Supporting information

An adaptable phospholipid membrane mimetic system for solution NMR studies of membrane proteins

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Expression and purification of Saposin-A

Saposin-A (SapA) was expressed using *E. coli* shuffle-T7 cells. Cells were grown at 30 ˚C in LB supplemented with 100 µg/mL ampicillin. Cells were induced with 1 mM IPTG at O.D. 0.6–0.8, and incubated overnight at 16 ˚C. The cells were harvested by centrifugation (5,000 g, 30 min). Cell pellets were resuspended in anion-exchange buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) with protease inhibitor cocktail (Sigma-Aldrich) and lysed by sonication. Soluble proteins were separated by centrifugation (40,000 g, 30 min, 4 ˚C). The supernatant was incubated at 85 ˚C for 15 min to denature other proteins. Precipitated proteins were pelleted by centrifugation (40,000 g, 30 min, 4 ˚C) and discarded. The anion-exchange purification was performed using a 5 mL HiTrap QSepharose column (GE Healthcare) equilibrated with anion-exchange buffer. Elution used a 0–50% linear gradient of elution buffer (50 mM Tris-HCl, pH 7.4, 1 M NaCl). Fractions were checked by SDS-PAGE and those containing SapA were pooled and concentrated using Amicon Ultra-15 (3,000 MWCO). The sample was further purified by gel-filtration using a HiPrep 16/60 Sephacryl S-200 (GE Healthcare)

Expression and purification of pSRII, OmpX, and β_1AR

pSRII and OmpX were expressed and purified as reported previously.[1,2] The sequence of the thermostabilised turkey β_1AR construct used in this study is:

MGAELLSQQWEAGMSLLMALVVLLIVAGNVLVIAAIGSTQRLQTLTNLFITSLACA
DLVMGLLVVPFGATLVVRGTWLWGSFLCELWTSLDVLCVTASIWTLCVIAIDRYLAI
TSPFRYQSLMTRARAKVIICTVWAISALVSFLPIMMHWWRDEDPQALKCYQDPGCC
DFVTNRAYAIASSIISFYIPLLIMIFVYLRVYREAKEQIRKIDRASKRKTSRVMAMREH
KALKTLGIIMGVFTLCWLPFFLVNIVNVFNRDLVPKWLFVAFNWLGYANSAMNPIIY
CRSPDFRKAFKRLLAFPRKADRRLHHHHHHHH

For β_1AR expression, Sf9 cells, grown in serum free SF4 media were centrifuged (500 g, 10 min) and washed with sterile PBS, to reduce the carry-over of unlabelled methionine. The washed cells were diluted to a density of $3x10^6$ cells/mL into methionine deficient SF4 media at half the intended final culture volume. The culture was then infected with 4 mL/L of high density viral stock, and incubated for 5 h, before supplementing the culture with 250 mg/L of ^{13}C-methyl methionine and diluting to a final density of $1.5x10^6$ cells/mL. The initial reduction in culture volume ensures optimal aeration in the initial phase of the viral infection. Cells were grown at 27 °C for 48 h and were harvested by centrifugation (3,500 g, 15 min).

The frozen insect cell pellet was thawed with solubilisation buffer (20 mM Tris-HCl pH 8.0, 350 mM NaCl, 3 mM imidazole, Complete Protease Inhibitor Cocktail (Roche), 1% DDM) and stirred for 1 h. The solubilised cells were clarified by centrifugation (175,000 g, 45 min) and the soluble fraction was loaded onto a nickel affinity column. The column was washed with equilibration buffer (20 mM Tris-HCl pH 8.0, 350 mM NaCl, 3 mM Imidazole, 0.06% DDM) and the protein was eluted with the same buffer

supplemented with 250 mM imidazole. The final sample was exchanged into 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.06% DDM.

Generation of Salipro nanoparticles

Lipid stock solution was prepared by dissolving an appropriate amount of dimyristoylphosphatidylcholine (DMPC) powder (Avanti Polar Lipids) in chloroform and evaporating the solvent under nitrogen gas. Dry lipids were resuspended in buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), vortexed, and sonicated to obtain a final 10 mM lipid stock solution (the solution remained cloudy because of the low solubility of DMPC). Nanoparticle assembly involved mixing lipid stock solution, SapA, and buffer at the desired pH (pH 4.8: 50 mM sodium acetate, 150 mM NaCl. pH 6: 50 mM sodium phosphate, 150 mM NaCl. pH 7.4: 50 mM Tris-HCl, 150 mM NaCl) to make a 500 µL mixture with a final SapA concentration of 120 µM. The final concentration of DMPC was in a range of 600 µM to 3.6 mM depending on the SapA:DMPC ratio used in the experiments. The mixture was then incubated at 37 ˚C for 10 min, followed by room temperature for 5 min. The sample was centrifuged to remove precipitate (21,130 g, 5 min) before analysing by size-exclusion chromatography using a Superdex 200 Increase 10/300 (GE Healthcare) equilibrated with the buffer used in the assembly mixture.

For Salipro nanoparticle formation in the presence of DDM (pH 6 or pH 7.4), an additional 0.2% DDM was included in the initial assembly mixture, and the cloudy solution due to insoluble DMPC turned clear immediately. The same incubation temperature and time was used as for the lower pH. The detergent removal process used 400 mg of washed Amberlite XAD2 beads (Sigma-Aldrich) per 500 µL sample and was performed at 25 ˚C for 2 h with gentle shaking. Beads were removed by centrifugation (2,000 g, 10 min).

Generation of mixed lipid SapA-DMPC/DMPG (dimyristoylphosphatidylglycerol) nanoparticles followed the same protocol as described above in the presence of DDM. 10 mM of mixed lipid stock solution (7.5 mM DMPC and 2.5 mM DMPG) was used to make the assembly mixture.

Quantification of SapA:DMPC ratio in empty Salipro nanoparticles using molybdate assay and 1D ^1H NMR spectroscopy

The concentration of SapA was determined by the absorbance at 280 nm using an extinction coefficient of 8855 $M^{-1}cm^{-1}$. To quantify the DMPC concentration, we used a molybdate assay described previously[3] to determine the phosphate concentration in the sample. Briefly, a 5 µL sample was mixed with 30 µL of 10% magnesium nitrate followed by ashing over a strong flame. The powder was dissolved in 350 µL of 0.5 N HCl, and 300 µL of the sample was transferred to a 1.5 mL Eppendorf tube and boiled for 15 min. After the sample cooled down to room temperature, 700 µL of the ascorbic acid/ammonium molybdate solution was added and incubated at 45 ˚C for 25 min. The concentration of phosphomolybdate complex was measured by absorbance at 825 nm. A standard curve was generated using DMPC solutions with known concentration. The SapA-DMPC nanoparticles were assembled in the presence of DDM at pH 6 followed by the detergent removal process as decribed above. The pH 6 buffer used in this experiment was 50 mM sodium acetate instead of 50 mM sodium phosphate to avoid high phosphate noise from the buffer.

Membrane protein incorporation into SapA-DMPC nanoparticles

Membrane protein incorporation used a similar protocol to making SapA-DMPC nanoparticles in the presence of DDM. The only difference was including detergent solubilised protein (pSRII in 0.1% DDM; OmpX in 0.5% DPC; β_1AR in 0.1% DDM) in the initial assembly mixture. Large-scale preparation for NMR experiments were performed

using the same setup in multiple Eppendorfs and a concentration step using an Amicon Ultra-15 (3,000 MWCO) was added before gel-filtration. The incorporation of pSRII and OmpX was done at pH 6 with the SapA:DMPC:MP ratio of 10:50:1 with a final membrane protein concentration of 12 μM. For β_1AR incorporation, pH 7.4 buffer was used because of the low pH tolerance of this protein. The SapA:DMPC:β_1AR ratio was 10:250:1 with a final β_1AR concentration of 12 μM. The same detergent removal process and size-exclusion chromatography were performed as described above. The fractions from the SEC were checked by SDS-PAGE, and the fractions containing membrane proteins embedded in Salipro nanoparticles were pooled and concentrated using an Amicon Ultra-15 (3,000 MWCO). The incorporation yield of pSRII was higher than 90% in general by comparing the characteristic absorbance at 498 nm before and after the incorporation. For OmpX and β_1AR, the incorporation yield was generally higher than 80%, estimated from absorbance at 280 nm with the contribution from SapA taken into account.

NMR experiments

NMR experiments were all recorded on a Bruker Avance AVIII 800 spectrometer (^{1}H frequency of 800 MHz) equipped with a 5 mm TXI HCN/z cryoprobe. All the spectra were processed with Azara (W. Boucher, unpublished) or NMRPipe[4] and analysed with SPARKY software.[5]

Spectra for OmpX and pSRII in pH 6 buffer (50 mM sodium phosphate, pH 6, 150 mM NaCl), were recorded at 318 K using a [^{1}H, ^{15}N]-SOFAST-TROSY experiment. The direct (^{1}H) dimension was acquired with 10,000 Hz spectral width and 1024 points, whereas 2,778 Hz spectral width and 128 points was used for the indirect dimension (^{15}N). Spectra were recorded with 3,078 scans, resulting in an experiment time of 36 h.

In the β_1AR experiment, 50 μM ^{13}C methyl-methionine labelled β_1AR in pH 7.4 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) was used, and 1 mM isoprenaline and 50 μM Nb80 were added sequentially into the sample to record NMR spectra. All the spectra were recorded at 308 K using a ^{13}C-SOFAST-HMQC experiment. The direct (^{1}H) dimension was acquired with 10,000 Hz spectral width and 1024 points. The indirect dimension (^{13}C) was acquired with 4,000 Hz spectral width and 200 points. Spectra were recorded with 128 scans, giving an acquisition time of 4 h.

Negative-stained electron microscopy

For the EM grid preparation, sample with a concentration of 10 μg/mL was applied to a glow discharged carbon-coated copper grid (400 mesh) (EMresolution) for 30 s. Washing was done with three drops of water before staining with 2% (w/v) uranyl acetate for 30 s. The grids were blotted dry and stored at room temperature. Negative-stain image data were collected with a FEI Tecnai F20 field emission gun transmission electron microscope at an accelerating voltage of 200 kV fitted with either a 1K × 1K or a 4K × 4K CCD camera at the Wolfson Electron Microscopy Suite, University of Cambridge.

Supplementary Tables

Table S1: Size determination of empty and occupied Salipro nanoparticles

	SEC			NMR$					
				translational diffusion#			rotational diffusion‡		
Salipro nanoparticle	V_{elu} (ml)	MW (kDa)	$2R_h$ (nm)	D_t * 10^{-10} (m^2/s)	$2R_h$ (nm)	MW (kDa)	τ_c (ns)	$2R_h$ (nm)	MW (kDa)
empty small (pH 6)	14.7	65	7	1.05	6.5	57			
empty large (pH 6)	12.9	171	9.3	0.75	9.4	165			
+ OmpX (pH 6)	14.1	84	7.6	0.90	7.6	84	28	7.6	87
+ pSRII (dimer) (pH 6)	12.2	200	9.7	0.71	9.6	190	57	9.6	191
empty small (pH 7.4)	13.9	91	7.8						
empty large (pH 7.4)	12.4	183	9.5						
+ β_1AR (pH 7.4)	12.8	156	9.1	0.76§	9.0	151			

$NMR measurements on empty nanoparticles and with OmpX and pSRII embedded were performed at 318 K, while β_1AR was measured at 308 K.
#Translational diffusion measurements were corrected for viscosity changes, relative to pure water.
§For comparison with the other measurements the translational diffusion coefficient was back-calculated to 318 K.
‡Rotational correlation times are based on 1D ^{15}N TRACT measurements (318 K) conducted on Salipro nanoparticles loaded with ^{15}N labelled OmpX or pSRII, respectively.

Table S2: Lipid content of Salipro nanoparticles and MSP1 nanodiscs

	empty#		OmpX#		pSRII (dimer) #		β_1AR#	
	MW	lipids$	MW	lipids$	MW	lipids$	MW	lipids$
Salipro small (pH 6)	61	25	85	31				
Salipro large (pH 6)	171	100			193	77		
Salipro small (pH 7.4)	91	40						
Salipro large (pH 7.4)	183	102					154	59
MSP1D1	156	80	168	76	164	46	173	64
MSP1D1ΔH5	106	50	114	44	109	13	118	31

$Lipid molecules per bilayer leaflet.
#For empty and protein-embedded Salipro nanoparticles the lipid content per bilayer leaflet was calculated from the average of the experimental weight estimates obtained in Table S1, assuming a MW ~ 0.67 kDa for DMPC and 9.2 kDa for SapA, 16.5 kDa for OmpX, 26.4 kDa for pSRII (monomer) and 38 kDa for β_1AR. For the empty MSP1-based nanodiscs, the number of lipid molecules per bilayer leaflet was taken from Hagn *et al.* 2013.[2] For the embedded nanodiscs the lipid content was calculated based on subtracting the area equivalent to the protein footprint πr^2 (r_{OmpX} = 1.1 nm, r_{psrII} 2.0 nm, r_{b1AR} = 2.05 nm) from the value of the empty disc.

Supplementary Figures

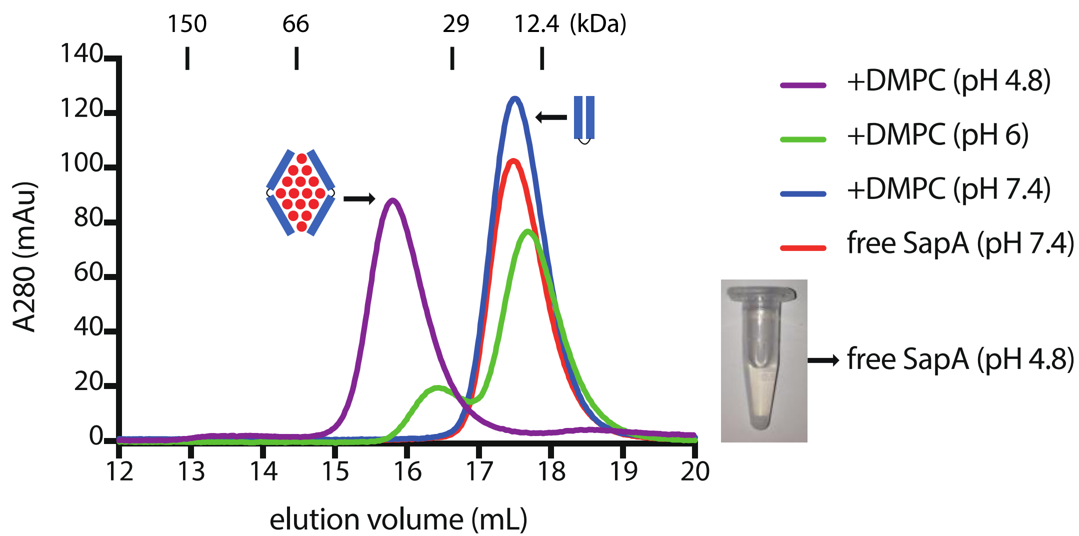


Figure S1. SEC chromatogram of SapA and SapA incubated with DMPC at various pHs. Free SapA in the absence of DMPC at pH 7.4 (red), after incubation with a 10-fold molar excess of DMPC at 37 ˚C for 10 min at pH 7.4 (blue), at pH 6 (green), and at pH 4.8 (purple). Molecular mass standards are indicated above the chromatogram in kilodaltons. The proposed SapA arrangement is indicated next to each peak. Most of the SapA remained in the closed conformation at pH 6 and pH 7.4, whereas a conversion to the open conformation was observed at pH 4.8. SapA is observed to be insoluble at pH 4.8 in the absence of DMPC (inset photograph).

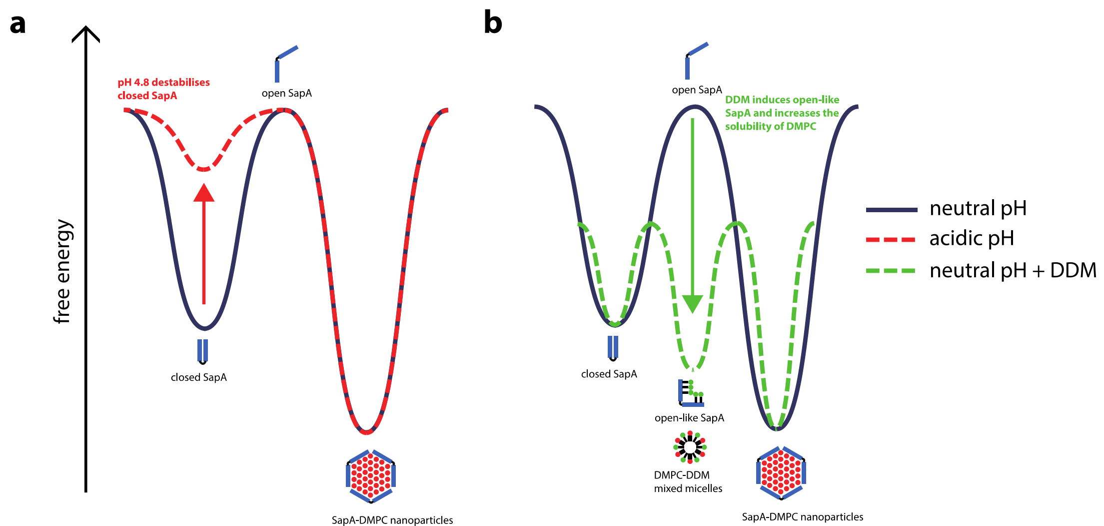


Figure S2. Proposed free energy diagram of SapA-DMPC nanoparticle formation process. The dark blue line shows two stable states: closed SapA and SapA-DMPC nanoparticles. The transition state between these two is the open SapA which is unstable without detergents or lipids. (a) The red dotted line shows that at pH 4.8 the closed form of SapA is destabilised, leading to a decrease in the kinetic barrier and nanoparticle formation with DMPC. (b) In the presence of DDM, the green dotted line shows the formation of a stable intermediate with SapA in the open form, maintained by interaction with DDM; hence DDM promotes Salipro nanoparticle formation. This intermediate state includes open-like SapA in complex with DDM and DMPC-DDM mixed micelles.

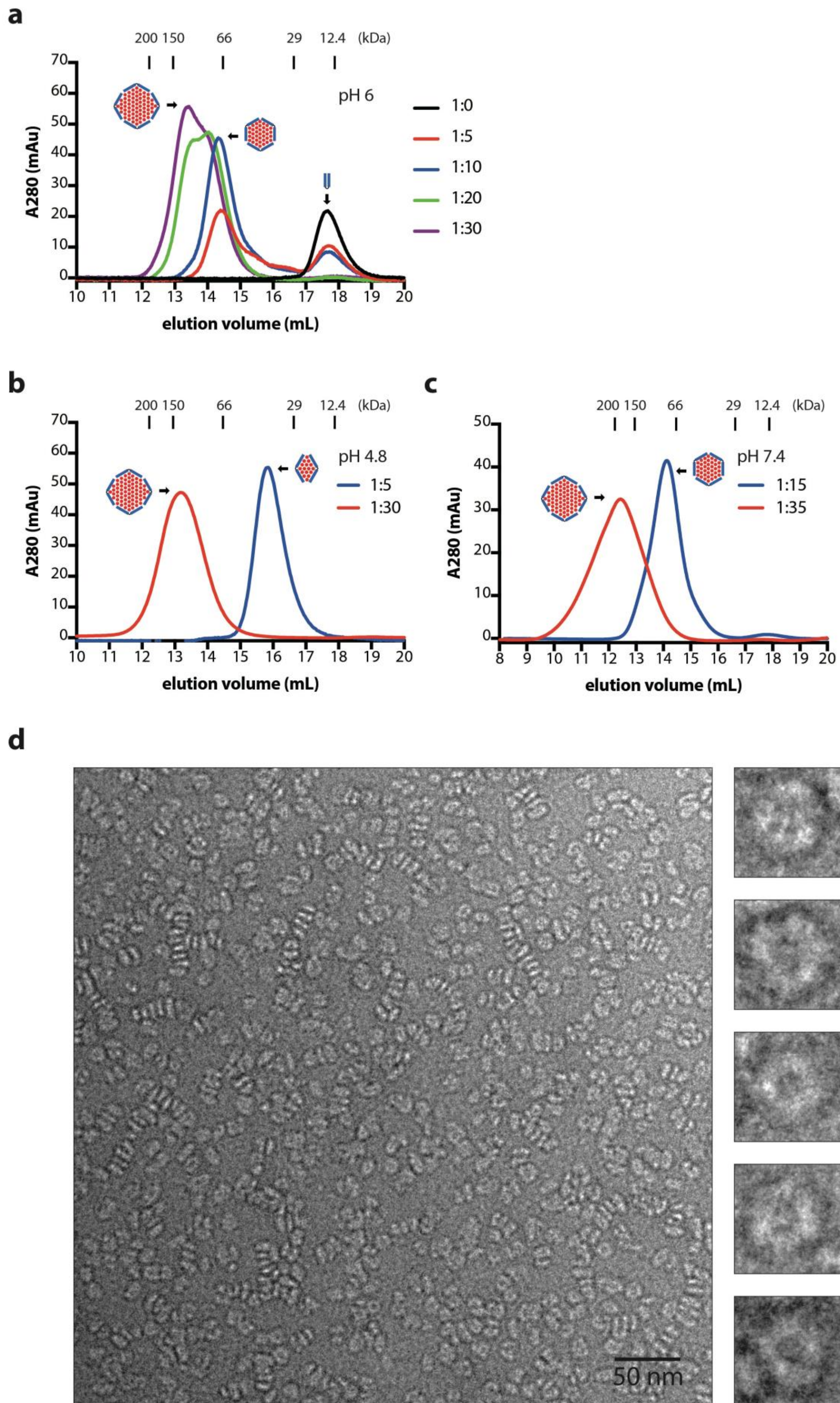

a
200 150 66 29 12.4 (kDa)
pH 6
1:0
1:5
1:10
1:20
1:30
A280 (mAu)
elution volume (mL)
b
200 150 66 29 12.4 (kDa)
pH 4.8
1:5
1:30
A280 (mAu)
elution volume (mL)
c
200 150 66 29 12.4 (kDa)
pH 7.4
1:15
1:35
A280 (mAu)
elution volume (mL)
d
50 nm

Figure S3. Size-exclusion chromatography (SEC) of SapA-DMPC generated in the presence of DDM followed by a detergent-removal procedure. (a) Screening procedure to optimize the SapA:DMPC ratio at pH 6: Various SapA:DMPC ratios were screened as indicated by the colour of the chromatograms. Two sizes of SapA-DMPC nanoparticles were formed for the range of ratios tested here. The results of the optimized ratios at pH 6, 1:15 and 1:45, are shown in Figure 2 in the main manuscript. SEC traces of the optimized nanoparticles are shown in (b) for pH 4.8 (1:5 and 1:30) and (c) for pH 7.4 (1:15 and 1:35). The proposed SapA arrangement is indicated for each peak in (a-c). The observation that Salipro nanoparticles are smaller in an acidic environment is consistent with Popovic et al. (2012).[6] (d) Negative-stain EM images of $Salipro_4$. Scale bar, 50 nm. The insets show top-down views of representative $Salipro_4$ nanoparticle images in a 20 nm box.

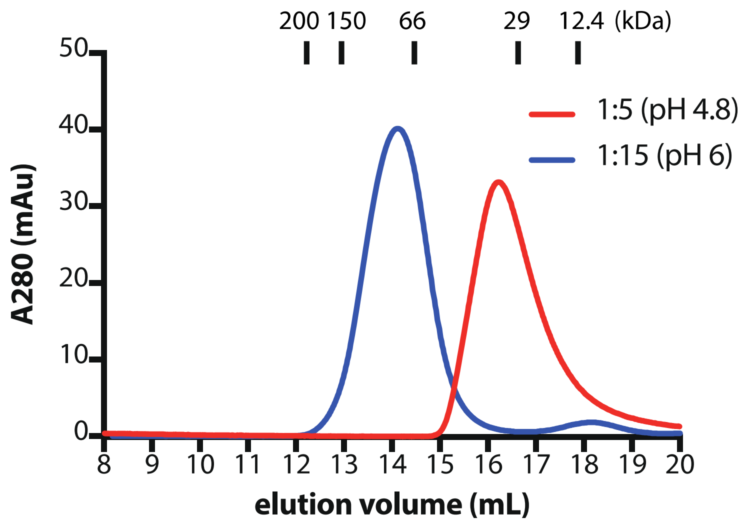


Figure S4. Size-exclusion chromatography of SapA-DMPC/DMPG nanoparticles at pH 4.8 and pH 6. Sample was prepared in the presence of DDM with a SapA:lipid (DMPC:DMPG = 3:1) ratio of 1:5 at pH 4.8 (red) and 1:15 at pH 6 (blue) followed by detergent removal.

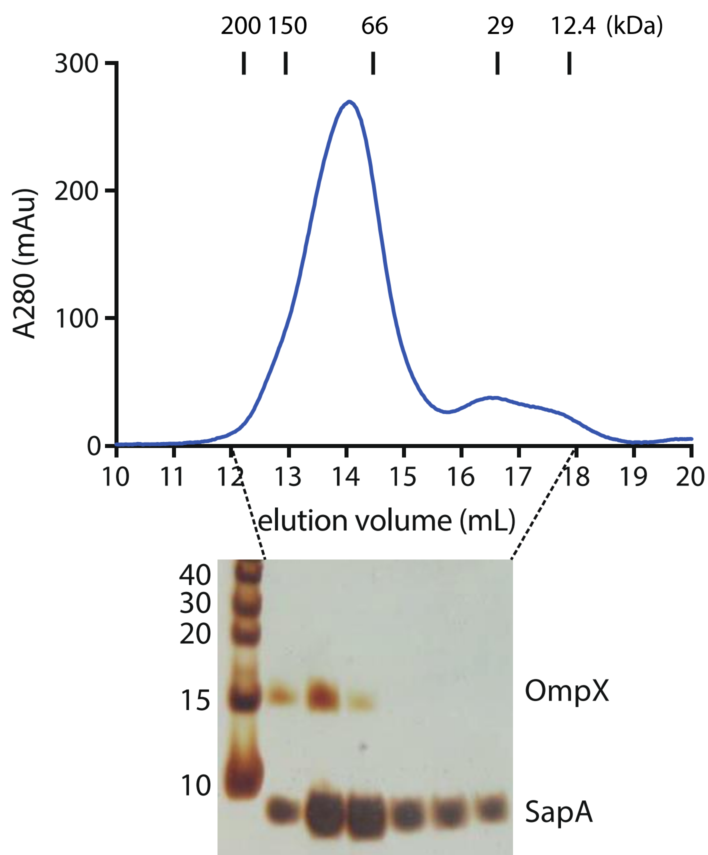
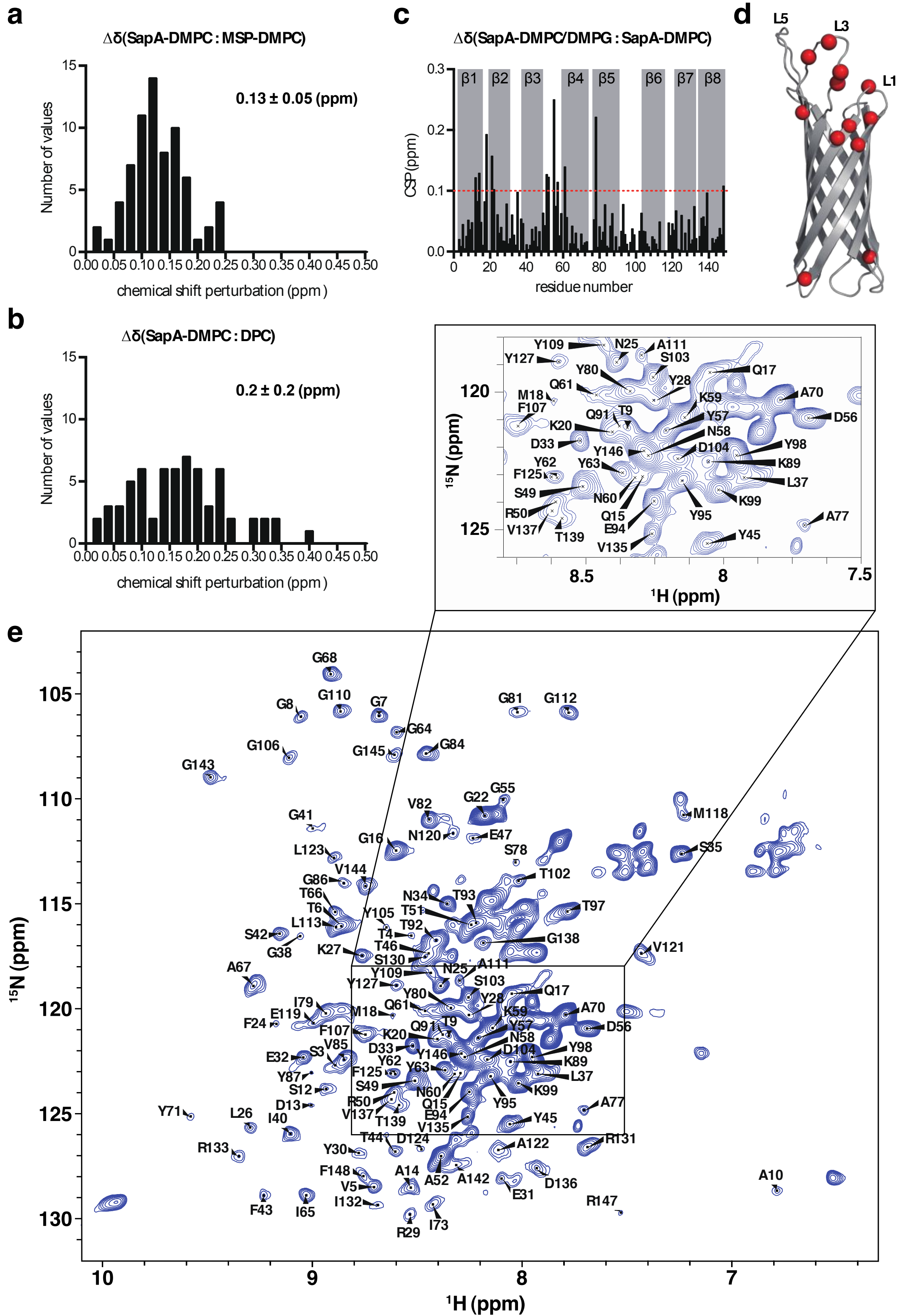


Figure S5. SEC of OmpX in SapA-DMPC nanoparticles with an SDS-PAGE analysis of the peak fractions. The fractions containing OmpX (12–15 mL) were pooled and concentrated for further investigation.


a
Δδ(SapA-DMPC : MSP-DMPC)
0.13 ± 0.05 (ppm)
Number of values
chemical shift perturbation (ppm)
b
Δδ(SapA-DMPC : DPC)
0.2 ± 0.2 (ppm)
Number of values
chemical shift perturbation (ppm)
c
Δδ(SapA-DMPC/DMPG : SapA-DMPC)
β1 β2 β3 β4 β5 β6 β7 β8
CSP (ppm)
residue number
d
L5
L3
L1
e
¹⁵N (ppm)
¹H (ppm)

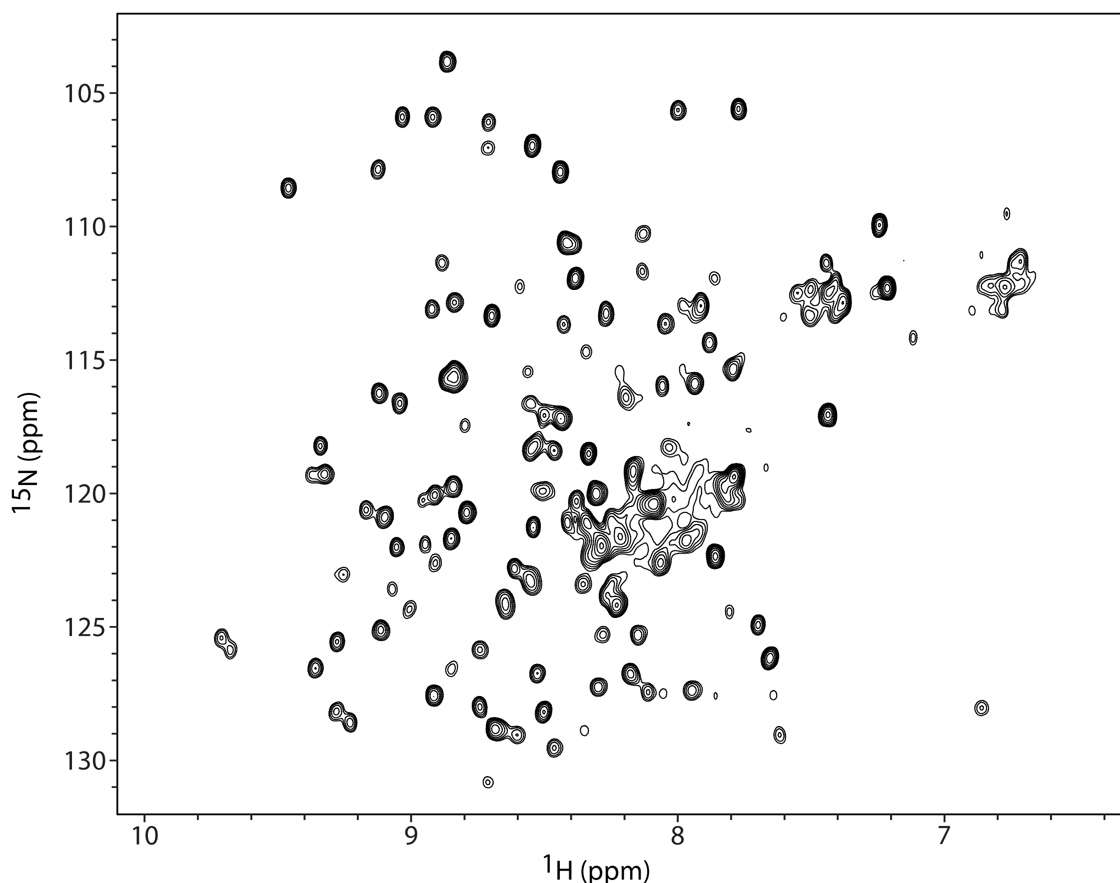


Figure S6. Comparison of backbone amide chemical shifts of OmpX in SapA-DMPC nanoparticles with OmpX solubilized in DPC micelles or in MSP1D1Δ5 nanodiscs. Histograms of chemical shift differences of 70 well resolved signals between OmpX in SapA-DMPC nanoparticles and (a) MSP1D1Δ5-DMPC nanodiscs (assignments kindly provided by Dr. Stefan Bibow)[7] and (b) DPC micelles (BMRB:18796).[2] The mean value and the standard deviation are shown in the histograms. The smaller chemical shift perturbations confirm that OmpX in SapA-DMPC nanoparticles is in a similar lipid bilayer environment to OmpX in MSP nanodiscs. (c) Chemical shift perturbation (CSP) between OmpX in SapA-DMPC nanoparticles and OmpX in SapA-DMPC/DMPG nanoparticles. Secondary structure is shown with grey bars indicating the β-sheet regions. (d) Red spheres on the OmpX structure (PDB:2M06)[2] show the residues with chemical shift perturbations higher than 0.1 ppm in (c). As the residues with the most significant CSP mainly reside in loops 1 and 3, this result indicates that the lipid head-group composition has a strong influence on specific regions of the membrane protein. (e) Enlarged size of the same 2D [^{1}H,^{15}N]-SOFAST-TROSY spectrum of non-deuterated ^{15}N-labelled OmpX in SapA-DMPC nanoparticles shown in Figure 3. The insert shows an expansion of the more overlapped center area of the spectrum. In this overlapped area of the spectrum in the absence of comprehensive sequential backbone connectivity the transfer of peak assignments based on proximity can result in errors. Accordingly, chemical shift analysis in (a) to (d) only rely on well resolved, unambiguously assigned signals. (f) 2D [^{1}H,^{15}N]-SOFAST-TROSY spectrum of non-deuterated ^{15}N-labelled OmpX in DPC, recorded at 800 MHz (^{1}H frequency) and 308 K.

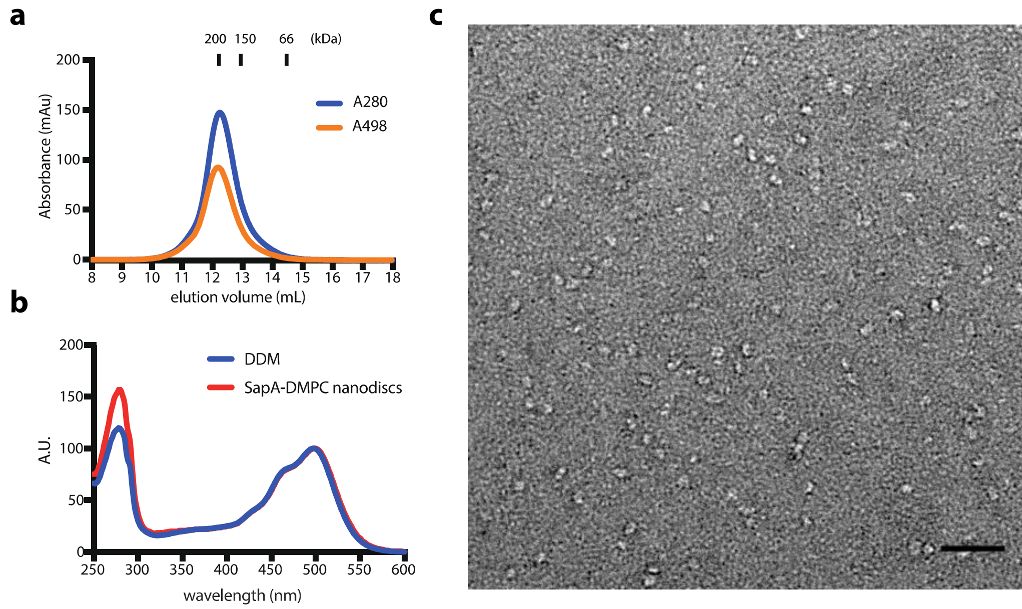


Figure S7. Biophysical characterization of pSRII in SapA-DMPC nanoparticles. (a) SEC of pSRII in SapA-DMPC nanoparticles. Absorbance at 498 nm (orange) was used to indicate the presence of correctly-folded pSRII. (b) Overlay of UV-Vis spectra of pSRII in DDM (blue) and in SapA-DMPC nanoparticles (red). The higher absorbance at 280 nm in SapA-DMPC nanoparticles originates from SapA scaffold proteins. (c) Negative-stain electron microscopy images of pSRII in SapA-DMPC nanoparticles. Scale bar, 50 nm.

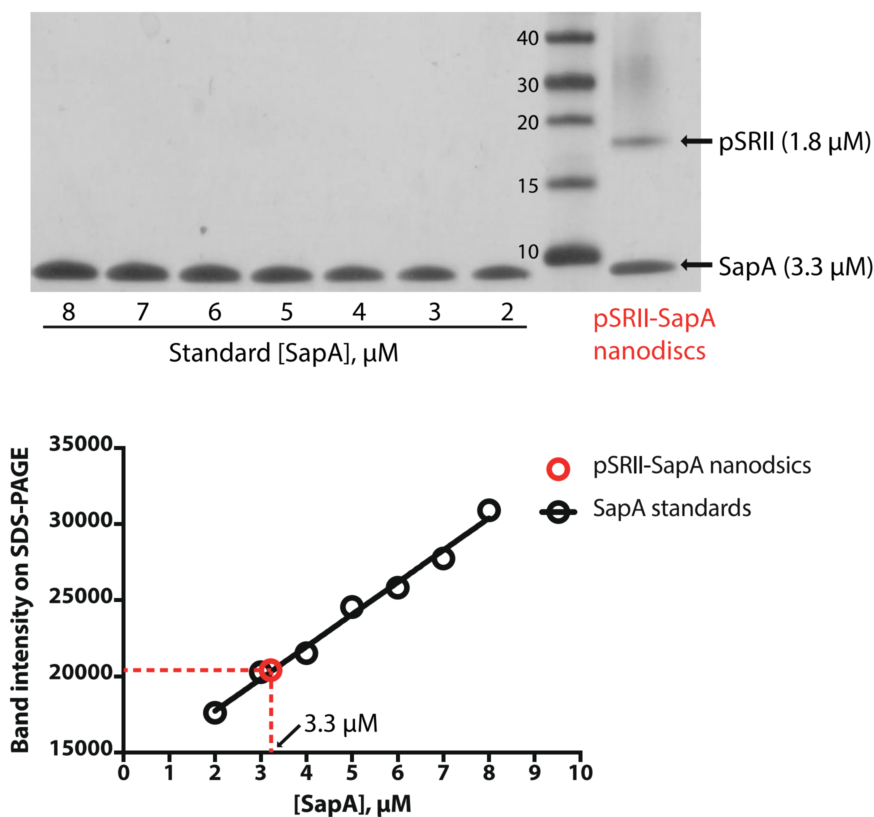


Figure S8. Determination of the SapA:pSRII ratio for pSRII in SapA-DMPC nanoparticles. Using the 498 nm absorption, which detects the bound retinal chromophore, and the extinction coefficient of 48000 $M^{-1}cm^{-1}$, the concentration of pSRII in the sample was determined to be 1.8 μM. The concentration of SapA in the sample was determined by comparing the band intensity to SapA standards with known concentration via SDS-PAGE (upper panel). The band intensity of the SapA standards was quantified using ImageJ,[8] plotted against the SapA concentration in black open circles, and fitted by linear regression (bottom panel). The SapA concentration in the pSRII-Salipro nanoparticles sample was calculated to be 3.3 μM using the fitted result. This result shows that the SapA:pSRII ratio is 2:1.

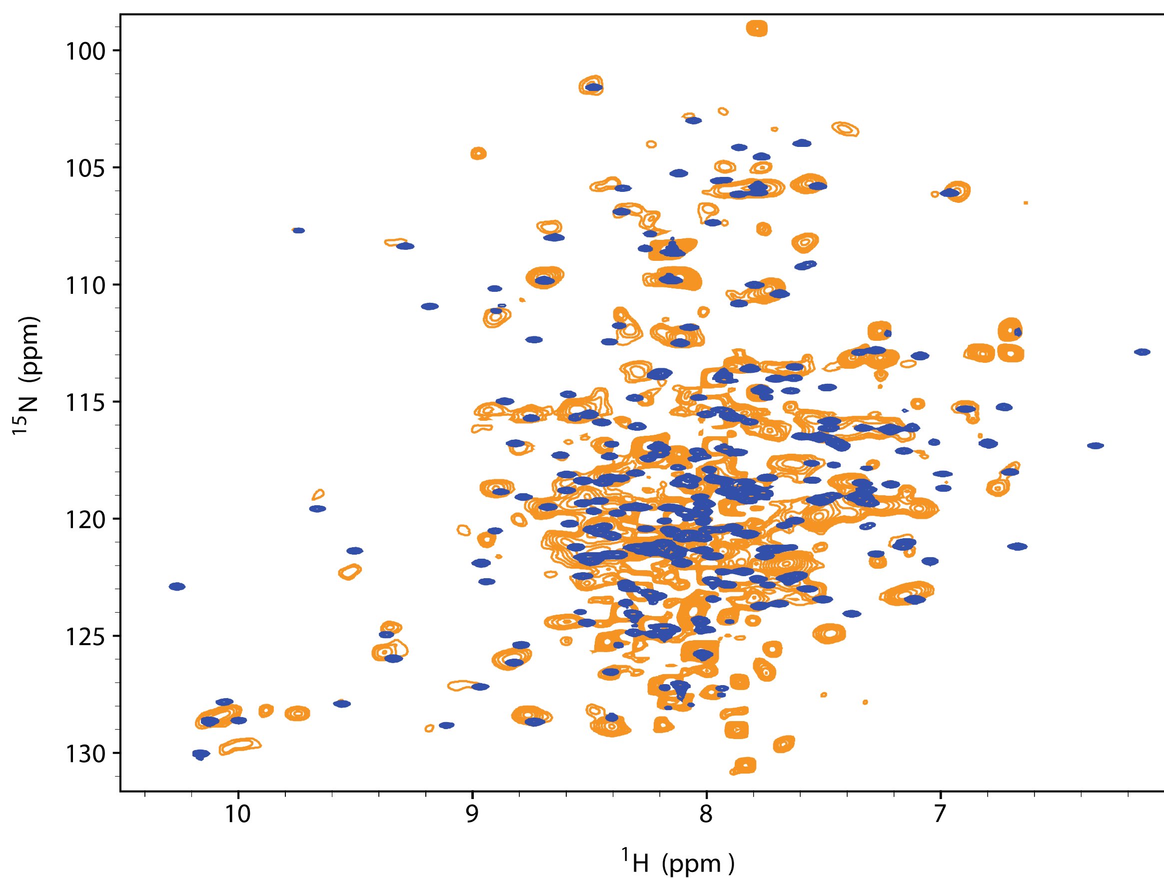


Figure S9. Overlay of 2D-[^{15}N,^{1}H]-SOFAST-TROSY spectra of ^{15}N-labelled pSRII in c7-DHPC micelles (blue) and in SapA-DMPC nanoparticles (orange). The spectra were recorded at 800 MHz (^{1}H) and 318 K.

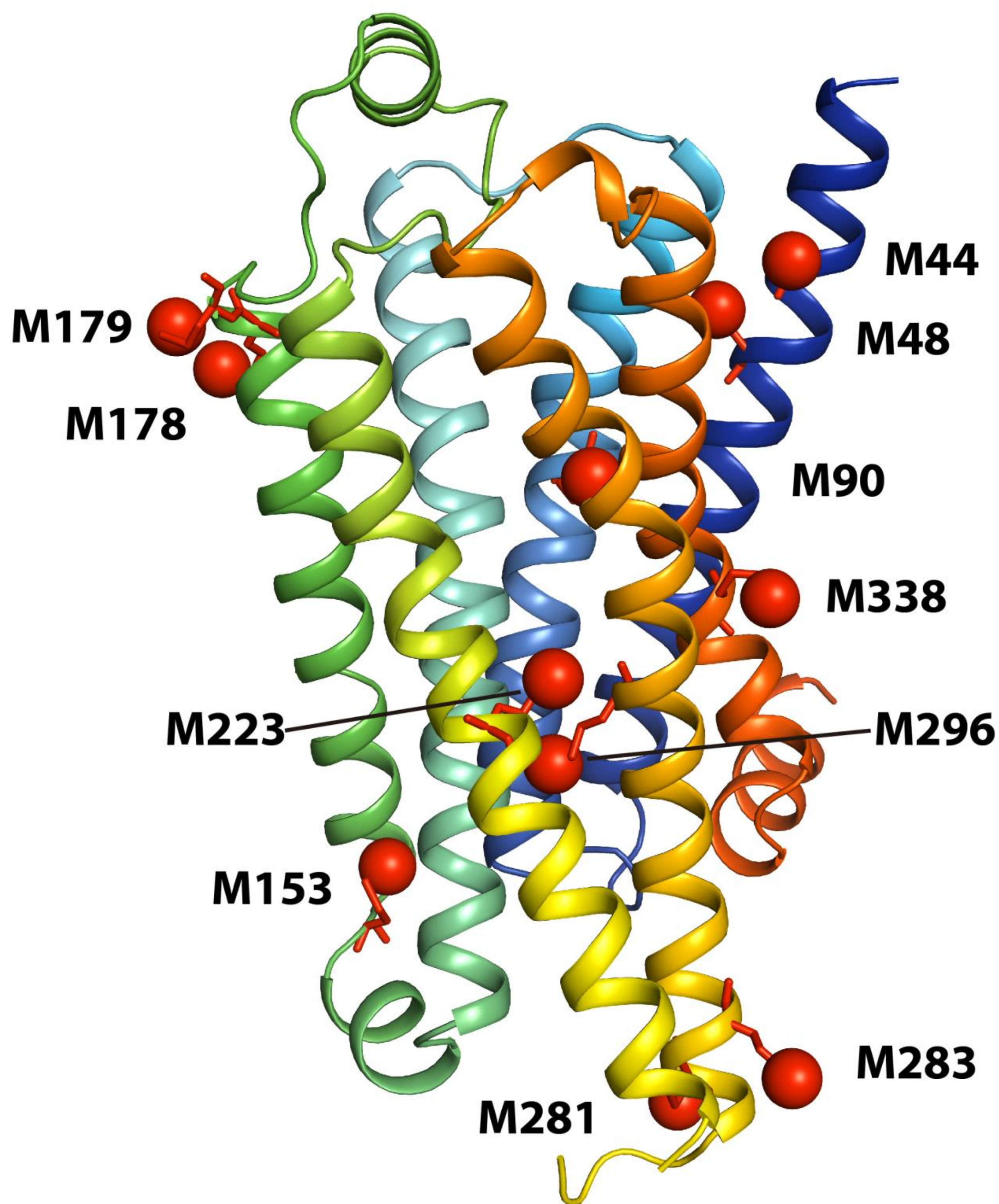


Figure S10. Crystal structure of turkey β_1 adrenergic receptor (β_1AR) (PDB: 4BVN).[9] Methionines are indicated by red spheres.

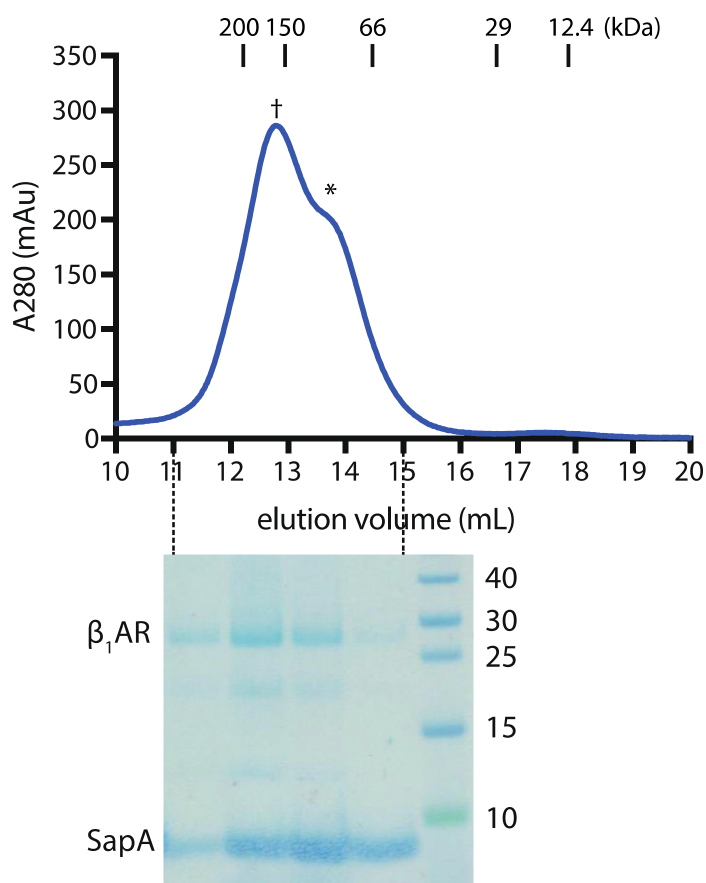


Figure S11. SEC of $β_1$AR in SapA-DMPC nanoparticles and the SDS-PAGE of the peak fractions. The peak indicated by † represents $β_1$AR in SapA-DMPC nanoparticles as judged by SDS-PAGE analysis; the peak indicated by * represents empty Salipro nanoparticles. Two faint bands at 13 kDa and 22 kDa, which we occasionally observe, correspond to fragments of $β_1$AR from protease cleavage at IL2. The fractions containing $β_1$AR in SapA-DMPC nanoparticles (11–14 mL) were pooled and concentrated for further investigation.

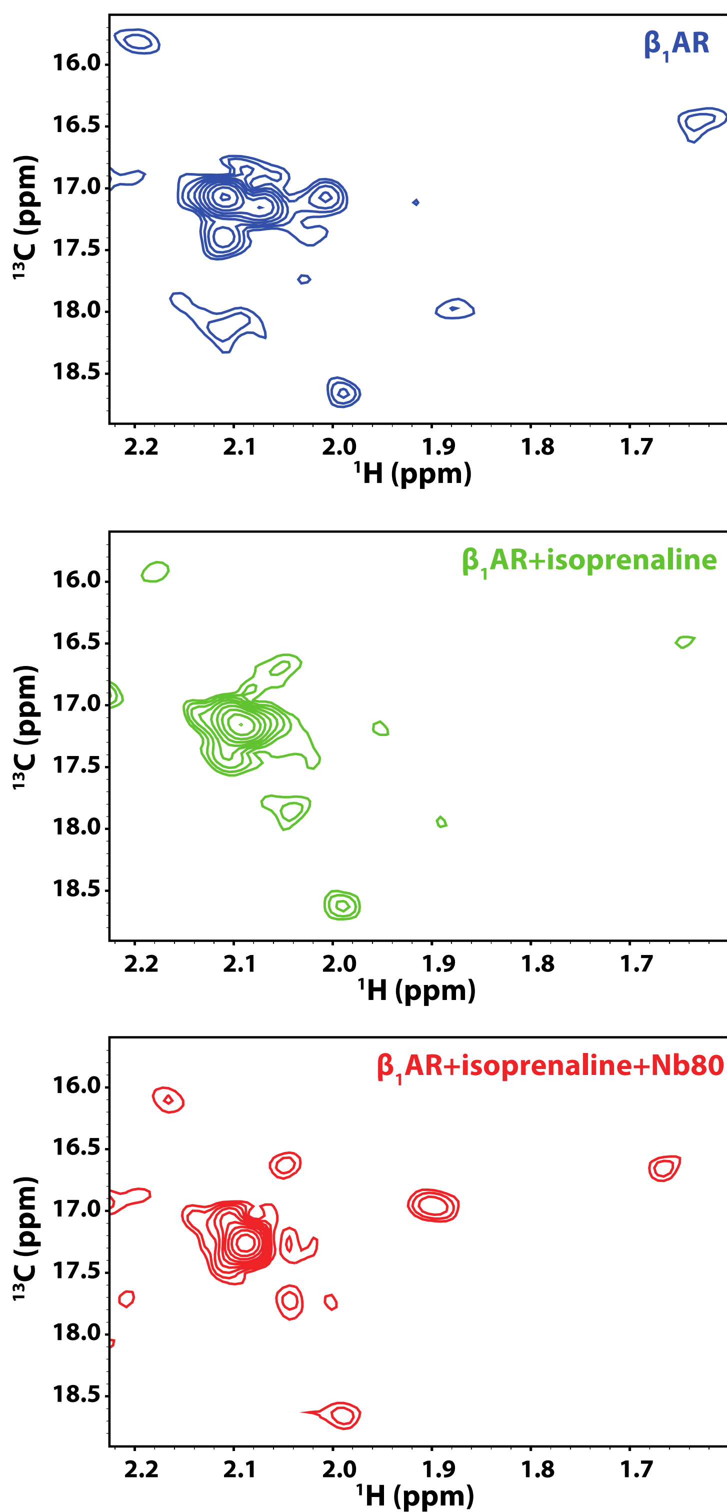


Figure S12. Separate views of the same 2D ^{13}C-SOFAST-HMQC spectra of [$^{13}C_{\varepsilon}$–methionine] β_1AR in the apo state (blue), the isoprenaline-bound state (green), and the isoprenaline-Nb80-bound ternary complex (red) shown in Figure 4 in the main manuscript.

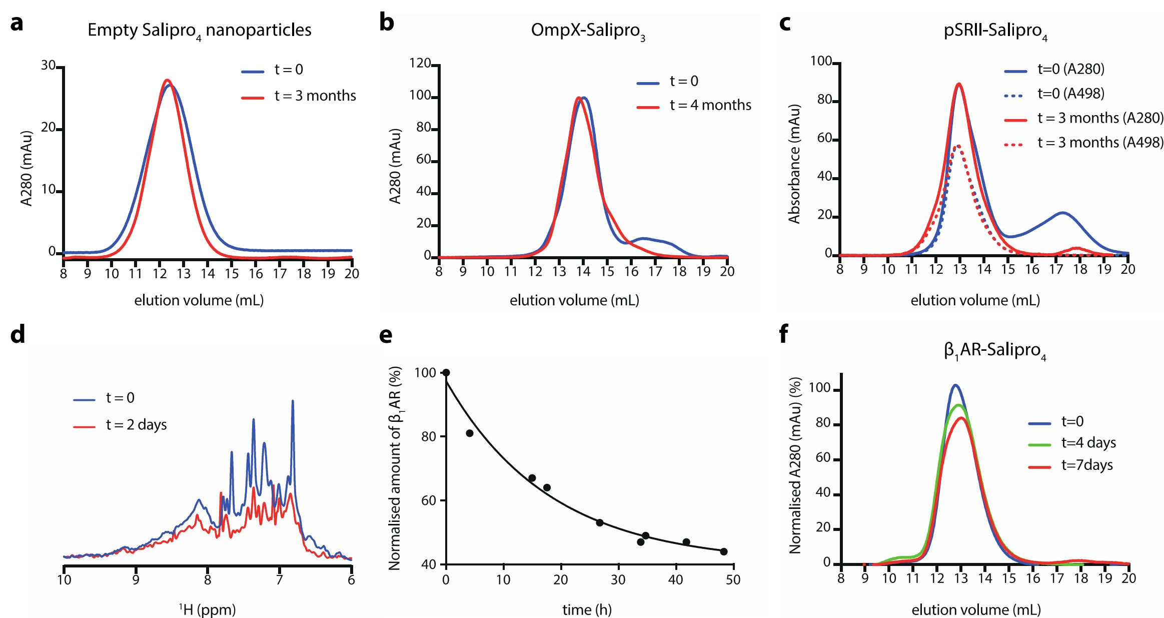


Figure S13. Stability test of Salipro nanoparticles and MP-incorporated Salipro nanoparticles. (a) Overlay of SEC traces of empty $Salipro_4$ nanoparticles after three months. (b) Overlay of SEC traces of OmpX in $Salipro_3$ nanoparticles after four months. (c) Overlay of SEC traces of pSRII in $Salipro_4$ nanoparticles after three months. The characteristic absorbance of the retinal chromophore of pSRII at 498 nm, is shown by a dotted line. (Note, that the peak at 17 mL in the blue SEC traces in (b) and (c) corresponds to closed SapA. It is absent from the red trace, as only the main peak fractions 12-15 mL were used for the time course.) (d) Overlay of the amide region of 1D ^{1}H NMR spectra (800 MHz ^{1}H) for β_1AR in LMNG micelles after two days at 25 ˚C. (e) The decay of β_1AR over a period of two days observed using 1D NMR spectra (as shown in (d)), displayed as normalised intensities (%). The half-life of β_1AR is ~35 hours at 25 ˚C. (f) Overlay of SEC traces of β_1AR in Salipro nanoparticles after four days (green, 90% of initial intensity) and seven days (red, 80% of initial intensity), displayed as normalised mAu intensities (%). The estimated half-life of β_1AR in nanoparticles is extended to ~30 days.

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