the last 10 ns of MD simulation were extracted to generate a STID map of *bo*$_3$ representative of its local dynamics in a lipid bilayer.

The docking starting point featured two *bo*$_3$ monomers centered at the origin. One monomer was kept fixed, while the other was allowed to translate in the *xy* plane and to rotate along its *z* axis between 0 and 2π radians. This axis of rotation was also permitted to precess into the *xy* plane between an angle of -0.05 and 0.05 radians. POW^{er} navigated this conformational space, looking for a region associated with two *bo*$_3$ monomers of maximal surface complementarity. Optimization was performed over 200 iterations using 60 randomly initialized particles. The "kick and reseed" procedure involves randomly reinitializing particles that have converged to a local minimum before placing a repulsive potential on the converged site to prevent oversampling. The optimization process was repeated three times, with memory of the previous landscape including the repulsion potentials kept for future iterations. The docking procedure thus evaluated 36000 docked poses, which were then clustered by *K*-means into 300 representative poses (cluster centroids). The dimer shown in Figure 2*C* is a representative model of the ensemble, relaxed in a lipid bilayer following the same MD simulation protocol adopted for monomeric *bo*$_3$.

The SASA of the equilibrated *bo*$_3$ monomer and all 300 predicted dimers were calculated using the Shrake-Rupley algorithm as implemented in VMD.[25] The mean and standard deviation of all dimeric models SASA was calculated as consensus value.

Native mass spectrometry. Mass spectra were recorded on prototype Orbitrap Q Exactive UHMR mass spectrometer (Thermo Fisher Scientific), equipped with a Nano Flex nanospray source and offline nanospray source head. OmpF prepared in 100 mM ammonium acetate at 2x CMC octyl β-D-glucopyranoside detergent (Anatrace) was transferred into the mass spectrometer using gold-coated, borosilicate glass capillaries prepared in house. The mass spectrometer was operated in positive ion polarity and in AGC prescan mode with a maximum inject time of 100 ms and target of 1e6. Capillary voltage was 1.4 kV, transfer capillary temperature 80 °C, inject flatapole 10 V, inter-flatapole lens 6 V, bent flatapole 4 V. Ion optics transmission was set to high *m/z*, and detector mode to low *m/z*. Pressure setting was 6-8 V. No in-source trapping or in-source activation was applied. Direct HCD voltage applied to remove the detergent micelle was 250 V. Microscans was set to 10, no averaging was applied, and transient time was 64ms, corresponding to a resolution of 12,500 at *m/z* 400. 25 scans were recorded and averaged using the Xcalibur software package v2.2-4.1 (Thermo Fisher Scientific).

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