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Corresponding Author:	Tobias Pirzer, Ph.D. Technische Universitat Munchen Garching, Bavaria GERMANY
Corresponding Author's Institution:	Technische Universitat Munchen
Corresponding Author E-Mail:	pirzer@tum.de
Order of Authors:	Kilian Vogele
	Thomas Frank
	Lukas Gasser
	Marisa A Goetzfried
	Mathias W Hackl
	Stephan A Sieber
	Friedrich C Simmel
	Tobias Pirzer, Ph.D.
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TITLE:

In Vesiculo Synthesis of Peptide Membrane Precursors for Autonomous Vesicle Growth

AUTHORS AND AFFILIATIONS:

Kilian Vogele[1], Thomas Frank[1], Lukas Gasser[1], Marisa A Goetzfried[1], Mathias W Hackl[2], Stephan A Sieber[2], Friedrich C Simmel[1,3], Tobias Pirzer[1]

[1]Physics of Synthetic Systems - E14, Physics-Department and ZNN, Technische Universität München, Garching, Germany
[2]Departmant of Chemistry, Center for Integrated Protein Science (CIPSM), Technische Universität München, Lichtenbergstrasse 4, Garching, Germany
[3]Nanosystems Initiative Munich, Munich, Germany

Corresponding Author:

Tobias Pirzer (pirzer@tum.de)

Email Addresses of Co-authors:

Kilian Vogele (ga25sod@tum.de)
Thomas Frank (ga95pax@mytum.de)
Lukas Gasser (lukas.gasser@tum.de)
Marisa A Goetzfried (mgoetzfried@mytum.de)
Mathias Hackl (mathias.hackl@tum.de)
Stephan A Sieber (stephan.sieber@tum.de)
Friedrich C Simmel (simmel@tum.de)

KEYWORDS:

synthetic cells, elastin-like polypeptides, vesicle growth, in vesiculo peptide expression, encapsulation, membrane growth, transcription-translation system

SUMMARY:

Presented here are protocols for the creation of peptide-based small unilamellar vesicles capable of growth. To facilitate *in vesiculo* production of the membrane peptide, these vesicles are equipped with a transcription-translation system and the peptide-encoding plasmid.

ABSTRACT:

Compartmentalization of biochemical reactions is a central aspect of synthetic cells. For this purpose, peptide-based reaction compartments serve as an attractive alternative to liposomes or fatty acid-based vesicles. Externally or within the vesicles, peptides can be easily expressed and simplify the synthesis of membrane precursors. Provided here is a protocol for the creation of vesicles with diameters of ~200 nm based on the amphiphilic elastin-like polypeptides (ELP) utilizing dehydration-rehydration from glass beads. Also presented are protocols for bacterial ELP expression and purification via inverse temperature cycling, as well as their covalent functionalization with fluorescent dyes. Furthermore, this report describes a protocol to enable the transcription of RNA aptamer dBroccoli inside ELP vesicles as a less complex example for a

biochemical reaction. Finally, a protocol is provided, which allows *in vesiculo* expression of fluorescent proteins and the membrane peptide, whereas synthesis of the latter results in vesicle growth.

INTRODUCTION:

The creation of synthetic living cellular systems is usually approached from two different directions. In the top-down method, the genome of a bacterium is reduced to its essential components, ultimately leading to a minimal cell. In the bottom-up approach, artificial cells are assembled de novo from molecular components or cellular subsystems, which need to be functionally integrated into a consistent cell-like system.

In the de novo approach, compartmentalization of the necessary biochemical components is usually achieved using membranes made from phospholipids or fatty acids[1-4]. This is because "modern" cell membranes mainly consist of phospholipids, while fatty acids are regarded plausible candidates of prebiotic membrane enclosures[5,6]. For the formation of new membranes or to facilitate membrane growth, amphiphilic building blocks must be provided from the exterior[7] or ideally through production within a membranous compartment using the corresponding anabolic processes[4,8].

While lipid synthesis is a relatively complex metabolic process, peptides can be produced quite readily using cell-free gene expression reactions[9,10]. Hence, peptide membranes formed by amphiphilic peptides represent an interesting alternative to lipid membranes as enclosures for artificial cell mimics that are able to grow[11].

Amphiphilic elastin-like di-block copolymers (ELPs) are an attractive class of peptides, which can serve as the building block for such membranes[12]. The basic amino acid sequence motif of ELPs is $(G\alpha GVP)_n$, where "α" can be any amino acid except for proline and "n" is the number of motif repeats[13-17]. ELPs have been created with a hydrophobic block containing mainly phenylalanine for α and a hydrophilic block mainly composed of glutamic acid[11]. Depending on α and solution parameters, such as pH and salt concentration, ELPs exhibit a so-called inverse temperature transition at temperature T_t, where the peptides undergo a fully reversible phase transition from a hydrophilic to hydrophobic state. The synthesis of the peptides can be easily implemented inside vesicles using the "TX-TL" bacterial cell extract[11,18-21], which provides all necessary components for coupled transcription and translation reactions.

The TX-TL system was encapsulated together, with the DNA template encoding the ELPs into ELP vesicles utilizing dehydration-rehydration from glass beads as a solid support. The formation of vesicles occurs through rehydration of the dried peptides from the bead surface[11]. Other methods[22] for vesicle formation can be used, which potentially show lower polydispersity and larger vesicle sizes (e.g., electro-formation, emulsion phase transfer, or microfluidics-based methods). To test the viability of the encapsulation method, transcription of the fluorogenic aptamer dBroccoli[23] can alternatively be used[11], which is less complex than gene expression with the TX-TL system.

Due to the expression of the membrane building blocks in vesiculo and their subsequent incorporation into the membrane, the vesicles start to grow[11]. Membrane incorporation of the ELPs can be demonstrated through a FRET assay. To this end, the ELPs used for formation of the initial vesicle population are be conjugated with fluorescent dyes in equal shares constituting a FRET pair. Upon expression of non-labeled ELPs in vesiculo and their incorporation into the membrane, the labeled ELPs in the membrane are diluted and consequently the FRET signal decreases[11]. As a versatile and common method for conjugation, copper catalyzed azide-alkyne cycloaddition is used. With the use of a stabilizing ligand such as tris(benzyltriazolylmethyl)-amine, the reaction can be carried out in an aqueous solution at a physiological pH without the hydrolysis of reactants[11], which is appropriate for conjugation reactions involving peptides.

The following protocol presents a detailed description of the preparation for growing ELP-based peptidosomes. The expression of the peptides and vesicle formation using the glass beads method are described. Furthermore, it is described how to implement transcription of the fluorogenic dBroccoli aptamer and the transcription-translation reaction for protein expression inside the ELP vesicles. Finally, provided is a procedure for the conjugation of ELPs with fluorophores, which can be used to prove vesicle growth through a FRET assay[11].

PROTOCOL:

1. Expression of elastin-like polypeptides

1.1. Day 1: Preparation of a starter culture and supplies for peptide expression

1.1.1. Prepare and autoclave expression culture flasks (4 x 2.5 L) and 3 L of LB medium. For 1 L of LB medium, add 25 g of LB powder to 1 L of ultrapure water.

1.1.2. Prepare a starter culture with 100 mL of LB medium, 50 µL of sterile-filtered (0.22 µm filter) chloramphenicol solution (25 mg/mL in EtOH), and 50 µL of sterile-filtered (0.22 µm filter) carbenicillin solution (100 mg/mL in 50% EtOH and 50% ultrapure water).

1.1.3. Add a small streak from a pre-made bacterial stock containing *E. coli* strain BL21(DE3)pLysS with the pET20b(+) expression vector encoding the polypeptide sequence MGHGVGVP((GEGVP)$_4$(GVGVP))$_4$((GFGVP)$_4$(GVGVP))$_3$(GFGVP)$_4$GWP (abbreviated as EF) to a pre-warmed 100 mL starter culture and incubate at 37 °C for 16 h with 250 rpm in a shaking incubator (*E. coli* strains and vectors are available upon request).

1.1.4. Pre-warm the expression culture flasks and the LB media at 37 °C overnight so that they are prepared for the next day.

1.2. Day 2: Protein expression

1.2.1. Add 750 mL of LB medium to each expression culture flask, 375 µL of sterile-filtered

(0.22 µm filter) chloramphenicol stock (25 mg/mL in EtOH), and 375 µL of sterile-filtered (0.22 µm filter) carbenicillin stock (100 mg/mL in 50% EtOH and 50% ultrapure water).

1.2.2. After agitation to distribute the antibiotics, take 2 mL of the media as a reference sample for the optical density measurement at 600 nm (OD600).

1.2.3. Add 7.5 mL of the starting culture to the expression flask and incubate for 1 h at 37 °C with 250 rpm.

1.2.4. After this step the OD600 of each flask will be checked every 20 min.

1.2.5. When the optical density reaches approximately 0.8 reduce the temperature to 16 °C and induce peptide expression by adding 750 µL of 1 M sterile-filtered β-isopropyl thiogalactoside (IPTG) in ultrapure water into each expression flask.

1.2.6. Incubate the expression flask with the induced bacteria at 16 °C for 16 h with 250 rpm.

1.3. Day 3: Extraction of the expressed polypeptide

1.3.1. Pre-weigh the centrifugation flask to enable determination of the mass of the cell pellet after harvesting.

1.3.2. Harvest all the bacteria from the expression flasks by centrifugation for 20 min using a pre-cooled centrifuge at 4,000 x *g* and 4 °C.

1.3.3. Pour out the supernatant and blot the centrifuge bottles on a sterile paper towel.

1.3.4. Weigh the centrifugation flasks and calculate the cell pellet mass.

1.3.5. Resuspend the cells, which are pelleted down from 750 mL of original cell culture, in 15 mL of phosphate-buffered saline (PBS, pH 7.4) by pipetting, and centrifuge at 4,000 x *g* for 20 min at 4 °C. Pour out the supernatant and blot the centrifuge bottles on a sterile paper towel.

1.3.6. Resuspend the cell pellet for the lysis step in 2 mL of buffer per 1 gram of cell pellet using PBS (pH 7.4) supplemented with lysozyme (1 mg/mL), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 0.5 U of DNase I.

1.3.7. Sonicate the cells for further lysis on ice with a sonicator at 8 W for 9 min with alternating 10 s sonication and 20 s pausing steps, followed by a 9 min pause during which the sample should be cooled on ice. Repeat the 9 min sonication step once more.

1.3.8. Add 2 mL of 10% (w/v) polyethyleneimine (PEI) per 1 L of original cell culture for nucleic acid precipitation.

1.3.9. Distribute the sample into 2 mL centrifuge tubes and incubate at 60 °C for 10 min followed by an incubation for 10 min at 4 °C.

1.3.10. Centrifuge the solution at 16,000 x *g* for 10 min at 4 °C and collect the supernatant containing the ELP.

1.4. Protein purification via inverse temperature cycling:

1.4.1. Adjust the supernatant to a pH of 2 to switch the hydrophilic part of the peptide to hydrophobic and induce aggregation. To adjust the pH, phosphoric acid and sodium hydroxide are used. pH stripes are sufficient to determine the pH level.

1.4.2. To improve the yield of the purification the sample, heat the sample to 60 °C and add sodium chloride up to 2 M.

1.4.3. Subsequently, centrifuge the sample at 16,000 x *g* for 10 min at room temperature (RT).

1.4.4. Discard the supernatant and re-dissolve the pellet in PBS (but only half of the initial volume is used compared to the previous step).

1.4.5. Adjust the pH to 7 with phosphoric acid and sodium hydroxide. pH stripes are sufficiently accurate to determine the pH.

1.4.6. Centrifuge the sample subsequently at 16,000 x *g* for 10 min at 4 °C.

1.4.7. Collect the supernatant and repeat steps 1.4.1–1.4.6 up to 3x total, except that in the last repetition of step 1.4.4, only water is added instead of PBS.

1.4.8. Measure the concentration of the peptides with an absorption spectrometer. Use an extinction coefficient of 5500/M/cm at 280 nm.

1.4.9. Adjust the concentration of the ELP to 700 µM.

2. Vesicle production using the glass beads method

2.1. Concentrate the ELP solution to 1.1 mM using a centrifugal vacuum concentrator.

2.2. Mix 200 µL of the concentrated ELP solution with 1250 µL of a 2:1 chloroform/methanol mixture followed by vortexing.

2.3. Add 1.5 g of spherical glass beads (212–300 µm in size) to a 10 mL round bottom flask.

2.4. Add the solution containing ELP and the chloroform/methanol mixture to the round bottom flask and mix by gentle shaking.

2.5. Connect the flask to a rotary evaporator. Adjust the speed to 150 rpm and regulate the pressure to -0.8 x 10^5 Pa (-800 mbar) for approximately 4 min until the liquid is evaporated at room temperature.

2.6. Place the round bottom flask into a desiccator for at least 1 h to ensure that remaining chloroform and methanol are evaporated. To avoid loss of the glass beads, loosely attached aluminum foil should be wrapped around the round bottom flask opening.

2.7. For a single experiment, mix 100 mg of the peptide covered glass beads with 60 µL of the swelling solution, such as PBS. Incubate this sample at 25 °C for 5 min.

2.8. Centrifuge the sample quickly with a table-top centrifuge to sediment the glass beads.

2.9. Use a pipette to collect the supernatant which contains the vesicles.

3. Transcription of RNA aptamer dBroccoli inside the vesicles

3.1. Clean the lab bench with wipes containing RNase decontamination solution to create an RNase-free working environment.

3.2. Mix the ssDNA (5 µM) comprising the T7 promotor and dBroccoli sequence (GAGACGGTCGGGTCCATCTGAGACGGTCGGGTCCAGATATTCGTATCTGTCGAGTAGAGTGTGGGCTC AGATGTCGAGTAGAGTGTGGGCTC), as well as the noncoding strand (5 µM) in nuclease-free water supplemented with RNAPol reaction buffer [40 mM Tris-HCl (pH = 7.9), 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 2 mM spermidine], to prepare the DNA template for the transcription reaction.

3.3. Incubate the solution at 90 °C for 5 min followed by a slow temperature decrease to 20 °C for 30 min.

3.4. Prepare a 1 mM (5Z)-5-(3,5-difluoro-4-hydroxybenzylidene)-2,3-dimethyl-3,5-dihydro-4H-imidazol-4-one (DFHBI) solution by dissolving 1.26 mg of DFHBI in 5 mL of DMSO.

3.5. Prepare the transcription reaction by mixing RNAPol reaction buffer [40 mM Tris-HCl (pH = 7.9), 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 2 mM spermidine] with 125 mM KCl, 15 mM $MgCl_2$, 4 mM rNTP, 10 µM DFHBI, 200 nM DNA template, 0.5 U/µL RNase inhibitor murine, and water.

3.6. Right before the reaction is started, add 4 U/µL T7 polymerase.

3.7. Use this reaction mix as the swelling solution in step 2.7 and incubate at 37 °C for the duration of the experiment, which is typically 1 h.

4. Transcription-translation (TX-TL) reaction

NOTE: For the transcription-translation reaction, a crude cell extract is required as well as reaction buffer and DNA. The crude cell extract is prepared as described in Sun et al.[18]. For a TX-TL reaction, use the following: 33% (v/v) of the crude *E. coli* extract, 42% (v/v) reaction buffer, and 25% (v/v) phenol-chloroform purified DNA plus additives. The final concentrations are approximately 9 mg/mL protein, 50 mM HEPES (pH = 8), 1.5 mM ATP, 1.5 mM GTP, 0.9 mM CTP, 0.9 mM UTP, 0.2 mg/mL tRNA, 26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 68 mM folinic acid, 1 mM spermidine, 30 mM PEP, 1 mM DTT, 2% PEG-8000, 13.3 mM maltose, 1 U of T7 RNA polymerase, and 50 nM plasmid DNA in ultrapure water.

4.1. Phenol-chloroform purification of the DNA template

4.1.1. Prepare six overnight cultures with 5 mL of LB medium and 5 µL of sterile-filtered (0.22 µm filter) carbenicillin solution (100 mg/mL in 50% EtOH and 50% ultrapure water).

4.1.2. Add a small streak from a pre-made bacterial stock containing DH5α *E. coli* with the pET20b(+) expression vector to each pre-warmed 5 mL overnight culture and incubate at 37 °C for 16 h with 250 rpm. Depending on which peptide (EF) or protein (YPet or mVenus) is to be expressed, a specific encoding plasmid is used.

4.1.3. Extract the plasmid with a mini-prep kit as described in the user manual or by following the protocol provided by Zhang et al.[24].

4.1.4. Prepare 100 µL of the pre-purified plasmid DNA (concentration can range from 200–600 ng/µL) and mix with 100 µL of Roti-phenol/chloroform/isoamyl alcohol (pH = 7.5–8.0) in a microcentrifuge tube to enable better phase separation.

4.1.5. Gently invert the tube up to 6x and centrifuge for 5 min at 16,000 x *g* at RT.

4.1.6. Add 200 µL of chloroform to the upper phase of the tube and invert the tube up to 6x.

4.1.7. Centrifuge the sample at 16,000 x *g* for 5 min at RT.

4.1.8. Pipet the supernatant to a separate tube and add 10 µL of 3 M of sodium acetate for ethanol precipitation.

4.1.9. Add 1 mL of -80 °C cold ethanol and store the sample at -80 °C for 1 h.

4.1.10. Centrifuge the sample at 16,000 x *g* for 15 min at 4 °C.

4.1.11. Decant the supernatant and add 1 mL of -20 °C cold 70% (v/v) ethanol.

4.1.12. Centrifuge at 16,000 x *g* for 5 min at 4 °C.

4.1.13. Remove the liquid by pipetting. Be careful to not disturb the DNA pellet.

4.1.14. Store the sample at RT for approximately 15 min to evaporate the remaining ethanol.

4.1.15. Add ultrapure water to the sample to adjust the sample concentration to approximately 300 nM, which is measured by absorption at 260 nm.

4.2. Preparation of the TX-TL reaction

4.2.1. Thaw the prepared crude cell extract and the reaction buffer on ice.

4.2.2. For a 60 µL reaction mix, add the plasmid DNA (phenol-chloroform purified) to 37.5 µL of the reaction buffer [50 mM HEPES (pH = 8), 1.5 mM ATP, 1.5 mM GTP, 0.9 mM CTP, 0.9 mM UTP, 0.2 mg/mL tRNA, 26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 68 mM folinic acid, 1 mM spermidine, 30 mM PEP, 1 mM DTT, 2% PEG-8000, and 13.3 mM maltose), followed by the addition of 28.7 µL of crude cell extract. Fill to a final volume with ultrapure water to 58.8 µL.

4.2.3. Right before the reaction starts, add 1.2 µL of the T7 RNA polymerase solution and mix the sample by pipetting up and down.

4.2.4. Use this reaction mix as a swelling solution in step 2.7 and incubate at 29 °C for the duration of the experiment, which is typically 4–8 h.

5. Conjugation of elastin-like polypeptides with fluorophores via copper catalyzed azide-alkyne Huisgen cycloaddition

5.1. Prepare a 20 mM NHS-azide linker (γ-azidobutyric acid N-hydroxysuccinimide ester) solution in DMSO.

5.2. Mix 250 µL of the ELP solution (600 µM) with PBS (8 g/L NaCl, 0.2 g/L KCl, 1.42 g/L Na_2HPO_4, 0.27 g/L K_2HPO_4, pH = 7.2) and NHS-azide (1.2 mM) and incubate for 12 h at RT.

5.3. Load the sample in a 10 kDa dialysis cassette and store it at 4 °C for 12 h in 1 L of ultrapure water to remove the salts and residual NHS-azide.

5.4. Mix the activated ELP (500 µM) with the alkyne-conjugated dye (500 µM), Cy5-alkyne, or Cy3-alkyne.

5.5. Mix this sample with 1 mM TBTA (tris(benzyltriazolylmethyl)amine), 10 mM TCEP (tris(2-carboxyethyl)-phosphine hydrochloride) and 10 mM $CuSO_4$ and incubate at 4 °C for 12 h.

5.6. Load the sample in a 10 kDa dialysis cassette and store it at 4 °C for 12 h in 1 L of

ultrapure water, to remove the salts and residual alkyne-conjugated dyes.

5.7. These dye-modified ELPs are used in a 1:1 mixture of the Cy3 labeled ELPs and the Cy5 labeled ELPs analogous to the peptides in part two.

REPRESENTATIVE RESULTS:

Vesicle production

Figure 1 shows transmission electron microscopy (TEM) images of vesicles prepared with different swelling solutions and the glass beads method (also see Vogele et al.[11]). For the sample in **Figure 1A,** only PBS was used as swelling solution to prove the formation of vesicles and to determine their size. When TX-TL was used as swelling solution (**Figure 1B**), the vesicles also formed. Dynamic light scattering (DLS) measurements were performed to show that the glass beads method has an effect on vesicle formation. **Figure 2** depicts the measured intensity autocorrelation curves of an EF sample prepared without glass beads in PBS (blue) and an EF sample prepared with the glass beads method with PBS as the swelling solution (red). The sizes were calculated from a cumulant fit[25] and resulted in a diameter of 134 nm with a polydispersity of 25% when the vesicles were prepared without the glass beads method. When the glass beads were used, the cumulant fit resulted in a diameter of 168 nm with a polydispersity of 21%.

In vesiculo transcription[11]

Figure 3 shows the fluorescence intensity profile over time for the transcription of the dBroccoli aptamer inside the EF vesicles (green). As a negative control, DNase I was added to the swelling solution and thus also incorporated during vesicle formation (blue). The measurements were performed in fluorescence plate reader with excitation at 480 nm and emission at 520 nm.

In vesiculo protein expression[11]

Figure 4 shows the fluorescence intensity of two fluorescent proteins which were expressed inside the ELP vesicles using a fluorescence plate reader. Excitation was carried out 500 nm and emission at 520 nm. Transcription-translation of mVenus (**Figure 4A**) was performed to investigate expression dynamics and expression level of proteins in the ELP vesicle. It is important to note that after vesicle formation, the contents of the vesicles and outer solution are the same. Hence, to suppress protein expression outside of the vesicle, the antibiotic kanamycin was added to the exterior solution. As a control, kanamycin was also added to the swelling solution, in which case protein expression inside of the vesicle was suppressed. This further indicates that the small molecule kanamycin stays inside the vesicle and suggests that the membrane is not permeable for this molecule over the time scale of these experiments. If kanamycin diffused through the membrane, the mVenus expression would not have been suppressed and at 5 h, the fluorescence would be higher. In the second experiment, the expression of YPet (**Figure 4B**) was carried out. Both proteins were chosen because they exhibit a faster maturation time than, for instance, GFP. Furthermore, the T7 promoter was used for mVenus transcription and a constitutive promoter used for YPet transcription to show that both inducible and continuous expression are possible.

FRET assay[11]

Figure 5A shows the results of the FRET assay performed to demonstrate ELP incorporation into the membrane. Therefore, the vesicles were produced using a mixture of two equally concentrated fluorophore-peptide constructs. These were Cy3-EF (donor) and Cy5-EF (acceptor). At time t = 0, the starting FRET level was measured. The signals of the donor and the FRET signal depend on the mean distance between the dyes in the membrane. Upon expression of the membrane ELPs, additional peptides incorporate into the membrane, which increases the average distance between the FRET pairs. The latter was measured through the increase in donor fluorescence and a decrease of the FRET signal at time t = 2.5 h. **Figure 5B** shows the negative control. Here, kanamycin was added to the swelling solution before vesicle formation. Kanamycin suppresses protein expression; therefore, no change in FRET was visible. It is important to note that this assay only shows EF incorporation.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative TEM images. (**A**) EF vesicles formed in the swelling solution PBS. (**B**) EF vesicles formed in the swelling solution TX-TL. Scale bars = 200 nm.

Figure 2: Representative DLS data. Intensity autocorrelation curves measured by DLS. The blue curve depicts EF peptides in PBS prepared without the glass beads method. The red curve depicts an EF solution produced with the glass beads method and PBS as the swelling solution.

Figure 3: dBroccoli transcription. Representative data of the transcription of the dBroccoli aptamer inside EF vesicles. The green curve depicts the fluorescence signal and the blue curve the control measurement with encapsulated DNase I.

Figure 4: Protein expression inside peptide vesicles. (**A**) A plasmid encoding mVenus and the TX-TL expression mix were encapsulated in the ELP vesicles. After approximately 5 h, the fluorescence saturated. When the antibiotic kanamycin was encapsulated as well, no fluorescence was observed. (**B**) Similar results were obtained upon encapsulation of a YPet plasmid. Error bars represent the standard deviation of the measured values at the indicated time during a time frame of 15 min.

Figure 5: FRET assay. (**A**) Starting fluorescence levels at time t = 0 for donor emission and FRET signal (acceptor emission). At t = 2.5 h, the donor showed increased emission, and the FRET signal decreased. (**B**) When only hydrophilic ELP were expressed inside the vesicles, the FRET signal and donor emission stayed constant. Error bars represent the standard deviation of the measured values at the indicated time during a time frame of 15 min.

Supplemental File: Contains all plasmid sequences resp. gene sequences.

DISCUSSION:

Film rehydration is a common procedure for the creation of small unilamellar vesicles. The main source of failure is the wrong handling of the materials used in the procedure.

Initially, the ELPs are produced by *E. coli* cells. The yield after ELP purification can vary significantly depending on how carefully the protocol is conducted during its crucial steps. These are the inverse temperature cycling (ITC) step and the resuspension of the ELPs after precipitation. For purification, we triggered the hydrophobic collapse of the peptide through the addition of phosphoric acid, which changes the pH to acidic and leads to protonation of the glutamic acid side chains in the hydrophilic block. After this step, both blocks are hydrophobic at RT. It is preferable to use an acid whose salt is already in the solution to keep the ionic strength constant, but other acids such as hydrochloric acid can be used, as well. To increase the yield of ELP production, additional salts can be added to enhance the coacervation process, because salt decreases the transition temperature T_t and makes the ELP more hydrophobic. Usually sodium chloride is used, but kosmotropic salts such as sodium sulphate are far better, whereas the salt concentration can be close to its solution limit. Additional heating using a water bath can increase the yield even further.

Another critical step is the redissolution of the ELP pellet. For maximum ELP solubility, the solution should be pre-cooled, and a quick pH adjustment can help to dissolve the pellet faster. The pH may need to be readjusted during this process several times. For further improvement, the sample can be stored in the fridge at 4 °C and shaking of the sample should be avoided. To exchange salts, the described dialysis step at the end of the purification protocol should be preferred. In principle, ITC can be used to separate the peptides from the salt-containing supernatant. But depending on the previous salt concentration, residual salt can be still found in this pellet. Furthermore, dialysis can be used to concentrate the peptides needed for the experiments without losses as mentioned before.

The next crucial step is the evaporation of the chloroform/methanol mixture. During evaporation at reduced pressures, a delay in boiling can cause splatters or turbulences, which may result in large loss of glass beads. This can be prevented using either filters or tissues. The same problem arises when the desiccator is used, but aluminum foil at the opening of the round bottom flask can be used to prevent this. For the handling of the ELP-coated glass beads, it is recommended to use disposable antistatic micro-spatula, which also reduces losses and simplifies the procedure.

The swelling of the vesicles using various swelling solutions such as PBS and transcription mix is straightforward and less error prone. However, for the TX-TL system, variations from batch-to-batch of up to 10% can occur. To minimize this problem, all experiments should be performed with one batch of cell-free extract only, which can be used for up to 3,000 reactions. After rehydration, the vesicle content and outer solution are the swelling solution. A purification of the outside solution should not be performed, because this may change the osmotic pressure difference between vesicle inside and outside. The result would be an uncontrolled size change or even bursting of the vesicles. Therefore, possible reactions at the outside should be only suppressed. Depending on the reaction, this can be done, for instance, by the addition DNase I

to digest present DNA or by adding antibiotics such as kanamycin, which inhibits translation.

Film rehydration from glass beads has several advantages when compared to other preparation methods. In contrast to solvent injection, emulsion phase transfer or microfluidics displacement of solvent is not required, since the initial solvent is completely evaporated, and the peptides are rehydrated in aqueous solution. Therefore, the glass beads method is particularly suitable for sensitive samples such as TX-TL, which can be significantly affected or even destroyed by residual organic solvents used in other methods. Furthermore, the protocol is straightforward, less error prone, and able to be easily scaled up. Because of the large surface to volume ratio only small amounts of glass beads are needed and allow a high degree of parallelization with high throughput.

As its main disadvantage, the glass beads method is only useful for the creation of small unilamellar vesicles, which cannot be observed via fluorescence microscopy. The described method is somewhat limited when microscopic observation of the dynamics of single vesicles is desired, which is sometimes necessary (e.g., when observing potential vesicle division processes). Furthermore, the small size of SUVs limits the encapsulation of low-concentrated components such as the peptide-encoding plasmid, which explains the relatively low expression of the peptides. The underlying encapsulation process is a Poisson process, and thus the concentration of any particular component must be at least 150 nM to guarantee an encapsulation probability of 95%. Therefore, it is quite remarkable that it is possible to observe such a large increase in vesicle size in these experiments.

The protocol presented here will enable researchers to create peptide-based vesicles from elastin-like polypeptides. It also opens up opportunities to produce artificial cell-like structures encapsulated by peptide membranes, which represent attractive alternatives to classical compartments made from fatty acids or phospholipids. Peptides are advantageous in that they can be easily expressed in a cell-free environment (for instance, in the TX-TL system used here), which allows coupling of membrane growth directly to a transcription-translation process inside the vesicle. Furthermore, ELPs can be designed and adjusted to specific physicochemical parameters such as contour length, hydrophobicity/hydrophilicity of the used deblock, and sensitivity to stimuli such as pH or ionic strength.

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DISCLOSURES:

The authors declare no competing financial interests.

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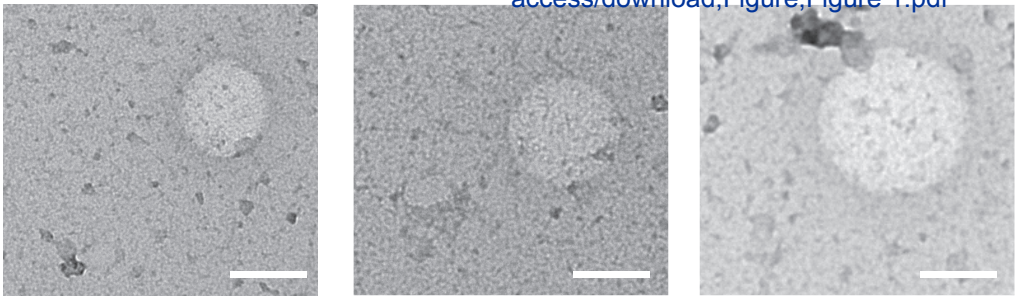
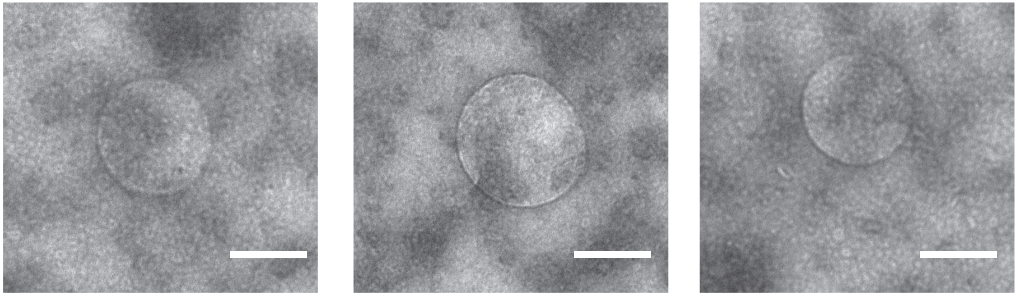
Figure

A in PBS



B in TX/TL



Figure

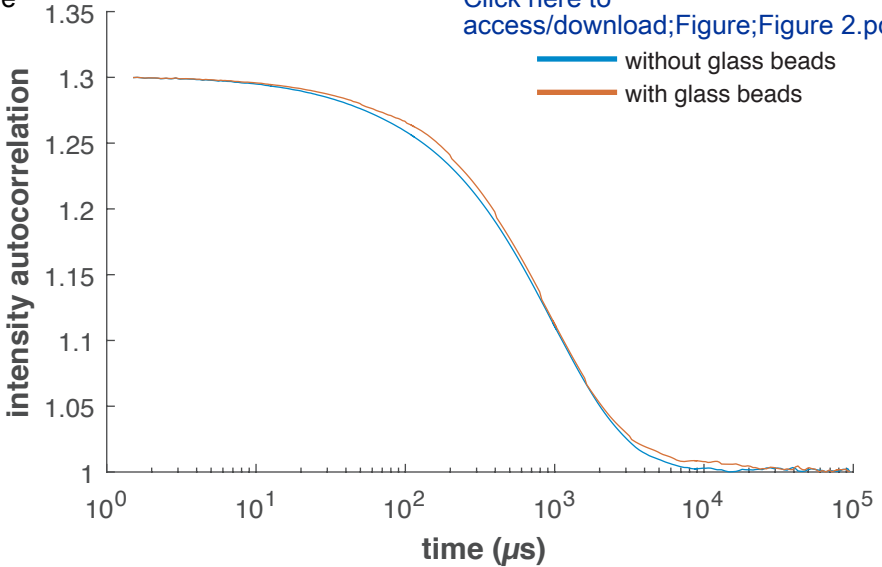
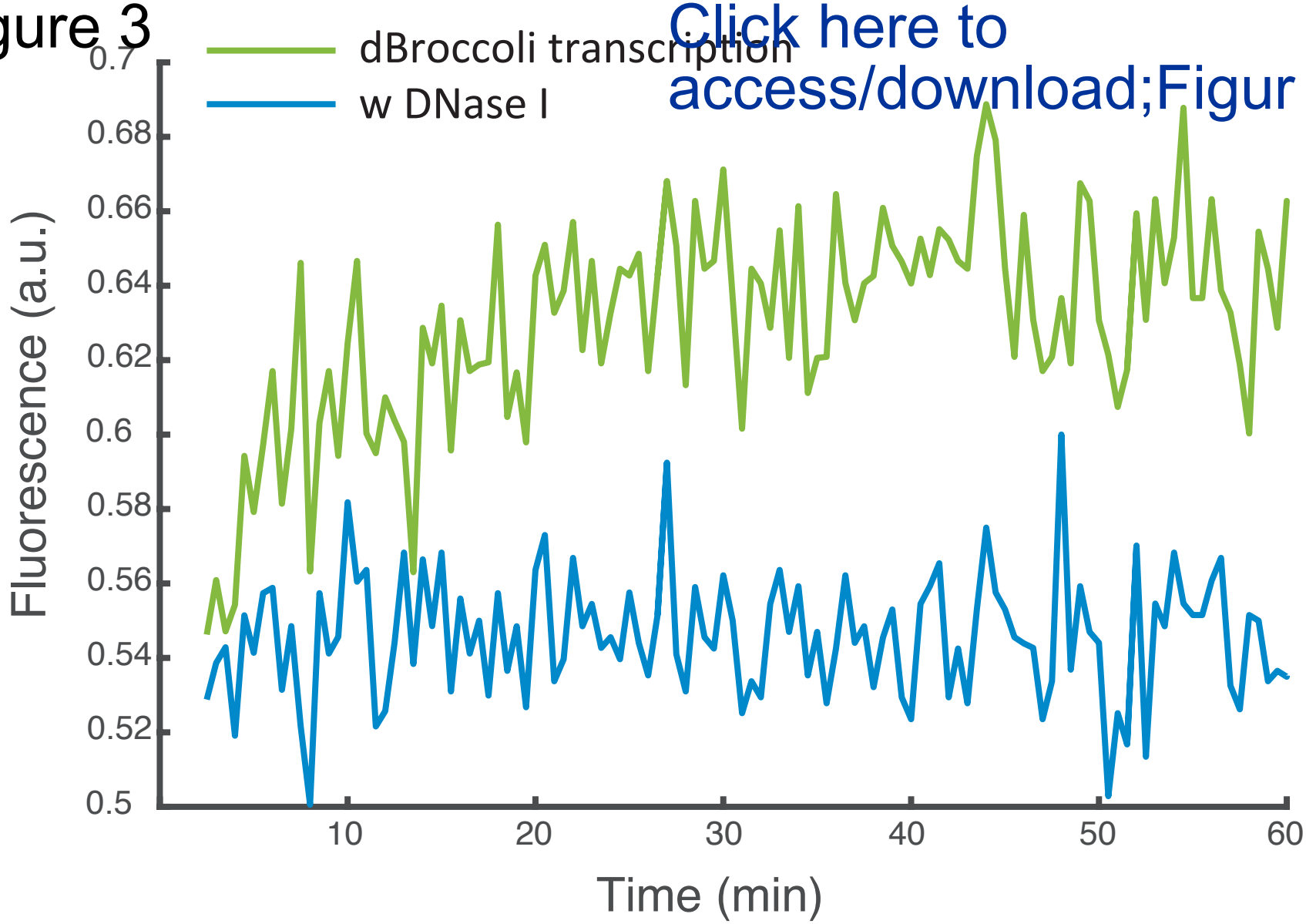
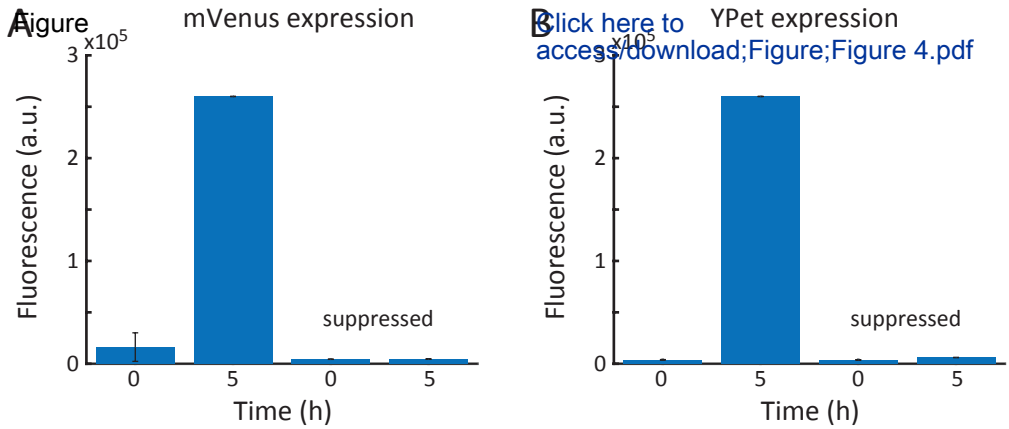


Figure 3





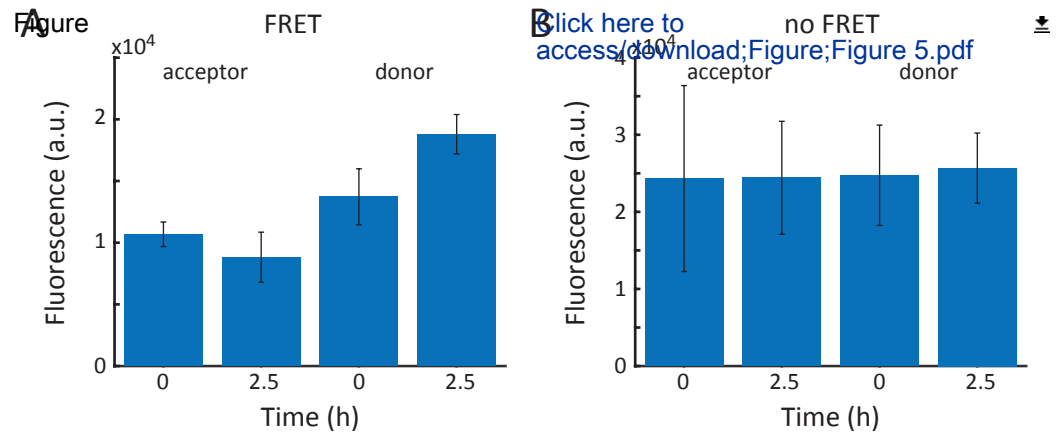


Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2xYT	MP biomedicals	3012-032	
3-PGA	Sigma-Aldrich	P8877	
5PRIME Phase Lock GelTM tube	VWR	733-2478	
alkine-conjugated Cy3	Sigma-Aldrich	777331	
alkine-conjugated Cy5	Sigma-Aldrich	777358	
ATP	Sigma-Aldrich	A8937	
benzamidin	Carl Roth	CN38.2	
BL21 Rosetta 2 E. coli strain	Novagen	71402	
Bradford BSA Protein Assay Kit	Bio-rad	500-0201	
cAMP	Sigma-Aldrich	A9501	
carbenicillin	Carl Roth	6344.2	
Chloramphenicol	Sigma-Aldrich	C1919	
chloramphenicol	Carl Roth	3886.3	
chloroform	Carl Roth	4432.1	
CoA	Sigma-Aldrich	C4282	
CTP	USB	14121	
$CuSO_4$	Carl Roth	P024.1	
DFHBI	Lucerna Technologies	410	
DMSO	Carl Roth	A994.1	
DNase I	NEB	M0303S	
DTT	Sigma-Aldrich	D0632	
Ethanol	Carl Roth	9065.2	
Folinic acid	Sigma-Aldrich	F7878	
Glass beads, acid-washed	Sigma-Aldrich	G1277	
GTP	USB	16800	
HEPES	Sigma-Aldrich	H6147	
IPTG (β-isopropyl thiogalactoside)	Sigma-Aldrich	I6758	
KCl	Carl Roth	P017.1	
K-glutamate	Sigma-Aldrich	G1149	
LB Broth	Carl Roth	X968.2	
lysozyme	Sigma-Aldrich	L6876	
methanol	Carl Roth	82.2	
$MgCl_2$	Carl Roth	KK36.3	
Mg-glutamate	Sigma-Aldrich	49605	
Micro Bio-Spin Chromatography Columns	Bio-Rad	732-6204	
NAD	Sigma-Aldrich	N6522	
NHS-azide linker (γ-azidobutyric acid oxysuccinimide ester)	Baseclick	BCL-033-5	
PEG-8000	Carl Roth	263.2	
pH stripes	Carl Roth	549.2	
phenylmethylsulfonyl fluoride	Carl Roth	6367.2	
phosphate-buffered saline	VWR	76180-684	
phosphoric acid	Sigma-Aldrich	W290017	
polyethyleneimine	Sigma-Aldrich	408727	
Potassium phosphate dibasic solution	Sigma-Aldrich	P8584	
Potassium phosphate monobasic solution	Sigma-Aldrich	P8709	
Qiagen Miniprep Kit	Qiagen	27106	
RNAPol reaction buffer	NEB	B9012	
RNase inhibitor murine	NEB	M0314S	
RNaseZap Wipes	ThermoFisher	AM9788	
rNTP	NEB	N0466S	
Roti-Phenol/Chloroform/Isoamyl alcohol	Carlroth	A156.1	
RTS Amino Acid Sampler	5 Prime	2401530	
Slide-A-Lyzer Dialysis Cassettes, 10k MWCO (Kit)	Thermo-Scientific	66382	
sodium chloride	Carl Roth	9265.1	
sodium hydroxide	Carl Roth	8655.1	
Spermidine	Sigma-Aldrich	85558	
sterile-filtered (0.22 µm filter)	Carl Roth	XH76.1	
T7 polymerase	NEB	M0251S	
TBTA (tris(benzyltriazolylmethyl)amine)	Sigma-Aldrich	678937	
TCEP (tris(2-carboxyethyl)-phosphine hydrochloride)	Sigma-Aldrich	C4706	
Tris base	Fischer	BP1521	
tRNA (from E. coli)	Roche Applied Science	MRE600	
UTP	USB	23160	

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Author(s): Tobias Pirzer

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Vineeta Bajaj, PhD.
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Munich, March 28th, 2019

Dear Vineeta Bajaja,

we hereby submit a thoroughly revised version of our manuscript “In vesiculo Synthesis of Peptide Membrane Precursors for Autonomous Vesicle Growth”, authored by Kilian Vogele, Thomas Frank, Lukas Gasser, Marisa Goetzfried, Mathias Hackl, Stephan Sieber, Friedrich Simmel and myself.

The reviewers’ comments were very constructive, and helped us to improve our work at various points. In the following you can find our point-to-point replies to editorial and peer review comments. We also provide a pdf of our manuscript where you can find our changes in the text.

We hope that after these improvements, our manuscript can be accepted for publication in JoVE.

With best wishes,

Tobias Pirzer

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Dr. Tobias Pirzer
Am Coulombwall 4a
85748 Garching

Tel. 089 289 11609
Fax 089 289 11612

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For example: RNaseZap, etc.
R: Thank you for this information. We removed all registered symbols and company names.
4. Please do not abbreviate journal titles.
R: We changed journal titles.

Reviewer #1:

Manuscript Summary:
The authors described detailed protocols for preparation of ELP-based vesicles that grow up in vesiculo expression of ELP. The protocols described in the article are: (1) expression and ICT purification of ELP, (2) vesicle preparation using the glass bead method, (3) transcription and (4) transcription-translation reactions in ELP vesicles (including the FRET assay for the vesicle growth by ELP expression). Overall, most of protocols are clear and provide detailed descriptions. However, there are some major and minor concerns that need to be addressed.

Major Concerns:

1. The protocols described in the manuscript are based on the author's previous work (Ref. 23; Nat. Commun. 9 (1), 3862, (2018)), but this was not clear due to missing citation at appropriate texts. For example, the authors' descriptions about the ELP design, vesicles formation, and growth by in-vesicle protein expression, which are provided in the introduction section, seem to require citation of the author's previous article. This would provide clear information about what have been done previously by the authors.
R: We agree with the reviewer on that crucial point. We included the citation of our previous work Nat. Commun. 9 (1), 3862, (2018) at several locations in the manuscript.

2. A part of protocols provided in the manuscript is the transcription of the RNA aptamer dBroccoli. However, no results for this are discussed in the main text, whereas the results for the transcription-translation (of mVenus and yPet) are provided. If there is any reason for not presenting the results, it should be provided.
R: We are very thankful for this remark. We added representative data regarding the aptamer transcription to the main text.

3. It would be critical to accurately quantify the amounts of vesicles produced from the glass beads method, if the vesicle yield is not close to 100%. The authors should comment on the vesicle yield or appropriate quantification methods.
R: The quantity of produced vesicles is indeed a valuable point. To our knowledge the glass beads method and other methods such as solvent evaporation (see Chem. Soc. Rev. 47 (23), 8571, (2018) produce solutions with vesicles, and micelles and monomers as well. Therefore, we couldn’t quantify the total number of vesicles. Peptide release experiments from glass beads would also not be sufficient, because they would only provide the amount of ELPs in solution, but not the form.

Minor Concerns:
The protocol for preparation of the TX-TL reaction (4.2) is confusing in some texts. It is likely that the protocols described in 4.2.2 seems to be repeated in 4.2.3, 4.2.4, and 4.2.5. The authors my need to clarify this or correct mistakes if there are any.
R: We changed these points to make the protocol clearer.

Reviewer #2:

Manuscript Summary:
This paper describes the expression, purification and dye labeling of ELP protein, and fabrication of vesicles from ELPs using solvent dehydration on glass beads and aqueous rehydration. It also describes preparation of reaction mixtures to express protein, fluorescent or ELP, and the procedure for loading these mixtures into the vesicles. Overall, the method description itself thorough, with only some minor details that are unclear. The major issues are the lack of documentation that vesicles were made from this procedure and that the loading procedure cannot place cargo only in the vesicles and not outside.

Major Concerns:
1. There are no TEM images or light scattering to actually prove that vesicles have been made from this method, as opposed to coacervates or micelles or some other sort of structure. What is their size and polydispersity? This should also be performed after the ELP synthesis vesical "growth" step, since the method claims to be able to grow vesicles and this is not demonstrated. The distance to change FRET is so small, that the overall vesicle size may not actually change significantly. I think a method paper should include verification that the steps were performed correctly and the reader made what was expected.
R: This is a very crucial point and we would like to thank this reviewer for mentioning this. We added TEM images to the manuscript, which show vesicles produced with the glass beads method using PBS as swelling solution and with TX-TL as swelling solution to see the differences. Additionally, we added DLS data for a sample in PBS produced without the glass beads method and a vesicle sample produced with the glass beads method with PBS as the swelling solution. If cell extract would be present it would influence the DLS measurement and a size determination would be error prone. But the size and the polydispersity can be determined from TEM measurements of the same sample at ELP expression start and after 240 min.
The reviewer is right, FRET cannot indicate the size or the size change of the vesicles. The FRET assay is only used to show the incorporation of ELPs into the membrane. For clarification we added this information to the main text.

2. It seems like a critical limitation of the method that the contents inside and outside the vesicle are the same, especially since some of the cargo components are quite expensive. The vesicles should be washed so the desired cargo is only inside, and if they cannot be washed, then it sounds like they are not actually stable. If this is the case, it should be made clear to the reader.
R: We agree to the reviewer that it is critical to have the contents inside and outside. But from our perspective this is not a limitation. Reactions at the outside can be suppressed and the wanted reactions at the inside observed. The suppression step does not interfere with the vesicles' integrity. We left out the washing step, because for all kinds of vesicles it is very important, that the osmotic pressure difference between inside and outside is not too much; otherwise they would change their size or even burst. And we cannot guarantee that the osmotic pressure stays constant through a washing step. We are now mentioning this in the main text, so that the reader is aware of this.

3. How was fluorescence of vesicles measured?
R: Thank you for commenting on that. The fluorescence was always measured using a fluorescence plate reader. We added this to the main text.

4. FRET assay is not described fully. What is the donor and what is the acceptor? Are they conjugated dyes or fluorescent proteins, or one of each?

R: We clearified these points in the main text.

Minor Concerns:
1.1.1 Provide actual components of your lysogeny broth, as there is significant confusion in the literature between it and Luria broth, also abbreviated LB.
R: We agree to this point. We now provide the components of the used LB.

1.3.5,6 How should the cells be resuspended? Pipet? Vortex? Please specify.
Why is PEI added during purification?
R: The cells can be resuspended by either way. We added this to the text. We also mentioned that PEI was added to precipitate DNA from cell lysates.

2.1 what kind of concentrator?
R: We added this to the main text.

3.4 What is DFHBI?
R: We mentioned this in the main text

4.1.15 How should DNA concentration be measured?
R: We mentioned this in the main text.

4.2.2 is confusing given the final concentrations of everything after mixing written at the beginning of step 4. Separate out what the cell lysate buffer should be and what the reaction buffer recipe is, since those are supposed to be made separately.
R: Thank you for this comment. We adjusted that point in the protocol.

5.7 Is the 1:1 mixture meant to be Cy3:Cy5, or dyed: undyed? It is not clear.
R: We clearified this point in the text.

Supplementary Information

In vesiculo Synthesis of Peptide Membrane Precursors for Autonomous Vesicle Growth

Kilian Vogele[1], Thomas Frank[1], Lukas Gasser[1], Marisa A Goetzfried[1], Mathias W Hackl[2], Stephan A Sieber[2], Friedrich C Simmel[1,3], and Tobias Pirzer[1]

[1]Physics of Synthetic Systems - E14, Physics-Department and ZNN, Technische Universität München, 85748 Garching, Germany.
[2]Departmant of Chemistry, Center for Integrated Protein Science (CIPSM), Technische Universität München, Lichtenbergstrasse 4, 85748 Garching, Germany.
[3]Nanosystems Initiative Munich, 80539 Munich, Germany.

Corresponding Author:
Tobias Pirzer
Pirzer@tum.de
Tel: +49-89-289-11609

1. Plasmid sequence for the plasmid encoding the membrane building block EF

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3. Plasmid sequence for the plasmid encoding the fluorescent protein mVenus

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4. Plasmid sequence for the plasmid encoding the fluorescent protein YPet

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