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Title: Preparation of Membrane Protein–Functionalized Polymer and Polymer/Lipid Hybrid Large and Giant Unilamellar Vesicles

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No.**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes.**

Lado Otrin, Team leader of the Artificial Life-Like Systems (ALLS) team in the Process Systems Engineering department

Current Protocol Length

Number of Steps: 24

Number of Shots: 43

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen. Please answer in *stand-alone full sentence with sufficient context***

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

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- 1.1. **Lado Otrin:** Our research focuses on building artificial cells from the bottom up — combining natural and synthetic materials to create functional, biomimetic systems.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

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- 1.2. **Lado Otrin:** Our protocol for building protein-functionalized polymer vesicles applies to drug delivery, biosensing, artificial cell construction, and bioinspired micro/nanoreactors.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

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- 1.3. **Nika Otrin:** This protocol allows researchers to measure membrane protein insertion efficiency, oxygen consumption, proton pumping activity, and vesicle size distribution in polymer compartments.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

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FINAL SCRIPT: APPROVED FOR FILMING



1.4. **Nika Otrin:** Partial dehydration is the most critical step while performing this protocol because over-dehydration impairs protein function, while LUV concentration and droplet size determine GUV yield.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

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1.5. **Nika Otrin:** Additional analyses after this procedure include cryo-TEM for structural insertion assessment and orientation studies using protein tags or selective fluorescence techniques.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What problem in your field of research does your article address?

- 1.6. **Lado Otrin, Team leader of the Artificial Life-Like Systems (ALLS) team in the Process Systems Engineering department,** : (authors will present their testimonial statements live)
 - 1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

How does the video format of your article help you to share your work with the broader scientific community?

- 1.7. **Lado Otrin, Team leader of the Artificial Life-Like Systems (ALLS) team in the Process Systems Engineering department,** : (authors will present their testimonial statements live)
 - 1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Preparation of Large Unilamellar Vesicles (LUVs)

Demonstrator: Wenqi Huang

- 2.1. To begin, use positive displacement pipettes with solvent-resistant tips or gas-tight glass syringes [1] to transfer the polymer, lipid, and fluorescent dye solutions dissolved in a 2 to 1 mixture of chloroform and methanol into a clean glass vial [2] [3].
 - 2.1.1. WIDE: Talent at the working bench with required reagents in the frame.
 - 2.1.2. Talent transferring the polymer, lipid, and fluorescent dye solutions into a clean glass vial.
 - 2.1.3. LAB MEDIA: Table 1 *Video editor: please show 2.1.2 and 2.1.3 side by side using split screen*
- 2.2. Mix the solution thoroughly by gentle vortexing while holding the glass vial upright to ensure homogeneity [1].
 - 2.2.1. Talent gently vortexing the glass vial while holding it upright.
- 2.3. Evaporate the organic solvents under a gentle stream of nitrogen in the fume hood for approximately 1 hour [1] until a uniform thin film forms on the glass vial wall [2].
 - 2.3.1. Talent placing the glass vial under a gentle stream of nitrogen in the fume hood.
 - 2.3.2. CU: Shot of the thin film formed on the glass vial wall.
- 2.4. If polymersomes are used for reconstitution, supplement the rehydration buffer with 0.1 percent sodium cholate [1]. Then, rehydrate the dried film with 1 milliliter of the rehydration buffer to obtain a final amphiphile concentration of 5 milligrams per milliliter [2].
 - 2.4.1. Talent adding sodium cholate to the rehydration buffer.
 - 2.4.2. Talent adding the rehydration buffer to the dried film in the glass vial.
- 2.5. Vortex the glass vial vigorously until the film is completely detached and suspended, forming multilamellar vesicles [1].
 - 2.5.1. CU: Talent vortexing the glass vial until the film is completely suspended.
- 2.6. Subject the hybrid multilamellar vesicle suspensions to five freeze-thaw cycles. Begin

by freezing the samples in liquid nitrogen for one minute [1]. Then thaw them in a water bath set to 35 degrees Celsius until completely melted [2]. Vortex the samples at 2500 to 3000 rpm for 30 seconds using a tube shaker [3].

- 2.6.1. Talent placing the sample tube into liquid nitrogen.
- 2.6.2. Talent placing the vial in the water bath.
- 2.6.3. Talent vortexing the tube using a tube shaker.
- 2.7. Transfer the vesicle suspension to a mini-extruder equipped with a 100-nanometer-pore-size polycarbonate membrane [1]. Extrude the vesicle suspension 21 times to obtain size-homogeneous Large Unilamellar Vesicles or LUVs (*L-U-Vees*)[2].
 - 2.7.1. Talent transfers the vesicle suspension into the mini-extruder fitted with a 100-nanometer-pore-size polycarbonate membrane.
 - 2.7.2. Talent adding the vesicle suspension to the membrane using the mini-extruder.
- 2.8. Store the vesicle suspension at 4 degrees Celsius and use it within one week to prevent aggregation and microbial growth in the sucrose buffer [1].
 - 2.8.1. Talent placing the large unilamellar vesicle suspension into refrigerated storage.

3. Reconstitution of Membrane Protein into Large Unilamellar Vesicles (LUVs)

Demonstrator: Wenqi Huang

- 3.1. Vortex the LUV (*L-U-V*) suspension before use to ensure it is well mixed and uniform [1]. Transfer 200 microliters of the LUVs into a 2-milliliter microcentrifuge tube [2]. For hybrid LUVs, add 1 microliter of 20% sodium cholate to reach a final concentration of 0.1% [3].
 - 3.1.1. Talent gently vortexing the large unilamellar vesicle suspension.
 - 3.1.2. Talent transferring 200 microliters of the large unilamellar vesicle suspension into a 2 milliliter microcentrifuge tube.
 - 3.1.3. Talent adding sodium cholate to the hybrid large unilamellar vesicle suspension.
- 3.2. Gently tap the microcentrifuge tube three times to mix, avoiding vigorous agitation to prevent foam and air bubbles [1].
 - 3.2.1. Talent lightly tapping the microcentrifuge tube 3 times with the finger. **NOTE:** (wrong clapperboard, take 3.1.3 take 4)
- 3.3. Add cytochrome bo3 (*B-O-Three*) oxidase–ATTO 643 (*Atto-Six-Four-Three*) to the detergent-destabilized vesicles while mixing gently and slowly to ensure even distribution throughout the solution and prevent local overconcentration. [1].
 - 3.3.1. Talent adding cytochrome bo3 oxidase–ATTO 643 to the detergent-destabilized

vesicle suspension while gently mixing the vesicle suspension

~~3.4. Add the protein slowly while gently mixing to distribute it evenly throughout the solution and avoid local overconcentration [1].~~

~~3.4.1. Talent slowly adding the protein while gently mixing the vesicle suspension.~~

NOTE: Part of 3.3.1 There is no 3.4.1

3.5. Incubate the mixture at 4 degrees Celsius for 30 minutes with mild agitation at 400 rpm to form protein–detergent–polymer assemblies before removing the detergent [1].

3.5.1. Talent placing the sample into the thermomixer.

3.6. Next, remove 30 milligrams of the prepared Bio-Beads SM-2 from the storage solution, ensuring the beads remain damp [1]. Add them to the sample [2] and incubate for 30 minutes at 4 degrees Celsius with gentle agitation at 600 rpm in a thermomixer [3]. Repeat this procedure 2 additional times for a total of 90 milligrams of Bio-Beads SM-2 and a total incubation time of 1.5 hours [4].

3.6.1. Talent removing 30 milligrams of damp Bio-Beads SM-2 from the storage solution.

3.6.2. Talent adding the Bio-Beads SM-2 to the sample.

3.6.3. Talent placing the tube into the thermomixer.

3.6.4. Talent adding the Bio-Beads SM-2 to the sample.

3.7. Pellet the Bio-Beads by centrifugation at 2000 g for 2 minutes using a benchtop mini centrifuge [1]. Then, carefully collect the supernatant without disturbing the Bio-Bead pellet [2].

3.7.1. Talent placing the sample tube into the benchtop mini centrifuge.

3.7.2. Talent carefully collecting the supernatant while leaving the Bio-Bead pellet undisturbed.

4. Preparation of Protein-Functionalized Giant Unilamellar Vesicles (GUVs)

Demonstrator: Chee Seng Man

4.1. Place the indium tin oxide-coated glass slides into the plasma cleaner with the conductive side facing upward [1]. Plasma-clean the slides for 1 minute at the high-power setting and a pressure of approximately 0.5 millibar [2].

4.1.1. Talent placing the indium tin oxide-coated glass slides into the plasma cleaner with the conductive side facing upward.

4.1.2. Talent starts the plasma cleaning program.

4.2. Mix 20 microliters of protein-free LUVs with 20 microliters of proteo-LUVs containing reconstituted bo3 oxidase–ATTO 643 [1]. Gently vortex to ensure homogeneous mixing [2].

- 4.2.1. Talent combining 20 ul protein-free large unilamellar vesicles and 20ul proteo-large unilamellar vesicles in a tube.
- 4.2.2. Talent gently vortexing the mixture.
- 4.3. Deposit seven 2-microliter droplets of the 5 milligrams per milliliter LUV and proteo-LUV suspension evenly across the 160.6 square-millimeter conductive surface of each electroformation slide [1], keeping the droplets separate to promote uniform film formation [2].
 - 4.3.1. Talent depositing the suspension as separate droplets onto the indium tin oxide-coated glass slide.
 - 4.3.2. Close-up of the evenly distributed droplets on the slide surface.
- 4.4. Allow the droplets to partially dehydrate at room temperature with approximately 20 percent humidity for 40 minutes, or until a white ring forms at the droplet perimeter [1].
 - 4.4.1. Shot of the droplets on the indium tin oxide-coated glass slide with a visible white ring forming around the perimeter. NOTE: Use the end of the shot
- 4.5. Next, assemble the electroformation chamber using two indium tin oxide-coated glass slides, separated by a 1.81-millimeter silicone spacer with a cut for buffer addition and removal [1].
 - 4.5.1. Talent assembling the electroformation chamber using 2 indium tin oxide-coated glass slides and a silicone spacer.
- 4.6. Fill the electroformation chamber with rehydration buffer, supplementing it with 10 micro molar pyranine for pH-sensitive experiments [1].
 - 4.6.1. Talent filling the electroformation chamber with the rehydration buffer.
- 4.7. Apply a sinusoidal alternating electric field at 50 hertz using sequential voltages of 50, 100, 200, 300, 500, 700, and 900 millivolts for 6 minutes each, followed by 1.1 volts for 2 hours [1].
 - 4.7.1. Talent connecting the electroformation chamber to the electrical setup and starting the electroformation program.
- 4.8. Then, carefully open the electroformation chamber using tweezers [1]. Using a FineTip Ultra-Micro Pasteur pipette or equivalent low-shear transfer pipette, collect the GUVs to minimize mechanical stress [2].
 - 4.8.1. Talent carefully opening the electroformation chamber using tweezers.
 - 4.8.2. Talent collecting the giant unilamellar vesicles using a FineTip Ultra-Micro Pastette pipette.
- 4.9. Acquire images of different GUVs throughout the chamber to obtain a representative population, followed by image analysis using appropriate software [1].

- 4.9.1. Talent at the microscope with acquired images displayed in the computer screen.
- 4.10. For proton-pumping analysis, activate cytochrome bo3 oxidase and monitor pyranine fluorescence [1]. Calculate the fluorescence ratio using the presented equation [2].
 - 4.10.1. Representative shot of talent monitoring the pyranine fluorescence on the computer screen.
 - 4.10.2. TXT ON A PLAIN BACKGROUND
$$R = I_{448}/I_{405}$$

Results

5. Results

- 5.1. For highly hydrophilic dyes such as ATTO 643, a 30-centimeter column achieved efficient separation of unbound dye from labeled bo3 oxidase [1]. The degree of labeling for bo3 oxidase labeled with ATTO 643 was 1.16 [2].
 - 5.1.1. LAB MEDIA: Figure 3A. *Video editor: Show the peak under the grey area*
 - 5.1.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the peak between 600-700 nm*
- 5.2. Following reconstitution, polymersome size increased only slightly [1], whereas hybrid vesicle size remained unchanged [2].
 - 5.2.1. LAB MEDIA: Figure 4A.
 - 5.2.2. LAB MEDIA: Figure 4B.
- 5.3. Protein activity was typically slightly higher in hybrid vesicles than in polymersomes [1].
 - 5.3.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the bar labelled 'PDMS-g-PEO/soy PC' and then the bar labelled 'PDMS-g-PEO'.*
- 5.4. Successful incorporation of labeled bo3 oxidase into the membrane was confirmed by confocal microscopy [1].
 - 5.4.1. LAB MEDIA: Figure 6A, B. *Video editor: Highlight the magenta circles*
- 5.5. Larger GUVs above 20 micrometers typically exhibited lower membrane-associated protein fluorescence than smaller vesicles [1].
 - 5.5.1. LAB MEDIA: Figure 6C, D. *Video editor: Highlight the bars after 20 um (X axis) mark*
- 5.6. Hybrid GUVs generally showed slightly higher membrane incorporation of bo3 oxidase than polymer GUVs [1].
 - 5.6.1. LAB MEDIA: Figure 6C, D. *Video editor: Compare the height of the bars in the PDMS-g-PEO/soy PC panels and then the PDMS-g-PEO panels.*
- 5.7. The intravesicular pyranine fluorescence ratio decreased after addition of dithiothreitol and ubiquinone 1 in hybrid GUVs containing bo3 oxidase [1], whereas protein-free GUVs showed a smaller decrease [2].
 - 5.7.1. LAB MEDIA: Figure 7B. *Video editor: Highlight the green trace.*
 - 5.7.2. LAB MEDIA: Figure 7B. *Video editor: Highlight the gray trace*

1. Positive displacement
Pronunciation link: <https://www.merriam-webster.com/dictionary/displacement>
IPA: /'pɑːzətɪv dɪs'pleɪsmənt/
Phonetic Spelling: PAH-zuh-tiv dis-PLAYS-muhnt
2. Chloroform
Pronunciation link: <https://www.merriam-webster.com/dictionary/chloroform>
IPA: /'klɔːrə,fɔːrm/
Phonetic Spelling: KLOR-uh-form
3. Methanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/methanol>
IPA: /'meθə,nɔːl/
Phonetic Spelling: METH-uh-nawl
4. Polymersomes
Pronunciation link: No confirmed link found
IPA: /'pɑːlɪmər,səʊmz/
Phonetic Spelling: PAH-lih-mer-sohmz
5. Reconstitution
Pronunciation link: <https://www.merriam-webster.com/dictionary/reconstitution>
IPA: /,rɪˌkɔːnstə'tuːʃən/
Phonetic Spelling: ree-kon-stuh-TOO-shuhn
6. Rehydration
Pronunciation link: <https://www.merriam-webster.com/dictionary/rehydration>
IPA: /,rɪˌhaɪ'dreɪʃən/
Phonetic Spelling: ree-hy-DRAY-shuhn
7. Sodium cholate
Pronunciation link: No confirmed link found
IPA: /'səʊdiəm 'kɒləɪt/
Phonetic Spelling: SOH-dee-uhm KOH-layt
8. Amphiphile
Pronunciation link: <https://www.merriam-webster.com/dictionary/amphiphile>
IPA: /'æmfəˌfaɪl/
Phonetic Spelling: AM-fuh-fyle
9. Multilamellar
Pronunciation link: No confirmed link found
IPA: /,mʌltɪ,læ'mələr/
Phonetic Spelling: mul-tee-lam-ELL-er
10. Vesicles
Pronunciation link: <https://www.merriam-webster.com/dictionary/vesicle>
IPA: /'vesɪkəlz/

Phonetic Spelling: VESS-ih-kuhlz

11. Polycarbonate

Pronunciation link: <https://www.merriam-webster.com/dictionary/polycarbonate>

IPA: /,pɑːliˈkɑːrbəˌneɪt/

Phonetic Spelling: pah-lee-KAR-buh-nayt

12. Unilamellar

Pronunciation link: No confirmed link found

IPA: /,juːniˌlæˈmɛlər/

Phonetic Spelling: yoo-nee-lam-ELL-er

13. Microcentrifuge

Pronunciation link: No confirmed link found

IPA: /,maɪkroʊˈsentrəˌfjuːdʒ/

Phonetic Spelling: my-kroh-SEN-truh-fyooj

14. Cytochrome

Pronunciation link: <https://www.merriam-webster.com/dictionary/cytochrome>

IPA: /ˈsaɪtəˌkroʊm/

Phonetic Spelling: SY-tuh-kroh-m

15. Oxidase

Pronunciation link: <https://www.merriam-webster.com/dictionary/oxidase>

IPA: /ˈɑːksɪˌdeɪs/

Phonetic Spelling: OK-sih-days

16. Cytochrome b₃ oxidase

Pronunciation link: No confirmed link found

IPA: /ˈsaɪtəˌkroʊm biː ɒʊ θriː ˈɑːksɪˌdeɪs/

Phonetic Spelling: SY-tuh-kroh-m bee-oh-THREE OK-sih-days

17. Detergent-destabilized

Pronunciation link: No confirmed link found

IPA: /dɪˈtɜːdʒənt diːˈsteɪbəlɪzd/

Phonetic Spelling: dih-TER-juhnt dee-STAY-buh-lyzd

18. Centrifugation

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifugation>

IPA: /ˌsentrəˈfjuːɡeɪʃən/

Phonetic Spelling: sen-truh-fyoo-GAY-shuhn

19. Supernatant

Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>

IPA: /ˌsuːpərˈneɪtənt/

Phonetic Spelling: soo-per-NAY-tuhnt

20. Indium tin oxide

- Pronunciation link: No confirmed link found
IPA: /'ɪndiəm tɪn 'ɑːksaɪd/
Phonetic Spelling: IN-dee-uhm tin OK-syde
21. Proteo-LUVs
Pronunciation link: No confirmed link found
IPA: /'proʊtiʊs ˌɛl juː 'viːz/
Phonetic Spelling: PROH-tee-oh el-yoo-VEEZ
22. Electroformation
Pronunciation link: No confirmed link found
IPA: /ɪ ˌɛktroʊfɔːr 'mɛʃən/
Phonetic Spelling: ih-lek-troh-for-MAY-shuhn
23. Pyranine
Pronunciation link: No confirmed link found
IPA: /'paɪrəˌniːn/
Phonetic Spelling: PY-ruh-neen
24. Sinusoidal
Pronunciation link: <https://www.merriam-webster.com/dictionary/sinusoidal>
IPA: /ˌsaɪnə'sɔɪdəl/
Phonetic Spelling: sy-nuh-SOY-duhl
25. Intravesicular
Pronunciation link: No confirmed link found
IPA: /ˌɪntrəvə'sɪkjələr/
Phonetic Spelling: in-truh-veh-SIK-yuh-ler
26. Hydrophilic
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrophilic>
IPA: /ˌhaɪdrə'fɪlɪk/
Phonetic Spelling: hy-druh-FIL-ik
27. Confocal
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kɑːn'fəʊkəl/
Phonetic Spelling: kon-FOH-kuhl
28. Ubiquinone
Pronunciation link: <https://www.merriam-webster.com/dictionary/ubiquinone>
IPA: /juː'bɪkwəˌnoʊn/
Phonetic Spelling: yoo-BIK-wuh-nohn
29. Dithiothreitol
Pronunciation link: No confirmed link found
IPA: /daɪˌθaɪəʊ'θriːɪ tɔːl/
Phonetic Spelling: dye-THY-oh-THREE-ih-tawl

30. Fluorescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>

IPA: /flʊˈresəns/

Phonetic Spelling: floo-RESS-uhns

31. Aggregation

Pronunciation link: <https://www.merriam-webster.com/dictionary/aggregation>

IPA: /ˌægrɪˈɡeɪʃən/

Phonetic Spelling: ag-rih-GAY-shuhn

32. Sucrose

Pronunciation link: <https://www.merriam-webster.com/dictionary/sucrose>

IPA: /ˈsuːkroʊs/

Phonetic Spelling: SOO-krohs

33. Reconstituted

Pronunciation link: <https://www.merriam-webster.com/dictionary/reconstitute>

IPA: /ˌriːˈkɔːnstə tuːtɪd/

Phonetic Spelling: ree-KON-stuh-too-tid

34. Homogeneous

Pronunciation link: <https://www.merriam-webster.com/dictionary/homogeneous>

IPA: /ˌhɒməˈdʒiːniəs/

Phonetic Spelling: hoh-muh-JEE-nee-uhs