Supplementary Information

Detergent-free isolation of CYP450-reductase's FMN-binding domain in *E. coli* lipid-nanodiscs using a charge-free polymer

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Table S1. The amino acid sequences of rat FMN-binding domain (FBD) of cytochrome P450 reductase containing a 6 His-tag. The molecular weight, pI, and net charge were calculated using Prot pi prediction Tool.[1]

Protein Name/Sequence	MW (kDa)	Isoelectric point (pI) at pH 7.4	Net charge (*z*) at pH 7.4
Rabbit FBD MKKTAIAIAVALAGFATVAQAGIPGDPTNMGDSHEDTSATMPE AVAEEVSLFSTTDMVLFSLIVGVLTYWFIFRKKKEEIPEFSKI QTTAPPVKESSFVEKMKKTGRNIIVFYGSQTGTAEEFANRLSK DAHRYGMRGMSADPEEYDLADLSSLPEIDKSLVVFCMATYGEG DPTDNAQDFYDWLQETDVDLTGVKFAVFGLGNKTYEHFNAMGK YVDQRLEQLGAQRIFELGLGDDDGNLEEDFITWREQFWPAVCE FFGVEATGEEHHHHHH	27.8	4.59	-24

Table S2. Lipid composition and the ratio of different lipids present in *E. coli* lipid extract (Avanti Polar Lipids) and polymer-nanodiscs samples.

	POPE (%)	POPG (%)	CL (%)	POPE:POPG:CL ratio
E. coli lipid extract	68	24	8	8.5:3:1
Polymer-nanodiscs (pentyl-inulin, *E. coli* lipids)	70	23	7	10:3.28:1

Table S3. Lipid composition and the ratio of POPE and POPG observed from *E.coli* lipid extract (Avanti Polar Lipids), polymer-nanodiscs, and native-nanodiscs containing FBD. CL was not detected in the native-nanodiscs (see Figure 3d [bottom trace]); hence for a comparison, only POPE and POPG ^{31}P NMR peaks' areas were integrated for the other two samples.

	POPE (%)	POPG (%)	POPE:POPG ratio
E. coli lipid extract	74	26	2.8:1
Polymer-nanodiscs (pentyl-inulin, *E. coli* lipids)	76	24	3.1:1
Native-nanodiscs (+FBD)	62	38	1.6:1

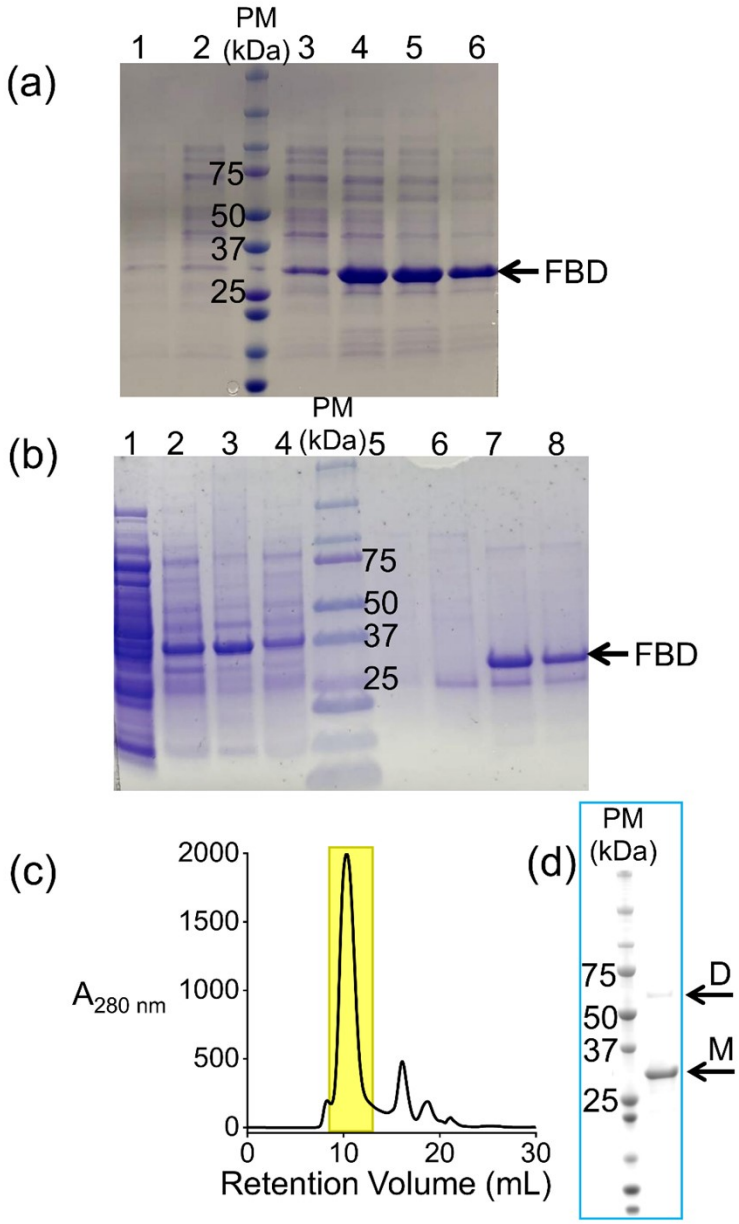


Figure S1. (**a**) SDS-PAGE analysis of protein fractions eluted from DEAE-resin using NaCl at 200 (lane 1), 300 (lane 2), 400 (lane 3), 500 (lane 4), 600 (lane 6) and 700 mM (lane 7) concentrations. The arrow indicates the protein band corresponding to the FBD. M, protein marker. **(b)** SDS-PAGE analysis of affinity (Ni^{2+}-NTA) purified the ^{15}N-labelled FBD in native-nanodiscs. Lane 1, unbound protein fraction after incubating solubilized membranes with Ni^{2+}-NTA resin; lanes 2-4, non-specifically-bound proteins removed by washing the resin with buffer; Lanes 5, 5 mM imidazole wash; Lane 6, 10 mM imidazole wash; and Lanes 7 and 8, the FBD eluted using a buffer containing 300 mM imidazole. M, protein marker. (**c**) Size-exclusion chromatography purification of the directly extracted ^{15}N-labelled FBD in native-nanodiscs. The peak between 8.5-12 mL highlighted with a yellow-colored shade corresponds to the FBD extracted in native-nanodiscs. The FBD fractions with yellow-colored solution are pooled together, analyzed by SDS-PAGE (d), and used in NMR experiments. The low-intensity peaks that are not consistently present in different batches of FBD purification by SEC might have appeared from impurities in the sample (see Figure 2b in the main text) were not characterized. M denotes protein monomer, and D denotes possibly a small fraction of protein dimer. PM: protein marker (kDa).

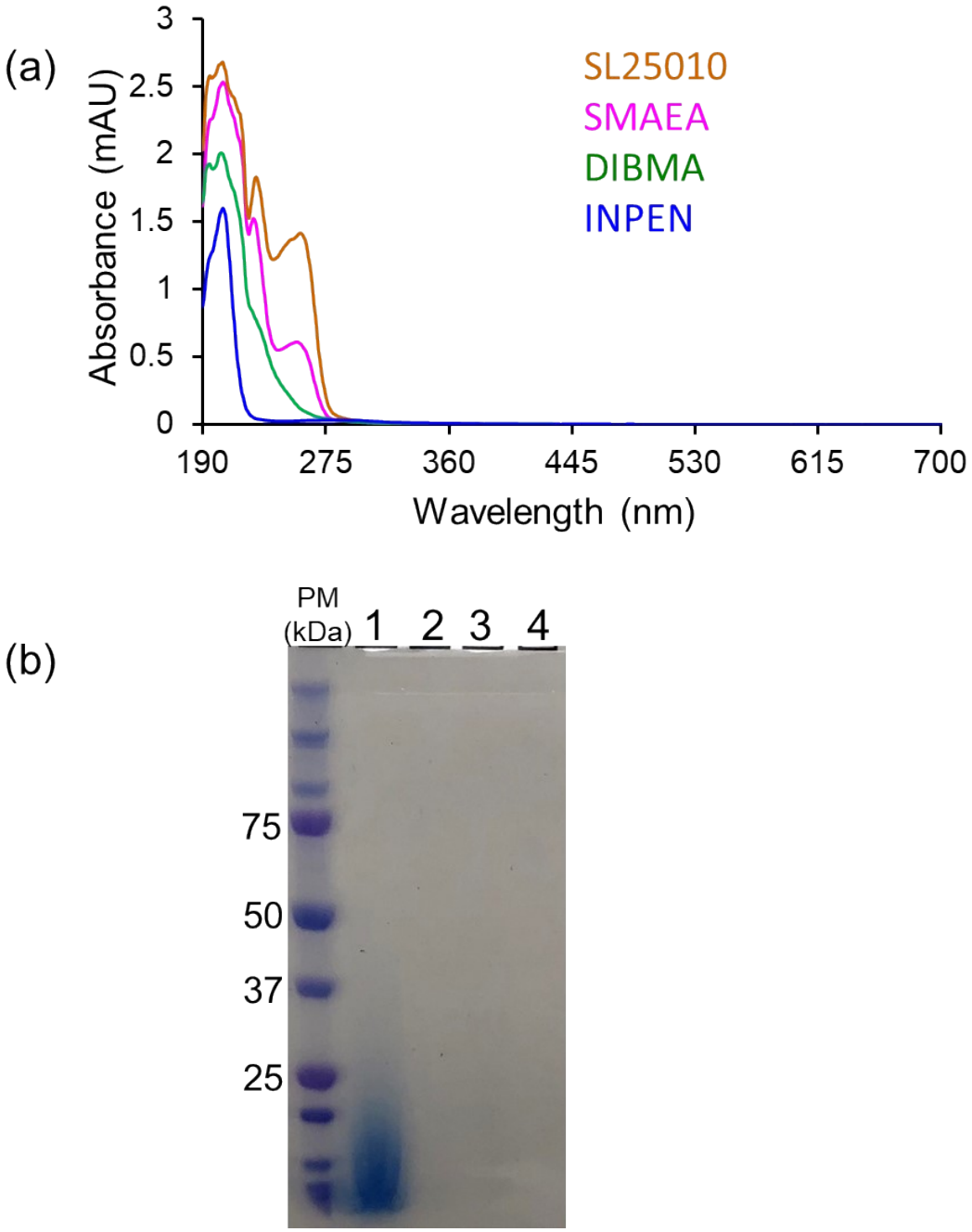


Figure S2. (**a**) Ultraviolet-visible absorbance spectra of pentyl-inulin, DIBMA, SMAEA, and SL25010 polymers. Pentyl-inulin and DIBMA showed no significant absorbance in the 230– 700 nm wavelength range. Due to aromatic rings, both SMA-based polymers SMAEA and SL25010 showed substantial absorbance near 260 nm wavelength. (**b**) SDS-PAGE analysis of (1) ~10 kDa SMA25010, (2) ~2 kDa SMA-EA, (3) ~12 kDa DIBMA, and (4) ~3 kDa pentyl-inulin. The samples were prepared in 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl. 50 µg of each polymer was loaded on an SDS-PAGE gel. PM: protein marker (kDa).

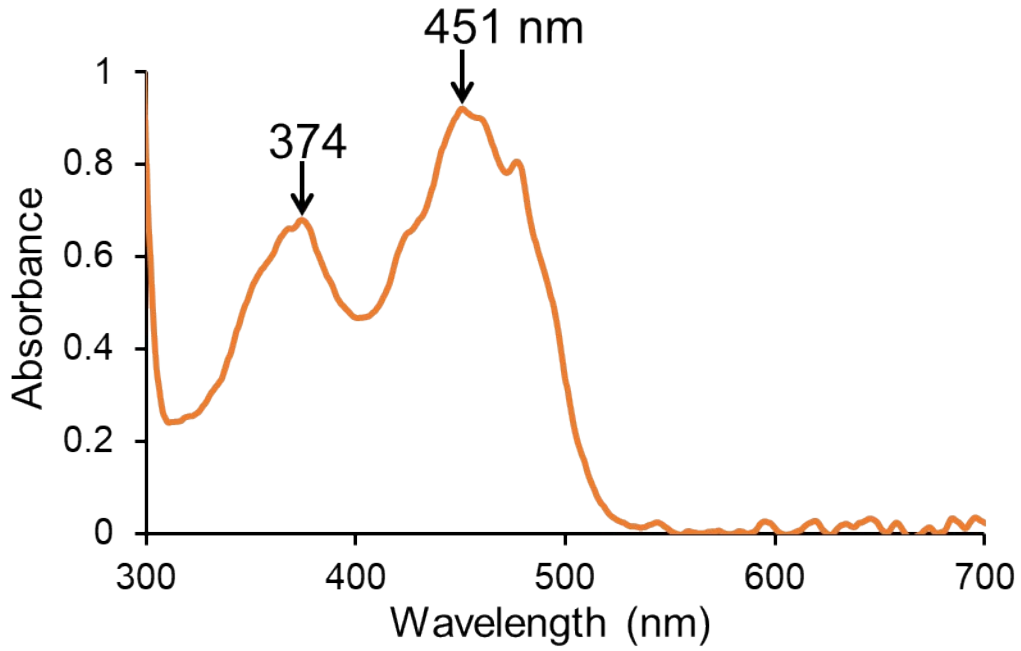
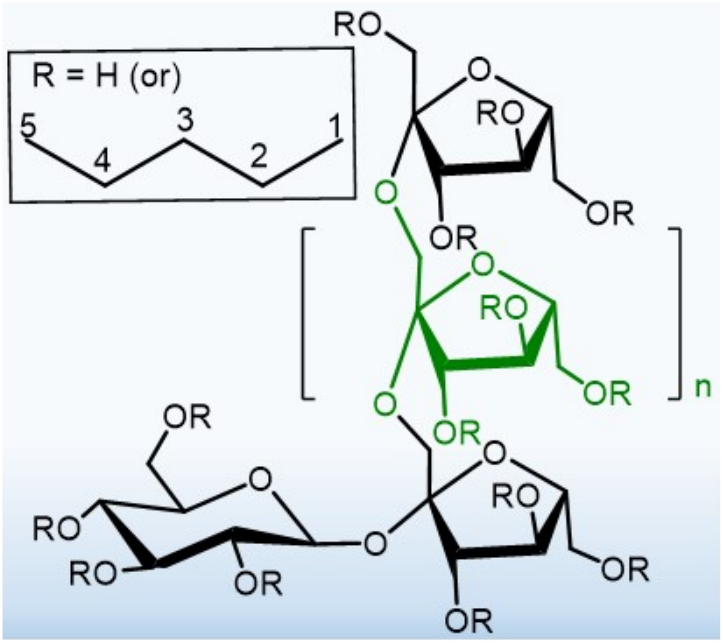
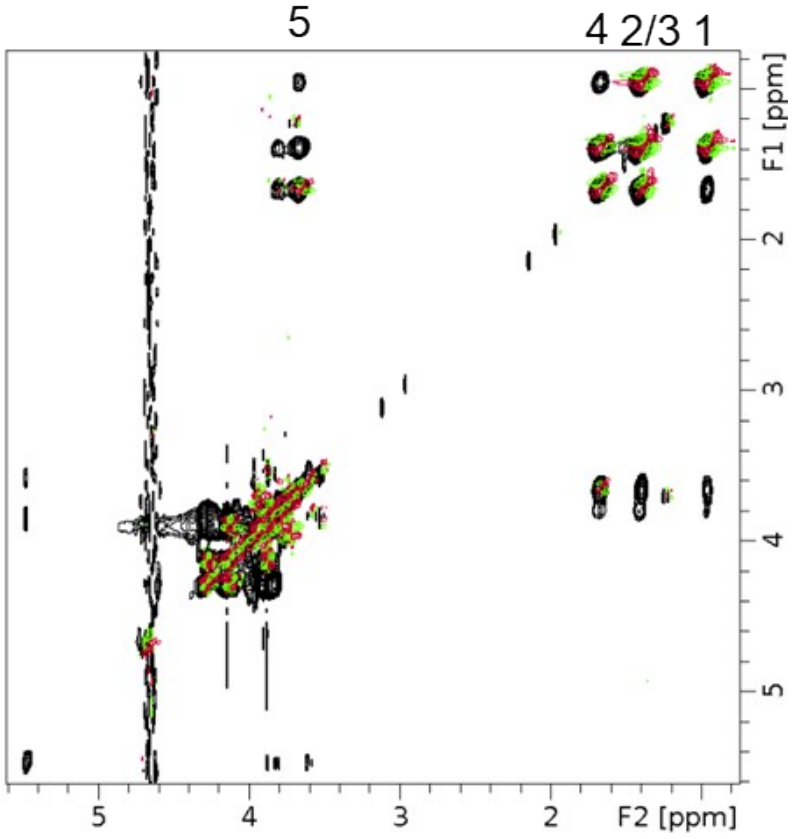


Figure S3. Characterization of FBD by UV-visible spectroscopy. The FBD in native *E.coli* lipid-nanodiscs, 25 mM sodium phosphate buffer (pH 7.4) showed a broadband with an absorption maximum at 451 nm.

(a)



(b)



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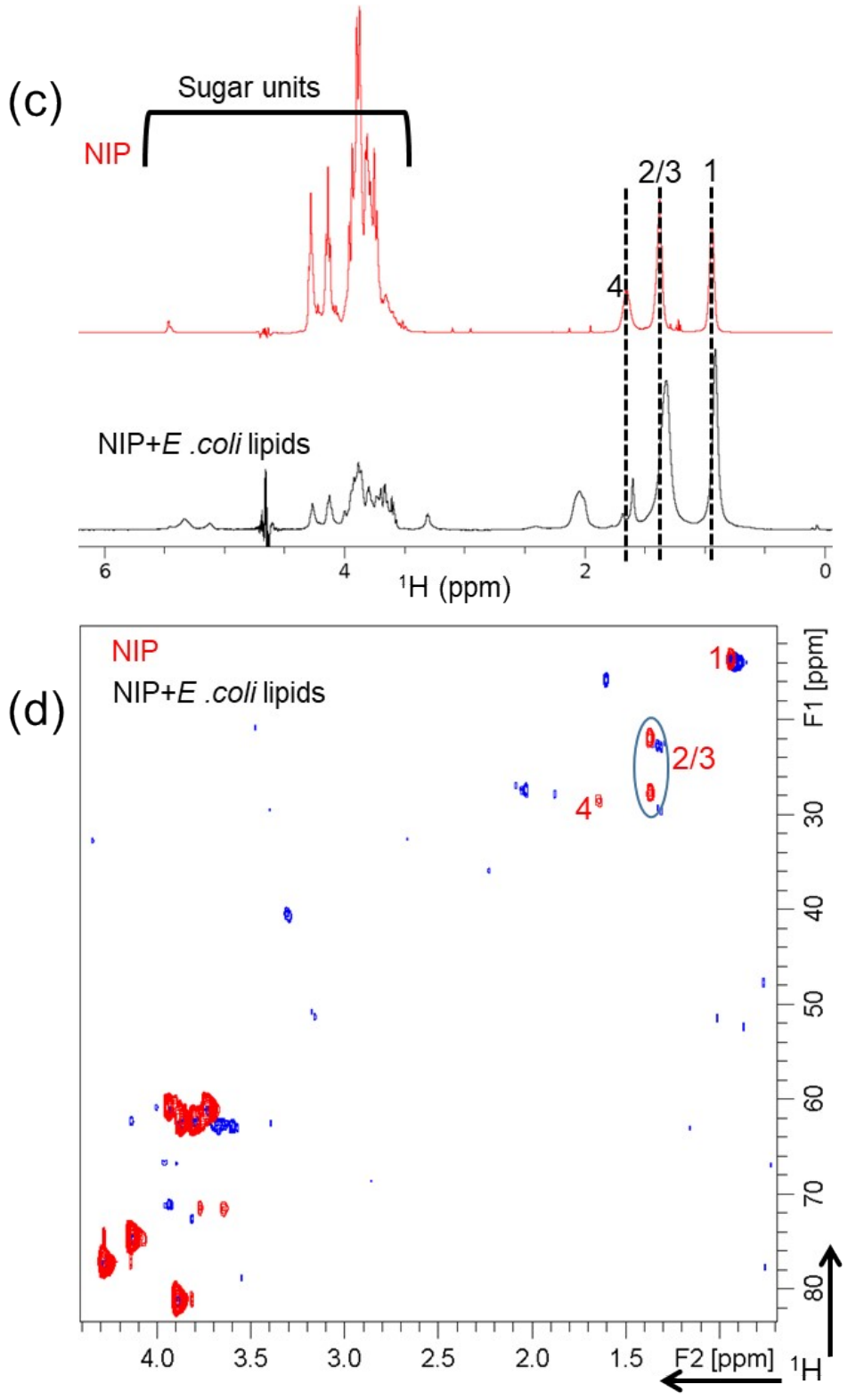

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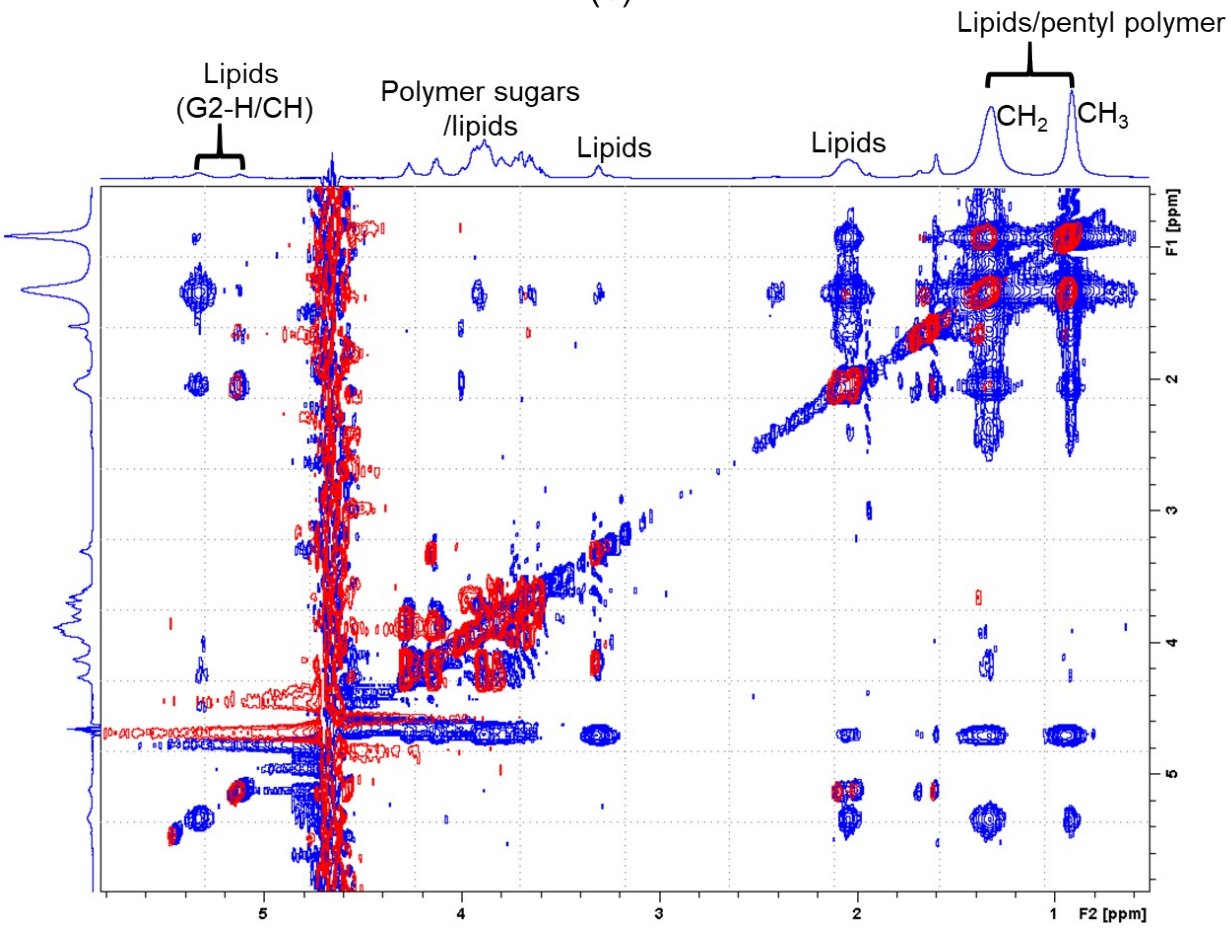
(e)



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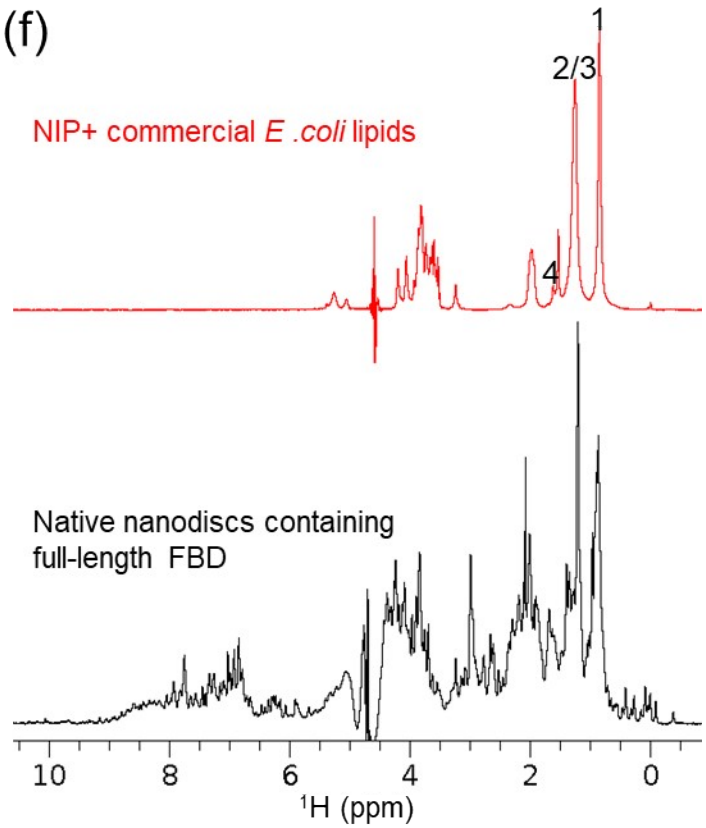


Figure S4. (**a**) Chemical structure of pentyl-inulin. (**b**) Chemical shift analysis of pentyl-inulin using 2D TOCSY (black) and 2D DQF-COSY (red/green). The assignments for the pentyl-group are labelled with numbers corresponding to protons/carbons, as shown in **(a).** The terminal methyl group is labelled as 1. (**c**) The ^{1}H and (**d**) ^{13}C NMR spectra of nanodiscs containing commercial *E. coli* lipids, and pentyl-inulin (black) overlaid with that of pentyl-inulin (red). The chemical shift differences are shown with broken lines. The additional peaks in the black spectrum originated from the *E. coli* lipids. The observed chemical shift differences in the presence of phospholipids indicate the interaction of the polymer with lipids. However, internuclear polymer-lipid NOE interactions could not be probed due to peak overlap (similar chemical shifts for protons from phospholipids and pentyl-inulin) in the 2D NOESY spectrum of pentyl-inulin-nanodiscs (Fig. S5). (**e**) Overlay of NOESY (blue) and TOCSY (red) spectra of pentyl-inulin-*E.coli* lipid nanodiscs recorded at 32 °C. The partial resonance assignments were made based on the published data.[2] Due to the overlap of severe resonances, the intermolecular interactions between polymer and lipids could not be resolved. (**f**) Overlay of nanodiscs containing pentyl-inulin and commercial *E. coli* lipids (red) and native-nanodiscs containing the FBD (black). The sample prepared using commercial lipids has cardiolipin, whereas in the native-nanodiscs, CL is not detected (See Figure 3, main text). All NMR data were collected on a Bruker 500 NMR spectrometer equipped with a TXI probe, and the samples were prepared in 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl.

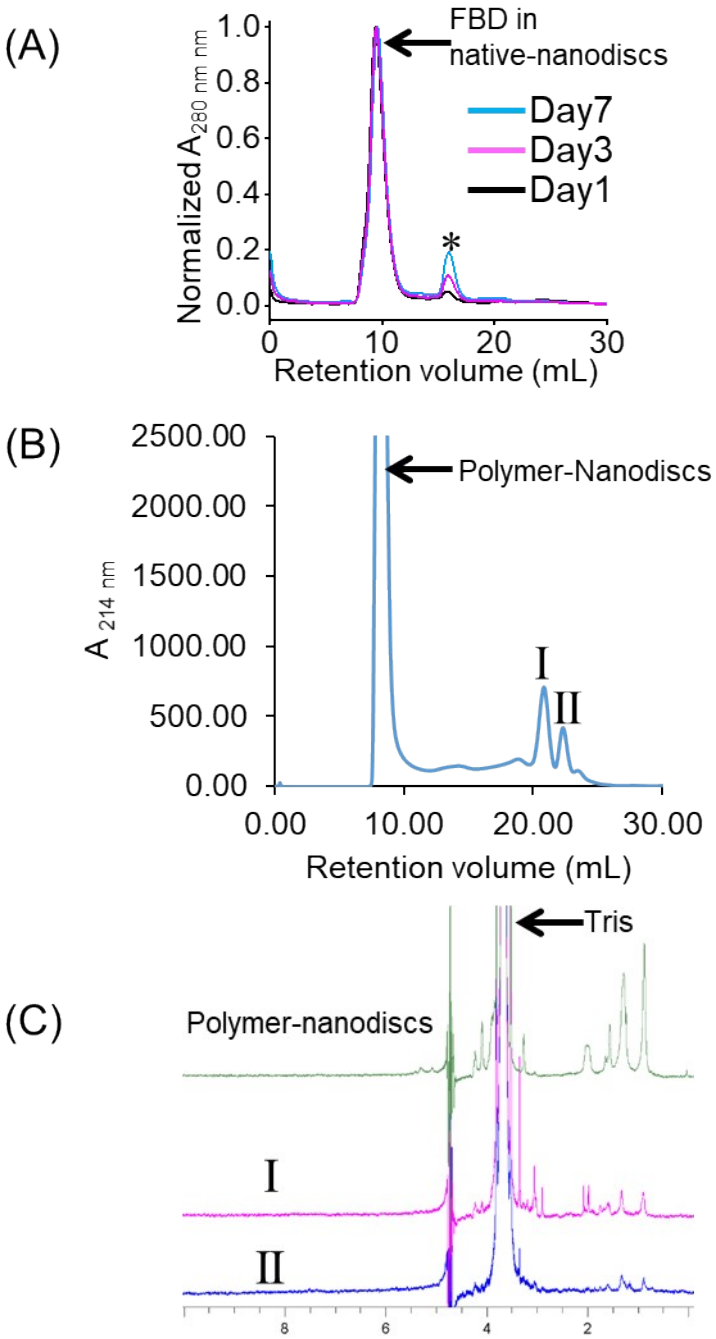
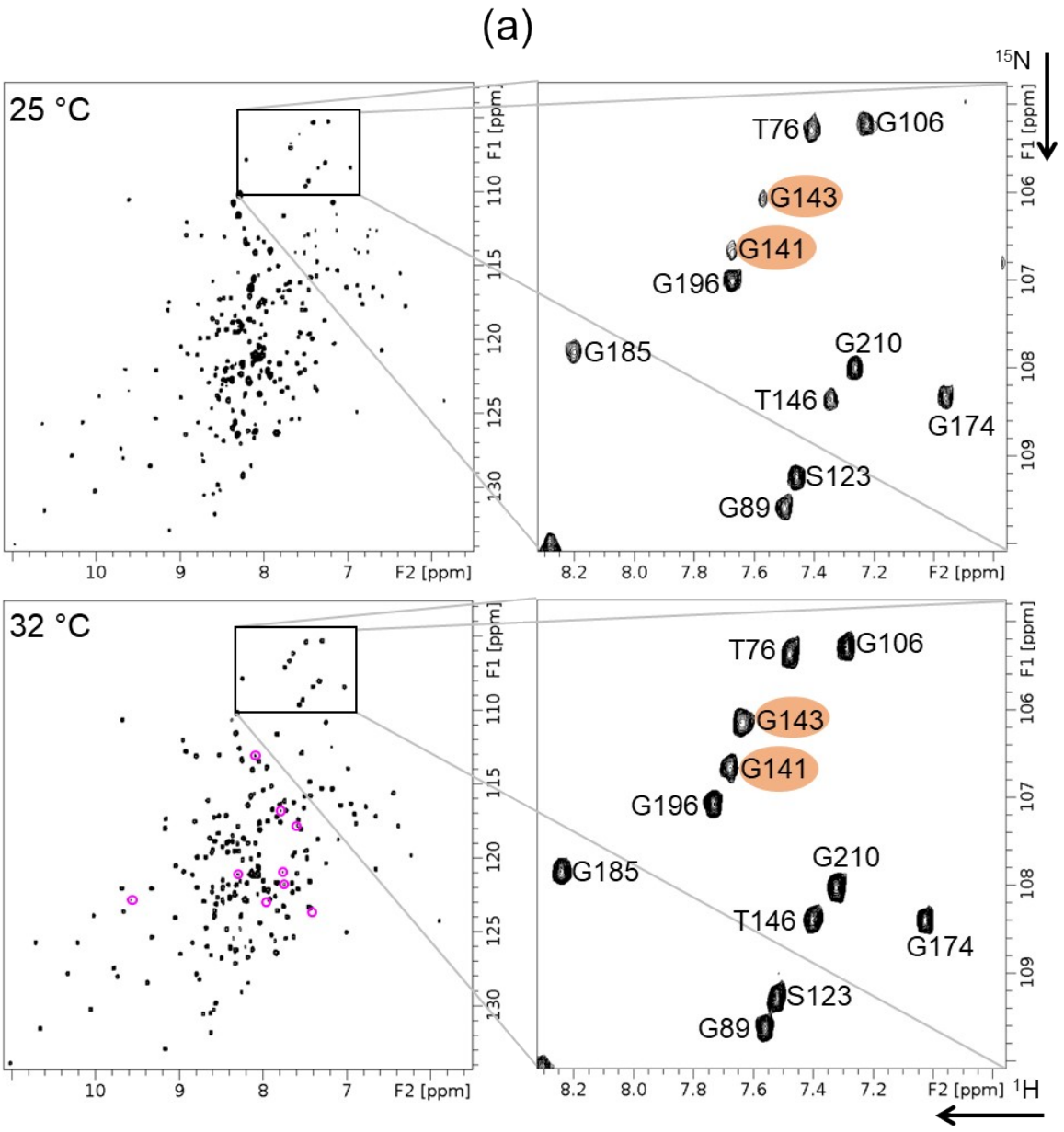


Figure S5. (**A**) Stability analysis of the FBD native-nanodiscs using size-exclusion chromatography (SEC). The SEC chromatograms collected at different time points showed the same elution times, indicating that the native-nanodiscs are stable at 4 °C. The elution peak at ~16 mL might have appeared from partial instability of nanodiscs over time (∗). The stability analysis was performed on the SEC column (10x300 Superdex 200) using 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl. The data were analyzed and plotted in OriginPro. (**B**) SEC purification profile of pentyl-inulin polymer-nanodiscs containing commercial *E. coli* lipids. The low-intensity peaks are most likely from the free polymer[3, 4] and impurities in the sample (labelled I, II). (**C**) ^{1}H NMR spectra of SEC fractions I, II shown in (**B**).



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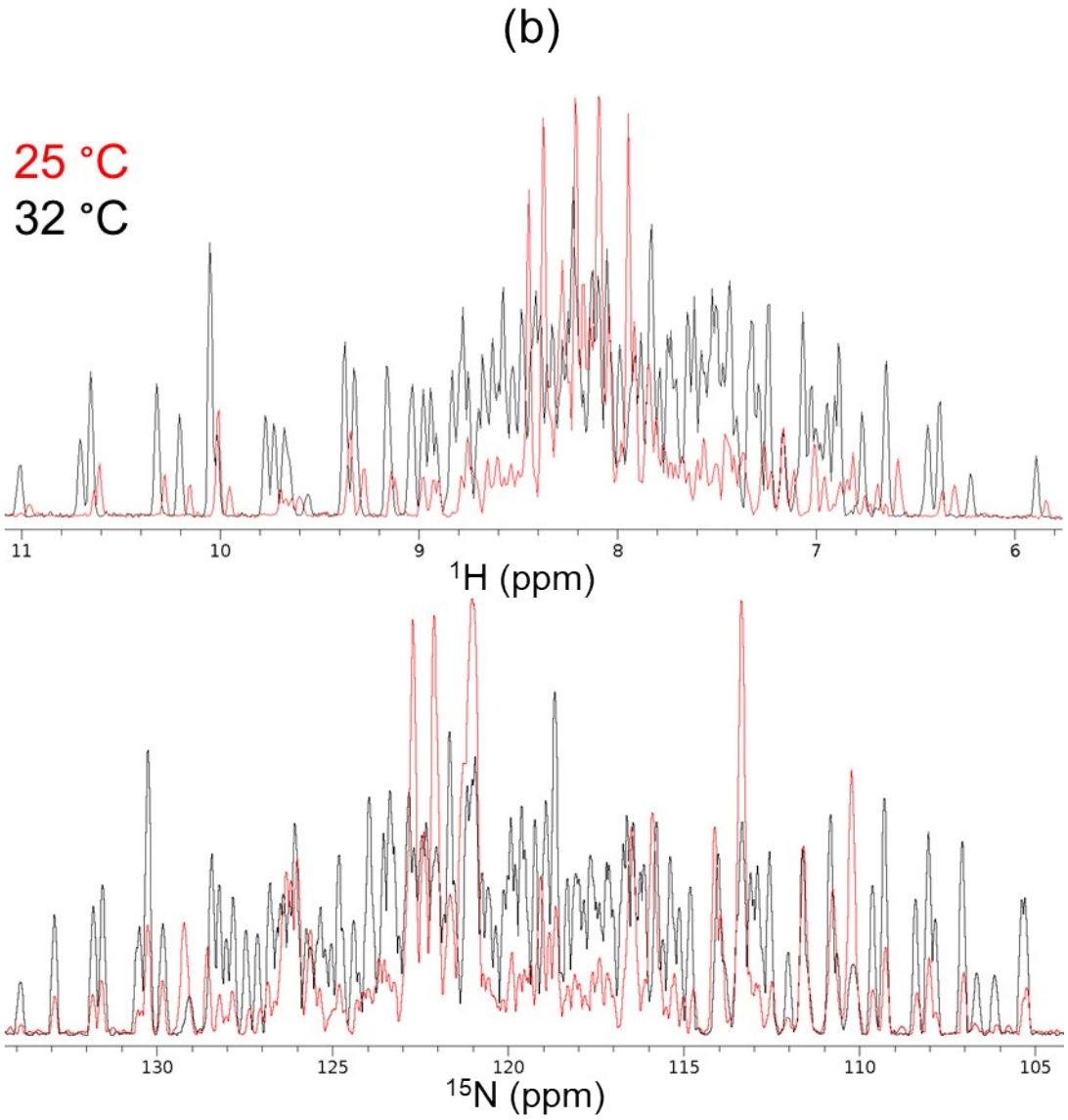

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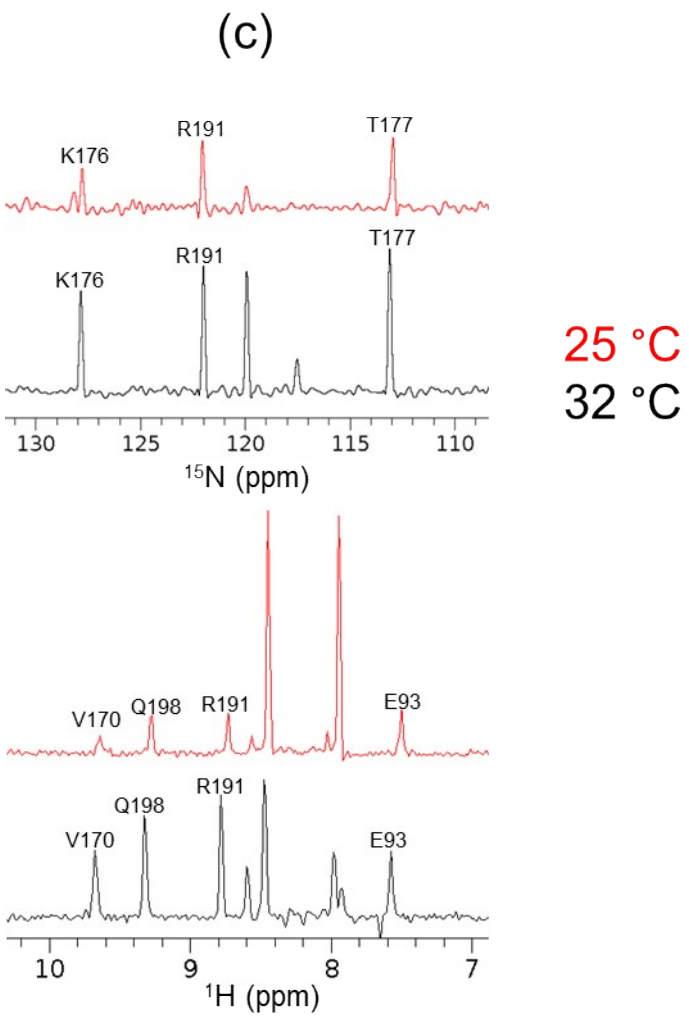

Figure S6. (**a**) 2D ^{15}N-^{1}H-TROSY-HSQC NMR spectra of 75 µM ^{15}N-labelled FBD in native-nanodiscs acquired at 25 °C (top) and 32 °C (bottom), respectively. The sample was prepared in 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl. The NMR data were collected using 64 scans at 200 t1 increments on an 800 MHz Bruker NMR spectrometer. The spectra were processed in Bruker TopSpin (4.0.6) using identical parameters. The quality of the NMR spectrum improved substantially when the temperature was increased from 25 °C to 32 °C. Expanded regions (right side) of spectra are shown for an easy reading, highlighting residues (G141, G143; loop3 residues surrounding the FMN prosthetic group) with a substantial signal improvement at the higher temperature. The low-intensity peaks are highlighted in magenta circles. (**b**) ^{1}H and ^{15}N projections are extracted from 2D ^{15}N-^{1}H-TROSY-HSQC NMR spectra of FBD recorded at 25 °C (red) and 32 °C (black). A substantial increase in the peak intensity at higher temperature (32 °C) indicates an increase in the mobility of the protein in nanodiscs compared to that at low temperature. (**c**) One-dimensional ^{1}H (bottom) and ^{15}N (top) slices extracted from 2D ^{1}H/^{15}N TROSY-HSQC spectra of FBD in native-nanodiscs, recorded at 25 °C (red) and 32 °C (black). The assignments are shown only for well-resolved resonances.

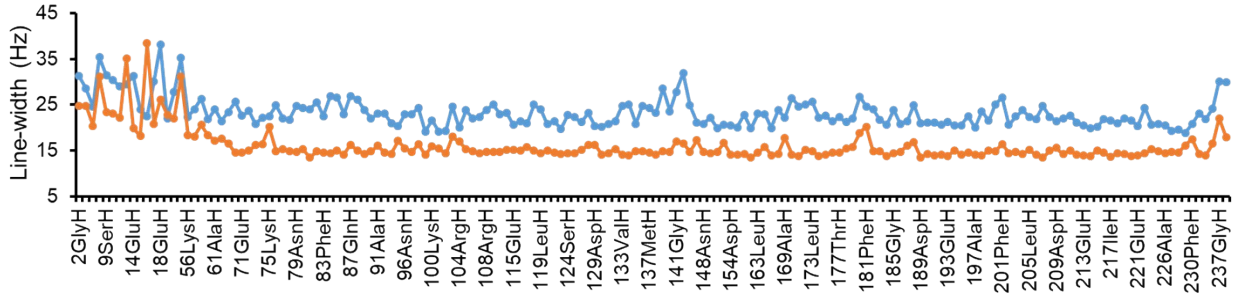

Figure S7. ^{1}H (blue) and ^{15}N (orange) line-widths, measured from the two-dimensional [^{15}N-^{1}H]–TROSY-HSQC (at 32 °C) NMR spectrum of a uniformly ^{15}N-labelled FBD reconstituted in *E.coli* native lipid-nanodiscs. The measured ^{1}H and ^{15}N line-widths are 20-28 Hz and 14-20 Hz, respectively. The peak-broadening was observed for the residues located close to the lipid bilayer, which may be due to their restricted motion. However, due to signal overlap, the line-widths for the N-terminal residues were not measured accurately.

Materials and Methods

Pentyl-inulin synthesis

Inulin (n=2-60)[5] extracted from chicory roots was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Pentyl-inulin (~3 kDa) polymer used in this study was synthesized by functionalizing inulin with hydrophobic pentyl groups using pentyl bromide as described elsewhere.[6]

Polymer stock solution preparation

The polymer powder was dissolved in 10 mM Tris (pH 7.4) containing 100 mM NaCl at 100 mg/mL concentration. The solution pH was adjusted to 7.4 by adding diluted HCl.

Protein expression and purification

The synthetic gene encoding for FBD was obtained from GenScript and expressed in *E. coli* C41 strain using pET28a(+) vector (restriction sites; *Nco*I/*Xho*I). The FBD was overexpressed at 30 °C for 16 hours. The overexpression of FBD was induced by adding 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma-Aldrich) when the optical density (O.D at 600 nm) of the culture was ~0.6. Kanamycin (Thermo Fisher Scientific, Waltham, MA, USA) was used as a selection marker. For the uniformly-^{15}N-labelled FBD, 25 mL of overnight culture grown in Terrific Broth (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 0.4 mM IPTG (Sigma-Aldrich). Buffers used for protein purification were cooled overnight at 4 °C. Cell pellets (at 1 g bacterial pellet/5 mL) were resuspended either in 50 mM Tris buffer or 50 potassium phosphate buffer (pH 7.4) containing 100 mM NaCl (Fisher Scientific) and cOmplete™ protease inhibitors (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 18,000 rpm, washed with the same buffer containing 500 mM NaCl (high-salt buffer), and repeated centrifugation. The membrane fragments were resuspended (to form liposomes) in either 50 mM Tris buffer or 50 potassium phosphate buffer (pH 7.4) containing 100 mM NaCl (Fisher Scientific) and cOmplete™ protease inhibitor cocktail (Sigma-Aldrich) at ~20 mg/mL concentration. The pentyl-inulin pre-dissolved in the same buffer was added at 1:0.5 (w/w) membranes:polymer ratio. The solution was incubated at 4 °C overnight with slow mixing on the racker and centrifuged at 5,000 rpm for 45 min. The supernatant was incubated with the pre-equilibrated DEAE anion-exchange resin, whereas for affinity-based purification, the supernatant was incubated with Ni^{2+}-NTA beads (GE-Healthcare, Chicago, USA) for 2 hours at 4 °C. Unwanted proteins from Ni^{2+}-NTA

resin were washed out using several column volumes of buffer with and without 5-10 mM imidazole. A NaCl gradient (200 – 700 mM) was used to elute FBD from the DEAE anion-exchange resin, whereas 300 mM imidazole was used to elute FBD from Ni^{2+}-NTA affinity-resin. DEAE ion-exchange and Ni^{2+}-NTA-affinity chromatography purification steps were carried out manually using 50 mL centrifuge tubes.

Size-exclusion chromatography (SEC)
The protein fractions (FBD in native-nanodiscs) obtained from ion-exchange or affinity-purification methods were concentrated to 1 mL using an Amicon Centricon concentrator (10-kDa cut-off). The SEC column (10x300 Superdex 200, GE Healthcare, Chicago, USA) was washed with milli-Q water and equilibrated with 2 column volumes of 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl. The protein was purified using a flow rate of 0.75 mL/min. All the solutions used in purification were filtered and degassed using a 0.2 µm Amicon membrane filter and Ultrasonic Cleaner (Cole-Parmer, Chicago, USA) before using them.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein and polymers
The protein fractions collected at different purification steps were analyzed by SDS-PAGE (Bio-Rad, Hercules, CA, USA). A protein ladder (10 kDa – 250 kDa) from Bio-Rad was used as a molecular weight marker. Polymer stock solutions ([1] SMA25010 (~10-kDa), [2] SMA-EA (~2-kDa), [3] DIBMA (~12-kDa), and [4] pentyl-inulin (~3-kDa)) were prepared at 2.5 mg/mL concentration in 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl. 50 µg of each polymer was tested on an SDS-PAGE gel to see whether pentyl-polymer forms any smearing.

UV-visible spectroscopy
The absorption spectra were recorded on a UV/vis spectrometer (DeNovix DS-11+[M/C], Wilmington, DE, USA) using a 1-cm-light path quartz cuvette. The concentration of the oxidized FBD was determined using an extinction coefficient of 12.2 mM^{-1} cm^{-1} at 451 nm.

Transmission electron microscopy (TEM)
TEM images were obtained using a Technai® T-20® machine (FEI®, Netherlands) with an 80 kV operating voltage. A dilute sample of SEC purified protein was dropped on a carbon-coated copper grid and dried overnight at room temperature in a desiccator before collecting images. The diameter of nanodiscs was measured using ImageJ software (version 1.53e, NIH, USA).

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. The DLS data were collected on the SEC purified native-nanodiscs containing FBD in 25 mM sodium phosphate buffer pH 7.4.

Protein NMR spectroscopy
The protein NMR measurements were performed on a 75 µM FBD sample prepared in 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl and 5 % $^{2}H_2O$ (Cambridge Isotope Laboratories [CIL], Inc., Andover, MA, USA). 1D ^{1}H and two-dimensional (2D) [^{15}N-^{1}H]-TROSY-HSQC NMR spectra were acquired at 25 and 32 °C on a Bruker 800 MHz NMR spectrometer equipped with a cryogenically cooled triple-resonance probe (Billerica, MA, USA). 1D ^{1}H and 2D [^{15}N-^{1}H]-TROSY-HSQC NMR spectra were recorded using 128 scans and 64 scans with 2048 (F2) and 200 (F1) increments, respectively. The NMR data were processed using Bruker TopSpin (4.0.6) and analyzed in CcpNmr Analysis (2.4.2). In addition, line-widths were measured in CcpNmr using the parabolic fit method.

Preparation of polymer-nanodiscs containing commercial *E. coli* lipids

E. coli lipid extract (in $CHCl_3$) was purchased from Avanti Polar Lipids (Alabaster, Alabama). The organic solvent was evaporated by applying a stream of low-pressure N_2-gas (takes 20-30 min) onto the lipid-solvents mixture. (*Caution*: N_2-gas at high pressure can spill the sample out of the centrifuge tube.) The dried lipid film was then incubated under vacuum for 1-2 hours to remove the residual solvents. Finally, the dried lipids were hydrated by resuspending them in 10 mM Tris (pH 7.4) containing 100 mM NaCl and by subjecting to freeze-thaw cycles (using liquid N_2 and hot water [~70 °C]) for three times. The lipids were mixed with polymer at a 1:1 (w/w) ratio and incubated at 4 °C overnight. The nanodiscs sample was centrifuged at 5000 rpm/45 min to remove insoluble material before purification. The polymer-nanodiscs were then purified by SEC using 10 mM Tris buffer (pH 7.4) containing 100 mM NaCl.

Lipid analysis by ^{31}P NMR spectroscopy

^{31}P NMR spectra were acquired on a Bruker 500 MHz NMR spectrometer equipped with a ^{1}H/^{31}P/^{2}H broad-band reverse probe operating at ambient temperature with a ^{31}P resonance frequency of 202 MHz. Spectra were recorded using broad-band proton decoupling using the WALTZ-16 during acquisition. The samples were prepared by adding 100 mM sodium cholate (Sigma-Aldrich) to the SEC purified nanodiscs. A reference spectrum was recorded on commercial *E. coli* lipids hydrated in $^{2}H_2O$ containing 100 mM sodium cholate for assignments. H_3PO_4 was used as an external reference (^{31}P peak frequency was set at 0 ppm) for calibrating ^{31}P NMR spectra. The percentage of each lipid type was estimated by measuring the peak areas (by peak integration) in each ^{31}P NMR spectrum using Topspin (Bruker NMR). Uncharacterized lipid peaks were not included in the data analysis.

References

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