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

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ABSTRACT

Towards creating a suitable compartment for synthetic cells, different cell chassis materials ranging from phospholipids to polymer to protein-polymer conjugates have been previously explored. Currently, the majority of cell-like compartments are made of lipid molecules as the resulting membrane resembles that of a natural cell. However, cell-sized lipid vesicles are sensitive to physical and chemical stresses and the resulting instability disrupts biochemical reactions within. Recently, peptide vesicles that are more robust and stable were developed as a new chassis material for synthetic cells. Here we demonstrate the facile and robust generation of giant peptide vesicles made of elastin-like polypeptides (ELPs) by using an emulsion transfer method. We show the robust nature of the peptide vesicles by challenging them with chemical (Triton-X 100) and physical (osmotic) stresses. We find that these peptide vesicles can stably encapsulate biomolecules and can host cell-free expression reactions. Lastly, we show incorporation of another cell-free expressed amphiphilic ELP into existing peptide vesicles' membrane, demonstrating a potential approach to grow ELP vesicles. Since ELPs are genetically encoded, the approaches presented here provide exciting opportunities to engineer synthetic cell membranes.

INTRODUCTION

Synthetic cells are cell-like compartments in the form of membrane vesicles that recreate some aspects of cellular biochemistry.[1,2] A synthetic cell provides a framework where internally encapsulated materials are protected from the external aqueous environment with minimum leakage. Ideally, the protecting boundary should be mechanically strong and semi-permeable for nutrients and gaseous exchange. Various amphiphilic molecules have been employed to create a robust cell-like system, including phospholipids,[3–5] fatty acids,[6] polymers,[7] double emulsion,[8,9] and a hybrid of protein-polymer[10] or lipid-polymer.[11] Phospholipid bilayer vesicles hold the most resemblance to natural cell membrane and have also been employed as drug delivery vehicles.[12,13] The application of these materials in applied synthetic cell research is impacted by their high susceptibility towards physical stresses like osmotic shock and mechanical deformation and chemical stress like oxidation in response to pH change and surfactants. Polymersomes with improved mechanical strength have been introduced,[14,15] and the use of synthetic polymer in building synthetic cells continues to grow.[16]

Recently, a genetically encoded elastin-like polypeptide (ELP) was introduced in a synthetic cell model by Pirzer and co-workers and they demonstrated cell-free synthesis of ELP in growing ELP vesicles.[17,18] ELP has a basic structure of a repeating pentapeptide entity Val-Pro-Gly-X-Gly, where the guest residue X can be any natural amino acid except proline. ELPs exhibit a reversible lower critical solution temperature (LCST) behavior and a change in phase above a transition temperature (T_t) due to selective assembly of hydrophobic blocks. ELPs remain soluble when the temperature is lowered below T_t.[19] Based on this feature, ELPs can be purified by an inverse transition cycling method.[20] ELPs are quite versatile and have been exploited for applications in drug delivery and tissue engineering. [21,22] Although ELPs have been

shown to form ~50 nm unilamellar vesicles,[23] there are few examples of generating cell-sized ELP vesicles. This is in part due to the propensity for ELPs to form an array of micellar structures. Here, we hypothesized that amphiphilic ELPs may be able to form bilayer vesicles when presented with a template.

In this work, we demonstrate a robust method for generating giant ELP vesicles as synthetic cells by using an emulsion transfer method originally developed by Pautot *et al.*[24] with modification. The formation of a peptide bilayer was subsequently confirmed by incorporation of a membrane labelling dye and a fluorescently labeled lipid. ELP vesicles are mechanically rigid with a Young's modulus of ~500 Pa. ELP vesicles are compatible with bacterial cell-free expression (CFE) and we show that they can host CFE reactions to express proteins of interest. Finally, we demonstrate membrane incorporation of another amphiphilic ELP into existing ELP vesicles as a proof of concept for growing ELP vesicles. The robust generation of ELP vesicles that support CFE reactions significantly advances synthetic cell engineering as it provides a new genetically encoded approach for engineering membrane without relying on lipid-producing enzymes.

MATERIALS AND METHODS

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. Vibrant 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

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)[20] 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).[25] 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.

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-PE 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 $\times$ 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, Vibrant 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

  ELP-coated silica beads were prepared using SUPER template method described by Pucadyil et al[26] with slight modification. Cell-free expressed S48I48 (10 μl) with 5 μl of silica bead solution (5 μm, 6.4 $\times$ 10^6 beads/ml) supplemented with 1 μL NBD-PE (1mg/mL) in high salt concentration buffer (PBS and 1M NaCl) incubated at RT for 30 minutes with intermittent tapping. Beads were washed twice with PBS by centrifugation at 500 $\times$ g for 3 minutes and observed under a fluorescence microscope.

Cell-free GFP expression

  Plasmid containing T7p14deGFP was expressed using homemade CFE components following the protocol by Sun *et al*.[27] 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.

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 polyacrylamide and 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. For polyacrylamide gels, indentations were performed in three distinct regions with 9 points taken within each region over a 100 x 100 μm grid (3 x 3). Polyacrylamide gels (Bio-Rad) were formed at a constant monomer acrylamide concentration of 3 w/v% while the bis-acrylamide crosslinker concentration was varied between 0.06 and 0.1 w/v%. The desired acrylamide and bis-acrylamide volumes were added to a solution containing 0.01M phosphate buffered saline (PBS, Sigma) and TEMED (1:1000 dilution, Bio-Rad) in deionized water. To initiate the polymerization reaction, 1 w/v% ammonium persulfate solution was added and the solution (1:100 dilution) and the polyacrylamide gel was added to a 3-aminopropyl-triethoxysilate-treated glass coverslip for gelation. Gels were stored in PBS solution overnight at 4 °C before nanoindentation testing. 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.[28] 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 were fit to the Hertz model for

polyacrylamide gel and 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

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.[29]

The all-atom structure was then coarse-grained using Martinize.py script[30] and all interactions

were modeled by Martini v2.2 force field.[30–32] 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[33]. The initial system size was 9 nm × 9 nm × 261 nm.

Molecular dynamics simulations were conducted using the Gromacs 2019.3 simulation suite.[34] 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.[35] Numerical integration of the classical equations of motion was conducted using the leapfrog algorithm[36] with a 20 fs time step. The temperature was maintained using a stochastic velocity rescaling thermostat with a time constant of 1 ps.[37] During the NPT equilibration the pressure was controlled using the Berendsen barostat[38] with a time constant of 12 ps, whereas during the NPT production run the pressure was controlled using the Parrinello-Rahman barostat[39] with a time constant of 12 ps. The bond constraints were handled by LINCS method[40] and electrostatics was handled by reaction-field method.[41]

RESULTS

Formation of giant peptide vesicle and peptide bilayer

We start with an amphiphilic ELP (denoted as S48I48) that comprises 48 repeats of pentapeptide (VPGXG) blocks of serine and isoleucine as the guest residue for the hydrophilic and hydrophobic domains, respectively (**Figure 1A**). S48I48 has a total of 483 amino acids with MW of 39.8 kDa, with each hydrophilic and hydrophobic domain containing 240 amino acids. S48I48 displays partial transition at 27°C due to self-assembly of isoleucine blocks and bulk phase transition at 75°C due to aggregation of serine blocks.[25] S48I48 was expressed and purified from *E. coli* by three cycles of ITC at 37°C and the purity of a lyophilized ELP was confirmed by SDS-PAGE (**Supplementary Figure 1**); the stock ELP solution is stored in a chloroform:methanol mixture.

There exist several methods for generating giant vesicles and many of them are based on generating large water-in-oil emulsion droplets that are stabilized by amphiphilic molecules.[16] A monolayer of amphiphilic molecules can self-assemble at the oil-water interphase between two bulk phases. Centrifugation of the emulsion droplets across the monolayer-stabilized interface generates bilayer vesicles. We applied this emulsion transfer method to generate giant ELP vesicles (**Figure 1B**). The S48I48 was dissolved in an oil mixture of mineral oil:silicone oil (1:4) and a trace amount of fluorescent lipid, NBD-PE, was used to label peptide vesicles. Giant S48I48 vesicles recovered from the water phase had a range of sizes from 3 to > 50 μm with an average size of 15.1 μm (**Figure 1C**).

To rule out the possibility that NBD-PE was somehow driving the formation of giant peptide vesicles, we made unlabeled S48I48 vesicles and asked if a membrane labeling dye can partition into the bilayer and label the peptide membrane. Following incubation of a cell membrane labelling dye, Vibrant DiO, we observed robust labeling of S48I48 vesicles (**Figure 1D**). When we performed emulsion transfer with the same concentration of NBD-PE used without including

S48I48, no vesicles were formed (data not shown). Together, these results demonstrate that amphiphilic ELPs can robustly form peptide vesicles and are compatible with emulsion transfer techniques.

A distinct advantage of an ELP-based compartment is that these peptides can be genetically encoded and thus we can leverage the power of cell-free protein expression[42] to synthesize ELPs *in situ*. However, we do not expect the expressed ELP to self-assemble into cell-sized vesicles. It is known that in the presence of hydrophilic silica microspheres, small lipid vesicles can rupture onto the silica surface thereby forming supported lipid bilayers. 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.[26,43] Following CFE of S48I48, we incubated the CFE reaction with 5 μm silica beads and a trace amount of NBD-PE at 37°C in a high salt solution. 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 **Figure 1E**. A solution of silica beads without cell-free expressed S48I48 and incubated with NBD-PE under the same condition did not yield any labeled membrane localized to silica beads, again signifying that NBD-PE alone cannot form bilayers at the concentration used.

To characterize the stiffness of these peptide vesicles, we used AFM nanoindentation and determined the Young's modulus of ELP vesicles and planar polyacrylamide gels of varied crosslinker concentrations (0.06 and 0.1 w/v%) as comparison. A force vs distance curve was plotted showing the AFM tip approaching a vesicle, force application, and deflection in cantilever, as shown in **Figure 2A**. Using the Maugis model to fit the data (**Figure 2B**), the

average Young's modulus of ELP vesicle was found to be 455 Pa which is higher than the planar polyacrylamide gel (223 Pa) with 0.06 % crosslinker but lower than the polyacrylamide gel (725 Pa) with 0.1 % crosslinker (**Figure 2C**).

We next conducted coarse-grained molecular dynamics simulations of the bilayer to predict its thickness and internal molecular structure. We present in **Figure 2D** a snapshot of the fully relaxed bilayer at 300 K and 1 bar together with the partial density profiles for the $(VPGSG)_{48}$ segment, $(VPGIG)_{48}$ segment and water across the bilayer. We observed the bilayer to quickly adopt a well-defined structure and morphology that remained stable over the course of the 1.8 μs production run, indicating that, consistent with experimental observations, the bilayer morphology is a stable morphology. The upper ~2.5 nm of each leaflet of the bilayer defines a transition region within which we observe limited incursion of water into the bilayer. Our calculations predict the thickness of the each of the two distal hydrophilic S48 regions to be (31.7 ± 0.1) nm, the central hydrophobic I48 region to be (67.9 ± 0.1) nm, and the transition region between S48 and I48 regions to be 3.0 ± 0.1 nm, for a total bilayer thickness of (134.4 ± 0.1) nm.

Next, we sought to test how tough these peptide vesicles are against chemical and physical stresses. Triton-X 100 is well known for lipid membrane solubilization with a critical micelle concentration of ~0.02% (w/v). As shown in **Figure 3A**, almost 50% of peptide vesicles were stable against 0.3% of Triton-X 100 while lipid vesicles were completely disrupted at 0.04% of Triton-X 100. To assess ELP vesicles physical stability, we challenge them with osmotic stress. In a hypo-osmotic condition, lipid vesicles were inflated and burst when an osmolarity difference of 193 mOsm (**Figure 3B**) was reached. In contrast, peptide vesicles were stable even at the osmolarity difference of 507 mOsm (**Figure 3B**).

Encapsulation of dye and proteins in peptide vesicles

Armed with a robust strategy to generate giant peptide vesicles, we next examined the capacity of these peptide vesicles for encapsulation of small molecules and proteins as this is an important feature of a synthetic cell model. As one would expect from an emulsion transfer method, encapsulation of a small molecule dye (e.g., Rhodamine-B) was robust in S48I48 vesicles (**Figure 4 A**), and we did not detect observable leakage of the encapsulated dye from overnight incubation at room temperature (data not shown).

We next examined the compatibility of bacterial CFE reactions with our newly developed peptide vesicle system and tested two scenarios: i) encapsulation of proteins expressed by a CFE reaction, and ii) encapsulation of a CFE reaction leading to *in situ* expression of proteins. Using GFP as a reporter, our home-made *E. coli* CFE robustly produced GFP over 4 hours (**Figure 4B**). Using this solution as the inner solution for making single emulsion droplets in S48I48-containing oil phase, as expected, GFP was detected in S48I48 peptide vesicles (**Figure 4C**). Similarly, cell-free expressed GFP was detected in S48I48 peptide vesicles when the CFE reaction was directly encapsulated and incubated (**Figure 4D**). These results demonstrate that giant S48I48 ELP peptide vesicles can host CFE reactions similar to giant lipid bilayer vesicles.

Membrane incorporation of cell-free expressed amphiphilic ELP

The growth of the vesicle, considered to be an important feature of synthetic cell development, can be achieved by the addition of membrane components[44–46] or by using lipid modifying enzymes either introduced as a purified protein[47] or by cell-free expression[48]. For ELP vesicles, a recent study reported by Frank *et al.*[49] showed the growth of peptide vesicles is driven

by a combination of osmotic-driven changes due to CFE reactions and fusion of membrane components fed from the outside. Here we sought to directly incorporate cell-free expressed ELP into pre-formed ELP vesicles. For this purpose, we selected another ELP termed 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. The free lysine at position 30 (**Supplementary Table 1**) can be fluorescently labelled *in situ* by including FluoroTect Green Lys during CFE. We hypothesized that Green Lys labeled RQ-F (RQ-F^{GreenLys}) can be incorporated into a pre-existing ELP vesicle membrane (**Figure 5A**). In a batch CFE reaction, we find that RQ-F^{GreenLys} was expressed very well (**Figure 5B**). When cell-free expressed RQ-F^{GreenLys} was incubated (without purification) with S48I48 peptide vesicles under iso-osmotic conditions at RT for 3 hours, we observed clear membrane incorporation of RQ-F^{GreenLys} (**Figure 5C**). The RQ-F^{GreenLys} insertion to the peptide bilayer appeared to be uniform. In distinct contrast, we did not find labeling of the membrane with only Green Lys alone in CFE reactions without expressing RQ-F.

DISCUSSION

Using an emulsion transfer method, we report micron-scale peptide vesicles made of amphiphilic S48I48 ELP. ELPs represent a desirable chassis material for synthetic cell construction for several reasons, including: (i) their stability in harsh physical and chemical conditions; (ii) inexpensive and relative ease of synthesis; (iii) ease of purification with high yield; (iv) and the ability to modify their polarity by introducing different guest residues. Unlike lipid vesicles, the S48I48 peptide vesicles appear to have a thick membrane, likely due to its

large hydrophobic and hydrophilic blocks each constituting 240 amino acids. To our knowledge, peptide vesicles comprised of S48I48 reported here are the largest polypeptide sequence reported to form giant peptide compartments.

The first demonstration of using an amphiphilic ELP to create self-assembled vesicular structures as reaction compartments for synthetic cells utilized a glass bead swelling method that yielded peptide vesicles ~200 nm in diameter.[50] Subsequently, the same group devised a solvent evaporation method[49] that consists of first dissolving lyophilized ELPs in tetrahydrofuran followed by adding the inner encapsulation solution via agitation. Evaporation of tetrahydrofuran followed by addition of outer solution resulted in two populations of ELP vesicles, ones that were tens of nanometer in diameter and others over 1 μm in size. Since the emulsion transfer method is commonly used for lipid vesicle encapsulation, its direct applicability to make peptide vesicles eliminates the use of harsh organic solvents like tetrahydrofuran. Since the size of emulsion droplets can be controlled by using microfluidics, an emulsion transfer method described here would allow for creation of peptide-based synthetic cells with homogeneous sizes. As with other emulsion-based methods for vesicle generation, we cannot rule out the possibility that there remains residual oil within the ELP bilayer.

Although we have demonstrated the facile and robust formation of giant peptide vesicles using only S48I48 as our model ELP, we believe this approach is generally applicable to other ELP sequences. The key conceptual advance in our approach is that ELPs can be templated on aqueous droplets or micron-sized solid supports comparable to strategies using amphiphilic lipid molecules. Since the ELP vesicles were cell-sized, our AFM measurements yielded Young's modulus of peptide vesicles and not the peptide membrane. Interestingly, the stiffness of ELP vesicles is comparable to whole cell stiffness.[51,52] Given that the stiffness of lipid and polymer

membranes is highly dependent on membrane thickness,[53] we expect the membrane stiffness of ELP membranes is higher than lipid membranes (typically ~20 k_BT). The mechanical strength of ELP vesicles could be tuned potentially an order of magnitude by cross-linking with a peptide-based polymer.[54]

As ELPs are genetically encodable and can be expressed by CFE, there is the potential for carrying out high throughput screening of ELP sequences with varying guest residues and block lengths to optimize their ability to form peptide vesicles. This could greatly expand the discovery of additional self-assembling ELPs that can be used as synthetic cell membranes. Our demonstration that a different cell-free expressed ELP can incorporate into existing ELP vesicles made of another ELP presents an exciting opportunity for generating composite, multi-functional peptide vesicles by leveraging peptide sequences that provide additional functionalities.

CONCLUSION

In summary, this study presents an emulsion transfer method as a facile approach for generating giant peptide vesicles and highlights the use of amphiphilic peptides as a chassis material for synthetic cell research. Using S48I48 as our model ELP, we show the robust generation of giant peptide vesicles that can be labeled with fluorescently labeled lipids or membrane dye molecules. These giant peptide vesicles can host bacterial CFE reactions and express proteins of interest. We further demonstrate that ELPs expressed by CFE can incorporate into existing an ELP membrane. These highly stable peptide vesicles could serve as a robust platform to advance research in bottom-up synthetic biology.

FIGURE CAPTIONS

Figure 1. Generation of giant peptide vesicles. (A) Amphiphilic ELP with 48 repeats of serine and isoleucine blocks constitute hydrophilic and hydrophobic domains, respectively. (B) Schematic representation of emulsion transfer method for giant peptide vesicles formation. Firstly, ELP S48I48 is dissolved in the oil mixture mineral oil:silicone oil (1:4) with NBD-PE dye. Oil-water interface was created for the directed assembly of amphiphilic polypeptide molecules (step 1). An emulsion containing water-in-oil droplets with an ELP monolayer is transferred to the oil phase (step 2). Giant peptide vesicles are collected by centrifugation at 10,000 × g for 10 minutes and removal of oil (step 3 and 4). (C) Peptide vesicle size distribution with average size 15.1 μm; inset image of a single peptide vesicle labelled with NBD-PE. $N_{vesicles}$ = 112. (D) Schematic illustrating peptide vesicle labeling with Vibrant DiO dye after its preparation and incubation and representative brightfield and fluorescence images of a labeled S48I48 peptide vesicle at least after 1-hour labeling. (E) 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 2. AFM and molecular dynamics characterizations of ELP vesicles. (A) Force-displacement graph from AFM nanoindentation of peptide vesicles (insert) showing both approach and withdrawal curves. Scale bar 10 μm. (B) Force-indentation curve fit to a Hertzian model. (C) Young's moduli calculated from AFM nanoindentation of polyacrylamide gels formed at a constant 3 w/v% monomer acrylamide solution with either 0.06 % (PA1) or 0.1 w/v% (PA2) bis-acrylamide crosslinker and single point nanoindentation measurements of 8 peptide vesicles. (D) The terminal snapshot from a coarse-grained molecular dynamics

simulation of the S48I48 bilayer together with the (VPGSG)$_{48}$ segment, (VPGIG)$_{48}$ segment and water partial density profiles calculated across the bilayer. The bilayer thickness is predicted to be (134.4 ± 0.1) nm.

Figure 3. Effect of chemical stress and osmotic shock on ELP vesicles. (A) Stability of peptide vesicles and lipid vesicles against increasing concentrations of Triton-X 100. All the data points were taken after 5 minutes of addition of Triton-X 100. Number of lipid vesicles and peptide vesicles analyzed were 26 and 19, respectively. (B) Representative images of lipid and peptide vesicles under hypo-osmotic shock, Δ is the difference in osmolarity between inner and outer solutions. Both chemical and physical stress experiments were repeated twice to ensure the reproducibility of the data. Scale bar 10 μm.

Figure 4. Peptide vesicles as a chassis for synthetic cell. (A) Encapsulation of small molecules like rhodamine dye in a S48I48 vesicle. (B) Bulk expression of GFP by an *E. coli* CFE reaction incubated for 4 hours at 30°C and measured on a fluorescence plate reader. (C) Schematic illustrating cell-free expressed protein (GFP) encapsulated inside peptide vesicles generated by emulsion transfer method and fluorescence images of cell-free expressed GFP encapsulated in a S48I48 vesicle. (D) Schematic illustrating encapsulation of CFE reaction to express GFP. Brightfield and fluorescence images of S48I48 vesicle after 4 hours of expression at 30°C. Scale bars are 10 μm.

Figure 5. Incorporation of ELP RQ-F[GreenLys] into the peptide membrane. (A) Fluorescent labelling of ELP RQ-F by incorporating green lysine during CFE by supplementing the reaction

with FluoroTect Green Lys. Labelled RQ-F was added to the outer solution of the peptide vesicles and incubated for at least 3 hours at room temperature. (B) SDS-PAGE of the fluorescently labelled RQ-F with a trail of unreacted reagent. (C) Brightfield and fluorescence images of the ELP S48I48 peptide vesicle with RQ-F^{GreenLys} inserted into the bilayer membrane. (D) Brightfield and fluorescence images of the ELP S48I48 peptide vesicle incubated with CFE reaction with green lysine but without RQ-F DNA. Scale bars are 10 μm.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

SDS-PAGE analysis of *E. coli* expressed ELP and its purification (PDF)

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Author Contributions

B.S., A.L.F, A.P.L conceived the study. B.S., A.P.L designed the experiments. B.S. performed the experiments. Y.M., A.L.F designed the simulation study, Y.M. carried out the simulation. H.H., B.M.B. designed the AFM characterization experiment, H.H. performed the

AFM experiments. B.S., A.P.L wrote the paper. All authors contributed to the manuscript revision and approved the final version of the manuscript.

CONFLICT OF INTEREST

ALF is a co-founder and consultant of Evozyne, LLC and a co-author of US Provisional Patents 62/853,919 and 62/900,420 and International Patent Applications PCT/US2020/035206 and PCT/US20/50466.

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