BROWN

ANITA SHUKLA
Assistant Professor
School of Engineering

Dr. Amit Krishnan
Review Editor, *Journal of Visualized Experiments (JoVE)*
1 Alewife Center #200
Cambridge, MA 02140

March 31, 2021

Dear Dr. Krishnan,

Enclosed please find our revised manuscript, "Assembly of cell mimicking supported and suspended lipid bilayer models for the study of molecular interactions" by C. M. Bailey-Hytholt,* V. LaMastro, and A. Shukla* (*corresponding authors). We thank the reviewers for their insightful comments and suggestions and have used these to improve our manuscript. The following are the details of the revisions we have made to the manuscript and our responses to the reviewer comments (all major changes have been highlighted in red in the manuscript):

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thank you for this opportunity. We have thoroughly proofread the manuscript.

2. Please provide an institutional email address for each author.

Response: Thank you, we have now included institutional email addresses for each author.

3. Please revise the following lines to avoid previously published work: 75-76, 434-437.

Response: Thank you, we have revised lines 75-76 and 434-437 within the text.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have revised the text to remove personal pronouns.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Trizma, Alconox, Milli-Q, Teflon, Kimwipe, etc.

Brown University Box D / 184 Hope Street Providence, RI 02912 TEL: 401 863-5719 FAX: 401 863-1238 Anita_Shukla@Brown.edu

BROWN

ANITA SHUKLA
Assistant Professor
School of Engineering

Response: Thank you for providing the examples. We have corrected these occurrences.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: Thank you, we have adjusted the protocol numbering.

7. For time units, use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the manuscript. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Line 140, 147, 188, 217, 245 etc.)

Response: Thank you for this note, we have corrected the time unit abbreviations in the text.

8. Line 165-166: Please mention the size/dimensions of the O-rings used.

Response: We have now measured the outer and inner diameters of the O-rings utilized and inserted these in the text.

9. Line 228-229: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: Thank you, we have ensured all text is written in imperative tense.

Reviewer comments:
Reviewer #1:

Minor Concerns:
1) The manuscript could benefit from mentioning additional relevant literature. A few interesting and relevant paper suggestions are:
https://doi.org/10.1016/j.cis.2020.102177 and https://doi.org/10.1016/j.cbpa.2007.09.020 - Review papers on model cell membrane for membrane interaction studies.
https://doi.org/10.1016/0079-6107(68)90019-9 - Bangham's well-established pioneering protocol for vesicle preparation using lipid extrusion.
https://doi.org/10.1016/j.matdes.2021.109486 - Recent research paper where membrane models

BROWN

ANITA SHUKLA
Assistant Professor
School of Engineering

are supported onto realistically-shaped substrates.
https://doi.org/10.1529/biophysj.104.053728 - Research paper where the use of QCM-D is supplemented by AFM and ellipsometry for a more thorough characterization of lipid bilayer formation (could complement or replace the current reference 4).

Response: Thank you for these suggested references. We have now incorporated several of the recommended references within our introduction section.

2) It might be very useful for the readers to have a full set of sample values to start from, so I suggest including this information throughout. The protocol starts by doing this, but stops after only a couple of steps. For example, 1.1.1 says "(...) For example, add 20 mL of chloroform to 200 mg of L-phosphatidylcholine egg, chicken) (egg PC)." and 1.1.2 says "(...) For example, to form 1 mL of egg PC vesicles at 2.5 mg/mL, pipette 250 µL of egg PC stock solution into the vial.", but subsequent sections do not include sample values. While it is true that the correct values for each step can be calculated individually from the information provided in the protocol, having a full set of sample values might make it easier for users to follow the protocol, at least as a first attempt.

Response: Thank you for this suggestion. We have included example values in the text in some important sections to increase clarity as noted. However, adding example values at every step would result in the protocol exceeding the maximum allowed page limit. Due to this, we have not included a full set of example values, but the detailed steps and values provided will help the reader through the protocol and any necessary calculations.

3) It could be good to provide the values shown in the various panels of Figures 2 and 4, in addition to the figures, perhaps as a separate table or included in the graphs? The approximate values can be estimated from the graph, but more accurate xx +/- yy values might be very useful for some of the protocol's users, especially for the hydrodynamic diameters shown in Fig 2a and 2b.

Response: Thank you for this suggestion. We have now included Table 1 that shows the average hydrodynamic diameters for each lipid vesicle composition corresponding with Figure 2.

Brown University Box D / 184 Hope Street Providence, RI 02912 TEL: 401 863-5719 FAX: 401 863-1238 Anita_Shukla@Brown.edu

BROWN

ANITA SHUKLA
Assistant Professor
School of Engineering

Reviewer #2:

1) Plastic pipettes should NEVER be used with chloroform as they are not resistent to this solvent. Chloroform solutions should ALWAYS be handled with hamilton syringes. Please correct that in step 1 of section 1.1.3

Response: Thank you for this comment. Yes, we agree that plastic pipette tips should never be used with chloroform. We use carbon fiber solvent safe pipette tips, which are compatible with chloroform, and have added this note to both the materials document and protocol section 1.1.1 as follows:

Page 3: "NOTE: All steps using chloroform need to be performed in a chemical fume hood. Chloroform should always be pipetted using solvent safe carbon fiber pipette tips. Solutions containing chloroform should always be stored in glass vials."

2) There are some papers out there using vesicle deposition to form suppported lipid bilayers that include membrane proteins. See Isaksson et al Nanoletters 2017, that used a mesoporous subtrate for facilitating vesicle fusion.

Response: We thank the reviewer for noting this interesting paper. We have incorporated this work into our discussion section where we mention the usefulness of further studies to add proteins into supported bilayers:

Page 17: "For example, a recent study incorporated proteins into supported lipid bilayers by using mesoporous silica substrates allowing for a tailored surface pore size to incorporate native transmembrane proteins.[52]"

52. Isaksson, S. *et al.* Protein-Containing Lipid Bilayers Intercalated with Size-Matched Mesoporous Silica Thin Films. *Nano Letters*. **17** (1), 476–485 (2017).

3) About simple lipid mixtures, the authors should mention that using vesicles of smaller sizes and in the absence of salts favors bilayer deposition even for complex mixtures such as extracts of bacterial lipids and yeast lipids. There are several examples of this in the literature that show that the use of divalent cations at a very fine concentration range favors vesicle fusion. Examples are many, see Lind et al ACS Omega 2019 and de Ghellinck et al PLoS One 2014

Response: Thank you for this suggestion. We have now added some discussion on additional methods to rupture multi-lipid vesicles to form supported lipid bilayers:

Page 16: "To form multi-lipid supported lipid bilayers we have noted the use of an AH peptide to induce vesicle rupture. Depending on the lipid composition, other methods may be explored to

Brown University Box D / 184 Hope Street Providence, RI 02912 TEL: 401 863-5719 FAX: 401 863-1238 Anita_Shukla@Brown.edu

BROWN

ANITA SHUKLA
Assistant Professor
School of Engineering

induce vesicle rupture, such as varying ionic strength, temperature, and flow. For example, altering the buffer salt concentration has been used to achieve multi-lipid bilayers, such as those mimicking bacterial membranes that include PE and phosphatidylglycerol (PG) in the composition.[40]"

40. Lind, T.K., Skida, M.W.A., Cárdenas, M. Formation and Characterization of Supported Lipid Bilayers Composed of Phosphatidylethanolamine and Phosphatidylglycerol by Vesicle Fusion, a Simple but Relevant Model for Bacterial Membranes. *ACS Omega*. **4** (6), 10687–10694 (2019).

4) Hamilton syringes are used for extrusion with hand extruder. There are syringes of volumes larger than 1 mL and therefore larger volumes than 1 mL can be extruded with hand extruders. Please correct the first NOTE in section 1.1.2.

Response: Thank you for this note. The manufacturer of the mini extruder suggests syringe sizes of 1 mL or less. We prefer to keep the syringe volume as 1 mL in the text to align with what is included with the purchased mini extruder kit and recommended. The polycarbonate membrane size for the mini extruder has a smaller diameter than the large extruder. This is likely be due to the lower volume that the mini extruder is capable of handling compared to the large extruder. However, we have added "recommended" into the NOTE in section 1.1.2, to clarify:

Page 3: "NOTE: The prepared volume may depend on the extruder process being used (see step 1.3). The mini extruder maximum recommended volume is 1 mL, while the large extruder volume range is 5-50 mL."

5) Dry lipid films can be stored in the -20 freezer for at least 6 months.

Response: We thank the reviewer for this comment. We have not previously stored the dry lipid films in the -20°C freezer for this amount of time, but have added a note regarding different storage durations to section 1.1.2:

Page 4: "NOTE: The process can be stopped here. If the lipid film will not be used immediately after vacuum drying, store in a desiccator until used. We have observed that these lipid films yield similar quality vesicles after 1 week or storage at these conditions; the vesicle quality following lengthier storage durations, if necessary, should be further explored."

Brown University Box D / 184 Hope Street Providence, RI 02912 TEL: 401 863-5719 FAX: 401 863-1238 Anita_Shukla@Brown.edu

BROWN

ANITA SHUKLA
Assistant Professor
School of Engineering

6) Detergents are typically avoided when working with lipids. See point 1, section 1.3.1.

Response: Thank you for this note. Yes, it is indeed important to make sure that the lipids do not come in contact with detergent. The manufacturer of the mini extruder recommends cleaning the extruder components with detergent as noted in the protocol, followed by thorough rinsing with ultrapure water. Thus, the extruder is free of any detergent prior to addition of the vesicle solution. It is noted in the protocol to rinse at least three times with water to ensure full removal of the detergent.

7) Why 21 extrusion steps? There is plenty evidence that small unilamellar vesicles are obtained after 5 extrusion steps, see Åkesson et al PCCP 2010

Response: Thank you for this note. We have found that 21 extrusion steps result in reproducible and successful formation of lipid vesicles at the target size. However, we do agree that this can be dependent on the lipid composition. We have included a note in the protocol text following section 1.2.1.7:

Page 5: "NOTE: The number of extrusions can be optimized depending on the lipid composition."

8) Extruded vesicles solutions should be used within 1-2 days, this is though highly dependant on the lipid preparation.

Response: We thank the reviewer for this comment. We agree that the length of time for storage of lipid vesicles is highly dependent on both the preparation as well as the lipid composition. For example, we have found no change in size or ability to form lipid bilayers with egg PC vesicles for at least two weeks. We have included a note regarding this following section 1.2.1.8.

Page 5: "NOTE: The recommended vesicle storage duration is highly dependent on the lipid composition, and the vesicle physicochemical properties (e.g., hydrodynamic diameter, zeta potential) should be monitored over time. For example, egg PC vesicles have been stored for at least two weeks with no change in vesicle size or bilayer formation capacity."

9) The cleaning of the modules in section 3.2 is not what is recommended by Q-Sense. The modules should be dissasembled and cleaned properly, by bath sonication in hellmanex 2% and water. Removing O-rings and the inner plastic piece that is in contact with the loop for temperature.

Brown University Box D / 184 Hope Street Providence, RI 02912 TEL: 401 863-5719 FAX: 401 863-1238 Anita_Shukla@Brown.edu

BROWN

ANITA SHUKLA
Assistant Professor
School of Engineering

Response: Thank you for this note. Q-Sense recommends two cleaning protocols. The daily protocol requires pumping 2% detergent (such as hellmanex or sodium dodecyl sulfate) followed by Milli-Q water. This is the protocol we note in the text. The thorough cleaning protocol, which requires the disassembly of the modules and bath sonication, is not required in between every measurement. However, we agree that it is important to note this cleaning should be performed as needed and have included a note regarding the thorough cleaning.

Page 8: "NOTE: The cleaning protocol above is used daily before and after every measurement. A thorough cleaning can be performed as needed. Briefly, to perform a thorough cleaning, disassemble the flow modules. All components except for the electrode side of the flow module should be immersed in 2% (w/v) SDS and bath sonicated, followed by thorough rinsing with ultrapure water and drying with a stream of N_2 gas. The component of the flow module containing the electrode pins should never be in contact with liquid."

10) An example for the permeation across suspended bilayers should be shown including a figure.

Response: Thank you, we have example results for permeation shown in Figure 4C for uni-lipid and multi-lipid bilayers and have noted P_{app} calculation results in the results section.

We again thank the reviewers for their questions and comments and have now addressed these and used them to improve our manuscript. We feel that the updated manuscript is now suitable for inclusion in the *Journal for Visualized Experiments (JoVE)* and ask that this manuscript be considered for publication.

Sincerely,

Anita Shukla

Anita Shukla, Ph.D. (corresponding author)
Assistant Professor of Engineering,
Assistant Professor of Molecular Pharmacology, Physiology, and Biotechnology (courtesy appointment)
Brown University, Providence, RI
anita_shukla@brown.edu

CBHytholt

Christina M. Bailey-Hytholt, Ph.D. (corresponding author)
Affiliate Assistant Professor of Chemical Engineering

BROWN
Worcester Polytechnic Institute, Worcester, MA
cmbailey@wpi.edu

ANITA SHUKLA
Assistant Professor
School of Engineering

Brown University Box D / 184 Hope Street Providence, RI 02912 TEL: 401 863-5719 FAX: 401 863-1238 Anita_Shukla@Brown.edu