TITLE:

Reconstitution of the Bacterial Glutamate Receptor Channel by Encapsulation of a Cell-Free Expression System

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SUMMARY:

This protocol describes the inverted emulsion method used to encapsulate a cell-free expression (CFE) system within a giant unilamellar vesicle (GUV) for the investigation of the synthesis and incorporation of a model membrane protein into the lipid bilayer.

ABSTRACT:

Cell-free expression (CFE) systems are powerful tools in synthetic biology that allow biomimicry of cellular functions like biosensing and energy regeneration in synthetic cells. Reconstruction of a wide range of cellular processes, however, requires successful reconstitution of membrane proteins into the membrane of synthetic cells. While expression of soluble proteins is usually successful in common CFE systems, reconstitution of membrane proteins in lipid bilayers of synthetic cells has proven to be challenging. Here, a method for reconstitution of a model membrane protein, bacterial glutamate receptor (GluR0), in giant unilamellar vesicles (GUVs) as model synthetic cells based on encapsulation and incubation of the CFE reaction inside synthetic cells is demonstrated. Utilizing this platform, the effect of substituting N-terminal signal peptide of GluR0 with proteorhodopsin signal peptide on successful co-translational translocation of GluR0 into membranes of hybrid GUVs is demonstrated. This method provides a robust procedure that will allow cell-free reconstitution of various membrane proteins in synthetic cells.

INTRODUCTION:

Bottom-up synthetic biology has gained increasing interest over the past decade as an emerging field with numerous potential applications in bioengineering, drug delivery, and regenerative

medicine[1, 2]. Development of synthetic cell as a cornerstone of bottom-up synthetic biology, in particular, has attracted a wide range of scientific communities due to the promising applications of synthetic cells as well as their cell-like physical and biochemical properties that facilitate *in vitro* biophysical studies[3–6]. Synthetic cells are often engineered in cell-sized giant unilamellar vesicles (GUVs) in which different biological processes are recreated. Reconstitution of cell cytoskeleton[7, 8], light-dependent energy regeneration[9], cellular communication[10, 11], and biosensing[12] are examples of efforts made to reconstruct cell-like behaviors in synthetic cells.

While some cellular processes rely on soluble proteins, many characteristics of natural cells such as sensing and communication often utilize membrane proteins including ion channels, receptors, and transporters. A major challenge in synthetic cell development is reconstitution of membrane proteins. Although traditional methods of membrane protein reconstitution in lipid bilayers rely on detergent-mediated purification, such methods are laborious, ineffective for proteins that are toxic to the expression host, or are often not suited for membrane protein reconstitution in GUVs[13].

An alternative method for protein expression is cell-free expression (CFE) systems. CFE systems have been a powerful tool in synthetic biology that allow *in vitro* expression of various proteins using either cell lysate or purified transcription-translation machinery[14]. CFE systems can also be encapsulated in GUVs, thus allowing compartmentalized protein synthesis reactions that can be programmed for various applications such as creation of light-harvesting synthetic cells[9] or mechanosensitive biosensors[15, 16]. Analogous to recombinant protein expression methods, membrane protein expression is challenging in CFE systems[17]. Aggregation, misfolding, and lack of post-translational modification in CFE systems are major bottlenecks that hinder successful membrane protein synthesis using CFE systems. The difficulty of bottom-up membrane protein reconstitution using CFE systems is due in part to the absence of a complex membrane protein biogenesis pathway that relies on signal peptides, signal recognition particles, translocons, and chaperoning molecules. However, recently, multiple studies have suggested that presence of membranous structures such as microsomes or liposomes during translation promotes successful membrane protein expression[18–21]. Additionally, Eaglesfield *et al.* and Steinküher *et al*. have found that inclusion of specific hydrophobic domains known as signal peptides in the N-terminus of the membrane protein can significantly improve its expression[22, 23]. Altogether, these studies suggest that the challenge of membrane protein reconstitution in synthetic cells can be overcome if the protein translation occurs in the presence of the GUV membrane and if proper N-terminal signal peptide is utilized.

Here, a protocol for encapsulation of the protein synthesis using recombinant elements (PURE) CFE reactions for membrane protein reconstitution in GUVs is presented. Bacterial glutamate receptor[24] (GluR0) as model membrane protein is selected and the effect of its N-terminal signal peptide on its membrane reconstitution is studied. The effect of proteorhodopsin signal peptide, which was shown to improve membrane protein reconstitution efficiency by Eaglesfield *et al*.[22], is investigated by constructing a mutated variant of GluR0 denoted as PRSP-GluR0 and its expression and membrane localization with wild type GluR0 (referred to as WT-GluR0 hereafter) that harbors its native signal peptide is compared. This protocol is based on the inverted emulsion

method[25] with modifications that make it robust for CFE encapsulation. In the presented method, the CFE reactions are first emulsified using a lipid-in-oil solution that generates micron-sized droplets that contain the CFE system and are stabilized by the lipid monolayer. The emulsion droplets are then layered on top of an oil-water interface that is saturated with another lipid monolayer. The emulsion droplets are then forced to travel across the oil-water interface via centrifugal force. Through this process, the droplets obtain another monolayer, thus generating a bilayer lipid vesicle. The GUVs containing the CFE reaction are then incubated during which the membrane protein is expressed and incorporated into the GUV membrane. Although this protocol is specified for cell-free expression of GluR0, it can be used for cell-free synthesis of other membrane proteins or different synthetic cell applications such as cytoskeleton reconstitution or membrane fusion studies[26].

PROTOCOL:

1. Bulk CFE reactions in the presence of small unilamellar vesicles (SUVs)

1.1. SUV preparation

NOTE: This step needs to be performed in a fume hood following the safety instructions for working with chloroform.

1.1.1. In a glass vial, prepare 5 mM 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) SUVs by transferring 76 µL of 25 mg/mL POPC stock solution dissolved in chloroform.

1.1.2. While gently rotating the glass vial, blow a gentle stream of argon into the glass vial to form a film of dried lipids at the bottom of the vial. Next, transfer the glass vial to a desiccator with its cap loosely screwed to evaporate excess chloroform.

1.1.3. Keep the glass vial in the desiccator for 1h. Then add 0.5 mL ultra-pure deionized water to dissolve the lipid film and vortex for approximately 2 minutes.

1.1.4. Set up a mini-extrusion apparatus by soaking two filter supports in deionized water and placing them on each of the internal membrane supports. Then soak a 100 nm polycarbonate filter and place it in between the two internal membrane supports held together by the extruder outer casing and the retainer nut. Place this setup in the extruder stand.

1.1.5. Flush two 1 mL gas-tight syringes 3 times with ultra-pure deionized water.

1.1.6. Load the sample of lipid-water mix into one of the 1 mL gas-tight syringes and place it in one end of the mini extruder using the swing arm clips to hold the syringe in place. Insert the second syringe in the other end of the mini-extruder and make sure it is fully depressed.

NOTE: When loading the syringe with the lipid-water mix, ensure there is no air in the syringe before passing it through the mini-extruder.

1.1.7. Gently pass the lipid-water mix from the original syringe to the empty syringe through the mini-extruder apparatus. Repeat this step 11 times to form SUVs. Transfer the SUVs to a 1.5 mL microcentrifuge tube.

NOTE: The SUV solution can be stored at 4 °C for up to two weeks.

1.2. CFE reaction assembly

1.2.1. Assemble the CFE reaction by following the cell-free expression protocol provided by the manufacturer with slight modifications detailed in the following. Mix 10 µL Solution 1 (containing amino acids, NTPs, tRNAs and substrates for enzymes, and necessary buffer), 1 µL Solution 2 (proteins in 20% glycerol), 2 µL Solution 3 (ribosome (20 µM)), appropriate amount of the DNA encoding for soluble sfGFP-sfCherry(1-10)[27] (referred to as soluble sfGFP hereafter), WT-GluR0-sfGFP, or PRSP-GluR0-sfGFP (10-60 ng/1000 base pairs), 1 µL murine RNAse inhibitor, and 4 µL 5 mM SUV solution for membrane proteins or 4 µL water for soluble proteins. Bring the reaction final volume to 20 µL by adding ultra-pure deionized water.

NOTE: When assembling the CFE system, all components must be kept on ice. All materials are temperature-sensitive and can degrade if they reach room temperature.

1.3. CFE reaction incubation and monitoring

1.3.1. Transfer the CFE solution to a 96-well conical V-bottom plate. To prevent evaporation during the course of the reaction, cover the plate using a sealing film.

1.3.2. Incubate the plate at 37 °C in a plate reader for 4-5 h while monitoring the CFE reaction by measuring the GFP signal with a gain of 100 at 488/528 excitation/emission wavelengths every two minutes.

2. CFE reactions encapsulated in GUVs

2.1. Preparation of GUV outer buffer solution

2.1.1. In a 1.5 mL microcentrifuge tube, mix 1.5 µL 1 M spermidine, 37.5 µL 100 mM ATP, 25 µL 100 mM GTP, 12.5 µL 100 mM CTP, 12.5 µL 100 mM UTP, 25 µL 1 M creatine phosphate, 18 µL 1 M magnesium acetate, 93.33 µL 3 M potassium glutamate, 50 µL 1 M HEPES KOH (pH 7.4), 1.15 µL 332 mM folinic acid, 100 µL 2 M glucose, and 50 µL from stock solution of 6 mM mixture of each of the 20 amino acids (prepared by following the protocol described by Sun *et al.*[28]). Bring the solution final volume to 1 mL by adding ultra-purified deionized water.

NOTE: All components must be kept on ice. Add the amino acid mixture at the end to avoid amino acid depletion[28].

NOTE: The solution can be aliquoted in 330 µL aliquots and stored at -20 °C until use.

2.2. Preparation of the lipid-in-oil mixture

NOTE: this step needs to be performed in a fume hood following the safety instructions for working with chloroform.

2.2.1. Under a fume hood, mix 17.3 µL of 25 mg/mL POPC stock solution and 1.08 µL of 50 mg/mL poly(butadiene)-b-poly(ethylene oxide) (PEO-b-PBD) copolymer in a 15 mL glass vial.

NOTE: The final lipid-in-oil solution contains 0.5 mM lipid with 95% and 5% POPC and PEO-b-PBD, respectively. PEO-b-PBD was used to enhance membrane stability during protein expression but was kept at a low molar ratio to reduce copolymer's tendency to aggregate into micelles separate from lipid molecules[29, 30].

2.2.2. Carefully blow a gentle stream of argon gas into the glass vial while rotating the vial to evaporate the chloroform.

2.2.3. Pipette 1.2 mL of light mineral oil to the 15 mL glass vial containing the dried lipids.

2.2.4. Mix the lipids and oil by vortexing at maximum speed for 10 – 20 seconds. The dissolved lipid-copolymer mix will look cloudy.

2.2.5. To ensure that all possible lipid aggregates in the oil are fully dissolved and dispersed throughout the oil, place the glass vial in an oven at around 50 °C for 20 minutes before vortexing for an additional 10-20 seconds at maximum speed.

2.3. **CFE reaction assembly and encapsulation**

Fig. 1 depicts a summary of the following steps.

2.3.1. Prepare 300 µL of the GUV outer solution in a 1.5 mL microcentrifuge tube by mixing 270 µL of CFE outer buffer solution prepared in step 2.1, 15 µL of 5 M NaCl, and 15 µL of 4.5 M KCl.

NOTE: At this step, add 0.45 µL of 1 M 1,4-dithiothreitol (DTT) to the GUV outer solution.

NOTE: The purpose of adding NaCl and KCl is to adjust the outer solution osmolality to match it with the inner CFE solution. The exact volume of NaCl and KCl depends on the desired osmolality adjustment. One can add either NaCl or KCl or both to adjust the osmolality.

2.3.2. Gently pipette 300 µL of lipid-in-oil mixture on top of the GUV outer solution.

NOTE: When adding the lipid-oil mix, it is important that the oil does not mix with the aqueous outer solution. After the addition, there should be a visible interface between the lipid-in-oil mixture and the GUV outer solution.

2.3.3. Incubate the oil-water interface at room temperature for 2 h to allow the lipid monolayer to form and stabilize at the interface.

2.3.4. Meanwhile, follow section 1.2.1 to assemble a CFE reaction containing the plasmid DNA encoding the membrane protein variants or soluble GFP. Replace the 4 µL 5 mM SUV solution or water with 4 µL 1 M sucrose. This reaction will be the GUV inner solution.

NOTE: An osmometer was used to measure the osmolality of the inner and outer solutions. The osmolality of the outer solution was then adjusted accordingly by addition of NaCl or water. The osmolality of the CFE reaction is typically around 1600 mOsm/Kg.

NOTE: Addition of sucrose to the reaction increases the inner solution density, thus allowing the vesicles to travel across the oil-water interface during the centrifugation step. An alternative to sucrose is Opti-Prep density gradient solution.

2.3.5. Add 600 µL of lipid-oil mixture to the microcentrifuge tube containing the CFE reaction and pipette up and down vigorously for ~1 min to emulsify the reaction in lipid-in-oil solution and form the lipid monolayer around synthetic cells.

NOTE: The final solution should not have any bubbles and should look opaque.

2.3.6. Gently pipette the inner solution emulsion on top of the oil layer in the 1.5 mL microcentrifuge tube where the oil-water interface was set up.
NOTE: Be careful not to disturb or destabilize the interface.

2.3.7. Centrifuge for 10 minutes at 2000 g at 4 °C.
NOTE: The centrifugation speed was optimized for this protocol. Adir *et al*.[31] reported a different centrifugation speed.

2.3.8. Once the centrifugation is over, carefully remove the excess oil and outer solution from the microcentrifuge tube using a pipettor. Remove the outer solution until the remaining volume is around 100 µL.
NOTE: A pellet of GUVs is usually visible at the bottom of the microcentrifuge tube. However, a lack of a visible pellet does not necessarily mean no GUV yield.
NOTE: Instead of using a pipettor, the excess oil on top of the outer solution can be removed by aspiration.
NOTE: It is critical to ensure the lipid-in-oil solution is completely removed. Oil contamination in GUV solution can cause low-quality images.

2.3.9. Resuspend the GUV pellet in the remaining 100 µL solution by gently pipetting up and down. Next, transfer the GUV solution to a clean 96 well clear flat bottom plate to incubate.

3. Encapsulated CFE reaction incubation and imaging

3.1. Cover the plate using a sealing film to prevent evaporation. Incubate the plate at 37 °C for 5-6 h. One can use a plate reader and follow step 1.3.2 to prepare the plate reader for the incubation step.

3.2. Once the incubation is over, place the 96 well plate on the imaging stage of an inverted microscope equipped with an iXON3 EMCCD camera (or a sCMOS camera), DAQ-MX controlled laser (or an integrated laser combiner system), and a Yokogawa CSU-X1 spinning disk confocal (or a laser scanning confocal). Focus on any ROI containing GUVs and capture images at excitation wavelength of 488 nm using a Plan-Apochromat 60 x/1.4 NA objective.

3.3. Save images of GUVs in .tiff format.

3.4. Open the images in an image processing software on ImageJ or Fiji. Open the **Brightness/Contrast** setting panel. Adjust the brightness and contrast to appropriate settings that make fluorescent proteins visible.

3.5. If the goal is to compare the signal intensity of different expressed proteins, first stack individual images of GUVs containing different proteins using **Images to Stack** panel located under **Image>Stacks** submenu. Then adjust the brightness and contrast of all images using the **Brightness/Contrast** panel.

REPRESENTATIVE RESULTS

Prior to encapsulation of the CFE reactions, two variants of GluR0-sfGFP harboring native and proteorhodopsin signal peptides (signal peptide sequences presented in **Table S1**) and the soluble sfGFP were individually expressed in bulk reactions and their expression was monitored by detecting the sfGFP signal using a plate reader (**Fig. 2A**). Membrane proteins were expressed in the absence or presence of 100 nm SUVs. Additionally, using a calibration curve that correlates sfGFP signal to its concentration (**Fig. S1**), concentrations of synthesized proteins were estimated (**Table S2**). Clearly, soluble sfGFP had the highest expression among all three proteins which suggests that the expression of membrane proteins imposes a burden on CFE system, thus slowing down the reaction and lowering its yield. In addition, on average, reactions expressing membrane proteins in the presence of SUVs showed higher sfGFP signal compared to reactions lacking SUVs. This observation aligns with the findings of Steinküher *et al*. who showed expression of membrane proteins reduces the capacity of the CFE systems to produce proteins[23]. Nevertheless, given the successful demonstration of protein expression in bulk CFE reaction, one can reason that encapsulated CFE will also synthesize proteins inside GUVs.

Next, individual CFE reactions were encapsulated in GUVs using the inverted emulsion method to express variants of GluR0, namely WT-GluR0 and PRSP-GluR0, and soluble sfGFP. While WT-GluR0, harboring GluR0 native signal peptide, demonstrated excellent expression and membrane localization (**Fig. 2B**, left panel), its counterpart, PRSP-GluR0 which has proteorhodopsin N-terminal signal peptide, did not show similar strong membrane localization. PRSP-GluR0 was found to be more prone to aggregation and punctate formation (**Fig. 2B**, middle panel). Expectedly, soluble sfGFP was expressed in GUVs and stayed in the GUV lumen (**Fig. 2B**, right panel; see **Fig. S2** for images of cohorts of GUVs).

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental steps of inverted emulsion. (**1**) Steps 2.3.1 through 2.3.3 of the protocol are visualized to demonstrate the assembly of the lipid monolayer at the interface of the lipid-oil mix and outer buffer solution. (**2**) Visualization of step 2.3.5 of the protocol is shown here to represent the formation of the lipid monolayer around emulsified droplets encapsulating the inner CFE solution. (**3**) Step 2.3.6 of the protocol shows the addition of the monolayer GUVs to the microcentrifuge tube with the lipid monolayer at the interface of a lipid-oil mix and outer buffer solution. (**4**) Step 2.3.7 is depicted here in which centrifugation leads to the formation of a GUV pellet in the outer solution. (**5**) Step 2.3.8 is shown here indicating the process of removing the excess lipid-in-oil mixture and outer solution. (**6**) Finally, step 2.3.9 is depicted here where the GUV pellet is resuspended in the outer solution and the GUVs are ready for incubation followed by imaging.

Figure 2: Protein expression in bulk CFE reactions and in GUVs encapsulating CFE reactions (**A**) Fluorescence readouts of individual bulk CFE reactions expressing WT-GluR0-sfGFP, PRSP-GluR0-sfGFP, and soluble sfGFP. The soluble sfGFP graph represents the signal from a 2.5 µL reaction (standard reaction volume is 20 µL) to avoid oversaturation of the plate reader measurements. Data is presented as mean ± S.D. n = 3. (**B**) Left: a representative confocal image of a GUV encapsulating CFE reaction expressing WT-GluR0-sfGFP. Middle: a representative confocal image

of GUVs encapsulating CFE reaction expressing PRSP-GluR0-sfGFP. Right: a representative confocal image of a GUV encapsulating CFE reaction expressing soluble sfGFP. Scale bars: 10 µm.

DISCUSSION

Virtually any cellular process that depends on transfer of molecules or information across the cell membrane like cell signaling or cell excitation requires membrane proteins. Thus, reconstitution of membrane proteins has become the main bottleneck in realizing various synthetic cell designs for different applications. Traditional detergent-mediated reconstitution of membrane proteins in biological membranes requires GUV generation methods such as gentle swelling or electroformation. Swelling approaches usually produce small-sized vesicles and electroformation yield significantly drops when complicated solutions, which is often the case when generating synthetic cells, are encapsulated[32]. Additionally, detergents solubilize the membrane protein and their removal during the reconstitution process can cause protein misfolding[33, 34]. On the other hand, the approach presented here relies on co-translational incorporation of the membrane protein into the lipid bilayer which resembles more the natural protein biogenesis pathway in cells[22].

From a technical point of view, the presented protocol is advantageous to other common encapsulation methods such as electroformation and continuous droplet interface crossing encapsulation[7, 8, 35, 36] (cDICE) for easier implementation as the only laboratory equipment required for GUV generation is a centrifuge. As opposed to electroformation, inverted emulsion method allows encapsulation of different combinations of molecules with various concentrations. Additionally, compared to the original inverted emulsion technique[25], this approach generates more stable GUVs that are suitable for encapsulation of CFE lysates or PURE systems. The higher GUV stability is owed to the presence of diblock copolymer in the composition of GUV membrane[37] as well as the long incubation of oil-water interface that allows the interface to be saturated with lipid molecules. Lastly, as opposed to microfluidics approaches, the protocol presented here does not require small channels and tubing. Therefore, the CFE reaction can be encapsulated as soon as it is assembled and the shorter time of GUV assembly due to lack of flow and possible clogging prevents premature start of the CFE reaction. While the demonstration of membrane protein expression in this protocol is exclusive to PUREfrex reactions, one can extend this method to synthesize proteins using different available CFE systems such as lysate-based bacterial or mammalian CFE systems.

The presented approach here has limitations that are caused by the oil-dependent nature of GUV formation process and the intent to have stable GUVs. This approach is typically longer compared to other methods such as cDICE or microfluidics methods, due to the long incubation time of the oil-water interface that is required for interface stabilization and high GUV yield. Additionally, lipid composition is primarily limited to POPC with small doses of other lipids or block copolymers while other methods such as electroformation are more suited for incorporation of lipids with different physical and chemical properties. While the GUV membrane composition in this method is a mixture of POPC and PBD-PEO to maximize CFE yield, possible variations in GUV membrane composition can be tested. However, further optimization of the parameters might be required for other membrane proteins. Since the droplet emulsification occurs through manual pipetting,

the GUVs generated via this method are polydisperse and quite heterogeneous in size. Further, the fact that lipids are dissolved in the organic phase may occasionally cause a layer of oil between the two leaflets of the GUV membrane or contaminate the imaging chamber with oil that can detriment image quality. A possible workaround for the challenge of residual oil is to replace mineral oil with a volatile organic solvent, such as diethyl ether as shown by Tsumoto *et al.*[38], to rely on solvent evaporation along with centrifugation during GUV formation.

While there is no demonstration of channel function in this work, inspired by previous assays used for probing reconstituted mechano- or light-sensitive channel functionality, a fluorescence microscopy-based assay is outlined. The opening of GluR0 channel is reported to increase the membrane conductivity for K^+ ions[24]. Because CFE reactions already contain a high concentration of K^+, typical potassium indicators will not be suitable for assessing channel functionality. However, because potassium influx changes the membrane potential, sensitive membrane potential indicators such as DiBAC4(3)[22] BeRST 1[39] could report GluR0 activity in the presence of glutamate.

Successful reconstitution of membrane proteins in synthetic cells opens up numerous possibilities for creating synthetic cells with unprecedented abilities that more closely mimic natural cells. A current major disadvantage of synthetic cells is their inability to reproduce and recycle energy. However, with light- and chemical-dependent energy regeneration schemes that rely heavily on membrane proteins, one can envisage long-lasting synthetic cells[40]. Utilizing CFE systems allows the reconstitution of multiple membrane proteins that can collectively perform certain tasks. For instance, reconstitution of a ligand-gated ion channel similar to GluR0 described here along with different voltage-gated ion channels can lead to the construction of an excitable neuron-like synthetic cell.

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DISCLOSURES

The authors declare no conflicts of interest.

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