Supporting Material for

Facile Formation of Giant Elastin-Like Polypeptide Vesicles as Synthetic Cells

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EXPERIMENTAL SECTION

Materials

S48I48 ELP was a kind gift from Andrew MacKay, University of Southern California, USA (Addgene plasmid no. 68394), RQF ELP was a kind gift from Tobias Pirzer, Technical University Munich, Germany. All the chemicals were procured from Sigma-Aldrich (St Louis, MI) unless otherwise specified. Vybrant DiO was purchased from Invitrogen. NBD-DPPE (NBD-PE) and Rho-DOPE (Rho-PE) were purchased from Avanti Polar Lipids. Mineral oil was purchased from Acros Organics. BLR(DE3) Competent cells-Novagen were procured from Millipore Sigma. Terrific Broth powder was purchased from Thermo Fisher. Slide-A-Lyzer™ MINI Dialysis Device, 10K MWCO, 2 mL was purchased from Thermo Fisher Scientific. FluoroTect™ $Green_{Lys}$ *in vitro* Translation Labeling System was purchased from Promega. Silica beads were procured from Bangs Laboratories (Fishers, IN).

Expression and purification

S48I48 ELP construct was acquired from Addgene (plasmid no. 68394, Andrew MacKay, University of Southern California). The plasmid pET-25b(+) encoding S48I48 ELP was transformed into *Escherichia coli* BLR(DE3) strain that is suitable for expressing recombinant proteins with repetitive sequences. A starter culture was inoculated and grown overnight (~14 hours) in 50 mL Terrific Broth supplemented with 100 μg/mL ampicillin at 37°C at 200 rpm. 1-2 volume % of the starter culture was then scaled up to 1 L culture and

grown for 24 hours at 37°C at 200 rpm in the presence of ampicillin. ELP was synthesized by leaky expression of T7 RNA polymerase without using IPTG.

Cell pellets were collected in a pre-weighed centrifuge tube by centrifugation at 6,000 × g for 10 minutes at 4 °C and resuspended in 30 mL PBS. The resuspended cells were washed with phosphate buffer saline (PBS) by centrifugation at 4,000 × g for 20 minutes at 4°C to obtain the cell pellet mass. For each gram of cell pellet, 2 ml of lysis buffer was used for cell lysis by sonication. Lysis buffer contains PBS with 1 mg/mL lysozyme, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 0.5 U of DNase I. Cells were sonicated using Branson Sonifier Sound Enclosure with settings of output power of 5, duty cycle 50%, and 10-second sonication with 20-second pause time for 6 minutes, followed by incubation on ice for 10 minutes. For complete lysis, the steps were repeated twice. For the precipitation of genomic DNA, 2 mL of 10% v/v polyethyleneimine (PEI) was added to the cell lysate. Supernatant containing ELP was collected after aliquoting the lysate in 1.5 mL Eppendorf tubes, followed by centrifugation at 16,000 × g for 10 minutes at 4°C. At least three rounds of inverse transition cycling (ITC)[1] were performed to purify S48I48. This method harnesses the phase transition temperature of ELP for its purification. S48I48 ELP exhibits two transition temperatures, one at 27°C (due to the isoleucine blocks) and another at 75°C (due to the serine blocks).[2] The lower transition temperature was used for the purification of ELP. Briefly, in hot-spin cycle of ITC, the ELP solution was heated above transition temperature (here 37°C) followed by dropwise addition of 5 M NaCl until the solution becomes cloudy. Immediately, the pellet was collected by centrifugation at 16,000 × g for 10 min. For a cold-spin cycle, pellet containing ELP was re-suspended in

cold PBS and incubated at ice for 10 minutes, followed by supernatant collection at 16,000 × g for 10 min. ELP transitions from pellet (hot-spin) to supernatant (cold-spin) in single round of ITC. In the final round of ITC, supernatant was then dialyzed using Slide-A-Lyzer MINI device by following the manufacturer's protocol to remove excess salt and undesired proteins. Lyophilized sample was stored at −20°C. Purity of ELP was determined by standard SDS-PAGE. S48I48 has a total of 483 amino acids with MW of 39.8 kDa, with each hydrophilic and hydrophobic domain containing 240 amino acids.

Peptide vesicles preparation and labeling

Peptide vesicles were prepared by using the emulsion transfer method. A stock solution of ~135 μM (5.44 mg/mL) concentration of ELP was prepared in a mixture of chloroform:methanol (2:7) and stored at −20°C in a sealed glass vial until use. 99.9 mol % of S48I48 (10 μM) along with 0.1 mol % NBD-DPPE were dissolved in an oil phase containing a mixture of mineral oil:silicone oil (1:4). For optical phase contrast, a combination of 200 mM sucrose and 200 mM glucose were used as inner and outer aqueous solutions, respectively. About 130 μl of water-in-oil emulsion was generated by vortexing 100 μL of oil mixture containing ELP followed by addition of 30 μL of sucrose solution. An interface was established by first adding 200 μl glucose solution in a 1.5 ml Eppendorf tube, followed by 400 μl of ELP-containing oil phase and left undisturbed for at least 15 min. The water-in-oil emulsion was then carefully transferred to the oil phase and centrifuged at 10,000 × g for 10 minutes at room temperature (RT). The oil phase was

carefully removed without disturbing the giant peptide vesicles at the bottom. Vesicles were retrieved by pipetting and observed under confocal fluorescence microscopy. Giant peptide vesicles were labelled by a cell membrane labelling dye, Vybrant DiO. Briefly, DiO was mixed with peptide vesicles at a ratio of 1:200 following the manufacturer's protocol and observed under a fluorescence microscope at least after 1 hour of incubation at RT.

ELP-coated beads

We next investigated whether nano-ELP vesicles could form supported peptide bilayers using the supported bilayers with excess membrane reservoir (SUPER) templating technique that has been used to study membrane-localized processes and membrane protein reconstitution.[3,4] Following CFE of S48I48, we incubated the CFE (10 µl) reaction with 5 µm silica beads and a trace amount of NBD-DPPE at 37°C in a high salt solution (PBS and 1M NaCl) at RT for 30 minutes with intermittent tapping. Beads were washed twice with PBS by centrifugation at 500 × g for 3 minutes and observed under a fluorescence microscope. This is above the T_t for S48I48 and we suspect that under high salt conditions, S48I48 micelles/nanovesicles fuse with the silica beads. A uniform labeling of peptide bilayer was observed on the silica beads as shown in **Fig. S2**. A solution of silica beads without cell-free expressed S48I48 and incubated with NBD-DPPE under the same condition did not yield any labeled membrane localized to silica beads, again signifying that NBD-DPPE alone cannot form bilayers at the concentration used.

Cell-free GFP expression

Plasmid containing T7p14deGFP was expressed using homemade CFE components following the protocol by Sun *et al*.[5] Briefly, a 10 µL CFE reaction was prepared by using S30 extract and protein synthesis buffer along with T7 RNA polymerase (21.25 ng/µl), RNase inhibitor, plasmid encoding GFP (1 nM) and water. All the components were vigorously mixed and incubated at 30 °C for 4 hours. Fluorescence intensity with end-point kinetic was recorded using a Biotek fluorescence plate reader. CFE-expressed GFP was then encapsulated in peptide vesicles by using emulsion transfer method in an iso-osmotic condition.

For *in situ* expression of GFP in peptide vesicles, the CFE components were assembled along with a GFP-expressing plasmid and encapsulated as the inner solution as described in previous paragraph. Vesicles were then incubated at 30°C for 4 hours and the expressed protein was observed under microscope.

Membrane incorporation of ELP RQ-F

In vitro synthesis of ELP RQ-F was carried out using in-house CFE reaction for 4 hours at 30°C, supplemented with FluoroTect Green Lys, by following the manufacturer's protocol. This labelling system incorporates the lysine-labeled with BODIPY®-FL into ELP RQ-F sequence, making it fluorescent. RQ-$F^{GreenLys}$ was encapsulated inside ELP S48I48 vesicles and incubated at RT for at least 2 hours for membrane incorporation.

RQ-F: an amphiphilic ELP with sequence $[(VPGRG)_5(VPGQG)_5]_2(VPGFG)_{20}$ with arginine (R) and glutamine (Q) blocks constituting the 20 pentapeptide repeat of the

hydrophilic block and phenylalanine (F) constituting the 20 pentapeptide repeat of the hydrophobic block

Microscopy

All the peptide vesicles images were acquired using an oil immersion 60×/1.4 NA Plan-Apochromat objective lens mounted on an Olympus IX-81 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan) equipped with a CSU-X1 spinning disc confocal head (Yokogawa Electric Corporation, Tokyo, Japan), AOTF-controlled solid-state lasers (Andor Technology, Belfast, UK), and an iXON3 EMCCD camera (Andor Technology, Belfast, UK). Fluorescence images were captured using MetaMorph software (Molecular Devices, San Jose, CA) with laser excitation at 488 nm for GFP, DiO, and Green Lys and 561 nm for Rho-PE and rhodamine. Image analysis was performed using NIH-ImageJ.

Atomic force microscopy: mechanical testing

To measure peptide vesicle tensile mechanical properties, atomic force microscopy (AFM) nanoindentation in contact mode was performed using a Nanosurf FlexBio atomic force microscope. A HYDRA6V-200NG (AppNano) probe tip with spring constant of 0.0322 N/m affixed with a 14.194 μm diameter glass microsphere (Fisher) was used. To adhere peptide vesicles to a rigid glass surface for nanoindentation testing, glass coverslips were treated with poly-L-lysine then 0.5 vol% glutaraldehyde. 1 mole % of cholesterol-PEG-Peptide K (cholesterol-PEG4-KKRRAKSQ(EKLAAIK)$_4$) was included in the peptide

vesicles during vesicle formation. Cholesterol-PEG-Peptide K was a gift from Nicholas Stephanopoulos and synthesized based on previously described chemistry.[6] ELP vesicles with exposed lysine were allowed to adhere to treated glass coverslips overnight. Single point nanoindentation measurements were performed at the apex of peptide vesicles across 8 vesicles. Using AtomicJ, force-displacement curves was fit to the Maugis model for peptide vesicle assuming a Poisson's ratio of 0.5 to determine Young's modulus values.

Chemical and physical stresses to peptide vesicles

To 50 µl samples of peptide vesicles or lipid vesicles, different concentrations of Triton-X 100 (10% stock) were added, and fluorescent images were captured after 5 minutes. For hypo-osmotic shock, samples were diluted by adding water successively to reduce the osmolarity of outer solution. Individual vesicles were observed in real time by fluorescence microscopy. Lipid vesicles were prepared by electroformation with 1 mM total lipid and a molar ratio of DOPC:Chol:Rho-PE 69.9:30:0.1.

Molecular dynamics simulation

The all-atom structure of $(VPGSG)_{48}(VPGIG)_{48}$ chain was prepared using PyMOL software.[7] The all-atom structure was then coarse-grained using Martinize.py script[8] and all interactions were modeled by Martini v2.2 force field.[8–10] The $(VPGSG)_{48}(VPGIG)_{48}$ bilayer membrane was initialized by first placing the $(VPGSG)_{48}(VPGIG)_{48}$ chains on a 10-by-10 grid where the centers of mass (COM) of neighboring chains were separated by 0.9 nm. This monolayer of peptide chains was then flipped across the x-y plane to obtain a

bilayer. The solvent-exposed N-termini of the hydrophilic $(VPGSG)_{48}$ blocks were set to have +1 charge while the buried C-termini of the hydrophobic $(VPGIG)_{48}$ blocks were set to be neutral. Coarse-grained water molecules and Cl^- ions were then added above and below the bilayer using the PACKMOL package[11]. The initial system size was 9 nm × 9 nm × 261 nm.

Molecular dynamics simulations were conducted using the Gromacs 2019.3 simulation suite.[12] The initial system configuration was first energy minimized using the steepest descent algorithm to eliminate all forces in excess of 1000 kJ/mol.nm. Initial atomic velocities were then sampled from a Maxwell-Boltzmann distribution at 300 K and the system was subjected to 40 ns of NVT equilibration at 300 K. We then performed a 60 ns NPT equilibration at 300 K and 1 bar. Finally, we conducted a 1.8 μs NPT production run at 300 K and 1 bar. We verified that the equilibration period was sufficiently long to relax the bilayer to its equilibrium structure by verifying that the thickness, structure, and density profiles of the bilayer remained stable over the course of the 1.8 μs production run. Simulation parameters were selected following best practices for coarse-grained simulations using the Martini model.[13] Numerical integration of the classical equations of motion was conducted using the leapfrog algorithm[14] with a 20 fs time step. The temperature was maintained using a stochastic velocity rescaling thermostat with a time constant of 1 ps.[15] During the NPT equilibration the pressure was controlled using the Berendsen barostat[16] with a time constant of 12 ps, whereas during the NPT production run the pressure was controlled using the Parrinello-Rahman barostat[17] with a time constant of

12 ps. The bond constraints were handled by LINCS method[18] and electrostatics was handled by reaction-field method.[19]

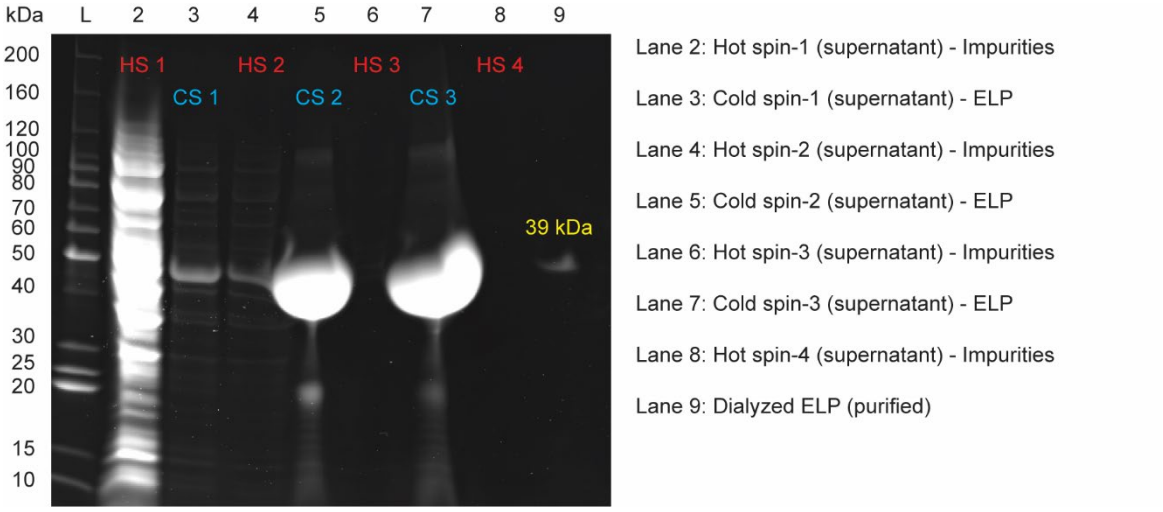

Figure S1: SDS-PAGE analysis of *E. coli* expressed ELP and its purification**: Lane L**: pre-stained protein marker 10-200 kDa, **Lane 2-4-6, and 8**: marked HS 1-4 (in red) show supernatant from hot-spin cycle at 37˚C, fractions carrying cell debris and impurities in protein fraction, **Lane 3-5-7**: marked CS 1-3 (in blue) show the supernatant from cold-spin cycle at 4˚C, fractions of purified ELP S48I48 at 39 kDa, and **Lane 9**: shows the purified and dialyzed ELP fraction.

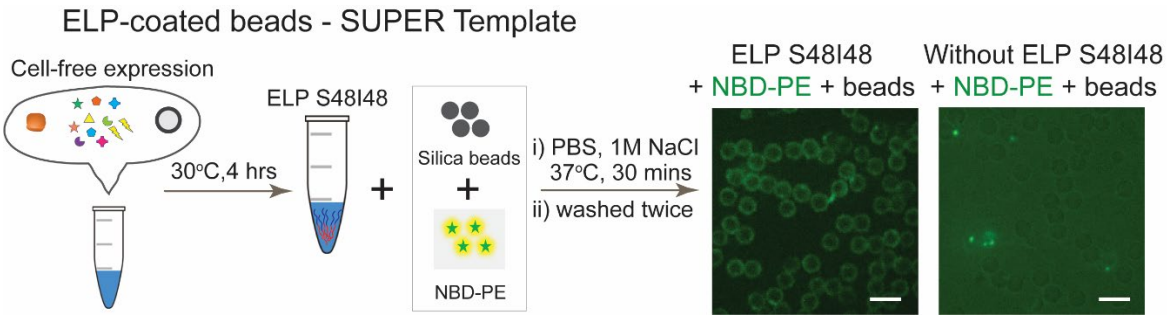

Figure S2: Schematic illustrating cell-free expressed S48I48 forming peptide bilayer on 5 µm silica beads using the SUPER template procedure. Beads were washed twice before observation. Fluorescence images of silica beads with or without cell-free expressed S48I48. Scale bars are 10 µm.

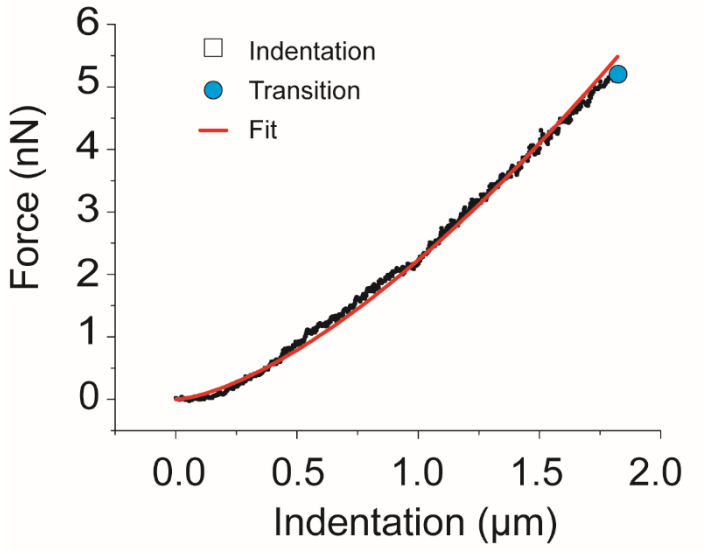

Figure S3: Force-displacement curve fit to the Maugis model using AtomicJ software to determine Young's Moduli values of peptide vesicles.

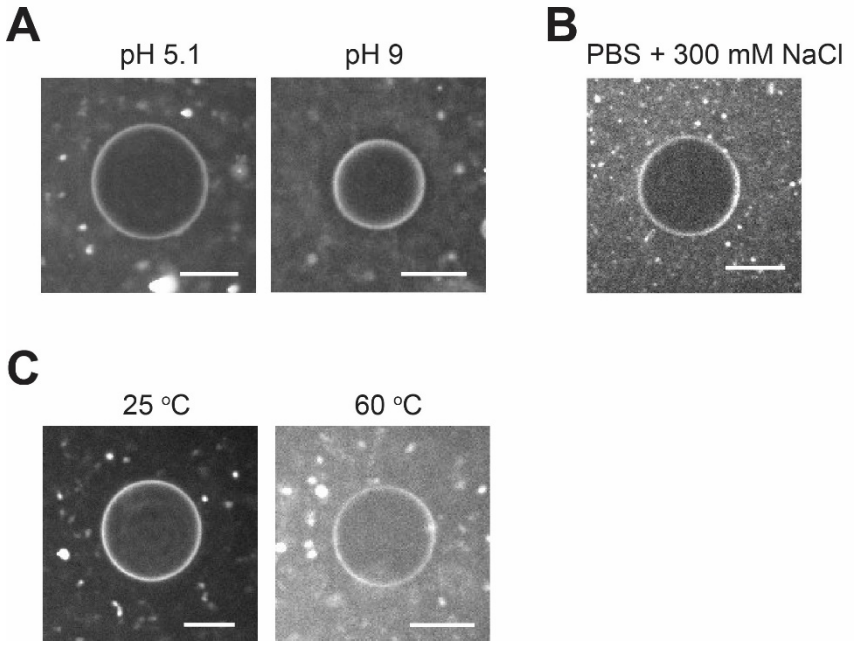

Figure S4: Stability of ELP peptide vesicles under different conditions. (A) Fluorescence images of peptide vesicle labeled with Rho-DOPE in acidic and basic conditions. (B) Fluorescence images of peptide vesicle labeled with Rho-DOPE in high NaCl concentration (437 mM total). (C) Temperature treatment of ELP peptide vesicles labeled with Rho-DOPE and their stability at 60 °C. Scale bars are 10 μm.

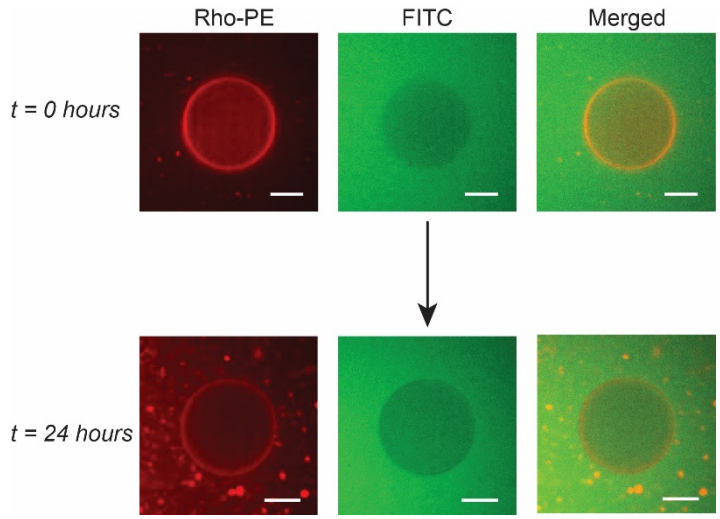

Figure S5: ELP peptide vesicles are impermeable to small molecules. 200 mM glucose in PBS, supplemented with trace amount of FITC was used as an outer solution during emulsion transfer method. No observable diffusion of FITC into the lumen of peptide vesicles was observed after 24 hours of incubation at room temperature. Scale bars are 10 μm.

Table S1

RQ-F Sequence: $[(VPGRG)_5(VPGQG)_5]_2(VPGFG)_{20}$
MVPGRGVPGRGVPGRGVPGRGVPGRGVPGKGVPGQGVPGQGVPGQGVPGQGV PGRGVPGRGVPGRGVPGRGVPGRGVPGQGVPGQGVPGQGVPGQGVPGQGVPG QGVGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFG VPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGW PHHHHHH

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