

Effect of polymer charge on functional reconstitution of membrane proteins in polymer nanodiscs

Thirupathi Ravula^a Nathaniel Z. Hardin,^a Jia Bai,^a Sang-Choul Im,^b Lucy Waskell,^b and Ayyalusamy Ramamoorthy^a *

Supporting Information

Materials and Methods

Poly(Styrene-co-Maleic Anhydride) cumene terminated (SMA), with a -1.3:1 molar ratio of styrene:maleic anhydride and average molecular weight of $M_n \sim 1600$ g/mol, Lipodisq® Styrene:Maleic Anhydride Copolymer 3:1, Pre-hydrolyzed anhydrous, N-Methyl-2-Pyrrolidone (NMP), 2-Aminoethanol (EA), Triethylamine (Et_3N), HEPES, potassium phosphate, acetic acid (HOAc), hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich®. 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Lipids Polar, Inc®. The ~16 kDa rabbit cytochrome b_5 was expressed in *E.coli* and purified as reported elsewhere.¹ Expression and purification of the cytochrome P450 2B4 (CYP2B4) were performed as described in the literature.^{2, 3}

SMA-EA copolymer synthesis: SMA-EA was synthesized as described previously.⁴ Briefly 5g of SMA was dissolved in anhydrous NMP, and then 2 ml of Ethanol amine (EA) was added. A total of 4.3 mL of Et_3N was added to the reaction, stirred at 70 °C for 2 hours, after which the polymer was precipitated by the addition of 0.1 M HCl. The resulting precipitate was separated by centrifugation, followed by several cycles of washing the pellet with 0.1 M HCl and centrifugation to remove traces of NMP and EA. The resulting pellet was re-dissolved in 100 ml 1 M NaOH and heated to 70° C and stirred for 2 hours. The resulting solution was cooled to room temperature, polymer was extracted by the addition of 1 M HCl. The precipitate was washed several times with water and lyophilized to give a white powder of the SMA-EA polymer in a quantitative yield.

Synthesis of SMA-QA: SMA-QA was synthesized as described previously.⁵ Briefly 1 g of SMA was dissolved in 30 ml of anhydrous DMF dried over molecular sieves. 1.3 g of (2-aminoethyl)

trimethylammonium chloride hydrochloride was then added to the solution and to this mixture 5 ml of trimethylamine was added causing the solution to turn dark yellow. The reaction mixture was then stirred at 100 °C for 12 hours. The solution was cooled to room temperature and precipitated with diethyl ether. The precipitate was washed 3 times with diethyl ether and dried under vacuum. The dried intermediate was then added to 30 ml acetic anhydride. 660 mg of sodium acetate and 200 mg of triethyl amine were then added. The reaction mixture was heated at 100 °C overnight and precipitated in ether. The precipitate was washed 3 times with ether and dried under vacuum. The product was then dissolved in water and passed through a saphadex LH-20 column. The product was collected and then lyophilized to give 850 mg brown powder.

Formation of nanodiscs: Nanodiscs with different polymers were prepared using DMPC (10 mg/ml) in 50 mM potassium phosphate at pH 7.4 as stock solution. Polymer stock solutions were prepared as 10 mg/ml in buffer for SMA-QA and SMALP, 0.1 M NaOH solution was used for SMA-EA polymer. SMALP nanodiscs were prepared by the addition of SMALP (10 mg/ml) and DMPC (10 mg/ml) for required amount to give 1.5:1 w/w ratio. The resulting mixture was incubated at 37 °C for 4 hrs, followed by the purification using size exclusion chromatography and the size was measured using dynamic light scattering. SMA-EA nanodisc were prepared by the addition of 2:1 w/w SMA-EA (10 mg/ml) and DMPC (10 mg/ml), followed by the incubation at 37 °C for 4 hrs. The prepared nanodiscs were purified using size exclusion chromatography and the size was measured using dynamic light scattering. SMA-QA nanodiscs were prepared by the addition of 1.5:1 w/w SMA-EA (10 mg/ml) and DMPC (10 mg/ml), followed by the incubation at 37 °C for 4 hrs. The prepared nanodiscs were purified using size exclusion chromatography and the size was measured using dynamic light scattering.

Reconstitution of ~57 kDa Cytochrome P450 2B4 in nanodiscs:

CytP450 was exchanged with 50 mM potassium phosphate (pH 7.4) buffer at 4 °C using size exclusion chromatography. The resulting CytP450 was immediately incubated with purified nanodiscs (100:1 DMPC: CytP450) at 25 °C for 12 hrs. Experiments with NaCl were performed by addition of a required amount of NaCl to purified nanodiscs, and incubating with CytP450. Concentration of CytP450 in nanodiscs was determined based on the CO-spectrum using an extinction coefficient of $\Delta\epsilon_{450-490} = 91 \text{ mM}^{-1} \text{ cm}^{-1}$.⁶

Carbon monoxide (CO) assay: A solution containing 3 μM CytP450 reconstituted in different nanodiscs were taken into a cuvette with 1 cm path length. UV-visible absorption spectra were recorded from 300 to 700 nm. The protein was then reduced by an excess of sodium dithionite powder, followed by bubbling of carbon monoxide (CO) gas over the solution for 1 min. UV-visible absorption spectra from 400 to 700 nm were recorded again.

Reconstitution of ~16 kDa rabbit cytochrome- b_5 in nanodiscs for NMR experiments: 300 μl of 100 μM ^{15}N -labeled-Cytb₅ was incubated overnight with nanodiscs solution (100:1 DMPC: Cytb₅) in 50 mM potassium phosphate buffer at pH 7.4. The resulting sample was concentrated to give 100 μM Cytb₅, and then 10% D₂O was added.

Static Light Scattering (SLS) Experiments: The time-dependent reconstitution of Cytb₅ in DMPC nanodiscs was monitored by the intensity of scattered light at 90° angle by using a Fluro Fluorimeter. Nanodiscs solution containing 0.1 mg/ml of DMPC stock solutions were taken in a 2 ml cuvette under stirring using a bar magnet. Then the solution was equilibrated for 5 min before the addition of 20 μl of 100 μM Cyt b_5 . The excitation and emission wavelengths were set at 400 nm and 404 nm respectively. The slit opening was set to 2 nm. All SLS experimental measurements were carried out using a FluoroMax 4® from Horiba Scientific®.

^1H - ^{15}N Heteronuclear Single Quantum Coherence Transverse Relaxation-Optimized Spectroscopy (HSQC-TROSY): Two-dimensional $^{15}\text{N}/^1\text{H}$ TROSY HSQC was recorded on a Bruker Avance II 600 MHz NMR spectrometer equipped with a cryoprobe. Spectra were obtained using 64 scans and 256 t_1 increments. Data were processed using TopSpin 2.0 (Bruker).

Size-Exclusion Chromatography (SEC): Superdex® 200 10/300 GL column attached to an AKTATM® purifier Fast Protein Liquid Chromatography (FPLC) purification system (GE Healthcare®) with a 1000 μL loop was used to elute the samples with a 50 mM potassium

phosphate buffer (pH 7.4) at a flow rate of 0.5 mL/min. Detection was done by collecting the absorbance at $\lambda = 254$ nm.

Dynamic Light Scattering (DLS): All DLS experiments were performed using Wyatt Technology® DynaPro® NanoStar® with a 1 μ L quartz MicroCuvette.

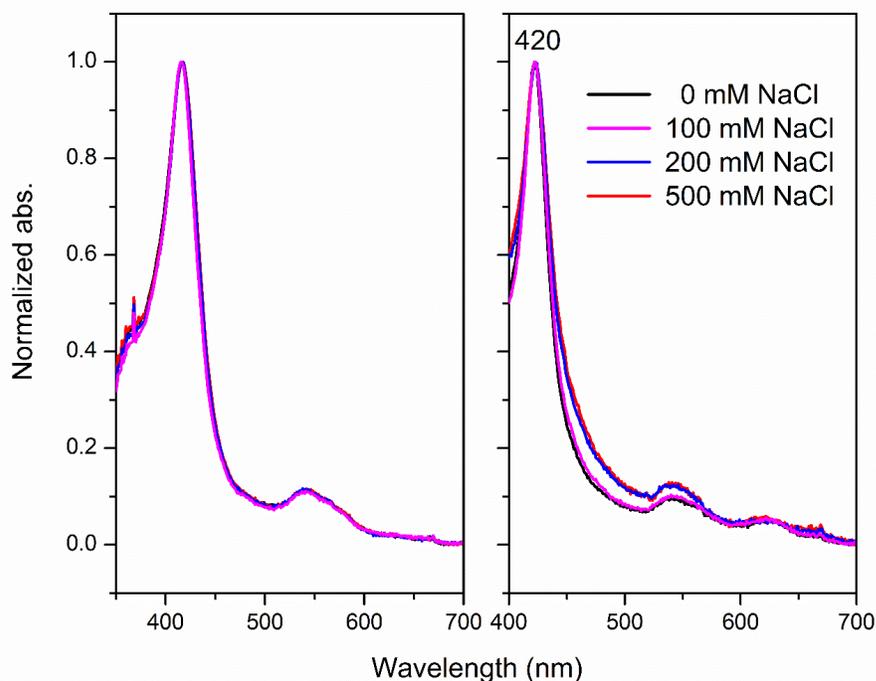


Figure S1. Destabilization of cytochrome P450 by a detergent. UV-vis absorption spectra of cytochrome P450 reconstituted in DPC micelles in its ferric state (left) and ferrous-carbon monoxide complex (right) at the indicated salt concentrations.

Polymer-belt possess a high charge density: The high affinity of a membrane protein (cytochrome P450 or cytochrome b5 used in this study) for the polymer can be attributed to the significant charge density of the polymer-belt that surrounds the lipid bilayer of the nanodisc; the negatively-charged carboxylate-groups of the polymer belt surrounds the lipid bilayer in a nanodisc. The charge density on the polymer-belt is expected to be very high as compared to the

lipids' charge density on the lipid bilayer. It is estimated that a polymer-nanodisc of ~10 nm diameter is made up of 3:1 lipid:polymer molar ratio (based on the 1:2 w/w ratio used to make the nanodiscs) with about ~75 polymer molecules and about ~224 lipid molecules; in this estimation, we assumed a molecular weight of ~2-kDa for SMA-EA polymer monomer (polydispersity of the polymer was not considered). This estimation suggests that each polymer-nanodisc consists of about ~523 negative charges from the carboxylate groups (each SMA-EA polymer consists of 7 negative charges; There are seven maleic anhydride groups each with a negatively charged carboxyl group and a -CO-NH-R group at neutral pH). It should be noted that these ~523 negative charges are crowded within the polymer-belt's surface area of about 125 nm² (assuming 10 nm diameter and 4 nm width) Therefore, the charge density on the polymer-belt is very high.

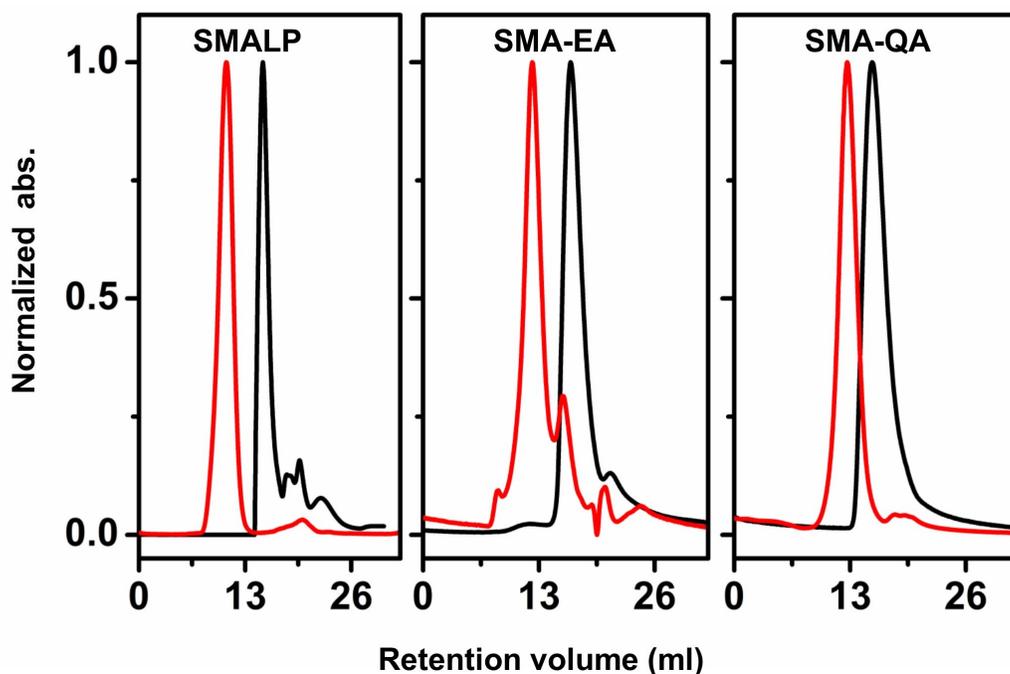


Figure S2. Effect of the polymer-belt's charge density. Comparison of SEC profiles of empty nanodiscs (black) and nanodiscs containing CytP450 (red).

References

- [1] S. B. Mulrooney, L. Waskell, *Protein Expr. Purif.*, **2000**, *19*, 173-178.
- [2] A. Bridges, L. Gruenke, Y.-T. Chang, I. A. Vakser, G. Loew, L. Waskell, *J. Biol. Chem.* **1998**, *273*, 17036-17049; S. Ahuja, N. Jahr, S.-C. Im, S. Vivekanandan, N. Popovych, S. V. Le Clair, R. Huang, R. Soong, J. Xu, K. Yamamoto, R. P. Nanga, A. Bridges, L. Waskell, A. Ramamoorthy, *J. Biol. Chem.* **2013**, *288*, 22080-22095.
- [3] A. S. Saribas, L. Gruenke, L. Waskell, *Protein Expr. Purif.*, **2001**, *21*, 303-309.
- [4] T. Ravula, S. K. Ramadugu, G. Di Mauro, A. Ramamoorthy, *Angew. Chem. Int. Ed. Engl.*, **2017**, *56*, 11466-11470.
- [5] T. Ravula, N. Z. Hardin, S. K. Ramadugu, S. J. Cox, A. Ramamoorthy, *Angew. Chem. Int. Ed. Engl.*, **2018**, *57*, 1342-1345.
- [6] J. B. Schenkman, I. Jansson, *Methods Mol. Biol.*, **2006**, *320*, 11-18.