Bioinspired, size-tunable self-assembly of polymer-lipid bilayer nanodiscs

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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~1600 g/mol, anhydrous N-Methyl-2-Pyrrolidone (NMP), 2-Aminoethanol (EA), Triethylamine (Et_3N), HEPES, phosphoric acid (H_3PO_4), acetic acid (HOAc), hydrochloric acid (HCl), sodium hydroxide (NaOH), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), and ytterbium chloride ($YbCl_3$) were purchased from Sigma-Aldrich®. All lipids used in this study 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (DMPE-rhodamine B) were purchased from Avanti Lipids Polar, Inc®. The ~15.7 kDa rabbit cytochrome b_5 was expressed in *E.coli* and purified as reported elsewhere [1].

SMA-EA copolymer synthesis: A 100 mg/mL solution of SMA was prepared in 50 ml by dissolving 5 g of SMA in 50 mL of anhydrous NMP. To this solution, an excess of 2 ml of EA was added. A total of 4.3 mL of Et_3N was added to the reaction, followed by incubation at 70 °C for a minimum of 2 hours, after which the polymer was precipitated by the addition of 0.1 M HCl. Then the polymer 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 for 2 hours. Then the 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. The SMA-EA polymer is used in this study as reported in the main text.

Reconstitution of ~15.7 kDa rabbit cytochrome-b_5 in macrodiscs for solid-state NMR experiments: 20 mg of SMA-EA was added to 20 mg of DMPC (10 mg/ml in 10 mM HEPES buffer), and the mixture was incubated for 30 min. The resulting mixture was subjected to 3 freeze-thaw cycles between liquid nitrogen and 50 °C. 1 ml of 200 μM (3.14 mg) of uniformly-^{15}N-labbeled-cytb$_5$ corresponds to 150:1 lipid to protein ratio was added to the macrodisc solution and incubated for 2 hours, followed by concentrating the total volume to 200 μl to give a highly viscous cytb$_5$ reconstituted macrodiscs. The degree of magnetic-alignment of the sample was measured using static ^{31}P NMR experiments and also examined by ^{14}N NMR experiments.

Reconstitution of ~15.7 kDa rabbit cytochrome-b_5 in nanodiscs for solution NMR experiments: 100 μM ^{15}N-labeled-Cytb$_5$ was incubated overnight with 1 mg DMPC and 3 mg of SMA-EA in 10 mM HEPES buffer at pH 7.4. The cytb$_5$ protein reconstituted nanodiscs sample was purified using size exclusion chromatography. The resulting sample was concentrated to give 100 μM cytb$_5$, and 10% D_2O was added.

Total Internal Reflection Fluorescence (TRIF) microscopy imaging: 17.5 μl of DMPE-rhodamine B stock solution (1 mg/ml) was mixed with 40 μl of DMPC in chloroform (25 mg/ml), dried under nitrogen gas to form a lipid film. Residual solvent was removed under high vacuum overnight. The lipidic film was hydrated with 1 ml of 10 mM HEPES buffer, pH 7.4. The resulting suspension was homogenized by vortex and three freeze-thaw cycles between liquid nitrogen and 50 °C. The resulting suspension was diluted 100 times to give a stock solution for visualizing under TIRF microscope. For TIRF microscopic experiments, a 10 μl of stock solution on a cover slip was placed under the microscope lens for visualization, and then 2 μl of 0.1 mg/ml polymer stock solution was added to it followed by the collection of TIRF images using an Olympus microscope.

Static Light Scattering (SLS) experiments: The time dependent solubilization of DMPC liposomes were monitored by the intensity of scattered light at 90^o angle by using Fluro Fluorimeter. 0.1 mg/ml DMPC sock 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 the SMA-EA polymer. Both excitation and emission wavelengths were set at 400 nm and the slit was set to 5 nm. All SLS experimental measurements were carried out using a FluoroMax 4® from Horiba Scientific®.

Fourier-Transform Infrared (FT-IR) Spectroscopy

The FT-IR spectra from 4000 cm^{-1} to 400 cm^{-1} were recorded using a Perkin-Elmer® Spectrum BX® spectrometer operating at room temperature and using the KBr pellet method.

Solid state NMR Spectroscopy

All solid-state NMR experiments were carried out on an Agilent/Varian 600 MHz solid-state NMR spectrometer using a 4 mm triple-resonance Magic Angle Spinning (MAS) probe under static sample conditions. The ^{1}H and ^{15}N resonance frequencies were 599.8 and 60.78 MHz respectively. Two-dimensional ^{1}H-^{15}N PISEMA (Polarization Inversion Spin Exchange at the Magic Angle) [2-4] spectra were obtained using the following parameters: 5μs 90° pulse, 25 t_1 increments, 7000 scans, 3s recycling delay, and 30 kHz ^{1}H decoupling. SPINAL-64 pulse sequence[5] was used to decouple protons during ^{15}N signal acquisition in the t_2 period. The ^{1}H decoupled ^{31}P NMR spectra were obtained to examine the magnetic alignment of macrodiscs using a single pulse experiment with a 4 μs 90^0 pulse and a 6s recycle delay. 64 scans, with a 20 ms acquisition time, were signal averaged to obtain each ^{31}P NMR spectrum. Continuous wave ^{1}H decoupling utilized a radio frequency field strength of 42 kHz during the acquisition period was applied. ^{31}P NMR spectra were referenced by setting the ^{31}P chemical shift of 100 % H_3PO_4 sample to 0 ppm.

^{31}P and ^{14}N NMR spectra were acquired using an Agilent/Varian 400 MHz solid-state NMR spectrometer using a 5 mm triple-resonance and double-resonance probes respectively. One-dimensional ^{1}H decoupled ^{31}P NMR spectra of flipped macrodiscs (by the addition of 0.5 mM, 1mM, 1.5mM or 2mM of $YbCl_3$ salt to macrodiscs) were acquired using the following parameters: 5 μs 90^0 pulse, 30 kHz ^{1}H CW decoupling, 64 scans, and a 6 s recycle delay. One-dimensional ^{14}N NMR spectra were recorded using the quadrupole-echo pulse sequence[6] with a pulse length of 4.5 μs and an echo-delay of 1.2 ms. ^{14}N signal was acquired using 30ms acquisition time, 4000 scans and a recycle delay of 0.2 s with no ^{1}H decoupling. ^{13}C CPMAS experiments on SMA and SMA-EA polymers were carried out on a Bruker 500 MHz solid-state NMR spectrometer under 8 kHz MAS using a 3.3 mm triple-resonance MAS probe operating at 500 MHz and 125.721 MHz for ^{1}H and ^{13}C respectively. Experimental parameters include 3 μs 90° pulse, 2 ms contact time of ramped-CP, 30 ms acquisition time, 64 scans, and a recycle delay of 4 s.

Solution NMR experiments

^{1}H-^{15}N Heteronuclear Single Quantum Coherence Transverse Relaxation-Optimized Spectroscopy (HSQC-TROSY): 100 μM ^{15}N-labeled-Cytb$_5$ was incubated overnight with 1 mg DMPC and 3 mg of SMA-EA in 10mM HEPES buffer at pH 7.4. The cytb$_5$ protein reconstituted nanodiscs sample was purified using size exclusion chromatography. The resulting sample was reconcentrated to give 100 μM cytb$_5$, and 10% D_2O was added. Two-dimensional ^{15}N/^{1}H TROSY HSQC[7] 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 was processed using TopSpin 2.0 (Bruker).

2D ^{1}H-^{1}H Nuclear Overhauser Effect Spectroscopy (NOESY): 5 mg of DMPC: SMAEA (1:3 w/w) lyophilized nanodisc sample was dissolved in 500 μl of 10 mM potassium phosphate buffer (pH 7.4). 10% D_2O was added. ^{1}H-^{1}H NOESY experiment was carried out on a Bruker Avance II 600 MHz NMR spectrometer equipped with a cryoprobe. Spectra were obtained using 64 scans and 256 t_1 increments, 200 ms mixing time, 0.25 sec acquisition time and 1 sec recycle delay. Data was processed using TopSpin 2.0 (Bruker).

Examining the solubilization of DMPC liposomes by ^{31}P NMR: 2 mg of DMPC dispersed in 10mM HEPES buffer was treated with different concentrations of SMA-EA and 10% D2O was added. The total volume of the sample was 600 μl. The samples were incubated at 35° C for overnight. NMR measurements were done using a triple-resonance broadband BBI probe on a Bruker 500 MHz NMR spectrometer operating at a ^{31}P resonance frequency of 202 MHz, 512 scans were acquired with an inverse-gated ^{1}H decoupling pulse sequence, an acquisition time of 0.5 s, 12175 Hz spectral width and a recycle delay of 3 s. Chemical shifts were referenced to H_3PO_4 in D_2O as an external standard. A 10 Hz line broadening was used in data processing.

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 10 mM HEPES buffer at a flow rate of 0.5 mL/min. Detection was done by collecting the absorbance at λ = 214 nm.

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

Transmission Electron Microscopy (TEM): The TEM micrographs were obtained using a Technai® T-20® machine (FEI®, Netherlands) with 80 kV operating voltage. A dilute solution was dropped on the carbon-coated copper grid and dried overnight at room temperature in a desiccator before using in the experiments.

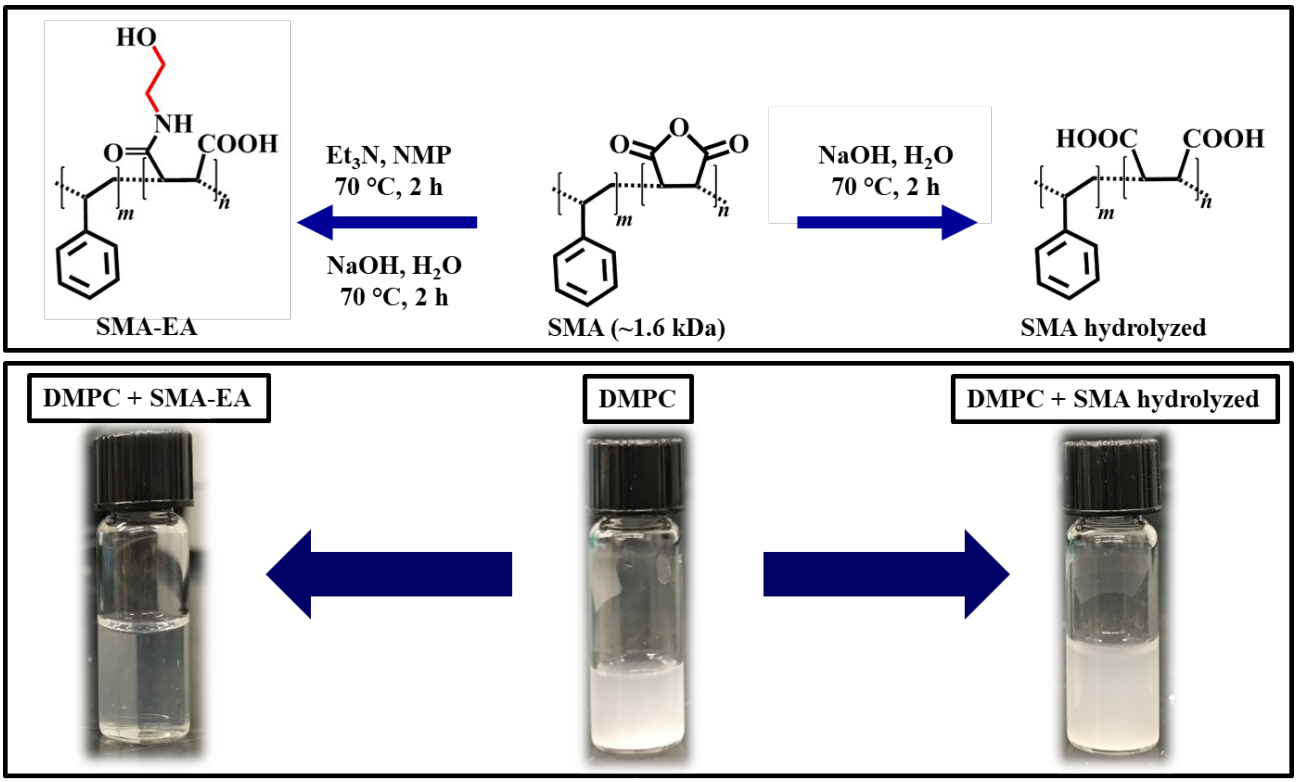

Figure S1. Chemical reaction showing the hydrolysis of the commercially obtained ~1.6 kDa SMA polymer. The photographs showing the inability to form a clear solution upon the addition of hydrolyzed SMA polymer.

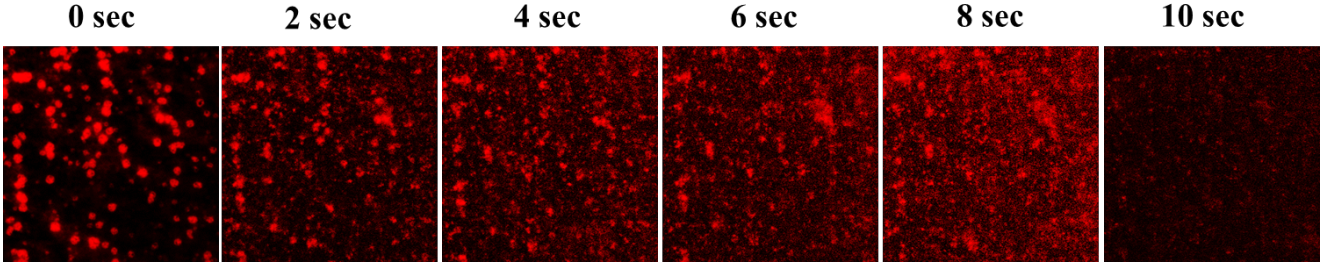

Figure S2. Total internal reflection fluorescence (TRIF) microscopy images of DMPC multilamellar vesicles (MLVs) containing DMPE-rhodamine B as a function of time after the addition of the SMA-EA polymer. At time=0, MLVs are visible, whereas the addition of SMA-EA polymer solubilizes MLVs as indicated by the disappearance of intense fluorescence signal as a function of time. This process solubilization of DMPC MLVs by the SMA-EA polymer can be better visualized in the included video.

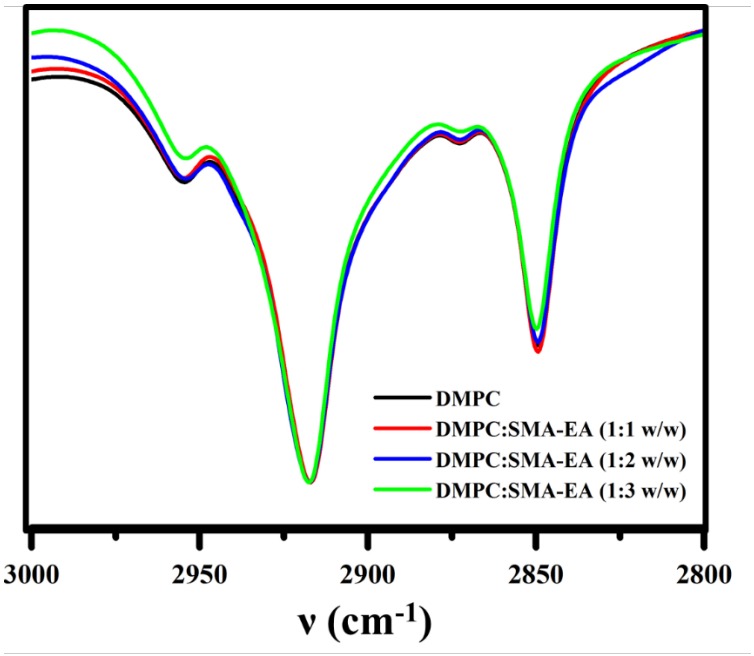

Figure S3. CH stretching region of FT-IR spectra for DMPC lipids annulated in SMA-EA polymer based nanodiscs at the indicated lipid:polymer ratio.

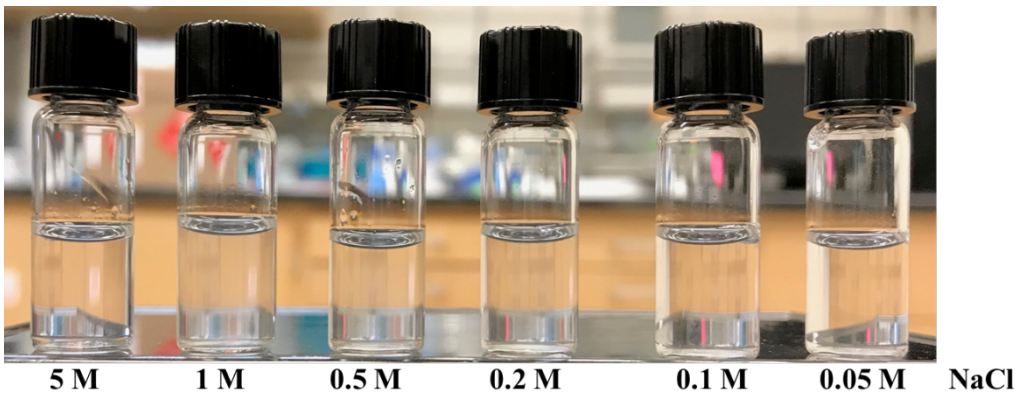

Figure S4. The photographs showing the stability of nanodiscs in different ionic strength buffer. 1 mg of lyophilized nanodisc (DMPC:SMAEA; 1:1 w/w) was dissolved in 10 mM HEPES buffer containing different concentration of NaCl at pH 7.4.

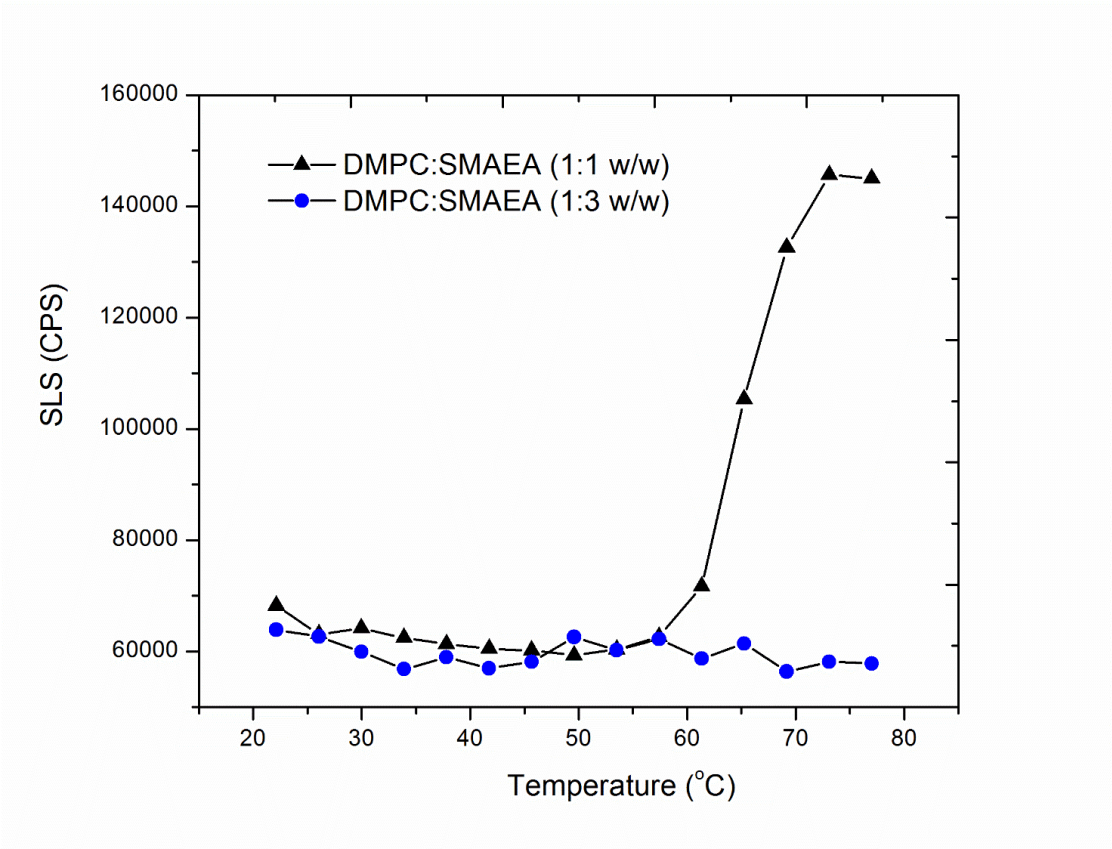

Figure S5. Thermal stability of nanodiscs: Static light scattering profiles of nanodisc at different temperatures showing that these macro-nanodisc are stable up to ~60 ^{o}C and nanodisc are stable up to 80 ^{o}C; 1 mg/ml of DMPC, 10 mM HEPES pH 7.4.

Effect of pH on SMA-EA nanodiscs: Since SMA-EA polymer contains acidic groups and pH plays an important role in biochemical assays, we titrated a 0.5 mg/mL solution of DMPC:SMA-EA nanodiscs in water with 2 M HCl. SMA-EA polymer was found to be soluble in basic solutions and the lowest pH condition usable to run experiments using the SMA-EA nanodiscs without any instability determined from experiments is shown in Figures S6.

The lipid:polymer ratio plays a role in the precipitation due to low pH condition or the presence of calcium and magnesium ions (Shown in Figure S6). Considering 1:1 lipid:polymer ratio as reference, our SMA-EA polymer based nanodiscs were found to be stable up to a pH value of 4.5 and in the

presence of 10 mM or larger concentrations of Ca^{2+} and Mg^{2+} ions. Each titration has been executed by adding 1 µL of the titrant solution to each sample solution. Instrumental condition used were the same as those for the static light scattering (SLS) experiments.

Effect of divalent metal ions on SMA-EA nanodiscs: Studies on previously reported Styrene:Maleic Acid copolymer based (SMA-derived) nanodiscs have been shown to be unstable and precipitate in the presence of low concentration of divalent metal ions (such as Ca^{2+} or Mg^{2+}) or low pH.[8] This property could affect several biochemical protein assays that involve any of these two divalent metals. In this study, we demonstrate that by reducing the number of free carboxylic group, the SMA-EA polymer based nanodiscs/macrodiscs render stability against the presence of divalent metal ions. To examine the stability of the newly developed nanodiscs, we titrated a 1 mg/mL solution of DMPC:SMA-EA nanodiscs in 10 mM HEPES buffer at pH = 7.4 with 4 M $CaCl_2$ or $MgCl_2$. Results obtained from an average of three independent measurements by fitting the observed data using a sigmoidal curve are shown in Figure S6.

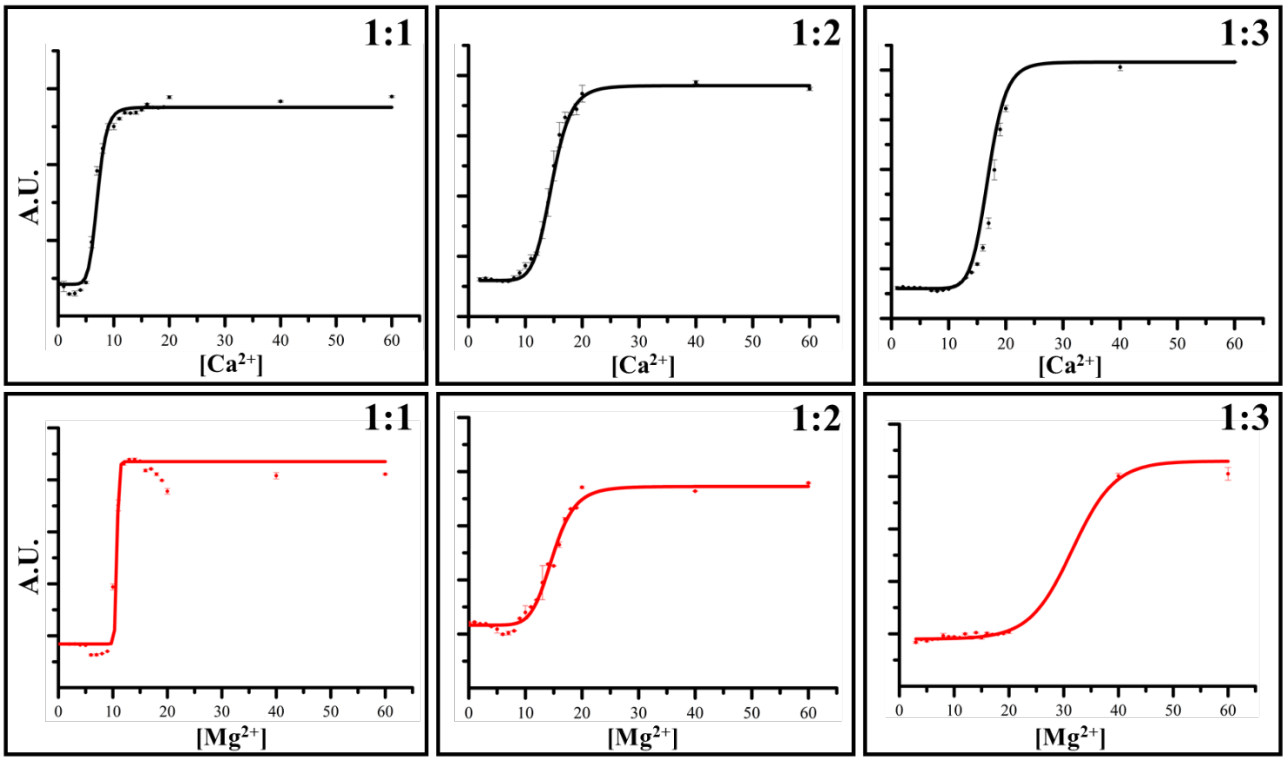

Figure S6. Static light scattering profiles of the indicated SMA-EA nanodiscs in the presence of divalent ions: Ca^{2+} (top row) or Mg^{2+} (bottom row); DMPC:SMA-EA (w/w) ratio is indicated. The experimentally measured data were fit into sigmoidal curves to obtain the minimum concentration of the salt required to destabilize the nanodiscs.

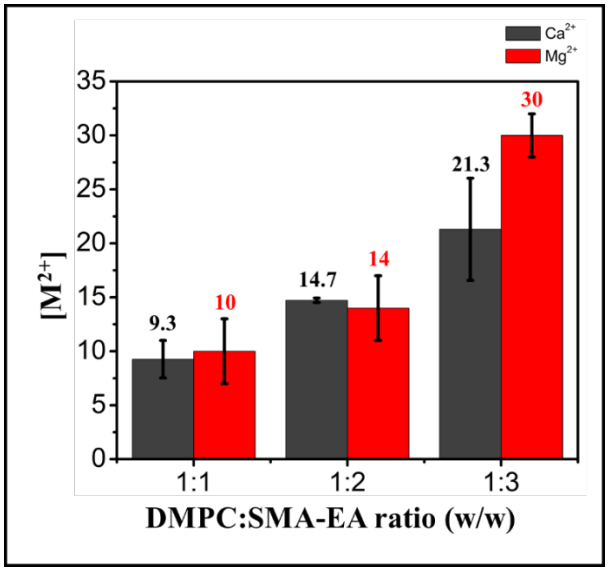

Figure S7. Highest concentration of divalent metal ions for which precipitation of SMA-EA polymer based DMPC lipid nanodiscs were observed using SLS experiments.

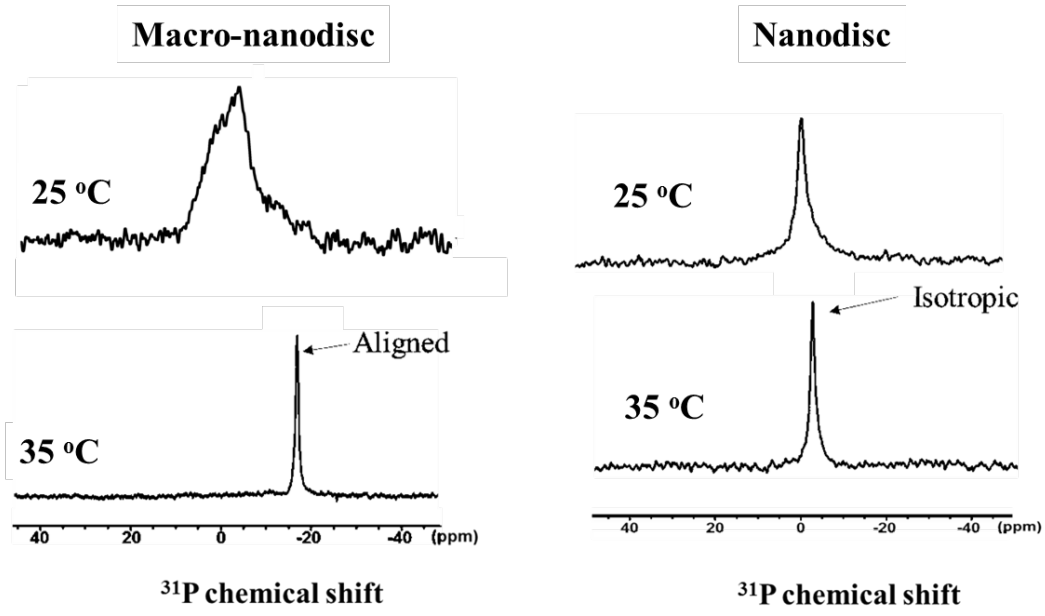

Figure S8. Static ^{31}P NMR spectra of DMPC:SMA-EA for lipid to polymer ratio of 1:1 w/w macrodiscs (left panel) and 1:3 w/w nanodiscs (right panel) obtained at the indicated temperature. As indicated, under fluid lamellar phase condition the isotropic nanodiscs (right panel) or magnetically-aligned macrodiscs (left panel) were observed. The ^{31}P chemical shift value for aligned macrodiscs confirms that the lipid bilayer in macrodiscs are aligned with their bilayer normal perpendicular to the direction of the external magnetic field of the NMR spectrometer. NMR studies on magnetically-aligned bicelles can found in the literature[8-13].

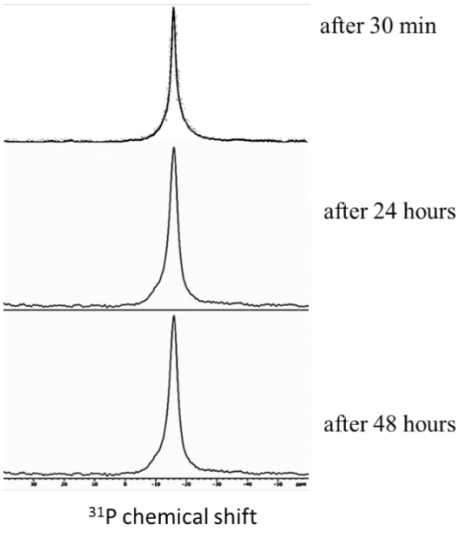

Figure S9. Stability of DMPC macrodiscs under magnetic-alignment condition. Static ^{31}P NMR spectra of DMPC:SMA-EA (1:1 w/w) macrodiscs measured as a function of time at 35 °C.

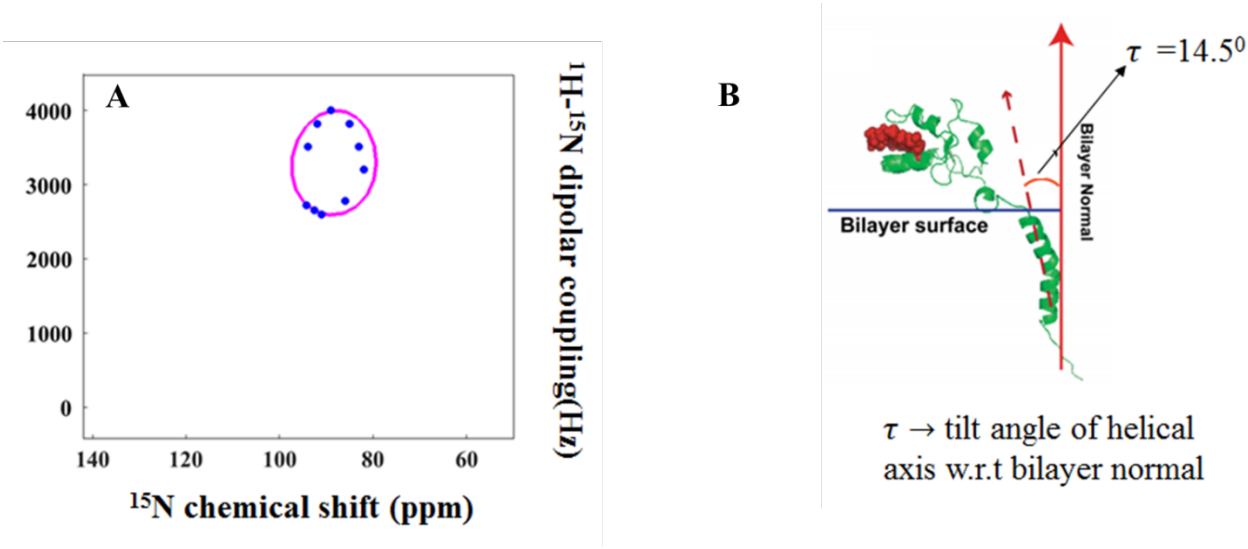

Figure S10. A) Simulated helical wheel (pink circle) of experimentally measured parameters (blue points). 2D PISEMA experiments were performed on magnetically-aligned macrodiscs of 1:1 DMPC:SMA-EA as mentioned in the main text and in the Experimental details above. ^{15}N anisotropic chemical shift and ^{1}H-^{15}N dipolar coupling values (represented as blue points in (A) were obtained from the 2D ^{1}H-^{15}N-PISEMA spectrum of ^{15}N-cytb$_5$ reconstituted in DMPC:SMA-EA macrodiscs (Figure 4H). B) A schematic representation of the transmembrane helix of cytb$_5$ that is tiled by 14.5° away from the lipid bilayer normal. PISEMA NMR spectroscopy has been used in the structural studies of a variety of membrane proteins reconstituted in aligned lipid bilayers.[4.12,14-19]

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