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Directed Assembly of Elastin-like Proteins into defined Supramolecular Structures and Cargo Encapsulation in vitro

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Dr. Stefan Schiller

Bionic Chemistry &
Synthetic Biology Lab

December 19th, 2019

Dear Dr. Steindel,

Please find enclosed the revised manuscript, **„Directed Assembly of Elastin-like Proteins into defined Supramolecular Structures and Cargo Encapsulation in vitro“** for publication in JOVE. As suggested, we revised the manuscript and addressed all points raised by the editor below. In an additional document we addressed all concerns raised by the reviewers point by point and also highlighted all changes made in the manuscript. We are looking forward to hearing from you.

Sincerely,

Dr. Andreas Schreiber

Editorial comments:	
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	We proofread the text for spelling or grammar issues.
2. Please revise lines 38-43 to avoid textual overlap with previous publications.	We changed this paragraph in the manuscript: “Here we provide two efficient protocols for guided self-assembly of amphiphilic ELPs into supramolecular protein architectures such as spherical coacervates, fibers and stable vesicles. The presented assembly protocols generate Protein Membrane-Based Compartments (PMBCs) based on ELPs with adaptable physicochemical properties. PMBCs demonstrate membrane fusion and phase separation behavior and are able to encapsulate chemically diverse fluorescent cargo molecules.”
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TITLE:

Directed Assembly of Elastin-like Proteins into defined Supramolecular Structures and Cargo Encapsulation In Vitro

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KEYWORDS

Elastin-like proteins, protein-based vesicles, protein fibers, drug-delivery systems, self-assembly, protein membrane

SUMMARY

At the interface of organic and aqueous solvents, tailored amphiphilic elastin-like proteins assemble into complex supramolecular structures such as vesicles, fibers and coacervates triggered by environmental parameters. The described assembly protocols yield Protein Membrane-Based Compartments (PMBCs) with tunable properties, enabling the encapsulation of various cargo.

ABSTRACT

Tailored proteinaceous building blocks are versatile candidates for the assembly of supramolecular structures such as minimal cells, drug delivery vehicles and enzyme scaffolds. Due to their biocompatibility and tunability on the genetic level, Elastin-like proteins (ELP) are ideal building blocks for biotechnological and biomedical applications. Nevertheless, the assembly of protein based supramolecular structures with distinct physiochemical properties and good encapsulation potential remains challenging.

Here we provide two efficient protocols for guided self-assembly of amphiphilic ELPs into supramolecular protein architectures such as spherical coacervates, fibers and stable vesicles. The presented assembly protocols generate Protein Membrane-Based Compartments (PMBCs) based on ELPs with adaptable physicochemical properties. PMBCs demonstrate phase separation behavior and reveal method dependent membrane fusion and are able to encapsulate chemically diverse fluorescent cargo molecules. The resulting PMBCs have a high application potential as a drug formulation and delivery platform, artificial cell, and compartmentalized reaction space.

INTRODUCTION

The assembly of supramolecular structures for biotechnological applications is becoming increasingly important[1–5]. For the assembly of functional architectures such as coacervates, vesicles, and fibers with desired physicochemical properties it is important to understand and control the physicochemical and conformational properties of the components. Due to the molecular precision of molecules found in nature, building blocks for supramolecular structures are increasingly based on lipids, nucleic acids or proteins. Compared to synthetic polymers, proteinaceous building blocks allow for precise control over emergent supramolecular structures[6] on the genetic level. The primary amino acid (aa) sequence of the individual protein building blocks intrinsically encodes the information for their assembly potential from the molecular up to the macroscopic level as well as the three dimensional shape and physical properties of the final supramolecular structure[7].

Reported methods for the assembly of different supramolecular structures often involve amphiphilic proteins such as temperature sensitive elastin-like proteins (ELP)[5,8,9], recombinant oleosin[10] and artificial protein amphiphiles[11]. Temperature triggered methods have led to the assembly of micelles[4,10,12], fibers[13], sheets[14] and vesicles[9,15,16]. Methods involving organic solvents have been applied for the formation of dynamic protein based vesicles[8,11,14]. So far, applied protocols for vesicle formation often lack assembly control over micrometer sized assemblies[16,17] or have limited assembly yield[5]. In addition, some reported ELP based vesicles have impaired encapsulation potential[12] or limited stability over time[9]. Addressing these drawbacks, the presented protocols enable the self-assembly of micrometer and sub micrometer sized supramolecular structures with distinct physiochemical properties, good encapsulation potential and long-time stability. Tailored amphiphilic ELPs assemble into supramolecular structures, spanning the range from spherical coacervates and highly ordered twisted fiber bundles to unilamellar vesicles depending on the applied protocol and associated environmental conditions. Large vesicular Protein Membrane-Based Compartments (PMBC) reveal all main phenotypes such as membrane fusion and phase separation behaviour analogous to liposomes. PMBCs efficiently encapsulate chemically diverse fluorescent cargo molecules which can be monitored using simple epifluorescence microscopy. The repetitive ELP domains used in this study are attractive building blocks for protein based supramolecular architectures[18]. The ELP pentapeptide repeat unit (VPGVG) is known to tolerate different aa besides proline at the fourth position (valine, V), while preserving its structural and functional properties[19]. The design of amphiphilic ELPs containing distinctive hydrophilic and hydrophobic domains was realized by inserting aa guest residues (X) in the VPGXG repeat with distinct hydrophobicity, polarity, and charge[20]. Amphiphilic ELP domains where equipped with hydrophobic phenylalanine (F) or

isoleucine (I) while the hydrophilic domain contained charged glutamic acid (E) or arginine (R) as guest residues. A list of eligible amphiphilic ELP constructs and corresponding aa sequences can be found in the supplementary information and references[8,21]. All building blocks where equipped either with small fluorescent dyes or fluorescent proteins for visualization via fluorescence microscopy. mEGFP and other fluorescent proteins were N-terminally fused to the hydrophilic domains of the ELP amphiphiles . Organic dyes were conjugated via copper-free strain promoted alkyne–azide cycloaddition (SPAAC) to a co-translationally introduced unnatural amino acid (UAA). The co-translational incorporation of the UAA *para*-azidophenylalanine (pAzF)[22] permits the N-terminal modification of the hydrophilic ELP domain. In this way the green fluorescent dye BDP-FL-PEG_4-DBCO (BDP) or any small fluorescent molecule with a strained cyclooctyne can be used as fluorescent probe. Successful incorporation of the UAA pAzF and cycloaddition of the dye via SPAAC can be easily confirmed via LC-MS/MS due to efficient ionization of the corresponding tryptic peptides[8]. This small organic dye was applied to broaden the solvent choice for assembly protocols, since fluorescent proteins are incompatible with most organic solvents. The two most efficient assembly protocols for supramolecular structures developed in our lab are described below. The THF swelling method is only compatible with organic dye modified amphiphilic ELP. In contrast, the 1-butanol (BuOH) extrusion method is compatible with many proteins as fluorescent probe e.g. mEGFP, since the described method fully preserves the fluorescence of these fusion proteins. In addition, the encapsulation of small molecules and vesicular fusion behavior works best by employing the BuOH extrusion method.

PROTOCOL

1. Design and cloning of amphiphilic elastin-like proteins (ELPs)

1.1. Clone and design the constructs as described elsewhere[8,20]. Plasmids are available upon request.

2. Protein expression, purification and preparation

2.1. Expression of F20E20-mEGFP and F20E20-mCherry

2.1.1. Inoculate main expression culture from overnight pre-culture to an OD_{600} of 0.3. Incubate at 37 °C, 200 rpm in sterile 400 mL LB medium supplemented with appropriated antibiotics in a 2 L flask.

2.1.2. Prepare IPTG stock solution (1 M) for induction of the expression culture in ultrapure water.

2.1.3. When OD_{600} 0.5–0.8 is reached, add IPTG to expression culture to a final concentration of 1 mM and reduce incubation temperature to 20 °C. Allow expression at 20 °C for approximately 20 h at 200 rpm.

2.2. Expression of amphiphilic ELP containing UAA pAzF

2.2.1. Inoculate main expression culture from overnight *E. coli* pre-culture containing the two plasmids pEVOL pAzF and e.g. pET28-NMBL-(TAG)R40F20-his or R40I30-his to an OD_{600} of 0.3 (see supplementary information for amino acid sequences). Incubate at 37 °C, 200 rpm in sterile 400 mL LB medium supplemented with kanamycin and chloramphenicol in a 2 L flask.

2.2.2. Prepare 100 mM pAzF stock solution in ultrapure water. For 10 mL of pAzF stock solution, weigh 206.2 mg pAzF and resuspend it in 8 mL of ultrapure water. To dissolve the pAzF raise the pH of the solution with 3 M NaOH and mix vigorously. When pAzF is dissolved, carefully lower the pH to 10.5 and add ultrapure water to a final volume of 10 mL. Use a sterile filter (0.22 µm) and aliquot the solution in 2 mL reaction tubes.

2.2.3. Prepare 1 M IPTG stock solution in ultrapure water and 20% arabinose stock solution in ultrapure water.

2.2.4. When OD_{600} 0.5–0.8 is reached, add pAzF to the expression culture to a final concentration of 2 mM. Incubate culture for 10 min, 37 °C, 200 rpm to allow for pAzF uptake.

2.2.5. Induce expression of target protein and expression of the necessary tRNA/t-RNA synthetase via simultaneous addition of IPTG (1 mM) and arabinose (2%) and reduce incubation temperature to 20 °C.

2.2.6. Allow expression at 20 °C for approximately 20 h at 200 rpm. Harvest expression culture by centrifugation at 4 °C, 4000 x *g*, 40 min.

2.3. Cell lysis and protein purification

2.3.1. Resuspend the *E. coli* pellet in lysis buffer (10 mL per liter of culture; 50 mM Tris-HCl pH 8, 500 mM NaCl, 4 M urea, 0.25% Triton X-100) containing lysozyme (0.1 mg/mL) and PMSF (0.1 mM). Incubate for 30 min on ice and freeze and thaw twice afterwards by submerging the sample in liquid nitrogen.

2.3.2. Sonicate the suspension (30%, 15 times, 30 s: 10 s break) and clear the lysate via centrifugation (4 °C, 10,000 x *g* for 40 min).

2.3.3. Purify protein using affinity chromatography (e.g. on a 1 mL nickel column using a FPLC system connected to a fraction collector; see **Table of Materials**). Elute the protein with elution buffer (50 mM Tris-HCl, 500 mM NaCl, 4 M urea, 250–500 mM imidazole) and store at 4 °C until further processing.

2.3.4. Analyze the purification efficiency via SDS-PAGE.

3. Dye-modification of ELPs via SPAAC

3.1. Roughly estimate the concentration of the ELP solution. A_{280} absorption for concentration evaluation is not valuable since pAzF-R40F20 and pAzF-R40I20 sequence are lacking amino acids absorbing in the UV range. Therefore, a previously lyophilized and weighted ELP amphiphile can be used as a reference for SDS PAGE band comparison. Through comparison of the summed gray value intesity of SDS PAGE bands from ELP solutions with known concentrations and your sample the rough concentration of your sample can be estimated.

3.2. Add 1 µL of fluorescent dye BDP-FL-PEG4-DBCO (10 mM stock solution; 20 µM final concentration) to 500 µL of ELP solution (~20 µM). Incubate the reaction for about 10 h at 15 °C, while shaking and protected from light.

3.3. For further use, dialyze the reaction to remove excessive BDP.

3.3.1. Equilibrate a dialysis membrane (e.g. 12 kDa cutoff) in ultrapure water for 10 min. Cut the dialysis membrane into the correct size to be placed on top of the opening of an reaction tube containing the clicked ELP solution. To fix the dialysis membrane in the opening, place a reaction tube lid with punched out core on the opening, thus closing the tube.

3.3.2. Place the reaction tube upside down in the chosen buffer. Exchange the buffer (2–5 L) twice after dialysis for at least 3 h every time. Remove any air bubbles trapped between the dialysis membrane and the buffer to ensure successful dialysis.

4. THF swelling protocol

4.1. Dialyze homogenous ELP solution against phosphate or tris buffer (10 mM) with stable pH 7.5 to remove salts and remaining compounds from his-tag purification.

4.2. Prepare the lyophilizer and cool down to starting temperature for freeze-drying.

4.3. Aliquot the dialyzed protein solution in 1.5 mL reaction tubes (50–500 µL per tube) and shock freeze in liquid nitrogen. To avoid unwanted mixing of different protein solutions during freeze-drying, caps with a small hole can be put on top of the reaction tube to seal it partially.

4.4. Take the frozen protein samples out of the liquid nitrogen and immediately place them in the lyophilizer to start freeze-drying. Freeze-drying is finished when the sample is completely dry (approximately 24–48 h). Subsequently, ventilate lyophilized amphiphilic ELPs with dry N_2, then immediately close the reaction tube lids to avoid contact with air moisture.

4.5. Add pure THF to the lyophilized samples (ELP, 5–10 µM) and place the solution in a water bath sonicator containing ice water for 15 min to allow for swelling of the ELP in THF.

4.6. Preheat a thermocycler to 30–60 °C for vesicle formation or up to 90 °C for fiber formation and prepare new reaction tubes containing either ultrapure water or buffer (50 mM NaH_2PO_4/Na_2HPO_4, 50 mM NaCl, pH 5–13). Spherical coacervates assemble predominantly at 20

°C within pH 9–13. Vesicle formation is favored at 50–60 °C between pH 7 and 9. Fiber formation is predominently induced above 60 °C between pH 5 and 12.

4.7. After the sonication step, place the ELP/THF solution as well as the prepared ultrapure water or buffer solution in the thermocycler and heat up to the desired temperature for 5 min. When temperature is reached the preheated ELP/THF solution should be carefully stratified on top of the preheated ultrapure water or buffer solution. A clear separation of the two phases with a distinct interface should be visible.

4.8. Place the mixture in the thermocycler again and incubate for 20 min to allow for vesicle or fiber formation at the interface. Afterwards, let the samples cool down to room temperature for 10 min before analysis via fluorescence microscopy or dialysis.

4.9. Dialyze solution containing the supramolecular structures against ultrapure water or buffer (50 mM NaH_2PO_4/Na_2HPO_4, 50 mM NaCl, pH 7–10).

5. BuOH extrusion protocol

5.1. Prepare a 1–50 µM ELP solution in ultrapure water or buffer (50 mM PB pH 7.5, 100 mM NaCl, may contain up to 4 M urea). The concentration of the amphiphilic ELP F20R20-mEGFP and F20R20-mCherry solution can be determined using the molar extinction coefficients (F20R20-mEGFP A_{280}=22015 M^{-1} cm^{-1} and F20R20-mCherry A_{280} = 34380 M^{-1} cm^{-1}) (see supplementary information for aa sequences).

5.2. Add 10%–20% (v/v) 1-butanol and immediately mix the solution by pipetting up and down or drawing it up through a syringe multiple times. A common 100 µL pipette or Hamilton syringe equipped with a 0.25 x 25 mm needle can be applied. The turbidity of the solution during mixing should increase, indicating vesicle formation. 1-octanol 5%–15% (v/v) can also be used for vesicle extrusion instead of 1-butanol.

5.3. In order to achieve a narrow size distribution, extrude vesicles using a mini extruder through a membrane with a pore size of 1 µm at room temperature. The membrane size used for extrusion determines the upper size cutoff of the vesicles.

5.4. Dialyze the vesicles as described above (step 3.3) to remove residual 1-butanol.

6. Dye encapsulation with the BuOH extrusion protocol

6.1. Mix approximately 40 µL ELP solution in 10 mM Tris-HCl, pH 8 with 1 µL Dextran Texas Red (0.0025 mg/mL final concentration).

6.2. Add 10 µL of BuOH to the solution and extrude 5–10 times through a syringe equipped with a 0.25 x 25 mm needle.

7. Analysis of supramolecular structures using fluorescence microscopy

7.1. Place a reinforcement ring on a glass slide and firmly press the adhesive side to the glass.

7.2. Add 5 µL of the sample to the inside of the reinforcement ring and place a cover slip on top.

7.3. Seal the sample with nail polish at the edges of the cover slip to avoid evaporation of the sample during analysis.

7.4. Carry out fluorescence microscopy as previously described[8].

REPRESENTATIVE RESULTS

Protocol development for vesicle production

Figure 1 outlines the two different vesicle preparation methods. The THF swelling method on the left side is composed of three successive steps and results in different supramolecular assemblies of the ELP depending on the temperature. In **Figure 1A** epifluorescence microscopy images show vesicles assembled from BDP-R20F20 and fibrillary structures assembled from BDP-R40F20. The BuOH method illustrated on the right side exclusively leads to the formation of ELP vesicles, about two orders of magnitude more compared to the THF swelling method. The schematic illustration shows the preparation process of BuOH vesicles. For vesicle preparation in **Figure 1B** BDP-R40I20 was mixed with 10%–15% (v/v) BuOH and vesicles were prepared via extrusion of the mixture.

Guiding supramolecular self-assembly into different structures

Figure 2 shows a schematic illustration and epifluorescence images of different supramolecular structures assembled from BDP-R40F20 via the THF swelling protocol. In this case lyophilized BDP-R40F20 was used for the different assembly protocols. The pH of the buffer and the temperature of the assembly process was adjusted to form either coacervates, fibrils or vesicles. The coacervates depicted in **Figure 2A** are 1–2 µm in diameter and were assembled from BDP-R40F20 at 20 °C and pH 13. Adjustment of the assembly temperature to 90 °C results in the formation of nanofiber bundles (**Figure 2B**) at pH 4–13 tested with BDP-R40F20. Stable vesicles could be assembled from the ELP at a temperature of 50 °C and pH 7 (**Figure 2C**). Small mistakes at one of the crucial steps in the assembly protocol can lead to the formation of aggregates depicted in **Figure 2D**.

Encapsulation of different cargo

Figure 3 shows the encapsulation of different cargo into the vesicle lumen of vesicles assembled from F20R20-mEGFP via the BuOH extrusion method. For the encapsulation of the positively charged dye Atto Rho13 in **Figure 3A**, the dye was mixed with the aqueous ELP solution before addition of (15% v/v) BuOH and syringe extrusion of the mixture. The confocal microscopy images show the vesicles formed from F20R20-mEGFP in the green channel, the red dye AttoRho13 in the red channel and the resulting merged channel shows the successful encapsulation inside the vesicle lumen.

The polysaccharide Dextran Red 3000 was successfully encapsulated using the BuOH extrusion method as described above. Images recorded in green channel depict the vesicles formed from F20R20-mEGFP while red channel shows the polysaccharide cargo. Merged green and red image in **Figure 3B** confirm the successful Dextran Red 3000 encapsulation in to the vesicle lumen.

Membrane component compatibility and phase separation of mixed BuOH vesicles before/after extrusion

Figure 4 shows the phase separation and fusion behavior of ELP amphiphiles upon mixing of single PMBC building blocks versus assembled PMBC populations. Mixing amphiphilic ELP building blocks (F20R20-mEGFP and F20R20-mCherry) prior to PMBC assembly leads to homogenously distributed molecules within the assembled PMBC membrane. The homogenous distribution of the fluorophores and associated ELP amphiphiles is evident upon merging the red and green channel of the respective fluorescence images. By mixing vesicle populations assembled from either F20R20-mEGFP or F20R20-mCherry clearly visible membrane patches of red or green fluorescence are visible immediately after mixing. This indicates that PMBC fusion events of differently labeled PMBCs occur and that these fusing membranes and their constituents stay phase separated for at least 20 min. A similar phase behavior is known from lipid rafts, within lipid membranes[23].

FIGURE LEGENDS

Figure 1: Illustration of the THF swelling method and the BuOH extrusion method for the guided self-assembly of amphiphilic ELPs into supramolecular structures such as vesicles or fibers. Schematic workflow and representative epifluorescence images of (**A**) the THF swelling method with BDP-R20F20 and BDP-R40F20 resulting in different supramolecular structures depending on temperature and pH and (**B**) the BuOH extrusion method exclusively yielding vesicles from BDP-R40I30 (scale bar 2 µm). This figure has been modified from Schreiber et al. 2019[8].

Figure. 2: By applying the THF swelling method, BDP-R40F20 self-assembles into different supramolecular structures. The environmental conditions applied during the assembly protocol (e.g. temperature or pH) determine the predominate supramolecular structure formed. Representative supramolecular structures at the respective conditions during the assembly were monitored via epifluorescence microscopy and range from (I) coacervates and (II) fibrils to (III) stable vesicles. (IV) Failure in the assembly of defined structures during the THF swelling protocol leads to the formation of unspecific aggregates (scale bar 2 µm). This figure has been modified from Schreiber et al. 2019[8].

Figure 3: Different cargos can be encapsulated within ELP vesicles using the BuOH extrusion method. (**A**) shows representative confocal images of F20R20-mEGFP vesicles with encapsulated positively charged dye AttoRho13 and (**B**) the encapsulation of the polysaccharide dextran red (scale bar 5 µm).

Figure 4: Membrane component compatibility and fusion behavior of vesicle membranes assembled from F20R20 via BuOH extrusion method. (**A**) Mixing of fluorescent F20R20-mEGFP

and F20R20-mCherry protein solution prior to syringe-extrusion leads to PMBC membranes with homogenously distributed amphiphilic proteins visible in green channel (left image), red channel (middle image), and merged channel (right image). (**B**) PMBCs assembled from either F20R20-mEGFP or F20R20-mCherry and mixed subsequently via syringe extrusion lead to visibly separated ELP amphiphile patches within the PMBC membranes. The separated ELP amphiphiles within the membrane are visible after PMBC fusion for at least 20 min in green channel (left image), red channel (middle image), and the merged channel (right image). Scale bars correspond to 5 µm. This figure has been modified from Schreiber et al. 2019[8].

DISCUSSION

A fault in the assembly of defined supramolecular structures following the described protocols mainly leads either to the formation of unspecific aggregates (**Figure 2**, IV) or to homogeneously distributed ELP-amphiphiles. Critical steps of the protocol are discussed below:

For high expression yield of the amphiphilic ELP, a relatively low temperature of 20 °C is optimal. For successful affinity based purification of the amphiphilic ELP an urea concentration of 4 M in the lysis buffer was proven to best solubilize the amphiphilic ELP and increase the protein yield in the soluble elution fraction. If lower urea concentrations in the lysis buffer are desired, affinity purification must be tested for the individual constructs. 2 M urea worked as well for some constructs, especially for those where the His-tag was fused to the hydrophilic domain and therefore still able to bind the resin. An additional purification step after His-tag purification via size exclusion chromatography can increase the vesicle yield as well.

In case of applying the THF-swelling protocol the amphiphilic ELP needs to be labeled with a fluorescent organic dye for visualization. Importantly for the BDP labeling of the amphiphilic ELP (see supplementary information for amino acid sequences containing UAA pAzF) via SPAAC is the absence of any reductant such as TCEP, DTT nor β-mercaptoethanol in all purification buffers. This is necessary to avoid the well reported azide to amine reduction of pAzF prior to the SPAAC reaction[24].

The exact reaction stoichiometry of dye to amphiphilic ELP (e.g. pAzF-R40F20) is not crucial since it is not necessary to label every ELP molecule for simple vesicle visualization via epifluorescence microscopy. Therefore, the correlation of a reference SDS gel band and the corresponding weighted lyophilized sample is only necessary once for each protein construct. However, if close to 100% labeling yield is desired an excess of 1:1 equivalents dye to ELP molecules is sufficient. Very similar amphiphilic ELPs were analyzed in our lab to be fully labeled at an equimolar addition of BDP (data not yet published).

For vesicle preparation using the THF swelling method, the most critical steps are the swelling of lyophilized amphiphilic ELP and subsequent stratification of this solution on top of the aqueous buffer phase. Therefore, the freshly lyophilized amphiphilic ELP should be as anhydrous as possible, which can be achieved by ventilation of the lyophilizer with dry N_2 and immediate closure of the reaction tube lids. If available, septum sealed dry THF should be used to increase the vesicle yield, but THF p.a. (>99.5%) without septum works as well. The stratification step upon

swelling the amphiphilic ELP in dry THF should be executed very carefully. Successful stratification of the two temperature-controlled solutions leads to a clearly visible phase boundary between organic and aqueous phase. The initial stratification step should be conducted slowly even though elevated temperature lead to thermal induced mixing of these phases. Emergent turbidity of the solution is due to light scattering of formed vesicles, fibers or coacervates. In control samples lacking the protein, no turbidity appears though small sized structures (up to 200 nm) are reported for the THF water-interface[25]. The THF stratification step is the most critical and failure prone step of the swelling protocol. After the incubation step the supramolecular structures can be dialyzed against buffer or ultrapure water. Preferentially the same aqueous solution which was used for initial assembly in order to maintain the osmolarity and prevent swelling or shrinking of the assembled vesicles. After dialysis, the vesicles, fiber and coacervates are usually stable for at least one week. Depending on the environmental parameters during assembly often a small proportion of other supramolecular structures besides the main structure are present if the THF swelling method is applied[8]. The described THF method increases the vesicle assembly yield by one order of magnitude while the BuOH extrusion improves the yield by three orders of magnitude compared to our previously published in vitro method[5].

The BuOH extrusion method is applied to obtain exclusively stable vesicular structures with high reproducibility, circumventing fibers and spherical coacervates. This method is less error prone and compatible with fluorescent proteins. Therefore F20R20-mEGFP or F20R20-mCherry can be applied as well as BDP-R40F20 or BDP-E20F20. The only critical step is the rapid mixing of the aqueous protein solution after addition of 10%–20% v/v BuOH. The F20R20-mEGFP or F20R20-mCherry concentration should be around 1–15 µM. By applying BuOH extrusion method vesicles can be assembled in ultrapure water or buffer containing up to 5 M NaCl or 4 M urea and pH ranging from 5 to 8. Extruded PMBCs in 20% v/v BuOH can be stored for at least 6 months at 4°C while preserving their vesicular structure. To narrow the vesicle size distribution, they can be extruded using a mini extruder through a membrane of 0.2-1 µm pore size. This pore extrusion can be done directly after BuOH addition to the amphiphilic ELP or after vesicle assembly. If PMBCs are too concentrated for imaging, assembled vesicles in BuOH can be diluted through rapid mixing using aqueous buffer containing 10%–20% v/v BuOH.

The major limitation of the BuOH extrusion method is that PMBC dialysis against aqueous buffers often results in poor vesicle yield. Further, the presence of residual BuOH within the membrane space cannot be excluded since simple fatty acids were able to incorporate into the PMBC membrane[21]. Therefore, PMBC membranes might be to some extent be composed of protein and alkanol moieties.

Encapsulation of chemically diverse cargo molecules works best using the BuOH extrusion method. Further, DMSO as solvent for the stock solution of the dye to be captured increases the dye encapsulation efficiency. For delicate cargo to be encapsulated, 5%–10% v/v 1-octanol can be used for PMBC assembly and has been proven to be better compatible, when compared to BuOH, with functional encapsulated enzymes such as DNA-ligase or TEV protease[21,26]. However, due to the shorter chain length of n-butanol it can be dialyzed against aqueous buffer in contrast to 1-octanol, which is not able to permeate the applied dialysis-membrane. Another method

limitation is that the applied temperatures and pH values needed to control the desired suprastructure formation can affect enzyme activity. In future work, affinity purification or size exclusion purification should be established to separate non-encapsulated versus encapsulated molecules without deteriorating vesicle membrane integrity.

In contrast to film rehydration methods[16,17] the herein described protocols enable the assembly of vesicles sizes greater than 600 nm. This allows monitoring of real time fusion events through simple epifluorescence microscopy and the observation of membrane phase separation[8]. Compared to temperature triggered vesicular assembly of amphiphilic ELP[9] the protocols described here yield PMBC with a long time stability of up to 6 month. However, the main disadvantage is the need of organic solvent for structure formation. Even though BuOH fully preserves the integrity and function of fluorescent proteins[27] (data not shown), the activity of encapsulated enzymes might be restricted by residual organic solvent and must be tested individually. However, catalytic reactions involving DNA- ligase, TEV-protease and lipase have been successfully conducted within the luminal space of the vesicles, assembled by 1-octanol or BuOH extrusion[26,21]. Additionally, even though THF dialysis after assembly is very unproblematic and vesicle integrity is preserved, the BuOH removal frequently results in loss of vesicle integrity due to unknown reasons.

The described protocols enable researchers to assemble micrometer and sub micrometer sized supramolecular structures with distinct physicochemical properties, good encapsulation properties, and long time stability. These supramolecular structures can be applied for the design of minimal cells[26] or artificial cell research[21], enzyme encapsulation, or drug formulation. The presented functional PMBCs are further promising candidates for drug delivery, since their building blocks are not immunogenic[28], exhibit dynamic fusion behavior, and allow for diverse cargo encapsulation.

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DISCLOSURES

The authors declare no competing financial interests.

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Figure 1

A

THF swelling method

lyophilization

THF swelling and sonication

20-90°C stratification of ELP-THF solution on top of same

50-60°C

60-90°C

B

BuOH extrusion method

addition of BuOH (10-20% v/v)

extrusion with mini extruder/ syringe extrusion

Figure 2

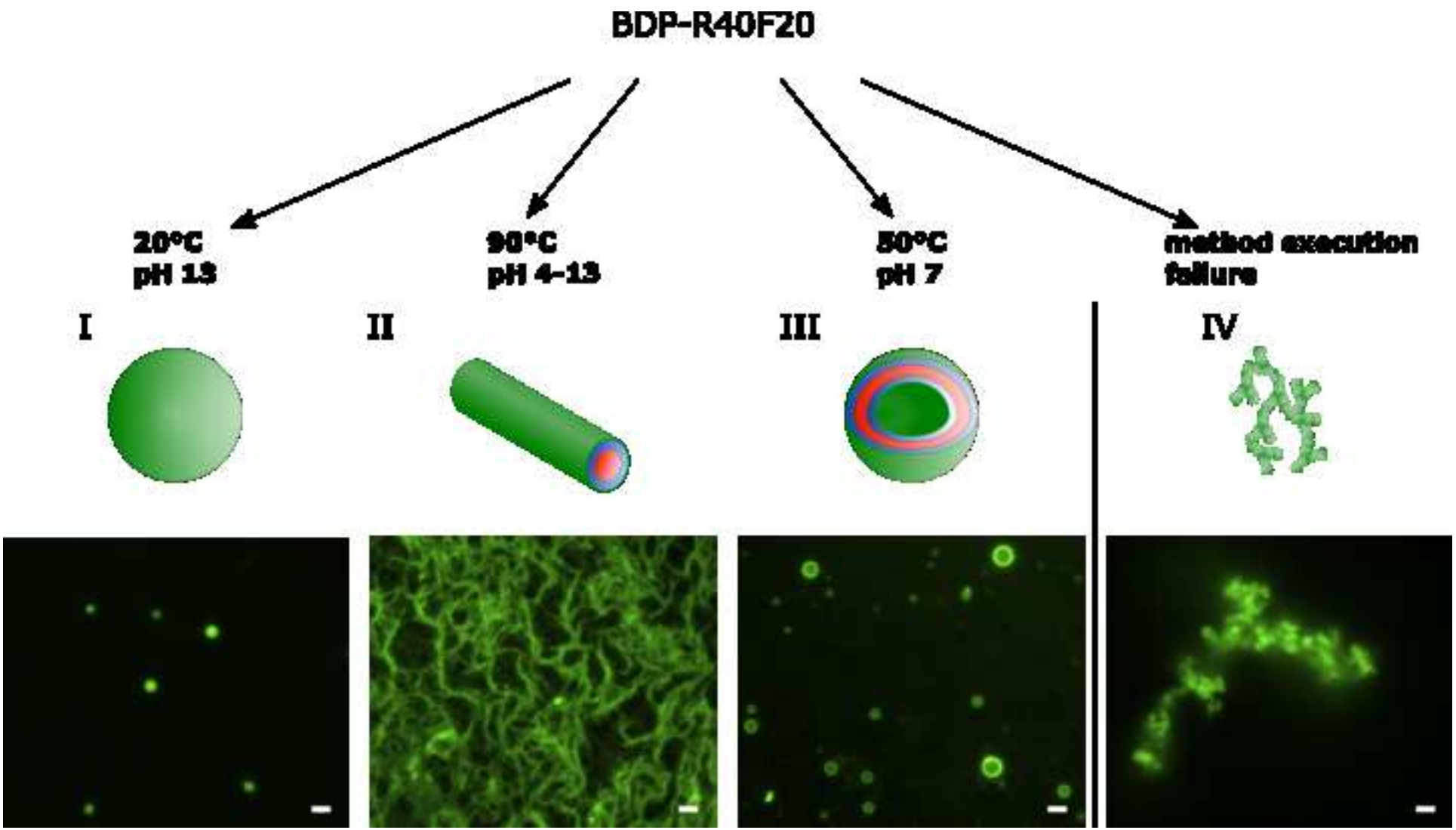
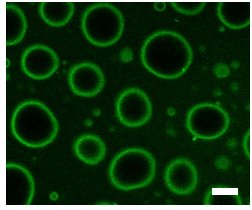
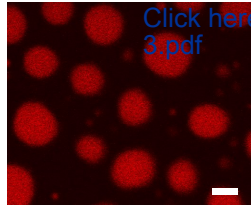


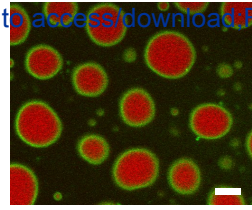
Figure 3

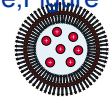
A

F20R20-mEGFP-his
n-BuOH extrusion





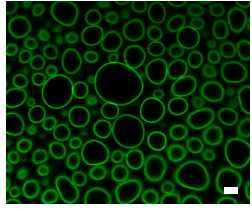


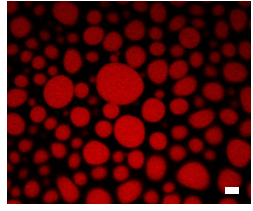


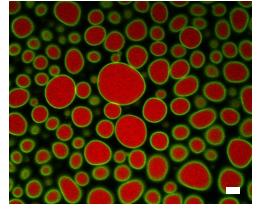
positively charged dye
ATTO Rho 13
MW 0.84 kDa

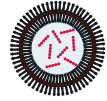
B

F20R20-mEGFP-his
n-BuOH extrusion



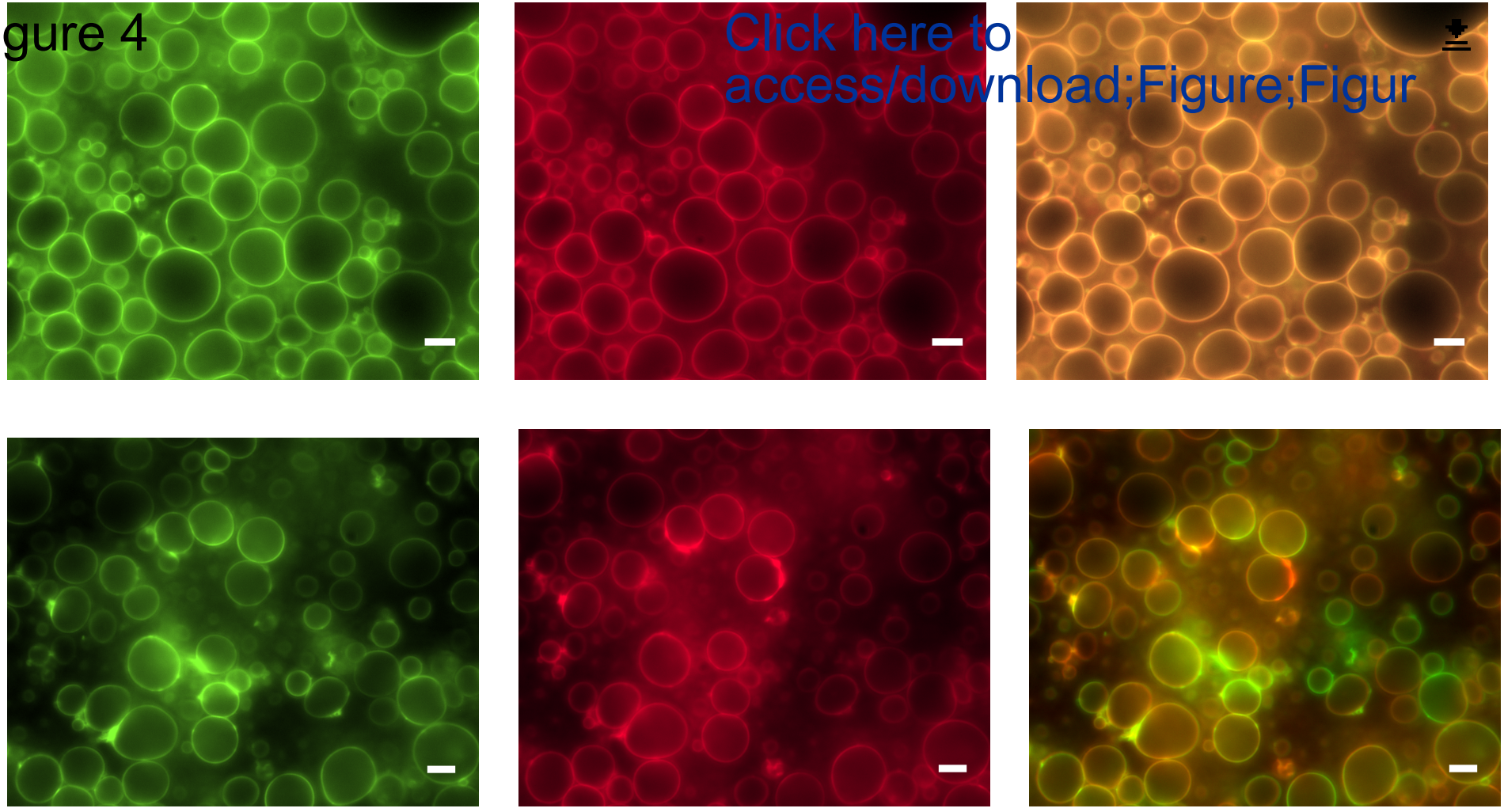






polysaccharide
dextran 3000
MW 3 kDa

Figure 4



Name of Material/ Equipment

1 µm and 0.2 µm Steril Filter
1,4-Dithiothreitol
1-butanol. >99.5% p.a.
2log DNA ladder
2-Mercaptoethanol
50 mL Falcon tubes
79249 Alkyne Mega Stokes dye
Acetic acid glacial
Acetonitrile, anhydrous, 99.8%
Ampicillin sodium-salt, 99%
BDP-FL-PEG4-DBCO
Biofuge
Bottle Top Filter with PES membrane (45 µm, 22 µm)
Brillant Blue G250 (Coomassie)
BspQI
Camera DS Qi1
Centrifuge 5417r
Centrifuge 5810r
CF-400-Cu square mesh copper grid
Chloramphenicol
CompactStar CS 4
Dextran, Texas Red, 3000 MW, neutral
Digital sonifier
Dimethylsulfoxide (DMSO)
Dnase I
EarI
EcoRI-HF
Environmental shaker incubator ES-20
Ethanol absolute
Ethidium bromide solution
Filter supports
Glass plates

Glycerol Proteomics Grade
Glycin
H4-Azido-Phe-OH
Heat plate MR HeiTec
HindIII
HisTrap FF crude column
Hydrochloride acid fuming, 37%, p.a.
Illuminator ix 20
Illuminator LAS-4000
Imidazole
Immersions oil for microscopy
Incubators shakers Unimax 1010
Inkubator 1000
IPTG, >99%
Kanamycinsulfate
L(+)-Arabinose
Laboratory scales Extend ed2202s/224s-OCE
LB-Medium
Lyophilizer Alpha 2-4 LSC
Lysozyme, 20000 U/mg
Microscope CM 100
Microscope Eclipse TS 100
Microscopy cover glasses (15x 15 mm)
Microscopy slides
Microwave
Mini-Extruder Set
NaCl, >99.5%, p.a.
Natriumhydroxid pellets
Ni-NTA Agarose, PerfectPro
Nucleopore Track-Etch Membrane
PH meter 766 calimatic
Phenylmethylsulfonylflourid (PMSF)
Polypropylene Columns (1 mL)

PowerPac basic
Propanol-2-ol
Protein ladder 10-250 kDa
Recirculating cooler F12
Reinforcement rings
SacI HF
SDS Pellets
Sodiumdihydrogen phosphate dihydrate, NaH_2PO_4
Sterile syringe filter 0.2 mm Cellulose Acetate
T4 DNA Ligase
TEMED
TexasRed Dextran-Conjugate
Thermomix comfort
THF, >99.5% p.a.
Triton X 100
Trypton/Pepton from Casein
Ultrasonic cleaner
Urea p.a.
Vacuum pump 2.5
XbaI
XhoI
ZelluTrans regenerated cellulose tubular membrane (12.0 S/ 3.5 S/ 1.0 V)

	Company	Catalog Number	Comments/Description
VWR			
Merck			
Roth			
NEB			
Roth			
VWR			
Sigma Aldrich			
VWR			
Sigma-Aldrich			
Roth			
Jena Bioscience			
Heraeus			
Thermo Scientific			
Roth			
NEB			
Nikon			
Eppendorf			
Eppendorf			
EMS			
Roth			
VWR			
Life Technologies			
Branson			
Applichem			
Applichem			
NEB			
NEB			
Biosan			
Roth			
Roth			
Avanti			
Bio-Rad			

Amresco	
Applichem	
Bachhem	
Heidolph	
NEB	
GE Life Sciences	Nickel column
Merck	
INTAS	
Fujifilm	
Merck	
Merck	
Heidolph	
Heidolph	
Roth	
Roth	
Roth	
Sartorius	
Roth	
Christ	
Roth	
Philips	
Nikon	
VWR	
VWR	
Studio	
Avanti Polar Lipids	
Roth	
Roth	
5 Prime	
Avanti	
Knick	
Roth	
Qiagen	

BioRad
Emplura
NEB
Julabo
Herma
NEB
Roth
VWR
VWR
NEB
Roth
MolecularProbes
Eppendorf
Acros
Roth
Roth
VWR
Roth
Vacuubrand
NEB
NEB
Roth

Reviewers' comments:	
Reviewer #1:	
The authors mention in the discussion section that a major limitation of the BuOH extrusion method is that it often results in poor vesicle yield of BuOH free PMBCs. Is the BuOH at the inside of the vesicles? Or is it inside the hydrophobic part of the membrane? Please clarify what "BuOH free" means. The reader should know where to expect residual BuOH.	We used 10-20% (v/v) 1-butanol for the vesicle assembly. It is likely that residual BuOH is left in the lumen or in the membrane space after dialysis. Since the purification of the vesicles is difficult it is so far not possible to determine the exact residual fraction of BuOH left. We added to the manuscript P11L383: "The major limitation of the BuOH extrusion method is that PMBC dialysis against aqueous buffers often results in poor vesicle yield. Further, the presence of residual BuOH within the membrane space cannot be excluded since we observed that also simple lipids are able to incorporate into the PMBC membrane.[1] Therefore, PMBC membranes might be to some extent composed of protein and alkanol moieties."
On page 10 line 376 the authors write that 1-octanol would be more compatible. Why is BuOH used instead of 1-octanol?	We changed in the manuscript P12L401: "However, due to the shorter chain length n-butanol can be dialysed against aqueous buffer in contrast to 1-octanol, which is not able to permeate the applied dialysis-membrane."
THF is also a non-polar solute. Could residual THF stay in the vesicles or inside the membrane?	We cannot exclude that residual THF stays in the vesicles or membranes. But it is very unlikely that residual THF is left after dialysis due to the low boiling point of THF and the efficient dialysis of THF.
Page 10 line 389 The authors mention that encapsulated enzymes might be affected by THF. This is a serious concern, because this would make this protocol not applicable for synthetic cells, drug transporters or any other application with proteins. Did the authors test that? Is it possible to provide references?	We added to the manuscript P12L406"Depending on the enzyme to be encapsulated its solvent compatibility must be tested individually. However, catalytic reactions involving DNA-ligase, TEV-protease and lipase have been successfully conducted within the luminal space of the vesicles assembled by 1-octanol or BuOH extrusion.[1,2]
It would be interesting for the readers if enzymes or fluorescent proteins are absolutely damaged or only to some degree (see my other comment).	The integrity of enzymes to be encapsulated must be tested individually. Regarding the fluorescent proteins upon 1-octanol or BuOH addition, their fluorescence intensity is fully preserved as stated in the manuscript P12L406: "Even though BuOH preserves the integrity and function of fluorescent proteins,...'
Furthermore, the different pH values and the temperatures needed to control the suprastructures will highly affect enzymes. Please mention this in your discussion as a crucial	We agree and therefore added to the manuscript P11: "Another limitation is that the applied temperatures and pH values needed to control the desired suprastructure formation can affect enzyme activity."

limiting factor.	
For their second protocol the ELPs are fused with megfp or mcherry. Is it possible that BuOH denatures the fluorescent proteins used? The authors state that BuOH preserves fluorescence, but to what degree?	See answer above.
The authors should provide control measurements or should provide relevant references. If only a small amount or even 50% are denatured it would be still ok for using fluorescence as a visualization method. But the reader should know what to expect.	As stated before, after BuOH addition the fluorescence intensity of mEGFP is fully preserved We therefore added to the manuscript p12: "However, the main disadvantage is the need of organic solvent for structure formation. Even though BuOH fully preserves the integrity and function of fluorescent proteins[3] (data not shown), the activity of encapsulated enzymes might be restricted by residual organic solvent and must be tested individually."
Again my question, why wasn't 1-octanol used instead of BuOH? Did the authors consider 1-octanol for the swelling method as well? Since 1-octanol is not used in the provided protocols but mentioned as more compatible, the reasons for this should be discussed.	For the swelling method 1-octanol, ethanol, methanol and butanol where tested but had lower vesicular assembly yield or dialysis to remove the organic solvent was less efficient compared to THF. Regarding the usage of BuOH instead of 1-octanol: We changed in the manuscript P11: However, due to the shorter chain length n-butanol can be dialysed against aqueous buffer in contrast to 1-octanol, not able to permeate the applied dialysis-membrane.
Page 8 line 324, page 9 line 365 and step 5.1 in the protocol the authors write that 4M urea was used? Is that correct? How can fluorescent proteins or possible encapsulated enzymes survive these conditions? It is also not very clear in which protocol urea was used. Maybe megfp does not denature, but enzymes fused with ELPs will be. In your chembiochem publication (which is cited) you used a protease which was fused to ELPs. The fact that the protease was still active should be discussed in this manuscript.	In the manuscript P9L365 we stated : 'By applying BuOH extrusion method, vesicles can be assembled in buffer containing **up to** 5 M NaCl or 4 M urea and pH ranging from 5 to 8.' This includes assembly of vesicles at the whole range of urea and salt concentrations. We added for clarification to the manuscript: 'By applying BuOH extrusion method vesicles can be assembled in ultrapure water or buffer containing up to 5 M NaCl or 4 M urea and pH ranging from 5 to 8. ' In the manuscript page 8 line 324 the purification of the ELP amphiphiles is described. Purification works with lower urea content as well: We added to the manuscript P 10: "If lower urea concentrations in the lysis buffer are desired, affinity purification must be tested for the individual constructs. 2 M urea worked as well for some constructs especially for those where the His-tag was fused to the hydrophilic domain and therefore still able to bind the resin."

The authors use THF and stratify THF and water. It is stated that it should be done carefully. The authors should absolutely discuss this as a very possible step for failure. Especially since the boiling point of THF is about 65 degree celsius. The sample gets heated to 50 and up to 90 degree celsius. I can't imagine that this sensitive interface between THF and water is stable enough at 90 degrees. Or does the THF get completely evaporated?	We added to the manuscript P11L357: "Successful stratification of the two temperature controlled solutions leads to a clearly visible phase boundary between organic and aqueous phase. The initial stratification step should be conducted slowly even though elevated temperature leads to thermal induced mixing of these phases. […] The THF stratification step is the most critical and failure prone step of the swelling protocol." For the THF swelling method it is unclear if a stable interface between THF and water phase is favourable for efficient vesicle or fiber assembly. However, during assembly the THF does not get completely evaporated.
Minor Concerns:	
In the abstract and in the manuscript the authors state that the two protocols are efficient and have a high yield. In the manuscript I couldn't find any given yields which would allow such a statement. Please provide measurements or provide references which show their efficiency.	In the abstract we stated: "Here we provide two efficient protocols for controlled self-assembly…" In this context "efficient" refers to the successful and repeated controlled assembly of distinct structures and is therefore in our opinion the correct description. In the manuscript P2L65 we state: 'So far, applied protocols for vesicle formation often lack assembly control over micrometer sized assemblies or have limited assembly yield[4]. Reference 4 refers to our previous method which produced vesicles with a yield two orders of magnitude lower than compared to the THF swelling method. The yield described is based on the vesicle number seen and counted via fluorescence microscopy. In our group we compared different assembly methods regarding their vesicle yield. This can be done by counting since the differences in vesicle number are in the range of multiple orders of magnitudes and are thereby clearly distinguishable. DLS might be statistically more appropriate to characterize absolute vesicle yield but has some limitations for our set up. In order to measure DLS samples vesicles need to be dialyzed in aqueous buffer to remove BuOH or THF and thereby reduce turbidity of the sample. During dialysis the number of vesicles changes and DLS data would not reflect the original assembly conditions. Additionally, the high scattering impact of the larger vesicles would mask the presence of small vesicles. We therefore decided

	to use epifluorescence microscopy to measure the number of vesicles. This gave significant differences for of vesicle yields comparing the three assembly methods. However, we changed the following sentences in the manuscript P7L246: "The BuOH method illustrated on the right side exclusively leads to the formation of ELP vesicles, about two orders of magnitude more compared to the THF swelling method." On 11L373 we changed "The BuOH extrusion method is applied to obtain exclusively stable vesicular structures with high reproducibility, circumventing fibers and spherical coacervates." On P11L370 we added to the manuscript: "The described THF method increases the vesicle assembly yield by one order of magnitude while the BuOH extrusion improves the yield by three orders of magnitude compared to our previously published *in vitro* method[4]."
In the introduction (page 2 line 59) the authors state that reported methods mainly involve amphiphilic molecules or polymers. I doubt that. For example supramolecular assemblies of DNA structures; DNA tiles can be used to create filamentous suprastructures (see a recent publication by K. Goepfrich). Some groups also use the assembly of F-actin from G-actin to create interesting suprastructures like F-actin bundles.	Since this manuscript focuses on amphiphilic proteins and polymeric assemblies, we did not include the multitude of DNA structures and DNA origami, which would go beyond the scope of this manuscript. Nevertheless, DNA structures play an important part in supramolecular assemblies. Therefore, we changed in the manuscript P2L59 the sentence: "Reported methods for the assembly of different supramolecular structures often involve amphiphilic proteins…"
page 2 line 65 The authors write "other applied protocols have limited assembly yield". If the yield is criticized, please provide references or compare measured values.	Please see the previous comment above. This comparison in yield refers to a different vesicle assembly method[4] previously published by and used in our group, which resulted in less vesicles compared to these methods. This was evaluated by comparing vesicle numbers via fluorescence microscopy.
Page 4 line 145 Why do the actors use an Aekta for purification? Is the inverse temperature cycling not sufficient enough? A brief statement in the manuscript might help the reader.	The amphiphilic nature of our di-block ELPs prevents us from using inverse temperature cycling since the transition temperature (Tt) is different for both blocks. So far, using UV Vis turbidity measurements we could only define a distinct Tt for single block ELPs but not for amphiphilic di-block ELPs. Therefore, it is easier to use an Aekta his-tag purification.
Page 6 line 218 The authors state that the size distribution can be narrowed by using an	The size distribution in figure 3 is indeed not narrow, because in this case we did not extrude

extruder. The vesicles' size in figure 3 do not seem narrow. Can you comment on that please?	the vesicles through an extrusion filter. These vesicles were prepared via syringe extrusion. To clarify this in the figure caption we added on P7L266: "For the encapsulation of the positively charged dye Atto Rho13 in Fig 3A, the dye was mixed with the aqueous ELP solution before addition of (15% v/v) BuOH and syringe extrusion of the mixture."
Please provide a brief description how you determined the ELP concentration using PAGE. And what concentrations did you get?	Samples with known concentration (lyophilized dry weight) were analysed on SDS PAGE and the protein band gray intensity values were used for a concentration correlation and as comparison to estimate the concentration of other samples. As stated, this is only a "rough" approximation since the dry weight determination and summed gray intensity values are error prone. However, it is valid enough to adjust the sample to a working concentration of ~ 20 µM of ELP solution.
Figure 1 and 2. Please use the term epi fluorescence to better differentiate between epi and confocal fluorescence microscopy in the figures.	We specified this in the manuscript as suggested.
Figure 2. Epi fluorescence is not sufficient to discriminate between vesicles and coacervates. Later the authors used confocal microscopy. A micrograph utilizing confocal microscopy would more convincing.	As can be seen on the epifluorescence pictures, there is a difference between coacervates and vesicles for sizes larger than about 600 nm. Coacervates appear as bright fluorescent spheres with no intensity dip in the middle. Vesicles appear as membrane bound spheres exhibiting an intensity dip upon drawing an intensity line plot across the spheres. Confocal fluorescent microscopy is not applicable for vesicles moving in solution and can only be conducted to analyse the fraction of vesicles attached to/ sitting on the glass slide.
Page 9 line 350 The authors state that visible turbidity originates from the suprastructures. That's right, but also THF droplets can appear in the water phase (see Li et al. J.Phys.Chem.B2011, 115, 7887-7895.) Please mention this in the manuscript.	Thank you for the hint. We added to the manuscript P11L360: "Emergent turbidity of the solution is due to light scattering of formed vesicles, fibers or coacervates. In control samples lacking the protein, no turbidity appears though small sized structures (up to 200 nm) are reported for the THF water-interface.[6]"
Figure 2 is in general misleading. Suprastructures II and III are nicely well controlled. Suprastructure I is a coacervate, which is - as I understood the literature - the natural form of ELPs in their hydrophobic state. Furthermore, Suprastructure	As we stated in the figure caption structures I-III are results of the correctly applied protocol. ELPs gradually adopt β-spiral structures upon temperature rise due to their LCST behaviour resulting in polymer-rich and water-rich phases.

IV seems to be a random aggregation. From my perspective these two should not described as controlled superstructures.	Unlike our coacervates these polymer-rich phases are not well-formed spherical particles. The coacervate formation in our case is a controlled process, similar to the self-assembly process of structure II and III. Structure IV represents the wrong outcome after mistakes made during the assembly protocol. This structure was included in the figure according to the author guidelines to help readers to identify mistakes after assembly. To clarify the design of the figure, we adapted it slightly.
Even when the authors change some solution parameters. And the authors on their own state that "the protocol often leads to the formation of unspecific aggregates". Often or is it controlled?	We stated in the manuscript that upon failure of the method the most frequent outcome is the formation of unspecific aggregates. We specified the misleading sentence by exchanging often by mainly. We added to the manuscript P10L317: "A fault in the assembly of defined supramolecular structures following the described protocols mainly leads either to the formation of unspecific aggregates (Figure 2 IV) or to homogeneously distributed ELP-amphiphiles."
Figure 4A nicely shows that two mixed ELPs form homogeneous membranes and that they stay mixed for the time measured. Furthermore, 4b nicely proofs vesicle fusion. But the authors should not write, that there is a phase separation. The latter would mean that in 4A the ELPs would de-mix, which is not shown. In 4b the ELPs stay phase separated. The actual process of phase separation was not measured by the authors. A simple explanation would be that the 2D diffusion of the ELPs used inside the membrane ist just too slow to be measured in the authors' experiment. Please change this, because it can confuse readers.	Thank you for this suggestion. We tried to clarify this point in the manuscript and added P10L314 : "PMBCs assembled from either F20R20-mEGFP or F20R20-mCherry and mixed subsequently via syringe extrusion lead to visibly separated ELP amphiphile patches within the PMBC membranes. The separated ELP amphiphiles within the membrane are visible after PMBC fusion for at least 20 min in green channel (left image), red channel (middle image), and the merged channel (right image)." This separation of ELP amphiphiles does not occur when ELP building blocks with different fluorescent proteins are mixed prior to vesicle formation. The visible separation of the membrane is only a snap shot in this case not an active process. The slow lateral 2D diffusion through the membrane or thermodynamic hindrance might be responsible.
The authors are varying the temperature and pH a lot. What transition temperatures have the ELPs used for this protocols? Maybe it makes the formation of the suprastructures more understandable. Do these ELPs have only this single transition temperature? As I know, all polymers have a LCST; is this transition	Yes, this transition temperature refers to the LCST of ELPs. ELP single blocks have a distinct Tt and show LCST behaviour [7]. If investigated as single block each of the two blocks of the amphiphilic ELP has their own Tt, which is different from the other. As mentioned above, the amphiphilic ELP di-block used for this protocols do not have a distinct Tt. Fused together, the Tts might

temperature an LCST?	influence each other, but we cannot know that for sure. We assume, that in the case of amphiphilic di-blocks the LCST behavior is still an immanent feature of the two single blocks fused together. But since the shift from soluble to insoluble probably occurs at different temperature points, the exact Tt is not measurable via UV Vis. As described in our Small publication[8] the temperature induced gradual conformational changes are responsible for the suprastructure formation. Therefore, the optimal temperature for controlled self-assembly was identified through multiple experiments.
Reviewer #2: Minor Comments:	
1) It is unclear if the orientation of his tag and fluorescent probe/protein (i.e conjugation to the hydrophobic or hydrophilic blocks) would have any differential effect on the assembly properties. It would be helpful if the authors discuss or provide data to address this.	We tested amphiphilic ELPs with different orientation of the his-tag, which did not have an influence on the self-assembly with both protocols. *In vivo,* the N-terminal or C-terminal orientation of hydrophobic vs hydrophilic is a critical parameter for suprastructure formation.[4] We did not alter the orientation of the fluorescent probe (BDP) for the THF swelling protocol, but we assume that this would not change the assembly properties. Fusion of the fluorescent protein to the hydrophobic part of the ELP was not tested since this would result in triblock copolymers which is potentially interesting as well. Currently we are working on the orientation of the membrane building blocks by altering the hydrophilic to hydrophobic ratio.
2) The authors claim the encapsulation protocols would work with diverse structurally and chemically different cargo. However, only two examples (Atto red, dextran red) have been demonstrated. Can the authors discuss more on the other cargo that would be compatible (in terms of charge, size, molecular class, hydrophobicity/hydrophilicity of the cargo etc.)	Since we focused on the assembly protocol in this manuscript (which is the intention of the editors) the detailed information is provided in Ref[8]. In this previously published paper[8] we showed the encapsulation of a positively charged dye, a hydrophobic dye, the polysaccharide Dextran 3000 and of whole proteins (GFP). Also, the encapsulation of functional enzymes could be shown[2]. Since the encapsulation efficiency was not the focus of this manuscript, we only included two encapsulation examples. Examples from the publications are discussed on P12 and include a cross reference for further information and better understanding of the encapsulation potential.

Major Concerns: 3) There is no quality control data showing that the intended proteins have been correctly expressed. Some combination of SDS-PAGE/Mass Spectrometry comparing expected and observed mass/Chromatography/DNA-Diagnostic Digestion/DNA sequencing results/percent purity.	The explicit proof for the correctness of the expressed and applied constructs is shown in Ref[8]. We focused on the assembly protocol in this manuscript as part of the editorial guidelines, therefore the detailed information is provided in Ref[8]. All DNA sequences were confirmed by standard DNA sequencing. The purified proteins were analysed via SDS PAGE and LC-MS/MS analysis and the correctness was fully confirmed.

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Supporting Information

Directed Assembly of Elastin-like Proteins into defined Supramolecular Structures and Cargo Encapsulation in vitro

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Table S2: Plasmids used for the expression of all amphiphilic ELP library proteins used in this study and the corresponding amphiphilic protein domains and sequences.

plasmid	expressed amino acid sequence
pET28-NMBL-F20R20-mEGFP-His	MSSSGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPG FGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGV PGFGVPGFGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPG RGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGR GVPGRGVPGRGVPGRGMVSKGEELFTGVVPILVELDGDVNGHKFSVSGE GEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHD FFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKED GNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQ NTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDE LYKGGREASSHHHHHH
pET28-NMBL-F20R20-mCherry-His	MSSSGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPG FGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGV PGFGVPGFGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPG RGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGR GVPGRGVPGRGVPGRGMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEF

	EIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPA DIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGT NFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDA EVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGM DELYKGGREASSHHHHHH
pET28-NMBL-mEGFP-E20F20-his	MDPMSSSGMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYG KLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPE GYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLE YNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPV LLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGVPGE GVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGV PGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPG EGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGV PGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGF GVPGFGGREASSHHHHHH
pET28-(TAG)NMBL-R20F20-his	MA*SSSGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRG VPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGV PGRGVPGRGVPGRGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPG FGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGV PGFGVPGFGVPGFGVPGFGGREASSHHHHHH
pET28-(TAG)NMBL-R40F20-his	MA*SSSGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRG VPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGV PGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVP GRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPG RGVPGRGVPGRGVPGRGVPGRGVPGRGVPGFGVPGFGVPGFGVPGFGVPGFG VPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPG FGVPGFGVPGFGVPGFGGREASSHHHHHH
pET28-(TAG)NMBXL-HTV-R40I20-his	MA*SSSGHHHHHHGENLYFQGVPGRGVPGRGVPGRGVPGRGVPGRGV PGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVP GRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPG RGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGR GVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGIGVPGIGVP GIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPG VPGIGVPGIGVPGIGVPGIGVPGIGVPG
pET28-(TAG)NMBXL-HTV-R40I30-his	MA*SSSGHHHHHHGENLYFQGVPGRGVPGRGVPGRGVPGRGVPGRGV PGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVP GRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPG

	RGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGR GVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGRGVPGIGVPGIGVP GIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIG VPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVPGIGVP GIGVPGIGVPGIGVPGIGVPGIGVPGIG
pET28-(TAG)NMBL-E20F20-his	MA*SSSGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEG VPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVPGEGVP GEGVPGEGVPGEGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGF GVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVPGFGVP GFGVPGFGVPGFGVPGFGGREASSHHHHHH
	* depicts for unnatural amino acid e.g. para-Azidophenylalanin (pAzF)

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