Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Detergent-free extraction, reconstitution and characterization of membrane-anchored cytochrome-b5 in native lipids

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(A)

GHHHHHHAAQSDKDVKYYTLEEIKKHNHSKSTWLILHHKVYDLTKFL
EEHPGGEEVLREQAGGDATENFEDVGHSTDARELSKTFIIGELHPDD
RSKLSKPMETLITTVDSNSSWWTNWVIPAISALIVALMYRLYMADD

Protein net charge at pH 7.4 is -8.8
Molecular mass ~16 kDa

(B)

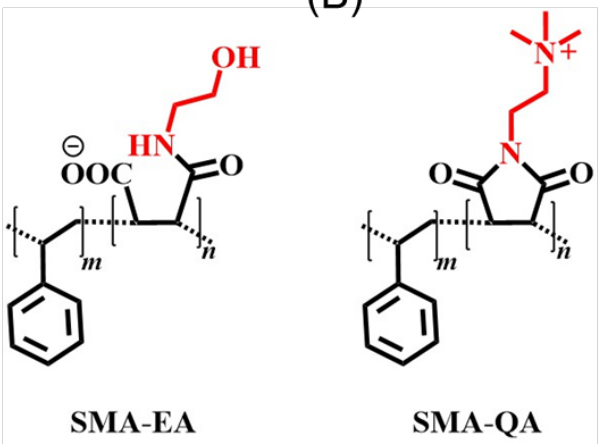

Figure S1. (**A**) The amino acid sequence of rabbit cytochrome-b5 containing a 6 His-tag for the affinity purification. The Gly residue at the N-terminus is coming from the cloning vector pET28a(+). (**B**) Molecular structures of negatively-charged SMA-EA and positively-charged SMA-QA polymers. Synthesis, characterization and ability to form lipid-nanodiscs of these polymers are reported in our previous publications.[1, 2]

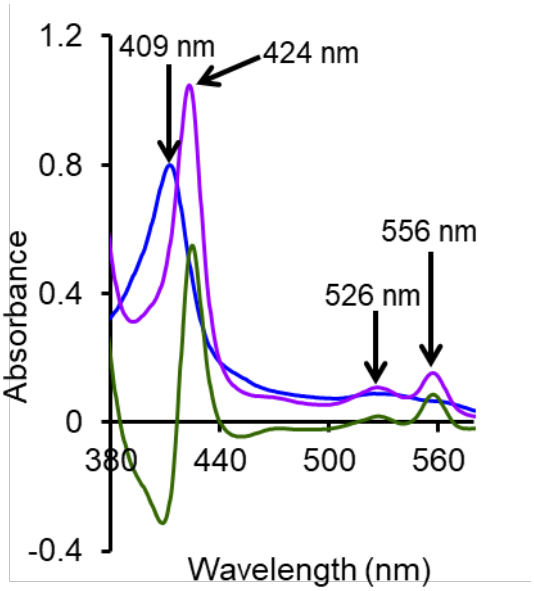

Figure S2. Absorbance spectra of recombinant cytochrome-b5 extracted in native *E.coli* lipids using SMA-EA polymer: oxidised form (409 nm) (blue), sodium dithionite-reduced form (424 nm, 526 nm and 556 nm) (magenta), difference spectra (reduced minus oxidised) (green).

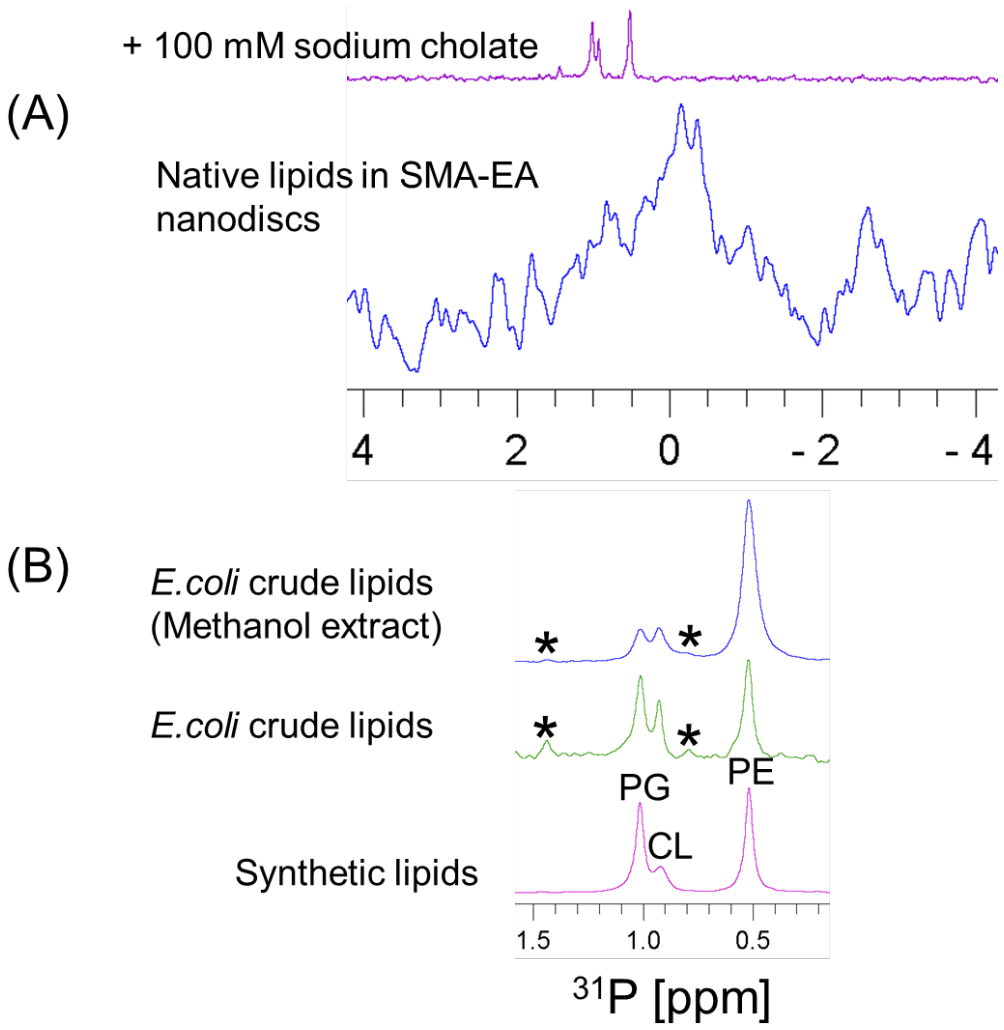

Figure S3. (**A**) ^{31}P NMR spectra of native *E.coli* lipids in the directly extracted SMA-EA polymer nanodiscs containing cytochrome-b5. The sample was prepared in 20 mM HEPES buffer (pH 7.4) containing 50 mM NaCl without (bottom) and with 100 mM sodium cholate (top). The ^{31}P NMR spectrum of cell lysates is broad possibly due to the presence of ^{1}H-^{31}P dipolar couplings even though a weak (~12.5 kHz) proton decoupling radio-frequency irradiation was used during signal acquisition. Other sources for line-broadening could be due to the unaveraged ^{1}H-^{1}H dipolar couplings as the ~10 nm size nanodiscs may not undergo completely isotropic tumbling to average the dipolar couplings and the lipids in the sample are not deuterated. The spectra were recorded at room temperature on a Bruker 500 MHz NMR spectrometer equipped with a broadband reverse probe. (**B**) The *E.coli* lipids were identified based on the chemical shifts in the reference spectrum of synthetic lipids. PE; phosphatidylethanolamine, CL; cardiolipin, PG; phosphatidylglycerol. * indicating unknown *E.coli* lipids. The samples were prepared in 20 mM HEPES buffer (pH 7.4) containing 50 mM NaCl and 100 mM sodium cholate.

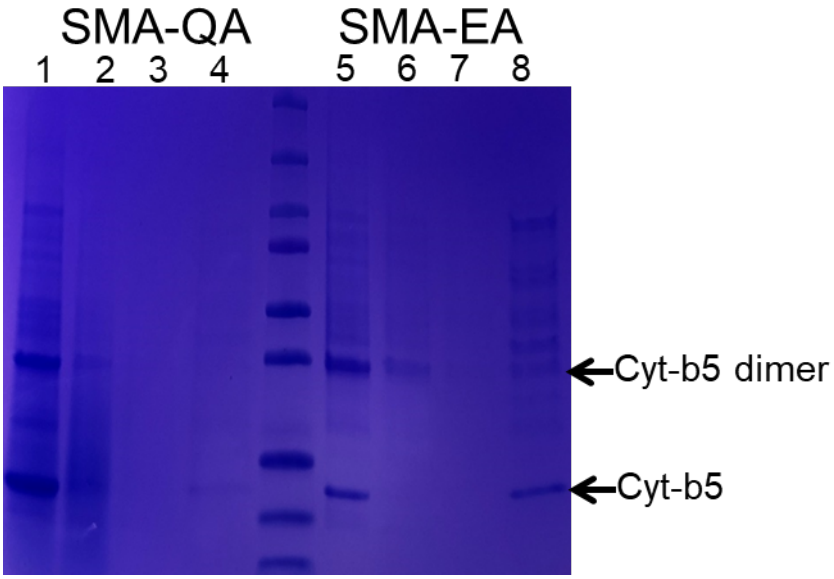

Figure S4. SDS-PAGE analysis of affinity-purified cytochrome-b5 that was extracted using SMA-QA (lanes 1-4) and SMA-EA (lanes 5-8) polymers. Lanes 1 and 5 from the pellet after solubilization of cell lysates using the polymer; 2 and 6, unbound protein fraction after binding to Ni-NTA resin; lanes 3 and 7 are from a buffer wash; lanes 4 and 8 from cytochrome-b5 elution using 300 mM imidazole. The protein bands appearing around 32-kDa (lanes 1, 2, 5, 6 and 8) are only from the initial purification steps and are expected to be a dimer of cytochrome-b5.

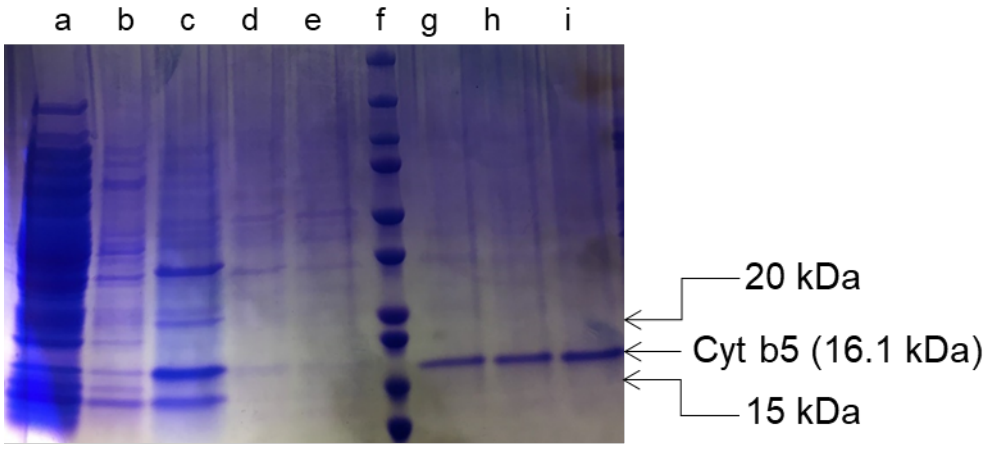

Figure S5. SDS-PAGE analysis of affinity-purified cytochrome-b5 in SMA-EA native bacterial lipid nanodiscs. Lane a is from the crude cell lysates; and lane b is from the supernatant of cell lysates after the centrifugation of cell lysates. Lane c is from the flow-through during the binding to the column; owing to non-specific polymer interaction with Ni-NTA resin, some of the cytochrome-b5 was not bound to the resin (**Fig. S4**), and therefore, the final protein yields are lower than expected. Lanes d and e are from the buffer wash of the column. Lanes g, h and i are from the protein eluted with 300 mM imidazole.

Experimental Details

Protein expression and purification

The synthetic gene encoding for cytochrome-b5 was obtained from GenScript and expressed in *Escherichia coli* using pET28a(+) vector (restriction sites; NcoI/XhoI). The *E.coli C41* cells were purchased from Lucigen Corporation (Wisconsin). The protein was expressed as described earlier.[3] Kanamycin (Thermo Fisher Scientific) was used as a selection marker. For the uniformly-^{15}N-labeled protein, 25 mL of overnight culture in TB medium was pelleted, and the cells were resuspended in 250 mL of M9 medium containing $^{15}NH_4Cl$ (Cambridge Isotope Laboratories). When the OD_{600} was ~0.8, the protein was overexpressed by adding 1 mM IPTG (Sigma-Aldrich). Cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 200 mM NaCl (Fisher Scientific) and a cOmplete™ protease inhibitor cocktail (Sigma-Aldrich)). Lysis was done in the presence of lysozyme (Sigma-Aldrich), Dnase (Sigma-Aldrich), $MgCl_2$ (Sigma-Aldrich) followed by sonication. The membranes were collected by centrifugation at 20,000 rpm and washed with the same buffer containing 500 mM NaCl (high salt buffer) and repeated centrifugation. The pellet was solubilized in high salt buffer using the SMA-EA polymer at 1:1 (w/w) pellet: polymer ratio. The solution was incubated at 4 °C overnight with slow mixing on racker and centrifuged at 3,500 rpm for 45 min. High-speed centrifugation might make the nanodiscs to settle along with insoluble membranes and subsequently can decrease the final protein yield. The supernatant was incubated with the pre-equilibrated Ni-NTA beads (GE-Healthcare) for 2 hours. The unwanted proteins were washed out using several column volumes of the buffer with and without 5 mM imidazole. The protein was eluted using 300-500 mM imidazole and analysed by SDS-PAGE (Bio-Rad).

Size-exclusion chromatography (SEC)

The affinity-purified cytochrome-b5 protein fractions in native lipid nanodiscs were concentrated and purified using SEC (10x600 Superdex 200, GE Healthcare). The purity of the protein was analyzed by SDS-PAGE.

Polymer synthesis

SMA-EA (~2 kDa) and SMA-QA polymers used in this study were synthesized and characterized as described earlier.[1, 2] The poly(styrene-co-maleic anhydride) cumene terminated (SMA from

Sigma-Aldrich) with a ~1.3:1 molar ratio of styrene:maleic anhydride (~M_n of 1600 g/mol) was used as stating material without any further purification. Due to the nature of functionalization, the polydispersity of the polymer does not change from the starting material.

UV-visible spectroscopy

The oxidative state of cytochrome-b5 was confirmed using UV-visible spectra obtained from a UV/vis spectrometer (DeNovix, DS-11+). Sodium dithionite (Sigma-Aldrich) was used as a reducing agent.

Transmission electron microscopy (TEM)

TEM images were obtained using a Technai® T-20® machine (FEI®, Netherlands) with an 80 kV operating voltage. A dilute extracted protein solution was dropped on a carbon-coated copper grid and dried overnight at room temperature in a desiccator before collecting images.

Dynamic light scattering (DLS)

DLS experiments for measuring the hydrodynamic radius of nanodiscs were performed using Wyatt Technology® DynaPro® NanoStar® using a 1 µL quartz MicroCuvette.

E.coli lipid extraction

Total lipids were extracted by the addition of MeOH (10 mL) to *E. coli* cell lysate (500 mg wet weight) and stirred for 5 min at room temperature. The resulting mixture was centrifuged for 10 min at 10000 rpm. The supernatant was taken, and methanol was removed under reduced pressure. The resulting crude lipids were used for NMR studies.

NMR spectroscopy

^{1}H and ^{31}P NMR NMR spectra were acquired on a Bruker 500 MHz NMR spectrometer operating with a ^{1}H and ^{31}P resonance frequencies of 500 MHz and 202 MHz, respectively, and equipped with TXI probe and $^{1}H/^{31}P/^{2}H$ BB reverse probe, respectively. For ^{31}P NMR, the samples were prepared in 20 mM HEPES buffer (pH 7.4) containing 50 mM NaCl and 100 mM sodium cholate (Sigma-Aldrich). H_3PO_4 used as an external reference for calibrating ^{31}P NMR spectra (set at 0 ppm). Commercial PE, PG and CL lipids were purchased from Avanti Polar Lipids (Alabaster,

Alabama). 2D ^{15}N-^{1}H-TROSY-HSQC NMR spectrum was acquired at 25 °C on a Bruker 800 MHz NMR spectrometer equipped with a cryogenically cooled triple-resonance probe.

References

1 T. Ravula, S. K. Ramadugu, G. Di Mauro and A. Ramamoorthy, *Angew Chem Int Ed Engl*, 2017, **56**, 11466-11470.

2 T. Ravula, N. Z. Hardin, S. K. Ramadugu, S. J. Cox and A. Ramamoorthy, *Angew Chem Int Ed Engl*, 2018, **57**, 1342-1345.

3 S. B. Mulrooney and L. Waskell, *Protein Expr Purif*, 2000, **19**, 173-178.