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Title: Synthesis of Compound Giant Unilamellar Vesicles: A Biomimetic Model of Nucleate Cells

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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? **Yes , done**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, done**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? It's just 50m

Current Protocol Length
Number of Steps: 22
Number of Shots: 52 (9 SC, 5 Scope)

Introduction

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

1.1. **Rochish Thaokar:** We have conducted an elaborate study on electrohydrodynamics of compound Giant Unilamellar Vesicles to establish them as bio-mimetic equivalents for eukaryotic cells. We use technologies involving the application of electric fields to biological cells, such as electroporation, electrodeformation, etc.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll:2.3.1*

What technologies are currently used to advance research in your field?

1.2. **Rupesh Kumar:** A combination of fluorescence and light microscopy, nanosecond pulse electric field treatment, oscilloscopes and power sources, and innovative synthesis techniques are employed to advance research in this area.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll:2.5.1*

What are the current experimental challenges?

1.3. **Rupesh Kumar:** The challenge in the synthesis of well-formed compound GUVs is the sensitivity of the method to temperature, types of lipids, and compositions used. In this work, we demonstrate this for the DMPC and cholesterol systems, but generalizing it remains challenging.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.8.1*

What research gap are you addressing with your protocol?

1.4. **Rupesh Kumar:** Simple GUVs that mimic anucleate cells have been synthesized in the literature. We synthesize compound Giant vesicles to biomimulate nucleated cells, with a well-defined structure resembling a half-sized inner vesicle.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll:3.2.1*

What research questions will your laboratory focus on in the future?

1.5. **Rochish Thaokar:** We will focus on studying the effect of micro and nanosecond pulses on the inner vesicles, which mimic nuclei of eukaryotic cells in the context of electroporation

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll:4.1.1*

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

Protocol

2. Electroformation for Synthesizing Simple Giant Unilamellar Vesicles (sGUVs)

Demonstrator: Rupesh Kumar

2.1. To begin, thoroughly clean the Indium Tin Oxide or ITO-coated slides using a dish soap solution **[1-TXT]** and rinse with deionized water with a conductivity of 0.055 microsiemens per centimeter **[2]**. Then, using a 100 percent ethanol solution, clean the slides again and rinse with deionized water **[3]**. Dry the slides in an oven set to 85 degrees Celsius **[4]**.

2.1.1. WIDE: Talent applying soap solution to ITO slide. **TXT: Prepare all the required reagents beforehand**

2.1.2. Talent holding the slide under distilled water being dispensed from a bottle.

2.1.3. Talent wiping the slide with ethanol-soaked lint-free tissue.

2.1.4. Talent placing the cleaned slide in an oven.

2.2. Identify the conductive side of each ITO-coated slide using a clamp meter **[1]**. Secure the cleaned slide onto the spin coater stage using a vacuum, ensuring that the conductive side is facing upward **[2]**.

2.2.1. Talent using a clamp meter to check conductivity of the slide.

2.2.2. Talent placing the slide on the spin coater and activating vacuum suction.

2.3. Apply 25 microliters of the lipid solution 1 as four droplets onto the conductive side of one ITO slide **[1-TXT]**. Take another slide and apply 25 microliters of the lipid solution 2 in the same manner **[2]**.

2.3.1. Talent dispensing four droplets of lipid solution from step 1.1 onto a slide using a micropipette.

2.3.2. Close-up Shot of the second slide with droplets placed.

2.4. Vacuum-dry both lipid-coated slides in a desiccator **[1]** stored in the dark for a minimum of 2 hours **[2]**. **NOTE**: VO edited

2.4.1. Talent placing the lipid-coated slides inside a desiccator.

Added shot: Talent Placing a dessicator in the dark.

2.5. Then, arrange a lipid-coated ITO slide with lipid 1 in parallel with a clean slide, ensuring the conductive sides face each other **[1]** and place a 3-millimeter-thick silicone rubber spacer between them **[2]**. Seal the setup with a clamp to create an electroformation chamber **[3]**.

2.5.1. Talent aligning the two slides face to face.

2.5.2. Talent placing a silicone spacer between the slides. **NOTE**: 2.5.1+2.5.2 clubbed together

2.5.3. Talent clamping the slides together to form a chamber. **TXT: Assemble the slides similarly for lipid 2**

2.6. Now, place both electroformation chambers in an incubator maintained between 38 and 40 degrees Celsius **[1]** and fill the chamber with 100 millimolar sucrose solution using a 2-milliliter syringe **[2]**. Connect the output cable of the function generator to the ITO-coated slides with the function generator **[3]**. Apply an alternating current electric field of 5 volts peak-to-peak at 10 hertz frequency using a dual-channel function generator for 4 hours **[4]**. **NOTE**: VO added

2.6.1. Talent placing both chambers into the incubator.

2.6.2. Talent filling the chambers with sucrose solution using a syringe.

Added shot: Connecting the output cable of the function generator to the ITO-coated slides with the function generator.

2.6.3. Talent adjusting settings to 5 Vpp and 10 Hz, and activating the electric field.

2.7. After incubation, disconnect the electroformation chambers from the function generator **[1]**. Remove them from the incubator and allow them to cool to room temperature **[2]**.

2.7.1. Talent disconnecting chambers from the generator.

2.7.2. Talent removing chambers from the incubator.

2.8. Using a syringe, harvest the synthesized giant unilamellar vesicles from each chamber and transfer them into separate 2-milliliter microcentrifuge tubes **[1]**. Incubate the tubes at room temperature between 25 and 27 degrees Celsius for 1 hour before proceeding to osmotic shock **[2]**.

2.8.1. Talent drawing vesicle solution from the chamber with a syringe and transferring into microcentrifuge tube.

2.8.2. Talent placing tube on the bench. **NOTE**: This shot was merged with 2.8.1

3. Shape Transition in Osmotic Shock (sGUV-to-Stomatocyte-to-cGUV)

3.1. Transfer 200 microliters of simple giant unilamellar vesicles or sGUVs *(S-G-U-Vees)* suspended in hydrating media containing 100 millimolar sucrose solution into the observation chamber **[1]** and introduce 125 microliters of 300 millimolar glucose solution to induce osmotic shock and initiate shape transitions **[2]**

3.1.1. Talent pipetting 200 microliters of sGUV suspension into the observation chamber.

3.1.2. Talent adding 125 microliters of glucose solution into the chamber containing sGUV suspension.

3.2. Allow the sGUVs, now undergoing osmotic shock, to rest for 1 hour inside the observation chamber placed on the microscopy stage **[1]**.

3.2.1. Talent placing the chamber on the microscope stage.

3.3. Perform differential interference contrast and epifluorescence microscopy using an inverted microscope equipped with a monochromatic camera **[1]**. Use Plan fluor, Extra Long Working Distance 20x by 0.45 and 40x by 0.60 objective lenses to observe shape transitions in the sGUVs **[2]**. For epifluorescence, use a green filter set with excitation at 510 to 560 nanometers, a dichroic mirror at 575 nanometers, and a barrier filter at 590 nanometers for Nile red-stained bilayers **[3]**.

3.3.1. Talent operating inverted microscope with monochromatic camera setup.

3.3.2. Talent adjusting the objective lenses.

3.3.3. Shot of Epifluorescence and DIC image settings being configured. **NOTE**: There are part 1 and part 2 of this shot

3.4. Monitor shape transitions within the observation chamber using Differential Interference Contrast and epifluorescence microscopy with Plan fluor objective lenses **[1]**.

3.4.1. SCOPE: 3.4.1

3.5. Observe the formation of stomatocytic vesicles **[1]** as the shape transition proceeds **[2]**. Monitor how the larger vesicles settle first, followed by an increase in the number of smaller vesicles over time **[2]**.

3.5.1. SCOPE: 3.5.1 00:22-00:35.

3.5.2. SCOPE: 33.5.2 0019-00:21.

3.5.3. SCOPE: 3.5.3 00:20-00:27.

3.6. Next, adjust the conductivity in the inner, annular, and outer regions of the confined giant unilamellar vesicles with the appropriate quantities of a 7.5 millimolar stock salt solution before inducing osmotic shock **[1]**.

3.6.1. Talent mixing the salt solution.

3.7. For example, to create higher conductivity in the outer and inner regions than the annular region, transfer 200 microliters of sucrose solution into the electrofusion chamber **[1]**. Add 20 microliters of 7.5 millimolar salt solution into the chamber **[2]** and induce osmotic shock with 125 microliters of 300 millimolar glucose solution **[3]**.

3.7.1. Shot of pipetting 200 microliters of sucrose solution into the chamber.

3.7.2. Shot of adding 20 microliters of salt solution to the same chamber.

3.7.3. Shot of initiating osmotic shock with 125 microliters of glucose solution.

3.8. Allow the osmotic-shocked sGUVs to rest in the chamber for 3 to 4 hours **[1]**. Confirm that the resulting confined GUVs have higher conductivity in the outer and inner regions compared to the annular region **[2]**.

3.8.1. Talent placing the chamber aside for incubation.

3.8.2. SCREEN: 3.8.2 00:53 **NOTE**: Use the screenshot at this timepoint

3.9. To perform electrodeformation of the confined GUVs, space the wire electrodes 500 micrometers apart **[1]** and apply an alternating current electric potential of 7.5 volts peak-to-peak at 100 kilohertz using a function generator **[2-TXT]**.

Added shot: Shot of Chamber with focus on two wire electrodes, spaced apart 500 micrometers. **NOTE: May have been slated as 3.9.0**

3.9.1. Talent configuring function generator and applying AC electric field across chamber. **NOTE: Use the shot named 3.9.2**

3.10. After the electric field is applied, capture video at 10 frames per second and observe the oblate deformation of the outer vesicles and the prolate deformation of the inner vesicles **[1]**.

3.10.1. SCREEN: 3.10.1. 00:10-00:20

4. Microscopic Analysis of Compound GUVs (cGUVs)

4.1. ~~For confocal microscopy, fill a custom imaging chamber, modified with coverslip glass, with an aqueous solution containing cGUVs **[1]**. Let the sample rest for 10 to 15 minutes for vesicles to settle **[2]**~~. Then, load the cGUV *(C-G-U-V)*-mixture into a cavity slide **[1]** and seal with a coverslip to prevent movement **[2]**.

~~4.1.1. Talent pipetting cGUV solution into custom chamber with coverslip bottom.~~

4.1.2. ~~Shot of the chamber~~. **Author's NOTE**: Shots of 4.1.1 and 4.1.2 were taken, but delete them as they are not required.

4.1.3. Talent loading the mix into the cavity slide.

4.1.4. Talent sealing the sample by placing a coverslip.

4.2. Use a laser scanning confocal microscope to analyze cGUV morphology through z-axis scanning with a step size of 1 micrometer **[1]**. Employ a Plan-Apochromat 40x by 1.3 oil DIC objective lens for imaging **[2]**. Use a red single-channel mode with 561 nanometers excitation and 561 to 695 nanometers emission to image cGUVs stained with Nile Red or Rhodamine-PE **[3]**.

4.2.1. Talent placing a cavity slide on the laser scanning confocal microscopy stage.

4.2.2. Talent pointing to the objective lens and positioning the objective lens for microscopical observation. **NOTE**: shots 4.2.1+4.2.2 were merged

4.2.3. Talent doing the z-scanning of cGUV **Author's NOTE**: file name given by videographer is **MVI 0127 and MVI0128** (videographer captured shot with focus on the screen)

4.2.4. SCOPE: Show video of cGUV with z scanning at an interval of 1 micron.

4.3. Modify and extract the .czi *(dot-C-Z-I)* image file using the microscope-linked software **[1]**. Insert graphics like scale bars and enable 2D and 3D views **[2]**. Then, go to

Processing, Method and **Parameters** to adjust the desired settings **[3]**, then click **Apply** to export the image in JPG format **[4]**.

4.3.1. SCREEN: 4.3.1_4.3.2_4.3.3.mp4 000:03-00:10.

4.3.2. SCREEN: 4.3.1_4.3.2_4.3.3.mp4 00:25-00:30 AND 00:39-00:42.

4.3.3. SCREEN: 4.3.1_4.3.2_4.3.3.mp4 01:10-01:19

4.3.4. SCREEN: 4.3.1_4.3.2_4.3.3.mp4 01:41-01:45.

4.4. Finally, open the file in ImageJ software **[1]**. To insert a scale bar, go to **Analyze** followed by **Set Scale** to calibrate **[2]**, then select **Analyze** followed by **Tools** and **Scale Bar** to apply it **[3]**.

4.4.1. SCREEN: 4.4.1_4.4.2_4.4.3.mp4 00:10-00:15.

4.4.2. SCREEN: 4.4.1_4.4.2_4.4.3.mp4 00:30-00:38.

4.4.3. SCREEN: 4.4.1_4.4.2_4.4.3.mp4 00:50-00:56 and 01:02-01:07

Results

5. Results

5.1. Confocal z-stack imaging confirmed that inner vesicles were fully separated from the outer vesicles and elevated in the z-plane due to lower density of the inner solution **[1]**.

5.1.1. LAB MEDIA: Figure 2.

5.2. The intermediate stomatocyte state showed a narrow neck connecting inner and outer vesicles before complete separation **[1]**.

5.2.1. LAB MEDIA: Figure 3 and 4. *Video editor: Emphasize the arrow pointing to the neck connection in figure 4*.

5.3. A diverse population of vesicular forms including multiple inner vesicles, star-shaped bodies, and tubular structures were observed 6 hours after osmotic shock **[1]**.

5.3.1. LAB MEDIA: Figure 5

5.4. A high abundance of cGUVs was formed using a lipid mixture of 1,2-dimyristoyl-sn-glycero-3-phosphocholine and cholesterol in a 63 to 37 molar ratio **[1]**.

5.4.1. LAB MEDIA: Figure 6.

5.5. Under an alternating current electric field, cGUVs showed deformation, with the outer vesicle forming an oblate shape and the inner vesicle forming a prolate shape **[1]**.

5.5.1. LAB MEDIA: Figure 7

Pronunciation guide:

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1. Indium

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/indium
- **IPA:** /ˈɪn.di.əm/
- **Phonetic Spelling:** IN-dee-um(merriam-webster.com)

2. Tin Oxide

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/tin%20oxide
- **IPA:** /ˈtɪn ˈɑkˌsaɪd/
- **Phonetic Spelling:** TIN OX-ide(merriam-webster.com)

3. Desiccator

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/desiccator
- **IPA:** /ˈdɛs.ɪˌkeɪ.tər/
- **Phonetic Spelling:** DES-ih-kay-ter(merriam-webster.com)

4. Stomatocyte

- **Pronunciation link:** https://www.howtopronounce.com/stomatocyte
- **IPA:** /ˈstoʊ.mə.təˌsaɪt/
- **Phonetic Spelling:** STOH-muh-toh-site(merriam-webster.com)

5. Epifluorescence

- **Pronunciation link:** https://www.howtopronounce.com/epifluorescence
- **IPA:** /ˌɛp.i.flʊˈrɛs.əns/
- **Phonetic Spelling:** EP-ee-flu-RES-ens(merriam-webster.com)

6. Plan Fluor

- **Pronunciation link:** https://www.howtopronounce.com/plan-fluor
- **IPA:** /plæn flʊər/
- **Phonetic Spelling:** PLAN FLUOR

7. Plan-Apochromat

- **Pronunciation link:** https://www.howtopronounce.com/plan-apochromat
- **IPA:** /plæn əˈpɒk.rə.mæt/
- **Phonetic Spelling:** PLAN uh-POK-ruh-mat

8. Rhodamine

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/rhodamine
- **IPA:** /ˈroʊ.dəˌmiːn/
- **Phonetic Spelling:** ROH-duh-meen(merriam-webster.com, merriam-webster.com)

9. cGUV

- **Pronunciation link:** No confirmed link found
- **IPA:** /si dʒi ju vi/

- **Phonetic Spelling:** see-GEE-you-vee(merriam-webster.com, merriam-webster.com)

10. sGUV

- **Pronunciation link:** No confirmed link found
- **IPA:** /ɛs dʒi ju vi/
- **Phonetic Spelling:** ess-GEE-you-vee
