FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 68594
Scriptwriter Name: Pallavi Sharma
Project Page Link: https://review.jove.com/account/file-uploader?src=20971543

Title: A Liposome Membrane Permeability Assay for Investigating the Effects of Phosphatidylinositol Phosphate Groups on Membranotropic Action of Venom PLA_2

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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? **Yes**

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

   The distance between Beijing, China and Reno, NV, USA. Filming will be done only in Beijing and OBS film recording will be done in Reno.

Current Protocol Length
Number of Steps: 23
Number of Shots: 50

Introduction

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

NOTE: 1.4, 1.5 and 1.6 are uploaded on the project page

1.1. **Edward Gasanoff:** In collaboration with Dr. Ruben Dagda, our research lab seeks the lowest concentration of aberrant phospholipase A_2 that increases membrane permeability—knowledge that could help treat inflammation-related diseases.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.7.3*

What significant findings have you established in your field?

1.2. **Zimu Zhou:** Our highly sensitive ligand substitution assay allowed us to establish a difference in activities of aberrant phospholipase A_2 in membranes with phospholipids of similar structure—a finding with significant pharmacological implications.

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

What advantage does your protocol offer compared to other techniques?

1.3. **Zhuoyan Zeng:** This cost-effective ligand substitution reaction assay we developed in our lab offers superior sensitivity for assessing membrane permeability. It also integrates easily with standard biophysical techniques in bio-membrane integrity studies.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.15.1*

How will your findings advance research in your field?

1.4. **Ruben Dagda:** Our JoVE article, developed from a 12-year collaboration with Dr. Gasanoff's laboratory, presents essential protocols designed to support students in low-resource settings and to foster international research and education collaborations.

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

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

1.5. **Ruben Dagda:** We would focus on expanding our techniques in molecular dynamics using GROMACS and HPC servers for drug discovery that can be easily followed by both undergraduate and high school students.

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.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Ruben Dagda: Associate Professor and co-director of UNR's T32 Interdisciplinary Training Program, Department of Pharmacology, University of Nevada Reno School of Medicine,**:

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

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Edward Gasanoff: Ph.D., Head of the Biochemistry Section, Advanced STEM Research Center, Beijing Chaoyang Kaiwen Academy:**

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

Protocol

2. In Silico Simulation of HDP-2P Interaction with PI and Phosphoinositides Using Molecular Modeling Software

Demonstrator: Maryam Barancheshmeh

2.1. To begin, install the chemical drawing editor that will be used to construct ligand molecules **[1]**. Then, install the molecular modeling software that supports ligand-receptor docking **[2]**. Proceed to install the automated docking tools that allow preparation of ligand and receptor structures **[3]**. Since the molecular modeling software requires a Python environment, install the latest version of Python **[4]**.

2.1.1. WIDE: 68594_screenshot_1.mp4: 00:04-00:06, 00:48-00:52, 01:08-01:10, 01:26-01:30

2.1.2. SCREEN: 68594_screenshot_1.mp4: 01:47-02:10

2.1.3. SCREEN: 68594_screenshot_1.mp4: 02:17-02:32

2.1.4. SCREEN: 68594_screenshot_1.mp4: 02:44-02:54

2.2. Using the chemical drawing editor, build the molecular structure of phosphatidylinositol **[1-TXT]**. Then, save the file in PDB *(P-D-B)* format **[2]**.

2.2.1. SCREEN: 68594_screenshot_2.mp4: 00:20-00:26 **TXT: Similarly, build structures for PI-4-P, PI-4,5-P_2, cardiolipin, and phosphatidylserine**

2.2.2. SCREEN: 68594_screenshot_2.mp4: 00:41-00:48

2.3. Now, open the saved PDB file in the automated docking tool software **[1]**. Add hydrogen atoms to the molecule in the docking software. Select either polar or all hydrogen atoms **[2]**.

2.3.1. SCREEN: 68594_screenshot_3.mp4: 00:03-00:15

2.3.2. SCREEN: 68594_screenshot_3.mp4: 00:16-00:32

2.4. Set the phosphatidylinositol molecule as the ligand in the docking software and save the ligand as a PDBQT *(P-D-B-Q-T)* file **[1-TXT]**.

2.4.1. SCREEN: 68594_screenshot_4.mp4: 00:04-00:16, 00:19-00:32. **TXT: Make sure to make all bonds rotatable.**

2.5. Now, download the PDB file for phospholipase A2 HDP-2 *(A-Two-H-D-P-Two)*, which contains two monomers: A, acidic and enzymatically inactive, and E, basic and enzymatically active **[1]**. Import the 2I0U *(Two-I-Zero-U)* file into the molecular modeling software **[2]**. Select all atoms of monomer A to isolate it **[3]**. Using the **Edit** menu, delete all atoms of monomer A to retain only monomer E, which will serve as the receptor **[4]**.

2.5.1. SCREEN: 68594_screenshot_5.mp4: 00:04-00:12, 00:18-00:21

2.5.2. SCREEN: 68594_screenshot_5.mp4: 00:43-00:52

2.5.3. SCREEN: 68594_screenshot_5.mp4: 00:53-01:03

2.5.4. SCREEN: 68594_screenshot_5.mp4: 01:04-01:10, 01:32-01:37

2.6. Next, open the processed HDP-2P receptor PDB file in the automated docking tool software **[1]** and remove all water molecules from the receptor structure **[1]**.

2.6.1. SCREEN: 68594_screenshot_6.mp4: 00:00-00:20

2.6.2. SCREEN: 68594_screenshot_6.mp4: 00:21-00:26

2.7. Now, add hydrogen atoms to the HDP-2P receptor **[1]**. Select only polar hydrogen atoms for improved visualization of polar and hydrogen bonds **[2]**.

2.7.1. SCREEN: 68594_screenshot_7.mp4: 00:00-00:14

2.7.2. SCREEN: 68594_screenshot_7.mp4: 00:15-00:20

2.8. Next, set the HDP-2P receptor in the docking tool as the receptor structure **[1]**. Save the receptor as a PDBQT file and ensure no rotatable bonds are assigned **[2]**.

2.8.1. SCREEN: 68594_screenshot_8.mp4: 00:00-00:13

2.8.2. SCREEN: 68594_screenshot_8.mp4: 00:14-00:28

2.9. For docking parameter setup, open both the ligand and receptor PDBQT files in the molecular docking software **[1]**.

2.9.1. SCREEN: 68594_screenshot_9.mp4: 00:00-00:25, 00:32-00:46

2.10. Access the grid box setup interface and define the grid box dimensions. Then, set the center coordinates to fully enclose the receptor molecule **[1]**.

2.10.1. SCREEN: 68594_screenshot_10.mp4: 00:03-00:20, 00:45-00:55

2.11. After that, close the grid box setup interface while keeping the defined settings **[1]** and

save the docking parameters into a file named config.txt *(Config-T-X-T)* **[2]**.

2.11.1. SCREEN: 68594_screenshot_11.mp4: 00:00-00:07

2.11.2. SCREEN: 68594_screenshot_11.mp4: 00:08-00:17, 00:25-00:27, 00:31-00:37

2.12. Launch the molecular docking software using the saved config.txt file **[1]** and execute the docking calculation using the defined parameters **[2]**.

2.12.1. SCREEN: 68594_screenshot_12.mp4: 00:00-00:10

2.12.2. SCREEN: 68594_screenshot_12.mp4: 00:11-00:12

2.13. Open and review the docking_log.txt *(Docking-Log-T-X-T)* file to assess the docking scores **[1]**. Then, select the docking result that shows the lowest energy conformation **[2]**.

2.13.1. SCREEN: 68594_screenshot_13.mp4: 00:00–00:21

2.13.2. SCREEN: 68594_screenshot_13.mp4: 00:22-00:27

2.14. Next, use the automated docking tool software to open the docking result PDBQT file **[1]**.

2.14.1. SCREEN: 68594_screenshot_14.mp4: 00:13-00:18, 00:28-00:32, 01:15-01:20

2.15. Analyze the ligand-receptor binding interactions, focusing specifically on the active center of the receptor and the types of intermolecular interactions, including ionic, ion-polar, polar, and hydrogen bonds, focusing on the key amino acid residues in the catalytic site of HDP-2P that bind to phosphatidylinositol **[1]**. For phosphatidylinositol or other phosphoinositides, prepare a table detailing the binding affinities in kilocalories per mole, charged and polar groups on the ligand's polar head, and the HDP-2P residues involved in each interaction, along with the bond types **[2-TXT]**.

2.15.1. SCREEN: 68594_screenshot_15.mp4: 00:17-00:22, 00:30-00:37, 01:30-01:33, 01:40-01:55, 02:10-02:12

2.15.2. SCREEN: 68594_screenshot_15.mp4: 02:26-02:30, 02:50-03:00**TXT: Repeat docking 3×/receptor-ligand pair to confirm binding site, affinity, interactions, residues, and bond type**

3. Effects of HDP-2P on Permeability of PC Liposomal Membranes Enriched with PI, PI-4-P, PI-4,5-P_2, PS, or CL

3.1. Prepare 10 milliliters of 0.06 molar stock solutions of six phospholipids **[1],** phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, cardiolipin, phosphatidylcholine, and phosphatidylserine **[2]**.

3.1.1. Talent adding phospholipids to the tubes.

3.1.2. Talent pipetting chloroform and methanol in a 2:1 volume ratio into tubes.

3.2. Prepare 100 milliliters of 0.10 molar Tris-HCl buffer at pH 7.4 **[1]** and add copper (II) *(two)* sulfate to attain a final concentration of 0.05 molar **[2]**.

3.2.1. Talent adding Tris-HCl buffer to a beaker.

3.2.2. Talent adding copper(II) sulfate to the buffer and mixing.

3.3. To prepare 4 milliliters of 1.5×10^{-3} molar HDP-2P stock solution, dissolve the calculated amount of HDP-2P in 0.05 molar copper (II) sulfate and 0.10 molar Tris-HCl buffer at pH 7.4 **[1]**.

3.3.1. Talent dissolving the required amount of HDP-2P into a tube containing the prepared buffer solution.

3.4. To prepare 100 milliliters of 0.5 molar ammonia solution, add 3.76 milliliters of 25% ammonia solution to a 100-milliliter volumetric flask **[1]** and bring the volume up to 100 milliliters with 0.10 molar Tris-HCl buffer at pH 7.4 **[2]**.

3.4.1. Talent transferring 25 percent ammonia solution using a pipette into a flask.

3.4.2. Talent adding Tris-HCl buffer to the flask up to the 100 milliliter mark and mixing gently.

3.5. To prepare liposomes with ammonia inside their inner volume, transfer 6.25 milliliters of the 0.06 molar phospholipid stock solution in chloroform and methanol into a glass tube **[1]**. Dry the phospholipids under a vacuum at 10 pascals to form a lipid film **[2]**.

3.5.1. Talent pipetting 6.25 milliliters of phospholipid solution into a clean glass tube.

3.5.2. Talent placing the tube in a vacuum chamber set to 10 pascals.

3.6. Hydrate the dried film with 1.5 milliliters of 0.5 molar ammonia in 0.10 molar Tris-HCl buffer at pH 7.4 **[1]** and incubate at 25 degrees Celsius for 1 hour in a thermostat **[2]**. Sonicate the resulting lipid suspension at a frequency of 22 kilohertz to produce sonicated liposomes **[3]**.

3.6.1. Talent adding the ammonia-buffer solution to the dried lipid film.

3.6.2. Talent placing the tube in a thermostat set to 25 degrees Celsius.

3.6.3. Talent sonicating the hydrated lipid suspension using a sonicator set at 22 kilohertz.

3.7. To remove ammonia located outside the liposomes, dialyze the liposome solution using a dialysis membrane with a molecular weight cut-off of 1.0 kilodalton **[1]**. Perform dialysis for 5 hours at room temperature against a 0.10 molar Tris-HCl buffer at pH 7.4, using a magnetic stirrer for constant agitation **[2]**.

3.7.1. Talent placing the liposome solution inside the dialysis membrane and sealing it securely.

3.7.2. Talent immersing the dialysis membrane in a beaker with Tris-HCl buffer and placed on a magnetic stirrer.

3.8. After dialysis, centrifuge the liposome solution at 200 *g* for 90 minutes to pellet the liposomes **[1]**. Discard the supernatant and resuspend the liposome pellet in 5.0 milliliters of 0.10 molar Tris-HCl, 0.05 molar copper(II) sulfate buffer at pH 7.4 **[2]**. Divide the resuspended solution into five separate samples of 1.0 milliliter each **[3]**.

3.8.1. Talent placing the liposome tubes in a benchtop centrifuge and setting the speed to 200 g for 90 minutes.

3.8.2. Talent discarding the supernatant and pipetting 5.0 milliliters of Tris-HCl/$CuSO_4$ buffer to resuspend the liposome pellet.

3.8.3. Talent pipetting 1.0 milliliter aliquots of the resuspended liposomes into five separate microtubes.

3.9. To the five samples, add varying volumes of Tris-HCl, copper sulfate buffer, and HDP-2P stock to achieve HDP-2P to phospholipid molar ratios of 0.000, 0.001, 0.002, 0.003, and 0.004 **[4-TXT]**.

3.9.1. Talent adding specific volumes of buffer and HDP-2P stock to each sample tube according to the molar ratio scheme. **TXT: Tris-HCl, 0.05 M CuSO4: 60, 40, 20, and 0 µL**; **HDP-2P stock buffer: 0, 20, 40, 60, 80 µL**

3.10. Incubate all liposome samples at 37 degrees Celsius in a thermostat for 30 minutes **[1]**. Measure the optical density of each sample at 600 nanometers using a spectrophotometer **[2-TXT]**.

3.10.1. Talent placing the sample tubes at 37 degrees Celsius.

3.10.2. Talent transferring sample into cuvettes and measuring absorbance at 600

nanometers using a spectrophotometer. **TXT: Repeat for 6 liposome types with 3 replicates each to ensure reproducibility**

Results

4. Results

4.1. Treatment of liposomes with HDP-2P showed that membrane permeability to ammonia was highest in phosphatidylcholine liposomes enriched with cardiolipin **[1]** and phosphatidylserine **[2].**

4.1.1. LAB MEDIA: Figure 7. *Video editor: Highlight the purple curve labelled "PC + CL".*

4.1.2. LAB MEDIA: Figure 7. *Video editor: Highlight the blue curve labelled "PC + PS".*

4.2 Treatment with HDP-2P of liposomes enriched with phosphatidylinositol **[1]** showed membrane permeability to ammonia lower than membrane permeability of liposomes treated with HPD-2P and enriched with cardiolipin **[2]** or phosphatidylserine **[3],** but higher than pure phosphatidylcholine treated with HDP-2P **[4].**

4.2.1. LAB MEDIA: Figure 7. *Video editor: Highlight the dark blue curve labelled "PC + PI".*

4.2.2. LAB MEDIA: Figure 7. *Video editor: Highlight the purple curve labelled "PC + CL".*

4.2.3. LAB MEDIA: Figure 7. *Video editor: Highlight the blue curve labelled "PC + PS".*

4.2.4. LAB MEDIA: Figure 7. *Video editor: Highlight the green curve labelled "PC".*

4.3. No increase in membrane permeability was observed in phosphatidylcholine liposomes enriched with phosphatidylinositol 4-phosphate **[1]** or phosphatidylinositol 4,5-bisphosphate after HDP-2P treatment **[2].**

4.3.1. LAB MEDIA: Figure 7. *Video editor: Highlight the dark green curve labelled "PC + PI-4-P" staying near the baseline.*

4.3.2. LAB MEDIA: Figure 7. *Video editor: Highlight the orange curve labelled "PC + PI-4,5-P_2" remaining flat at the bottom.*

1. **phosphatidylinositol**
   Pronunciation link: https://www.merriam-webster.com/medical/phosphatidylinositol (Merriam-Webster)
   IPA: /ˌfɑs fəˌtɪd ɪˈlɪ noʊ zə tɑl/
   Phonetic Spelling: fos-fa-ti-di-li-no-zi-tol
2. **phospholipase**
   Pronunciation link: https://www.howtopronounce.com/phospholipase (howtopronounce.com)
   IPA: /ˌfɑsfoʊˈlaɪˌpeɪz/
   Phonetic Spelling: fos-foh-lye-paze
3. **permeability**
   Pronunciation link: https://dictionary.cambridge.org/us/pronunciation/english/permeability (Cambridge Dictionary)
   IPA: /ˌpɝː.mi.əˈbɪl.ə.ti/
   Phonetic Spelling: per-mee-uh-BIL-i-tee
4. **liposomal**
   Pronunciation link: https://howjsay.com/how-to-pronounce-liposomal (howjsay.com)
   IPA: /lɪˈpɑːsəməl/ (or /lɪˈpɒsəməl/)
   Phonetic Spelling: li-poh-suh-mul
5. **dialysis**
   Pronunciation link: https://www.merriam-webster.com/dictionary/dialysis
   (You can check there)
   IPA: /daɪˈæləsɪs/
   Phonetic Spelling: dy-AL-uh-sis
6. **sonicate / sonication**
   Pronunciation link: https://www.merriam-webster.com/dictionary/sonicate
   IPA: /ˈsɑnɪˌkeɪt/ (for “sonicate”)
   Phonetic Spelling: SON-ih-kate
7. **automated**
   Pronunciation link: https://www.merriam-webster.com/dictionary/automated
   IPA: /ˈɔːtəˌmeɪtəd/
   Phonetic Spelling: AW-tuh-may-tid
8. **configuration**
   Pronunciation link: https://www.merriam-webster.com/dictionary/configuration
   IPA: /kənˌfɪgəˈreɪʃən/
   Phonetic Spelling: kun-fig-yuh-RAY-shun
9. **intermolecular**
   Pronunciation link: https://www.merriam-webster.com/dictionary/intermolecular
   IPA: /ˌɪntərmoʊˈlɛkjələr/
   Phonetic Spelling: in-ter-moh-LEK-yuh-ler

10. **conformation**
    Pronunciation link: https://www.merriam-webster.com/dictionary/conformation
    IPA: /ˌkɑnfərˈmeɪʃən/
    Phonetic Spelling: kon-fer-MAY-shun
11. **affinity**
    Pronunciation link: https://www.merriam-webster.com/dictionary/affinity
    IPA: /əˈfɪnəti/
    Phonetic Spelling: uh-FIN-i-tee