Supporting Information

Detergent-free functionalisation of hybrid vesicles with membrane proteins using SMALPs

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Methods:

Cytochrome bo₃ extraction and purification. Membrane protein cytochrome bo₃ (cyt bo₃) was expressed in Escherichia coli GO105/pJRhisA as previously described.[1] A colony of fresh plated E. coli GO105/pJRhisA was inoculated in LB (Lysogeny Broth) medium supplemented with 100 µg/mL carbenicillin and cultured at 37 °C at an agitation rate of 200 rpm for ∼16 h. This starter culture was then inoculated in LB medium (2% v/v) supplemented with 100 µg/mL carbenicillin and 0.1 mM CuSO₄. E. coli was grown to mid-logarithmic phase at 37 °C at an agitation rate of 200 rpm for ∼6 h (approximately until optical density of a sample measured at a wavelength of 600 nm, OD₆₀₀nm, reached 1.5). E. coli cells were harvested by centrifugation at 7,000 g for 20 min at 4 °C and resuspended in W1 buffer (20 mM MOPS, 5 mM Mg₂SO₄, 30 mM Na₂SO₄) at a concentration of 0.25 g of wet cells per mL. E. coli cells suspension was passed twice through a cell disrupter (Constant Systems) at 30 kPsi. Cell debris was removed by centrifugation at 17,500 g for 10 min at 4 °C. Cell membranes in the supernatant were spun down by ultracentrifugation at 200,000 g for 90 min at 4 °C.

For purification of cyt bo₃ in SMA nanodiscs, the membrane pellet was resuspended in 50 mM Tris-HCl (pH 8), 500 mM NaCl and 10% glycerol at a ‘wet weight’ concentration of 40 mg/mL (protein content ~ 4 mg/mL). Styrene maleic acid (SMA) copolymer (Cray Valley, SMA
2000 – MW 7.5 kDa) was added at a concentration of 2% w/v; the suspension was incubated for 2 h on a rotary shaker at RT and then centrifuged at 100,000 g for 45 min at 4 °C to remove any non-solubilised proteins. The supernatant was used directly for complex membrane proteins mixture (SMA-MPs) reconstitution or further purified for isolation of SMA-cyt bo₃.

For purification of cyt bo₃ via DDM, the membrane pellet was resuspended in 20 mM Tris-Cl (pH 8), 5 mM MgSO₄, 300 mM NaCl, 20 mM imidazole, 10% glycerol and 1% DDM at a protein concentration of 4 mg/mL. The suspension was incubated for 2 h on a rotary shaker at 4 °C and then centrifuged at 200,000 g for 45 min at 4 °C. Both SMA-solubilised proteins and DDM-solubilised proteins were incubated with pre-equilibrated Ni²⁺-NTA resin for ~16 h and 1 h, respectively, on a rotary shaker at 4 °C. The resin suspensions were loaded onto a gravity column, and either the SMA-cyt bo₃ nanodiscs or the DDM-cyt bo₃ were eluted with 200 mM imidazole (supplemented with 500 mM or 100 mM NaCl, respectively), which was removed immediately after the elution by 3 cycles of dilution in imidazole-free storage buffer and concentration using 100 kDa MW cut-off concentrator (VivaSpin), and stored at -20 °C until use.

The protein concentration of the cytoplasmic membrane preparation was determined using a bicinchoninic acid assay (BCA) assay. Protein concentration of purified cyt bo₃ was determined via Soret Band at 409 nm (Nanodrop DeNovix DS-11) using extinction coefficient value $\varepsilon_{408 \text{nm}} = 188 \text{mM}^{-1} \text{cm}^{-1}$. [2]

**Vesicles preparation.** Hybrid vesicles were prepared as previously described.[3] Dry E. coli polar lipids extract (Avanti Polar Lipids) and block-copolymer poly(butadiene-ethylene oxide) (PDB₂₂-b-PEO₁₄; Polymer Source, P9089-BdEO) were solubilised in chloroform and mixed in glass vials at 1:1 molar ratio. The solution was then dried in a vacummed desiccator for 2 h to give a thin lipid-copolymer film at the bottom of the glass vial. The lipid-copolymer film was resuspended in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM NaCl buffer (pH7.4) via repeated incubation at 50 °C for 5 min and vortex for 1 min followed by five freeze-thaw-vortex cycles and subsequently extruded 11 times through a 100 nm pore size polycarbonate membrane filter using an Avanti Mini-Extruder to form nanovesicles.
Liposomes were prepared by a similar procedure.\textsuperscript{[4]} 5 mg E. coli lipids polar extract (Avanti Polar Lipids) was resuspended in 40 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 60 mM K\textsubscript{2}SO\textsubscript{4} (pH 7.0) to a concentration of 20 mg/mL. The suspension was sonicated for 15 s on /45 s off for 6 cycles, and flash frozen and thawed with liquid nitrogen three times. The liposomes were then extruded as described for the hybrid vesicles.

**SMA-copolymer-vesicles stability studies.** SMA-copolymer was added to achieve a final concentration of 0.5 mM, 0.9 mM, 1.5 mM to separate aliquots of HVs or liposomes at 0.9 mM of total PDB\textsubscript{22-b-PEO\textsubscript{14}} polymer and lipids mixture. Stability of the vesicles was assessed following 4 h incubation at RT with gentle shaking. The samples were analysed using DLS (see methods below).

**Vesicles destabilisation studies.** Vesicles (0.94 µmol of total PDB\textsubscript{22-b-PEO\textsubscript{14}} polymer and lipids mixture) were suspended in a total volume of 1.0 mL. The optical density was measured at 540 nm (OD\textsubscript{540} nm) using a Shimadzu UV-2450 UV-Vis spectrophotometer. Sequential small volumes containing 0.15 µmol of SMA or Triton X-100 were added to the cuvettes and mixed thoroughly, and the OD\textsubscript{540} was recorded.

**Incorporation of SMA-cyt bo\textsubscript{3} into vesicles.** SMA-cyt bo\textsubscript{3} and vesicles, either hybrid vesicles or liposomes, were incubated on ice for 30 min at a protein to lipids content ratio (w/w) of ~1:100 (2:8 protein mass to polymer and lipids mass ratio for SMA-MPs directly incubated with HVs). MgCl\textsubscript{2} was added to a concentration of 10 mM and incubated with gentle shaking overnight at 4 °C. The samples were then spun at 17,000 g for 15 min, and the supernatant taken forward for other experiments. Reconstituted protein concentration was analysed by solubilising the vesicles in 0.3 % Triton X-100, incubated for 30 min at RT with shaking, and measuring the absorbance band at 409 nm.

**Protein reconstitution from DDM.** DDM-cyt bo\textsubscript{3} was reconstituted as described by Khan et al.\textsuperscript{[3]} The vesicles were titrated with 10% triton X-100 to 5 x 1 µL aliquots beyond \( R_s \), as indicated by absorbance at 540 nm. DDM-cyt bo\textsubscript{3} was added in a protein to lipids ratio of ~1:100 (w/w), followed by incubation for 15 min at 4 °C. Four rocker incubations at 4 °C with Biobeads were used to remove the detergent (30 mg Biobeads for 1 h, 30 mg Biobeads 2 h, 30 mg Biobeads overnight, 30 mg Biobeads 2 h). The vesicles were then stored at 4 °C. The vesicles were spun at 17,000 g for 20 min. Reconstituted protein concentration was analysed
by solubilising the vesicles in 0.3 % Triton X-100, incubated for 30 min at RT with shaking, and measuring the absorbance band at 409 nm.

**Dynamic Light Scattering.** Vesicle size distributions were characterised using a Zetasizer Nano ZSP (Malvern Instruments). Samples were equilibrated at 25 °C for 90 s, and a refractive index of 1.45 was used for the hybrid vesicles and 1.37 for the liposomes.[3]

**Stepped Mg$^{2+}$ addition to vesicles.** Liposomes were diluted to 1.5 mg/mL of lipids, and hybrid vesicles to 0.75 mg/mL lipids and the molar equivalent of polymer. 100 µL of vesicles and liposomes were incubated with 8 µl of 1.4 mg/mL cyt bo$_3$ on ice for 30 min. MgCl$_2$ was added to a concentration of 1 mM and the vesicles incubated overnight at 4 °C with gentle shaking. The following day, at 2 h intervals, MgCl$_2$ was added to a final concentration of 2 mM, 3 mM, 4 mM, 5 mM and 10 mM, with incubation at 4 °C and gentle shaking between. At each step samples were collected for analysis via SDS-PAGE.

**Clark electrode oxygen consumption measurement.** A 0.5 mL buffer of 50 mM KH$_2$PO$_4$ (pH 7) was equilibrated on a Clark-type electrode (Hansatech) at room temperature (-0.6 V). The oxygen concentration in the solution under ambient conditions was taken to be 250 µM. DTT and UQ$_1$ were added to concentrations of 2 mM and 200 µM respectively. A known concentration of soluble or reconstituted cyt bo$_3$ was added and the enzyme turnover determined from the initial oxygen consumption rate.[5,6] When the protein was solubilised in DDM, the buffer also contained 0.05% DDM. When Mg$^{2+}$ was added, the initial buffer also contained the relevant concentration of MgCl$_2$. All measurements were done while stirring.

**Detection of cyt bo$_3$ proton pumping.** Vesicles were prepared as described above with the difference that the lipid-copolymer films were resuspended in 0.5 mL of 20 mM MOPS, 30 mM K$_2$SO$_4$ buffer containing 5 mM HPTS. Unencapsulated HPTS was removed by size-exclusion chromatography using a Nap-5 G25 column (GE Healthcare) according to the manufacturer’s instructions. The vesicles with encapsulated HPTS were diluted to about ~40 µg/mL lipid in a cuvette and the ratiometric fluorescence was monitored in real-time by recording the emission at 510 nm as a ratio of excitations at 405 and 455 nm using a Horiba Quantamaster 8075 spectrofluorometer. Proton pumping was activated by addition of DTT and UQ$_1$ to a final concentration of 2 mM and 200 µM respectively. A calibration curve
(ratiometric fluorescence vs known pH) of HPTS in 20 mM MOPS, 30 mM K$_2$SO$_4$ buffer was used to convert the ratiometric fluorescence data to lumen pH values (Figure S5).

**SDS-PAGE gel analysis.** For SDS-PAGE gel analysis, trichloroacetic acid in dH$_2$O (100% w/v) and protein samples were mixed in a 1:4 v/v ratio and incubated for 10 min at 4 °C. The proteins were then precipitated *via* centrifugation at 17,000 g for 5 min. The pellets were washed and centrifuged twice with 200 µL cold acetone. The pellets were then solubilised in 14 µL of dH$_2$O, and 4 µL of 4X SDS-PAGE sample loading buffer was added. The solutions were incubated for 1 hour at 37 °C and then loaded onto a 15% polyacrylamide gel.

**Figure S1.** SDS-PAGE (15%) analysis of cyt $b_{0.3}$ using two different solubilisation approaches. Purified cyt $b_{0.3}$ solubilised by SMA (A) or solubilised by DDM (B). Both purification methods were followed by purification *via* Ni$^{2+}$-NTA-agarose column. Protein bands were visualised by Coomassie Blue staining. SU-I = Subunit 1; SU-II = Subunit 2. Subunit 3 and 4 from cytochrome $b_{0.3}$ are too small and not visible on these SDS-PAGE.
Figure S2. Destabilisation profiles of HVs (A and B) and liposomes (C and D) by SMA (A and C) and Triton X-100 (B and D). Absorbance (optical density (OD)) of vesicles was measured at 540 nm in a 1.0 cm path length quartz cuvette.
Figure S3. Dynamic light scattering (DLS) volume profiles of HVs titrated with increasing SMA copolymer concentration.

Figure S4. Oxygen consumption traces for cyt bo3 solubilised by DDM (A) or by SMA (B). The traces show the activity before and after MgCl2 treatment. The activity retention of cyt bo3 (C) was determined via comparison of the oxygen consumption rate (determined via regression of the first 30 seconds from the slope and normalised by the protein concentration) of the soluble fractions before and after overnight incubation with 10 mM MgCl2 and centrifugation.
Figure S5. Standard curve for HPTS in 20 mM MOPS, 30 mM K₂SO₄ buffer. The linear fitting of the steep region of the graph (R² = 0.9904) used for the calibration is shown in the figure.

Figure S6. SDS PAGE (15%) analysis of membrane protein samples contained either in SMA-MPs or SM-MPs HVs before and after treatment with MgCl₂ and separation of the insoluble part via precipitation. To allow bands visualisation, the concentration of *E. coli* membrane-proteins fraction (MPs) was halved in comparison to the other loaded samples.
Figure S7. Oxygen consumption traces for cyt $b_{o_{2}}$ activity in (A) SMA-MPs and (B) SMA-MPs HVs before and after treatment with MgCl$_2$ to remove soluble SMA and SMA-MPs. Traces are representatives of three independent experiments.

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