Journal of Visualized Experiments

Assembly of cell mimicking supported and suspended lipid bilayer models for the study of molecular interactions

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62599R2
Full Title:	Assembly of cell mimicking supported and suspended lipid bilayer models for the study of molecular interactions
Corresponding Author:	Christina M Bailey-Hytholt Worcester Polytechnic Institute Worcester, Massachusetts UNITED STATES
Corresponding Author's Institution:	Worcester Polytechnic Institute
Corresponding Author E-Mail:	cmbailey@wpi.edu
Order of Authors:	Christina Bailey-Hytholt
	Veronica LaMastro
	Anita Shukla
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Bioengineering
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Providence, RI, USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	

TITLE:

Assembly of Cell Mimicking Supported and Suspended Lipid Bilayer Models for the Study of Molecular Interactions

AUTHORS AND AFFILIATIONS:

Christina M. Bailey-Hytholt*,[1] Veronica LaMastro,[2] Anita Shukla*[2]

1. Department of Chemical Engineering, Worcester Polytechnic Institute, Worcester, MA
2. Center for Biomedical Engineering, School of Engineering, Brown University, Providence, RI

Author institutional email addresses:
Christina M. Bailey-Hytholt: cmbailey@wpi.edu
Veronica LaMastro: veronica_lamastro@brown.edu
Anita Shukla: anita_shukla@brown.edu

*Corresponding authors: anita_shukla@brown.edu, cmbailey@wpi.edu,

SUMMARY:

This protocol describes the formation of cell mimicking uni-lipid and multi-lipid vesicles, supported lipid bilayers, and suspended lipid bilayers. These *in vitro* models can be adapted to incorporate a variety of lipid types and can be used to investigate various molecule and macromolecule interactions.

ABSTRACT:

Model cell membranes are a useful screening tool with applications ranging from early drug discovery to toxicity studies. The cell membrane is a crucial protective barrier for all cell types, separating the internal cellular components from the extracellular environment. These membranes are composed largely of a lipid bilayer, which contains outer hydrophilic head groups and inner hydrophobic tail groups, along with various proteins and cholesterol. The composition and structure of the lipids themselves, play a crucial role in regulating biological function, including interactions between cells and the cellular microenvironment, which may contain pharmaceuticals, biological toxins, and environmental toxicants. In this study, methods to formulate uni-lipid and multi-lipid supported and suspended cell mimicking lipid bilayers are described. Previously, uni-lipid phosphatidylcholine (PC) lipid bilayers as well as multi-lipid placental trophoblast-inspired lipid bilayers were developed for use in understanding molecular interactions. Here, methods for achieving both types of bilayer models will be presented. For cell mimicking multi-lipid bilayers, the desired lipid composition is first determined via lipid extraction from primary cells or cell lines followed by liquid chromatography-mass spectrometry (LC-MS). Using this composition, lipid vesicles are fabricated using a thin-film hydration and extrusion method and their hydrodynamic diameter and zeta potential are determined. Supported and suspended lipid bilayers can then be formed using quartz crystal microbalance with dissipation monitoring (QCM-D) and on a porous membrane for use in a parallel artificial membrane permeability assay (PAMPA), respectively. The representative results highlight the reproducibility and versatility of *in vitro* cell membrane lipid bilayer models. The methods presented can aid in rapid, facile assessment of the interaction mechanisms, such as permeation, adsorption, and embedment, of various molecules and

macromolecules with a cell membrane, helping in the screening of drug candidates and prediction of potential cellular toxicity.

INTRODUCTION:

The cell membrane, composed primarily of phospholipids, cholesterol, and proteins, is a crucial component of all living cells[1]. With organization driven by lipid amphiphilicity, the cell membrane functions as a protective barrier and regulates how the cell interacts with its surrounding environment[2]. Several cellular processes are dependent on the lipid and protein composition of the membrane[1,2]. For example, cell membrane interactions are important for effective drug delivery[3]. Pharmaceuticals, biologics, nanomaterials, biological toxins, and environmental toxicants can impact the integrity of a cell membrane, thereby affecting cellular function[4]. The construction of *in vitro* cell mimicking membrane models based on the lipid composition of cell membranes has the potential to provide facile tools to greatly enhance the study of the potential impact of these materials on cells.

Model lipid bilayers include lipid vesicles, supported lipid bilayers, and suspended lipid bilayers. Supported lipid bilayers are a model of the phospholipid cell membrane commonly used in biotechnology applications where lipid vesicles are ruptured on a supported substrate material[5–9]. One common technique used to monitor bilayer formation is quartz crystal microbalance with dissipation monitoring (QCM-D), which examines the adsorption of vesicles in comparison to the bulk liquid properties *in situ*[8,10–14]. Previously QCM-D has been used to demonstrate that under flow conditions, once a critical vesicle coverage of phosphatidylcholine (PC) lipid vesicles is achieved on the surface, they spontaneously rupture into rigid lipid bilayers[15]. Previous work has also investigated supported lipid bilayer formation with varying lipid compositions[16], incorporation of lipid proteins[17–19], and utilizing polymer cushions[20], yielding supported lipid bilayers capable of mimicking various aspects of cell membrane function.

Lipid bilayers have been used to mimic various biological barriers from sub-cellular to organ levels including mitochondrion, red blood cell, and liver cell membranes by altering the phospholipid, cholesterol, and glycolipid components[21]. These more complex multi-lipid vesicles may require additional methods to achieve vesicle rupture, depending on the lipid composition. For example, previous studies have utilized an α-helical (AH) peptide derived from the hepatitis C virus's nonstructural protein 5A to induce bilayer formation by destabilizing the adsorbed lipid vesicles[22,23]. Using this AH peptide, supported lipid bilayers mimicking placental cells have previously been formed[24]. The great potential of supported lipid bilayers for biomedical applications has been demonstrated with investigations spanning molecular and nanoparticle transport[25, 26], environmental toxicant interactions[27], protein assembly and function[17–19], peptide arrangement and insertion[28, 29], drug screening[30], and microfluidic platforms[31].

Suspended lipid bilayers have been used for pharmaceutical screening studies via a parallel artificial membrane permeability assay (PAMPA) where a lipid bilayer is suspended across a porous hydrophobic insert[32–35]. PAMPA lipid models have been developed for different biological interfaces including the blood-brain, buccal, intestinal, and transdermal interfaces[36]. By combining both the supported lipid bilayer and PAMPA techniques, adsorption, permeability, and embedment of compounds within lipid components of a desired tissue or cell type can be thoroughly studied.

This protocol describes the fabrication and application of *in vitro* cell membrane lipid bilayer models to investigate several molecular interactions. Preparation of both uni-lipid and multi-lipid supported and suspended lipid bilayers is detailed. To form a supported lipid bilayer, lipid vesicles are first developed using thin-film hydration and extrusion methods followed by physicochemical characterization. Formation of a supported lipid bilayer using QCM-D monitoring and fabrication of suspended lipid membranes for use in PAMPA is discussed. Finally, multi-lipid vesicles for the development of more complex cell mimicking membranes are examined. Using both types of fabricated lipid membranes, this protocol demonstrates how this tool can be used to study molecular interactions. Overall, this technique constructs cell mimicking lipid bilayers with high reproducibility and versatility.

PROTOCOL:

1. Developing uni-lipid vesicles

1.1. Thin-film hydration method

1.1.1. Preparation and storage of lipid stock solutions

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.

1.1.1.1. Prepare a 10 mg/mL lipid stock solution by adding the appropriate volume of chloroform into the vial containing the lipid powder and mix well. For example, add 20 mL of chloroform to 200 mg of L-α-phosphatidylcholine (egg, chicken) (egg PC). The stock solution may be made at a different concentration if needed.

NOTE: If the powder lipid was stored in an ampule, after adding chloroform transfer to a glass vial with a polytetrafluoroethylene (PTFE) lined cap.

1.1.1.2. Seal the vial cap with Parafilm and store at –20 °C for up to 6 months.

1.1.2. Formation of a dry-lipid film

1.1.2.1. Add the appropriate volume of lipid stock solution into a clean glass vial needed for a final vesicle concentration of 2.5 mg/mL. 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.

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.

1.1.2.2. Remove chloroform from lipid stock solution using a stream of N_2 gas (ultrapure 5.0 Grade).

1.1.2.3. To ensure full removal of chloroform, connect the dried lipid film to vacuum and leave for at least 4 h.

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 of storage at these conditions; the vesicle quality following lengthier storage durations, if necessary, should be further explored.

1.1.3. Performing freeze-thaw-vortex cycles

1.1.3.1. Prepare a Tris sodium chloride buffer solution containing 10 mM of Tris base and 100 mM of NaCl. Rehydrate the dried lipid film with the required volume of Tris NaCl buffer to yield a final vesicle concentration of 2.5 mg/mL and vortex for approximately 15-30 s.

1.1.3.2. Transfer the vesicle suspension into a container with dry ice until frozen, approximately 30 min. After the sample is completely frozen, thaw the suspension in a 30-40 °C water bath. Vortex the thawed vesicle suspension.

NOTE: Liquid N_2 may be used in place of dry ice. Transfer the vesicle suspension into liquid nitrogen for 30 s, and then immediately thaw in an 80°C water bath.

1.1.3.3. Repeat Step 1.1.3.2 an additional 4 times, for a total of 5 freeze-thaw-vortex cycles.

1.2. Extrusion

NOTE: After freeze-thaw-vortex cycles, multilamellar vesicles are formed. Extrusion aids in reducing size and developing large unilamellar vesicles.

1.2.1. Mini (1 mL) extruder process

1.2.1.1. Thoroughly clean all components of the extruder using a mild detergent in ultrapure water and rinse at least three times with ultrapure water ensuring all detergent is removed. Dry with N_2 gas.

1.2.1.2. Assemble the two internal membrane supports and O-rings (inner diameter of 12.7 mm; outer diameter of 15.2 mm). Position each membrane support so the O-ring is facing up.

1.2.1.3. Pre-wet a filter support with ultrapure water. Place it on the membrane support surface inside the O-ring. Repeat for the second internal membrane support.

1.2.1.4. Position one internal membrane support into the extruder outer casing. Place one 100 nm polycarbonate membrane onto the internal membrane support, directly over the filter support.

NOTE: The polycarbonate membranes are stored separately between pieces of blue colored paper. Remove the separating paper before inserting onto the membrane support.

1.2.1.5. Position the second internal membrane support into the extruder outer casing with the O-ring and filter support side facing the polycarbonate membrane. Attach the PTFE bearing into the retainer nut and screw closed with the extruder outer casing. Clip the extruder into the heating block.

1.2.1.6. Load the lipid vesicle suspension into one of the syringes and position the syringe into the extruder heat block, inserting the needle fully into one end of the extruder. Insert the second, empty syringe into the opposite side and lock in both syringes using the arm clips on the heat block.

NOTE: If needed, place the extruder heat block on a hot plate and set the temperature to a value above the transition temperature of the lipid. Insert a thermometer into the holder built into the heat block for accurate temperature readings and wait until required temperature is reached (~15 min). Egg PC lipid vesicles do not require heat during extrusion.

1.2.1.7. Slowly push the vesicle suspension into the empty syringe, and then back into the original syringe. Monitor for pressure-changes throughout the extrusion that indicate a leak. Repeat 20 more times for a total of 21 passes through the polycarbonate membrane. Transfer the lipid vesicles into to a clean glass vial for storage.

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

1.2.1.8. If heat was used, allow for extruded vesicle suspension to reach room temperature. Store the extruded lipid vesicles at 4 °C until further use.

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.

1.2.2. Large (5-50 mL) extruder process

NOTE: Follow steps 1.2.2.1-1.2.2.5 if heat is required for chosen lipid. Skip to step 6 if heat is not needed. Steps 1.2.2.1-1.2.2.5 are not required for egg PC.

1.2.2.1. Fill a 1 L flask with reverse osmosis (RO) water.

NOTE: Do not use ultrapure water to circulate through the 50 mL system as it can cause metal ions to leach from the extruder cylinder.

1.2.2.2. Place the 1 L flask in a water bath on a hot plate and set the hot plate to a temperature above the transition temperature of the lipid.

1.2.2.3. Attach the sample cylinder to the flask with flexible tubing via the inlet on the sample cylinder. Attach tubing on the outlet of the cylinder to the top of the 1 L flask. Secure

tubing at both the inlet and outlet, as needed. This will create a uni-directional flow of the water through the sample cylinder.

1.2.2.4. Turn on the pump to start water circulation. If heat is needed, allow for approximately 30-45 minutes for sample cylinder to reach desired temperature.

1.2.2.5. Connect the cap of the sample cylinder to a nitrogen tank via the flexible connector attached to the pressure relief valve unit.

1.2.2.6. Clean all parts of the 50 mL extruder with 70% (v/v) ethanol.

1.2.2.7. Assemble the extruder by placing the large hole screen support, sintered disk, drain disks, and polycarbonate membrane into the space in the extruder lower support. Connect the extruder upper and lower supports using the four screws and tighten.

1.2.2.8. Attach the extruder unit to the sample cylinder by screwing to the bottom and tightening with a wrench to secure.

~~Fill the sample cylinder with ultrapure water.~~NOTE: If heat is used, place a thermometer into the cylinder and wait until water has reached desired temperature before continuing. This will ensure that the sample temperature is maintained throughout the entire extrusion process.

1.2.2.9. Fill the sample cylinder with ultrapure water. Extrude the water through the extruder unit prior to adding the sample into the sample cylinder. This is done to pre-wet the membranes, similar to the mini extruder.

NOTE: Ensure the cap is fully screwed on and the pressure relief valve is closed completely before turning on the nitrogen. Minimal pressure is required for this step (~5-10 psi).

1.2.2.10. Add the lipid vesicle suspension into the sample cylinder and screw the top closed. Slowly increase the pressure until the sample begins to drip from the extruder unit at a rate of approximately 2-3 drops/s into a clean glass vial.

NOTE: Do not increase the pressure quickly at this step, as too much pressure may negatively impact the membranes and lead to unsuccessful extrusion.

1.2.2.11. Once all sample has been extruded, turn off the N_2 supply and release the pressure in the sample cylinder by opening the pressure relief valve slowly. Pour the lipid vesicles back into the sample cylinder and repeat step 1.2.2.11, 9 more times for a total of 10 extrusions.

NOTE: The required pressure for extrusion may decrease with increasing number of extrusions, as the sample becomes more homogeneous and closer in size to the polycarbonate membrane pore size.

1.2.2.12. Store extruded lipid vesicle suspension at 4 °C until further use.

2. Characterizing lipid vesicles

2.1. Hydrodynamic diameter measurement using dynamic light scattering (DLS)

2.1.1. Vortex lipid vesicles and pipette 50 µL of the lipid vesicle suspension into a disposable low-volume cuvette. Cover to prevent contamination with dust and debris.

2.1.2. Load the vesicle suspension into the DLS instrument, input the sample details, and perform the measurement using the associated software.

2.2. Zeta potential

2.2.1. Prepare a folded capillary zeta cell by washing with ultrapure water, 70% ethanol, and ultrapure water using syringes that connect to the inputs of the cell. Gently push the liquid through the cell 3-4 times and empty the cell completely before switching to the next solution.

2.2.2. Vortex the lipid vesicles and prepare a 1:10 (v/v) dilution of lipid vesicles in ultrapure water.

2.2.3. Load the diluted lipid vesicle suspension. Remove air bubbles by pushing the suspension back and forth between the syringes. Attach the stoppers to each inlet.

NOTE: It is crucial to remove all bubbles, as this will impact the measurement.

2.2.4. Place the zeta cell in the sample chamber, ensuring that the electrodes are in contact. Close the sample chamber top. In the associated software, input the sample details and collect measurement.

3. Forming a uni-lipid supported lipid bilayer using QCM-D

3.1. Solution preparations

3.1.1. Prepare a 2% (w/v) sodium dodecyl sulfate (SDS) solution in ultrapure water. Mix on a stir plate until completely dissolved. Aliquot working solutions of at least 10 mL of ultrapure water, 2% SDS, and Tris NaCl.

3.1.2. Prepare a dilution of lipid vesicles in Tris NaCl buffer. The concentration of vesicles is dependent on the application. For egg PC, concentrations in the range of 0.01-0.5 mg/mL have been shown to result in successful supported lipid bilayer formation.

3.2. Cleaning silica-coated quartz crystal sensors

NOTE: Cleaning QCM-D crystals is dependent on the surface material of the sensor being used. To form supported lipid bilayers, silica-coated quartz crystals are used in this protocol and detailed below as adapted from the manufacturer's standard operating procedure.

3.2.1. Insert the silica-coated quartz crystal sensor into the flow module ensuring that the "t" on the crystal aligns with the "t" on the module. Screw the flow module closed.

NOTE: If the QCM-D utilized allows for multiple flow modules to be connected and run simultaneously, repeat the following procedures for the additional modules as needed.

3.2.2. Insert the flow module into the base of the instrument with the electrodes from the flow module connecting with the analyzer system. Lock the module into place.

3.2.3. Connect the inlet and outlet tubing to the flow module and pump. Place the tubing into the holding guards and close the lid of the analyzer system. Place a waste container at the outlet of the pump to collect spent solutions.

3.2.4. To perform the cleaning, first turn on the pump. Set the flow speed to 400 µL/min. Insert the inlet tubing into ultrapure water and flow 5-10 mL through the module.

3.2.5. Switch the inlet tubing into 2% SDS and flow 5-10 mL through the module. Switch the inlet tubing back into ultrapure water and flow 10-20 mL through the module. Remove the inlet tubing from the solution and flow air through the tubing until all liquid is ejected.

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.

3.2.5. Remove the sensor from the flow module and rinse the sensor with ultrapure water. Dry the sensor with an N_2 gas stream. Dry the flow module with an N_2 gas stream. Ensure the electrode always remain free of any liquid.

3.2.6. In a chemical fume hood, insert the silica-coated quartz crystal sensor into an ultra-violet (UV)/ozone cleaning instrument. Turn on the instrument and allow treatment for at least 2 min. Remove the sensors carefully and return into the flow module.

3.3. Forming a Tris NaCl baseline

3.3.1. Turn the analyzer instrument on to connect to the associated software and set temperature to the desired value for the supported lipid bilayer. Allow the temperature to stabilize to the desired input.

NOTE: If the set temperature is above room temperature, all solutions should be heated to the same temperature using a heat block.

3.3.2. Configure the measurement and find all sensor resonance frequencies and dissipations for overtones 3, 5, 7, 9, 11, and 13 before starting the measurement.

NOTE: The 1st overtone can be disregarded as this harmonic is overly sensitive and produces noisy data.

3.3.3. Turn on the pump and set the flow rate to 175 µL/min or the desired experimental flow rate.

3.3.4. Wipe the inlet tubing with ethanol prior to inserting into Tris NaCl. Start the measurement and begin flowing Tris NaCl.

NOTE: Data is collected and monitored in real-time. The change from air to liquid in the flow module will be observed in the data collection software by a rapid dissipation change (ΔD) increase and frequency change (ΔF) decrease.

3.3.5. Allow Tris NaCl to flow through the module for 5-10 min, ensuring that the baseline ΔF and ΔD values in liquid remain stable.

3.4. Forming a uni-lipid supported lipid bilayer

3.4.1. Stop the pump and remove the inlet tubing from the Tris NaCl solution and carefully insert into the lipid vesicle solution. Back flow for 5 s to remove any air bubbles from the inlet tubing, and then continue forward flow. Restart the measurement in the software to zero the baseline.

NOTE: Be careful to avoid air bubbles in the tubing, which can flow through the module and disrupt bilayer formation and the data recording.

3.4.2. Flow lipid vesicles until bilayer formation is observed in real-time in the data acquisition software (at least 8 min for egg PC vesicles).

3.4.3. Repeat step 3.4.1 to change the inlet tubing from lipid vesicles back into Tris NaCl buffer.

NOTE: If the desired application is to study molecular interactions, continue directly to step 6.1 without stopping the solution flow or data acquisition. If bilayer formation is the endpoint, proceed to step 3.4.4.

3.4.4. In the software, stop the measurement and save the file. Stop the pump.

3.4.5. Clean the flow module and silica-coated quartz crystal sensor following the protocol steps 3.2.4 and 3.2.5.

4. Forming a suspended lipid bilayer

NOTE: The protocol for forming a suspended lipid bilayer is adapted from the parallel artificial membrane permeability assay (PAMPA) protocol provided by the filter plate manufacturer[37].

4.1. Solubilize desired lipid in dodecane at 20 mg/mL (e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)).

4.2. Add 5 µL of the lipid solution to the donor compartment, which is a porous polyvinylidene difluoride (PVDF) 96-well multiscreen filter plate (0.45 µm pore size).

4.3. Immediately submerge the filter plate into the acceptor compartment, which is a transport receiver plate containing 300 µL of 1× phosphate buffered saline (PBS). Add 200 µL of 1× PBS to the transport receiver plate.

NOTE: Controls of filters with lipid only and untreated filters exposed to 1× PBS may be included.

4.4. Continue directly to section 7.2 to investigate molecular interactions with the suspended lipid bilayer. It is recommended to complete the study within 16 h of forming the suspended bilayer.

5. Developing multi-lipid cell mimicking vesicles and bilayers

5.1. Lipid extraction from mammalian cells

NOTE: Lipid extraction follows the Bligh-Dyer approach[38].

5.1.1. Culture the desired cell line as appropriate. After achieving 70-80% confluence (T75 flask), detach cells using trypsin-ethylenediaminetretaacetic acid at 37 °C for 5 min.

5.1.2. Centrifuge cells at 200 × *g* for 5 min. Remove the supernatant and resuspend the cell pellet in 1 mL of ultrapure water.

5.1.3. Add 3.75 mL of a 1:2 (v/v) mixture of chloroform:methanol to the cell suspension and vortex for 15 min. Then, add 1.25 mL of chloroform and vortex for 1 min. Finally, add 1.25 mL of water and vortex for 1 min.

5.1.4. Centrifuge cell mixture at 1000 x *g* for 10 min. Collect the bottom layer of liquid, which contains lipids in the organic phase. Dry under a stream of N_2 gas.

5.1.5. Quantify the lipid content using liquid chromatography-mass spectrometry (LC-MS) using a C18 reverse phase, 3.5 µm × 50 mm column.

5.1.6. For the mobile phase, prepare two solutions, the first with 60:40 (v/v) acetonitrile:water and the second with 90:10 (v/v) isopropanol:acetonitrile. Ammonium formate should be added to both solutions at a final concentration of 10 mM. Over 60 min, increase the mobile phase gradient from 35% (v/v) of the second solution to 95% (v/v).

5.1.7. Detect the effluent in negative ionization mode, with consecutive full-scan MS and tandem MS/MS. Identify the individual phospholipid species from their mass-to-charge (m/z) ratios. Analyze the mass spectra from collision-induced dissociation fragmentation, using LIPID MAPS

mass spectrometry analysis tools. Obtain extracted ion chromatograms to integrate the area under the curve, determining the abundance of each lipid species.

5.1.8. Perform steps 5.1.5-5.1.7 for a lipid standard containing the major lipid classes to determine the relative sensitives of detection for each different phospholipid class.

5.2. Developing multi-lipid vesicles

5.2.1. Follow steps 1.1.1.1-2 to prepare lipid stock solutions for lipids representing each desired bilayer component, as identified in step 5.1.

5.2.2. Based on the lipid compositions obtained from step 5.1, add the appropriate volume of lipid/chloroform stock into a clean glass vial needed for a final vesicle concentration of 2.5 mg/mL. Remove bulk chloroform drying solution under a stream of N_2 gas.

5.2.3. Follow steps 1.1.4, 1.2, and 1.3 to form multi-lipid vesicles. Follow step 2 for vesicle characterization.

5.3. Forming a multi-lipid supported lipid bilayer using QCM-D

NOTE: Some multi-lipid vesicles can result in spontaneous lipid vesicle rupture and bilayer formation similar to uni-lipid PC vesicles presented in step 3. However, more complex multi-lipid vesicles may require external input to aid in vesicle rupture. Here, the AH peptide is used to destabilize the outer leaflet of the vesicle resulting in bilayer formation. Other methods to achieve destabilization and vesicle rupture may be considered if desired.

5.3.1. Follow step 3 to form the multi-lipid supported lipid bilayer utilizing the multi-lipid vesicles formed in step 5.2.

5.3.2. If spontaneous rupture of the vesicles into a bilayer is not observed, attempt vesicle destabilization using the AH peptide. Prepare the AH peptide (peptide sequence: H-Ser−Gly−Ser−Trp−Leu−Arg−Asp−Val−Trp−Asp−Trp−Ile−Cys−Thr−Val−Thr−Asp−Phe−Lys−Thr−Trp−Leu−Gln−Ser−Lys−Leu−Asp−Tyr−Lys−Asp-NH_2) solution at 13 μM in Tris NaCl with 1% (v/v) dimethylsulfoxide, DMSO.

5.3.3. Follow steps 3.4.1-3.4.3. After step 3.4.3, change the inlet tubing into the AH peptide solution. Introduce the solution into the flow module until ΔF and ΔD are observed from the new solution addition. Stop the pump and allow the AH peptide to incubate with the vesicles for 10 min.

5.3.4. Switch the inlet tubing into Tris NaCl and start the flow to remove the AH peptide from the ruptured vesicles leading to successful formation of a lipid bilayer.

NOTE: If the desired application is to study molecular interactions, continue direction to step 6.1 without stopping the solution flow or data acquisition.

5.3.5. In the software, stop the measurement and save the file. Stop the pump.

5.3.6. Clean the flow module and silica-coated quartz crystal sensor following the protocol steps 3.2.4 and 3.2.5.

5.4. Suspended multi-lipid bilayers

5.4.1. Solubilize mixture of desired lipids in dodecane at 20 mg/mL.

5.4.2. Make up a 5 μL lipid mix solution using the desired cell mimicking composition.

5.4.3. Follow steps 4.2 and 4.3.

NOTE: Continue directly to step 6.2 to investigate molecular interactions with the suspended lipid bilayer.

6. Molecule interaction studies with uni-lipid and multi-lipid bilayers

6.1. Studying molecular interactions with a supported lipid bilayer using QCM-D

6.1.1. Prepare a solution of the desired molecule to investigate adsorption with a supported lipid bilayer. For example, prepare a solution of 200 μM di(2-ethylhexyl) phthalate (DEHP) in Tris NaCl with 1% (v/v) DMSO.

6.1.2. If the molecule solution is prepared in Tris NaCl it can be flowed directly following step 3.4.3 for a uni-lipid bilayer or 5.3.4 for a multi-lipid bilayer. If the molecule must be prepared in a different solvent, instead insert the inlet tubing into the desired solvent alone ~~the desired solvent alone~~ for at least 5 min (e.g., Tris NaCl with 1% (v/v) DMSO for DEHP).

NOTE: Viscosity changes due to the solvent can be monitored and considered by flowing it prior to and after introduction of the molecule of interest.

6.1.3. Switch the inlet tubing into the solution containing the molecule of interest and flow for at least 5 min. The flow may also be stopped and liquid containing the desired molecule allowed to incubate with the bilayer if desired.

6.1.4. Change the inlet tubing back to the molecule solvent alone if something other than Tris NaCl. Flow for at least 5 min. Then, switch the inlet tubing into Tris NaCl and flow for at least 5 min.

6.1.5. In the software, stop the measurement and save the file. Stop the pump.

6.1.6. Clean the flow module and silica-coated quartz crystal sensor following the protocol steps 3.2.4 and 3.2.5.

6.2. Studying molecular interactions with suspended lipid bilayers using PAMPA

6.2.1. Prepare a solution of the desired molecule. For example, prepare a 200 µM DEHP in 1× PBS with 1% (v/v) DMSO.

6.2.2. Transfer the solution containing the desired molecule into a new transport receiver plate with 300 µL per well.

6.2.3. Immediately following step 3.3 for a uni-lipid suspended bilayer or 4.4.3 for a multi-lipid suspended bilayer, remove the 1× PBS from the filter plate compartment and replace with 200 µL of the test solution. Immediately submerge in the transport receiver plate prepared in step 6.2.2.

6.2.4. Incubate with gentle rocking for a desired amount of time (e.g., 2 h) at 25 °C.

6.2.5. After incubation, collect 150 µL of the solution from the donor and acceptor compartments. Measure the molecule concentration in both samples using an appropriate method based on properties of this molecule.

6.2.5.1. For example, use a microplate spectrophotometer with the appropriate absorbance wavelength, such as 280 nm for DEHP, and compare with a standard curve of the molecule of interest.

6.2.6. Calculate the apparent permeability (P_{app}) of the molecule of interest using the following equations:

$$\log P_{app} = \log\{C \cdot -\ln\left(1 - \frac{[test\ compound]_{acceptor}}{[test\ compound]_{initial}}\right)\} \quad (1)$$

$$\text{Where } C = \frac{V_D \cdot V_A}{(V_D + V_A)A \cdot t} \quad (2)$$

NOTE: $[\text{test compound}]_{\text{acceptor}}$ is the concentration of the molecule of interest (e.g., DEHP) at time, t, in the acceptor compartment; and $[\text{test compound}]_{\text{initial}}$ is the initial concentration of the molecule. A is the membrane area, t is the time, V_D is the donor compartment volume, and V_A is the acceptor compartment volume.

REPRESENTATIVE RESULTS:

This protocol details methods for forming supported and suspended lipid bilayers (**Figure 1**). The first step to forming a supported lipid bilayer is to develop lipid vesicles. The mini extruder allows for small volumes of lipid vesicles to be prepared (1 mL or less), while the large extruder allows for 5-50 mL of lipid vesicles to be prepared in one batch. Size distributions of uni-lipid vesicles formed by either the mini or large extruder are shown in **Figure 2A.** As the large extruder uses high pressure N_2 gas to push the vesicle solution through the polycarbonate membrane, lipid vesicles result in an average size distribution at the target 100 nm hydrodynamic diameter. The mini extruder also results in a uniform distribution, although the vesicle hydrodynamic diameter is slightly larger than the polycarbonate pore size, which is typical for this manual method of extrusion.

Figures 2B-D compare size, polydispersity, and zeta potential of uni-lipid egg PC vesicles and two multi-lipid vesicle compositions. **Table 1** compares the average hydrodynamic diameter of each lipid vesicle composition. The composition of the first multi-lipid vesicle (ML1) is representative of placental trophoblast inspired lipid vesicles with a composition of 57:15:8:8:12 % (w/w) of PC: phosphatidylethanolamine (PE): phosphatidylinositol (PI): phosphatidylserine (PS): sphingomyelin (SPH). The size distribution of the egg PC vesicles and ML1 vesicles are highly uniform and nearly identical, with small differences in the average polydispersity (**Figure 2A,B**). As expected, due to differences in composition, the zeta potential of the egg PC uni-lipid vesicles and the ML1 vesicle were found to differ (**Figure 2D**). The second multi-lipid vesicle (ML2) is 60% egg PC and 40% 1,2-distearoyl-sn-glycero-3-ethylphosphocholine (EPC). The positive charge of EPC led to a positive zeta potential for these vesicles (**Figure 2D**), and an increase in polydispersity index of ML2 vesicles was also observed compared to egg PC or ML1 vesicles, likely a result of the specific composition of these vesicles.

QCM-D can be used to form supported lipid bilayers via vesicle rupture on a silica-coated sensor, and ΔF and ΔD during this process are monitored in real time. ΔF are inversely related to mass changes, and increased ΔD indicated increase in the structure fluidity. As vesicles adsorb to the sensor, ΔF decreases and ΔD increases. As the vesicle reach a critical vesicle coverage on the surface, there will be a plateau of the ΔF and ΔD. Finally, as the vesicles rupture, a ΔF increase and ΔD decrease is observed, due to release of encapsulated water from ruptured liposomes and formation of rigid bilayer, respectively. **Figure 3** shows the ΔF and ΔD occurring as the vesicles adsorb and rupture on the surface for uni-lipid and multi-lipid bilayer formations. Uni-lipid egg PC vesicles readily adsorb to the surface as shown by the ΔF decrease and ΔD increase. Critical vesicle coverage is reached within 5 min, after which the vesicles begin to rupture. The overall ΔF observed upon supported egg PC bilayer formation is ~-25 Hz, with ΔD of ~0.

ML1 vesicles take longer to adsorb compared to egg PC vesicles and unlike these vesicles, they do not spontaneously rupture, but remain stable on the surface. Instead, an AH peptide is allowed to incubate with the adsorbed vesicles causing their rupture when the AH peptide is removed with Tris NaCl. During rupture and bilayer formation, the ΔF increase and ΔD decrease is observed, similar to the egg PC vesicles. The ΔF of this multi-lipid bilayer results in approximately -28 Hz and ΔD of approximately 1×10^{-6}. Although comparable to the egg PC lipid bilayer, these slight differences in ΔF and ΔD likely indicate the increased fluidity of the bilayer due to the multiple lipid types present in the structure.

After bilayer formation, these structures can be used to study interactions with different compounds. With supported lipid bilayers, ΔF and ΔD can be analyzed before and after introduction of the compound. As an example, DEHP interaction with supported uni- and multi-lipid (ML1) bilayers are shown in **Figure 4A**,**B**. In this case, similar levels of DEHP adsorption are observed for both lipid bilayer types (**Figure 4A**). However, differences in ΔD were observed between the bilayers, with a larger ΔD seen for the egg PC bilayer compared to the ML1 bilayer (**Figure 4B**). While the supported lipid bilayer allows for the study of the adsorption and potential embedment of compounds of interest along with potential lipid removal, suspended lipid bilayers can provide information on the permeability across the bilayer using a PAMPA. In the case of

DEHP, little permeation was observed for both uni- and ML1 bilayers (**Figure 4C**). P_{app} calculated for DEHP across a uni-lipid (~5.5×10^{-11} cm/s) and multi-lipid bilayer (~6.5×10^{-6} cm/s) was characteristic of low permeability. However, other compounds may result in greater permeability, which can be investigated using this technique.

FIGURE AND TABLE LEGENDS:

Figure 1: Process of forming supported lipid bilayers (top) and suspended lipid bilayers (bottom).

Figure 2: Uni-lipid and multi-lipid vesicle characterization. (A) Hydrodynamic diameter distribution of egg PC vesicles formed using a mini extruder and large extruder. **(B)** Hydrodynamic diameter distribution of uni-lipid vesicles containing egg PC and two multi-lipid formulations, ML1 (57:15:8:8:12 % (w/w) PC:PE:PI:PS:SPH) and ML2 (60:40 % (w/w) egg PC:EPC). **(C)** Polydispersity indices of the uni-lipid and multi-lipid vesicles. **(D)** Zeta potentials of the uni-lipid and multi-lipid vesicles. Results are shown as mean ± standard deviation. Statistical significance was calculated using one-way analysis of variance (ANOVA) with Tukey's post hoc analysis (α=0.05, $p<0.05$ was considered to be statistically significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

Figure 3: **Uni-lipid egg PC bilayer formation (*ΔF* in light blue and *ΔD* in light red) and a multi-lipid bilayer formation (57:15:8:8:12 % (w/w) PC:PE:PI:PS:SPH) (*ΔF* in dark blue and *ΔD* in dark red) monitored over time using QCM-D.** Dashed lines indicate solution changes for uni-lipid bilayer formation (light blue) and multi-lipid bilayer formation (dark blue). The egg PC bilayer is formed by ~15 min, while the multi-lipid bilayer takes ~45 min and requires addition of the AH peptide.

Figure 4: Molecule interactions with supported and suspended lipid bilayers. (A) *ΔF* due to DEHP interaction with uni-lipid and multi-lipid (57:15:8:8:12 % (w/w) PC:PE:PI:PS:SPH) bilayers. **(B)** *ΔD* due to DEHP interaction with uni-lipid and multi-lipid bilayers. **(C)** Percent of DEHP permeated across the uni-lipid and multi-lipid suspended bilayers. Results are shown as mean ± standard deviation. Statistical significance was calculated using a student's *t*-test (α=0.05, $p<0.05$ was considered to be statistically significant, *$p<0.05$).

Table 1: Lipid vesicle hydrodynamic diameters.

DISCUSSION:

This protocol allows for the formation of lipid vesicles, supported lipid bilayers, and suspended lipid bilayers. Here, critical steps are presented to form each of these structures. When forming lipid vesicles, it is important to extrude above the transition temperature of the lipid[39]. When below the transition temperature, the lipid is physically present in its ordered gel phase[39]. In this ordered phase the hydrocarbon lipid tails are fully extended allowing for close packing, making extrusion challenging[39]. When heated above the transition temperature, the lipid becomes more disordered resulting in the liquid crystalline phase[39]. The hydrocarbon tails of the lipid are more fluid at these temperatures, allowing for successful extrusion[39]. Lipid characteristics such as the head group composition, saturation, and charge will impact their transition temperature. It is also important to

remove all chloroform when forming the lipid film, as residual chloroform will negatively affect vesicle formation and properties after rehydration.

During supported lipid bilayer formation using QCM-D it is crucial for the silica-coated quartz crystal sensor to be in pristine condition. The sensors may be re-used, but must be checked each time for any scratches, debris, or other wear and discarded if any imperfection is found, as sensor imperfections can affect vesicle adsorption and bilayer formation. The fundamental frequency of the piezoelectric quartz sensor is 5 MHz, with ΔF and ΔD monitored at odd overtones (3, 5, 7, 9, 11, and 13). Ensuring that the fundamental resonance frequencies for each overtone found prior to measurement are similar to the expected theoretical values can help identify a possible crystal issue. Collecting measurements from multiple overtones is important for viscoelastic modeling using the data obtained. It is also important to ensure that air is not introduced into the system during fluid flow through the QCM-D flow modules. Air will cause an air-liquid shift to occur which will be observed in the real-time data collection and result in loss of integrity of the lipid bilayer. 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 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] During suspended lipid bilayer formation, it is important that lipids are chosen that are soluble in dodecane, such as DOPC[38,39]. Depending on the application and particularly for cell membrane mimicking bilayers, it may be advisable to perform comparison permeability studies between the suspended lipid bilayers and cell monolayers formed on porous inserts[42–44].

There are many steps in this protocol that may be adapted or modified for a particular application. The lipids and the compositions used, concentration of the lipid vesicle suspension, volume of vesicles prepared, vesicle rehydration buffer, number of passes through the extruder, polycarbonate membrane pore size, quartz crystal substrate material, QCM-D flow rate, the molecular interactions studied, length of time for interaction, and temperature may all be adapted, making this a versatile approach. The extrusion processes detailed here can also be utilized to form therapeutic liposomes. For example, both the mini extruder and large extruder have been used to form antifungal liposomes that remain stable for at least 140 days at 4 °C in ultrapure water[45]. Other methods for vesicle or lipid bilayer formation and characterization may also be considered for comparison or additional verification. For example, sonication is another technique that is used to form lipid vesicles[46], and techniques such as cryo-transmission electron microscopy may be used to confirm lamellarity[15, 45]. Atomic force microscopy[47], surface plasmon resonance[48], and neutron reflectivity[49] can also be used in combination with QCM-D to study supported lipid bilayers[50]. Suspended lipid bilayers have also been formed in microfluidic devices[30, 51] in addition to using the filter and receiver well plates discussed in this protocol.

While the method described here can provide facile lipid bilayers that mimic cellular lipid composition, they only provide information about molecular interactions with these lipids. Proteins are also key components of the cell membrane and can influence adsorption, permeability, and active and passive transport properties. Thus, advancing these model lipid bilayers to incorporate proteins will result in additional cell mimicking properties, that can enhance the information obtained from these approaches. 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] Applications where these bilayers will be particularly importance include toxicity testing and pharmaceutical screening studies[24, 27]. Overall, the versatility and reproducibility of these cell mimicking models adds to their utility for a range of investigations.

ACKNOWLEDGMENTS:

This material is based upon work supported by the National Science Foundation under Grant No. 1942418 awarded to A.S., and a National Science Foundation Graduate Research Fellowship awarded to C.M.B.H., under Grant No. 1644760. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. The authors thank Dr. Noel Vera-González for lipid vesicle characterization data acquisition. The authors thank Professor Robert Hurt (Brown University) for the use of his Zetasizer. The authors thank the Brown University Mass Spectrometry Facility, in particular, Dr. Tun-Li Shen for assistance with quantifying lipid composition.

DISCLOSURES:

The authors declare that they have no conflict of interest or competing financial interests.

REFERENCES:

1. Lucio, M., Lima, J.L.F.C., Reis, S. Drug-Membrane Interactions: Significance for Medicinal Chemistry. *Current Medicinal Chemistry*. **17** (17), 1795–1809, doi: 10.2174/092986710791111233 (2010).
2. Mayne, C.G. *et al.* The cellular membrane as a mediator for small molecule interaction with membrane proteins. *Biochimica et Biophysica Acta - Biomembranes*. **1858** (10), 2290–2304, doi: 10.1016/j.bbamem.2016.04.016 (2016).
3. Bunea, A.I., Harloff-Helleberg, S., Taboryski, R., Nielsen, H.M. Membrane interactions in drug delivery: Model cell membranes and orthogonal techniques. *Advances in Colloid and Interface Science*. **281**, 102177 (2020).
4. Peetla, C., Stine, A., Labhasetwar, V. Biophysical interactions with model lipid membranes: Applications in drug discovery and drug delivery. *Molecular Pharmaceutics*. **6** (5), 1264–1276, doi: 10.1021/mp9000662 (2009).
5. Richter, R., Mukhopadhyay, A., Brisson, A. Pathways of Lipid Vesicle Deposition on Solid Surfaces: A Combined QCM-D and AFM Study. *Biophysical Journal*. **85** (5), 3035–3047, doi: 10.1016/S0006-3495(03)74722-5 (2003).
6. Lind, T.K., Cárdenas, M., Wacklin, H.P. Formation of supported lipid bilayers by vesicle fusion: Effect of deposition temperature. *Langmuir*. **30** (25), 7259–7263, doi: 10.1021/la500897x (2014).
7. Mingeot-Leclercq, M.-P., Deleu, M., Brasseur, R., Dufrêne, Y.F. Atomic force microscopy of supported lipid bilayers. *Nature protocols*. **3** (10), 1654–9, doi: 10.1038/nprot.2008.149 (2008).
8. Richter, R.P., Bérat, R., Brisson, A.R. Formation of solid-supported lipid bilayers: an integrated view. *Langmuir : the ACS journal of surfaces and colloids*. **22** (8), 3497–505, doi: 10.1021/la052687c (2006).
9. Chan, Y.-H.M., Boxer, S.G. Model membrane systems and their applications. *Current Opinion in Chemical Biology*. **11** (6), 581–587, doi: 10.1016/j.cbpa.2007.09.020 (2007).

10. Edvardsson, M., Svedhem, S., Wang, G., Richter, R., Rodahl, M., Kasemo, B. QCM-D and reflectometry instrument: applications to supported lipid structures and their biomolecular interactions. *Analytical chemistry*. **81** (1), 349–61, doi: 10.1021/ac801523w (2009).
11. Rodahl, M. *et al.* Simultaneous frequency and dissipation factor QCM measurements of biomolecular adsorption and cell adhesion. *Faraday Discussions*. **107**, 229–246, doi: 10.1039/a703137h (1997).
12. Keller, C.A., Glasmästar, K., Zhdanov, V.P., Kasemo, B. Formation of Supported Membranes from Vesicles. *Physical Review Letters*. **84** (23), 5443–5446, doi: 10.1103/PhysRevLett.84.5443 (2000).
13. Keller, C.A., Kasemo, B. Surface specific kinetics of lipid vesicle adsorption measured with a quartz crystal microbalance. *Biophysical journal*. **75** (3), 1397–1402, doi: 10.1016/S0006-3495(98)74057-3 (1998).
14. Cho, N.-J., Frank, C.W., Kasemo, B., Höök, F. Quartz crystal microbalance with dissipation monitoring of supported lipid bilayers on various substrates. *Nature protocols*. **5** (6), 1096–106, doi: 10.1038/nprot.2010.65 (2010).
15. Bailey, C.M., Tripathi, A., Shukla, A. Effects of Flow and Bulk Vesicle Concentration on Supported Lipid Bilayer Formation. *Langmuir*. **33** (43), 11986–11997, doi: 10.1021/acs.langmuir.7b02764 (2017).
16. van Meer, G., Voelker, D.R., Feigenson, G.W. Membrane lipids: where they are and how they behave. *Nature reviews. Molecular cell biology*. **9** (2), 112–24, doi: 10.1038/nrm2330 (2008).
17. Rossi, C., Chopineau, J. Biomimetic tethered lipid membranes designed for membrane-protein interaction studies. *European Biophysics Journal*. **36** (8), 955–965, doi: 10.1007/s00249-007-0202-y (2007).
18. Hatty, C.R. *et al.* Investigating the interactions of the 18 kDa translocator protein and its ligand PK11195 in planar lipid bilayers. *Biochimica et Biophysica Acta - Biomembranes*. **1838** (3), 1019–1030, doi: 10.1016/j.bbamem.2013.12.013 (2014).
19. Min, Y., Kristiansen, K., Boggs, J.M., Husted, C., Zasadzinski, J. a, Israelachvili, J. Interaction forces and adhesion of supported myelin lipid bilayers modulated by myelin basic protein. *Proceedings of the National Academy of Sciences of the United States of America*. **106** (9), 3154–3159, doi: 10.1073/pnas.0813110106 (2009).
20. Heath, G.R. *et al.* Layer-by-layer assembly of supported lipid bilayer poly-l-lysine multilayers. *Biomacromolecules*. **17** (1), 324–335, doi: 10.1021/acs.biomac.5b01434 (2016).
21. Alberts, B., Lewis, J. The Lipid Bilayer. *Molecular Biology of the Cell*. 6–11 (2013).
22. Cho, N.J., Wang, G., Edvardsson, M., Glenn, J.S., Hook, F., Frank, C.W. Alpha-helical peptide-induced vesicle rupture revealing new insight into the vesicle fusion process as monitored in situ by quartz crystal microbalance-dissipation and reflectometry. *Analytical Chemistry*. **81** (12), 4752–4761, doi: 10.1021/ac900242s (2009).
23. Hardy, G.J., Nayak, R., Munir Alam, S., Shapter, J.G., Heinrich, F., Zauscher, S. Biomimetic supported lipid bilayers with high cholesterol content formed by α-helical peptide-induced vesicle fusion. *Journal of Materials Chemistry*. **22** (37), 19506–19513, doi: 10.1039/c2jm32016a (2012).
24. Bailey-Hytholt, C.M., Shen, T.L., Nie, B., Tripathi, A., Shukla, A. Placental Trophoblast-Inspired Lipid Bilayers for Cell-Free Investigation of Molecular Interactions. *ACS Applied Materials and Interfaces*. **12** (28), 31099–31111, doi: 10.1021/acsami.0c06197 (2020).
25. Domenech, O., Francius, G., Tulkens, P.M., Van Bambeke, F., Dufrêne, Y., Mingeot-Leclercq, M.-P. Interactions of oritavancin, a new lipoglycopeptide derived from vancomycin,

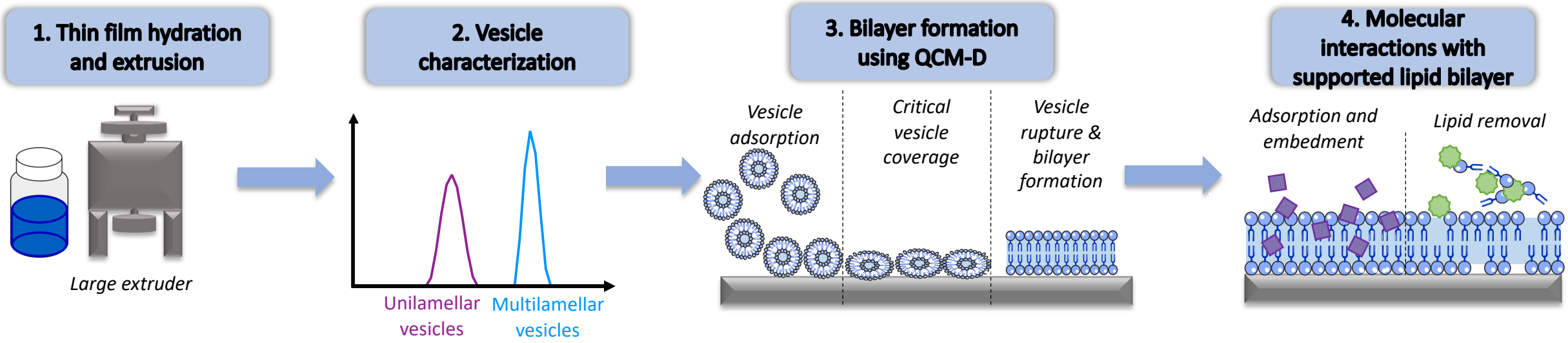
with phospholipid bilayers: Effect on membrane permeability and nanoscale lipid membrane organization. *Biochimica et biophysica acta*. **1788** (9), 1832–40, doi: 10.1016/j.bbamem.2009.05.003 (2009).
26. Bailey, C.M., Kamaloo, E., Waterman, K.L., Wang, K.F., Nagarajan, R., Camesano, T. a. Size dependence of gold nanoparticle interactions with a supported lipid bilayer: A QCM-D study. *Biophysical Chemistry*. **203**–**204**, 51–61, doi: 10.1016/j.bpc.2015.05.006 (2015).
27. Bailey-Hytholt, C.M., Puranik, T., Tripathi, A., Shukla, A. Investigating interactions of phthalate environmental toxicants with lipid structures. *Colloids and Surfaces B: Biointerfaces*. **190**, 110923, doi: 10.1016/J.COLSURFB.2020.110923 (2020).
28. Wang, K.F., Nagarajan, R., Camesano, T.A. Antimicrobial peptide alamethicin insertion into lipid bilayer: a QCM-D exploration. *Colloids and surfaces. B, Biointerfaces*. **116**, 472–81, doi: 10.1016/j.colsurfb.2014.01.036 (2014).
29. Lozeau, L.D., Rolle, M.W., Camesano, T.A. A QCM-D study of the concentration- and time-dependent interactions of human LL37 with model mammalian lipid bilayers. *Colloids and Surfaces B: Biointerfaces*. **167** (1), 229–238, doi: 10.1016/j.colsurfb.2018.04.016 (2018).
30. Kongsuphol, P., Fang, K.B., Ding, Z. Lipid bilayer technologies in ion channel recordings and their potential in drug screening assay. *Sensors and Actuators B: Chemical*. **185**, 530–542, doi: 10.1016/j.snb.2013.04.119 (2013).
31. Ren, X. *et al.* Design, fabrication, and characterization of archaeal tetraether free-standing planar membranes in a PDMS-and PCB-based fluidic platform. *ACS Applied Materials & Interfaces*. **6** (15), 12618-12628. (2014).
32. Seo, P.R., Teksin, Z.S., Kao, J.P.Y., Polli, J.E. Lipid composition effect on permeability across PAMPA. *European Journal of Pharmaceutical Sciences*. **29** (3–4), 259–268, doi: 10.1016/j.ejps.2006.04.012 (2006).
33. Avdeef, A. The rise of PAMPA. *Expert Opinion on Drug Metabolism & Toxicology*. **1** (2), 325–342, doi: 10.1517/17425255.1.2.325 (2005).
34. Avdeef, A., Artursson, P., Neuhoff, S., Lazorova, L., Gråsjö, J., Tavelin, S. Caco-2 permeability of weakly basic drugs predicted with the Double-Sink PAMPA method. *European Journal of Pharmaceutical Sciences*. **24** (4), 333–349, doi: 10.1016/j.ejps.2004.11.011 (2005).
35. Campbell, S.D., Regina, K.J., Kharasch, E.D. Significance of Lipid Composition in a Blood-Brain Barrier–Mimetic PAMPA Assay. *Journal of Biomolecular Screening*. **19** (3), 437–444, doi: 10.1177/1087057113497981 (2014).
36. Berben, P. *et al.* Drug permeability profiling using cell-free permeation tools: Overview and applications. *European Journal of Pharmaceutical Sciences*. **119**, 219–233, doi: 10.1016/J.EJPS.2018.04.016 (2018).
37. Schmidt, D., Lynch, J. Evaluation of the reproducibility of Parallel Artificial Membrane Permation Assays (PAMPA). *EMD Millipore Corporation*.
38. Bligh, E.G., Dyer, W.J. A Rapid Method of Total Lipid Extraction and Purification. *Canadian Journal of Biochemistry and Physiology*. **37** (8), 911–917, doi: 10.1139/o59-099 (1959).
39. Nayar, R., Hope, M.J., Cullis, P.R. Generation of large unilamellar vesicles from long-chain saturated phosphatidylcholines by extrusion technique. *BBA - Biomembranes*. **986** (2), 200–206, doi: 10.1016/0005-2736(89)90468-9 (1989).
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).

41. Berben, P. *et al.* Drug permeability profiling using cell-free permeation tools: Overview and applications. *European Journal of Pharmaceutical Sciences*. **119** (April), 219–233, doi: 10.1016/j.ejps.2018.04.016 (2018).
42. Bermejo, M. *et al.* PAMPA—a drug absorption in vitro model: 7. Comparing rat in situ, Caco-2, and PAMPA permeability of fluoroquinolones. *European Journal of Pharmaceutical Sciences*. **21** (4), 429–441, doi: 10.1016/J.EJPS.2003.10.009 (2004).
43. Kerns, E.H., Di, L., Petusky, S., Farris, M., Ley, R., Jupp, P. Application of parallel artificial membrane permeability assay and Caco-2 permeability. *Journal of Pharmaceutical Sciences*. **93** (6), 1440–1453, at <http://www3.interscience.wiley.com/journal/107640027/abstract%5Cnpapers://5aa36326-f23d-4d75-b12e-bf93c65dd1bd/Paper/p1889> (2004).
44. Masungi, C. *et al.* Parallel artificial membrane permeability assay (PAMPA) combined with a 10-day multiscreen Caco-2 cell culture as a tool for assessing new drug candidates. *Pharmazie*. **63** (3), 194–199, doi: 10.1691/ph.2008.7327 (2008).
45. Vera-González, N. *et al.* Anidulafungin liposome nanoparticles exhibit antifungal activity against planktonic and biofilm Candida albicans. *Journal of Biomedical Materials Research - Part A*. **108** (11), 2263–2276, doi: 10.1002/jbm.a.36984 (2020).
46. Barenholz, Y., Gibbes, D., Litman, B.J., Goll, J., Thompson, T.E., Carlson, F.D. A simple method for the preparation of homogeneous phospholipid vesicles. *Biochemistry*. **16** (12), 2806–2810, doi: 10.1021/bi00631a035 (1977).
47. El Kirat, K., Morandat, S., Dufrêne, Y.F. Nanoscale analysis of supported lipid bilayers using atomic force microscopy. *Biochimica et Biophysica Acta - Biomembranes*. **1798** (4), 750–765, doi: 10.1016/j.bbamem.2009.07.026 (2010).
48. Tawa, K., Morigaki, K. Substrate-supported phospholipid membranes studied by surface plasmon resonance and surface plasmon fluorescence spectroscopy. *Biophysical Journal*. **89** (4), 2750–2758, doi: 10.1529/biophysj.105.065482 (2005).
49. Koenig, B.W. *et al.* Neutron Reflectivity and Atomic Force Microscopy Studies of a Lipid Bilayer in Water Adsorbed to the Surface of a Silicon Single Crystal. *Langmuir*. **12** (5), 1343–1350 (1996).
50. Lind, T.K., Cárdenas, M. Understanding the formation of supported lipid bilayers via vesicle fusion—A case that exemplifies the need for the complementary method approach (Review). *Biointerphases*. **11** (2), 020801, doi: 10.1116/1.4944830 (2016).
51. Castellana, E.T., Cremer, P.S. Solid supported lipid bilayers: From biophysical studies to sensor design. *Surface Science Reports*. **61** (10), 429–444, doi: 10.1016/j.surfrep.2006.06.001 (2006).
52. Isaksson, S. *et al.* Protein-Containing Lipid Bilayers Intercalated with Size-Matched Mesoporous Silica Thin Films. *Nano Letters*. **17** (1), 476–485 (2017).

Figure 1

Click here to access/download;Figure;Figure 1.pdf

Supported lipid bilayers



Suspended lipid bilayers

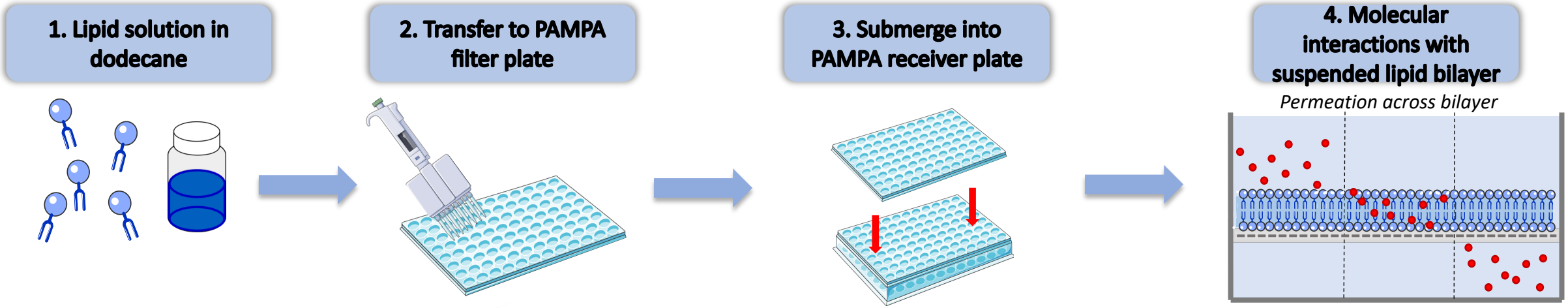


Figure 2

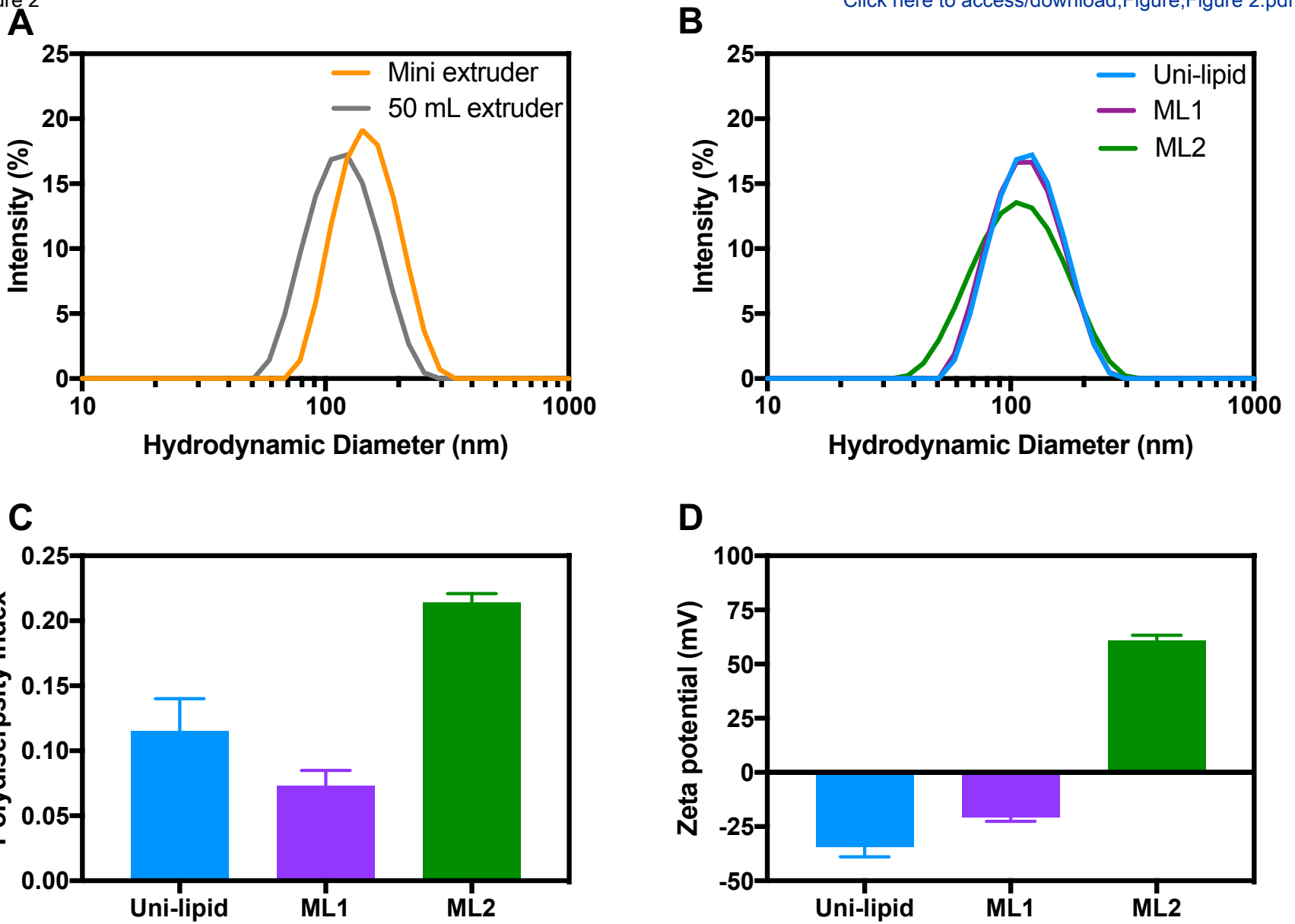


Figure 3

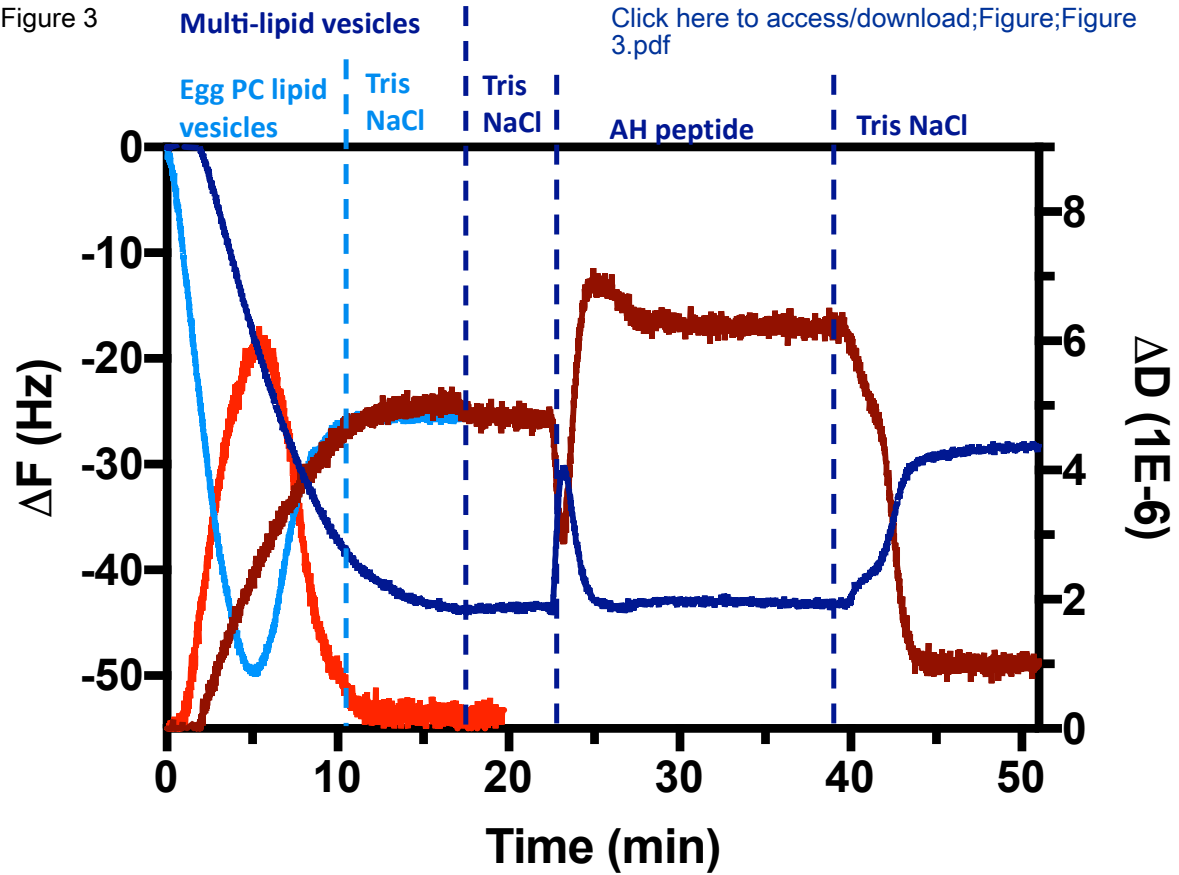
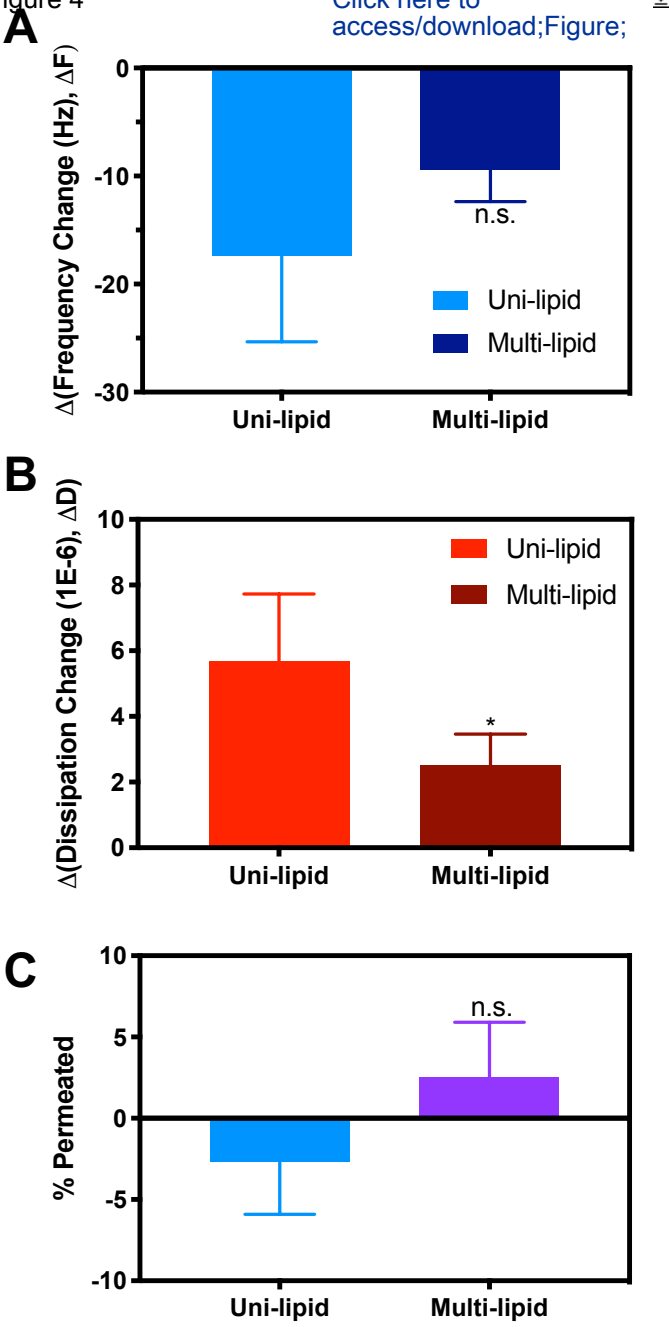


Figure 4

Click here to access/download;Figure;



Uni-lipid vs. multi-lipid	Composition	Extruder	Hydrodynamic diameter (nm)
Uni-lipid	100% egg PC	Mini	165 ± 1
Uni-lipid	100% egg PC	Large	108 ± 2
Multi-lipid	57:15:8:8:12 % (w/w) PC:PE:PI:PS:SPH	Large	109 ± 1
Multi-lipid	60:40 % (w/w) egg PC:EPC	Large	91.0 ± 0.2

Name of Material/ Equipment	Company	Catalog Number
1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC, 16:0-18:1 PC)	Avanti Polar Lipids	850457
1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS, 16:0-18:1 PS)	Avanti Polar Lipids	840034
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (16:0-18:1 PE)	Avanti Polar Lipids	850757
1,2-dioleoyl-sn-glycero-2-phospho-L-serine (DOPS, 18:1 PS)	Avanti Polar Lipids	840035
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 18:1 (Δ9-Cis) PC)	Avanti Polar Lipids	850375
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, 18:1 (Δ9-Cis) PE)	Avanti Polar Lipids	850725
1,2-distearoyl-sn-glycero-3-ethylphosphocholine (chloride salt) (18:0 EPC (Cl Salt))	Avanti Polar Lipids	890703
3 mL Luer-Loc syringes	BD	309657
40 mL sample vial, amber with polytetrafluoroethylene (PTFE)/rubber liner	Duran Wheaton Kimble	W224605
Acetonitrile	Sigma-Aldrich	271004
Alconox	Fisher Scientific	50-821-781
Ammonium formate	Millipore Sigma	LSAC70221
C18, 3.5 um x 50 mm column, SunFire	Waters	186002551

Chloroform	Millipore Sigma	LSAC288306
Cuvette UV Micro LCH 8.5 mm, 50 um, RPK	Sarstedt	67.758.001
Di(2-ethylhexyl) phthalate (DEHP)	Millipore Sigma	36735
Dimethyl sulfoxide (DMSO)	Millipore Sigma	LSAC472301
Ethanol	Pharmco	111000200
Filter supports, 10 mm	Avanti Polar Lipids	610014
Folded capillary zeta cell	Malvern Panalytical	DTS1070
Isopropanol	Sigma-Aldrich	190764-4L
Kimwipes	Kimberly Clark	34256
L-α-phosphatidylinositol (soy) (Soy PI)	Avanti Polar Lipids	840044
L-α-phosphitidylcholine (Egg, Chicken)	Avanti Polar Lipids	840051
LiposoFast ® LF-50	Avestin, Inc.	
Methanol	Sigma-Aldrich	179337 - 4L
Mini-extruder set with holder/heating block	Avanti Polar Lipids	610000
MultiScreen-IP Filter Plate, 0.45 µm, clear, sterile	Millipore Sigma	MAIPS4510

Nitrogen gas, ultrapure	TechAir	NI T5.0
Nuclepore hydrophilic membranes, polycarbonate, 19 mm, 0.1 um	Whatman	800309
Nuclepore hydrophilic membranes, polycarbonate, 25 mm, 0.1 um	Whatman	110605
Parafilm	Bemis	PM999
Phosphate buffer saline (PBS), 10x	Genesee Scienfitic	25-507X
Qsoft 401 software	Biolin Scientific	
Quartz Crystal Microbalance with Dissipation Q-Sense Analyzer	Biolin Scientific	
Scintillation vials, borosilicate glass vials, 20 mL	Duran Wheaton Kimble	986561
Silicon Dioxide, thin QSensors	Biolin Scientific	QSX 303
Sodium chloride (NaCl)	Millipore Sigma	LSACS5886
Sodium dodecyl sulfate (SDS)	Fisher Scientific	BP166-100
Solvent Safe pipette tips	Sigma-Aldrich	S8064
Sphingomyelin (Egg, Chicken)	Avanti Polar Lipids	860061
Trizma base	Millipore Sigma	LSACT1503
Trypsin-ethylenediaminetretaacetic acid	Caisson Labs	TRL01-6X100ML
Whatman drain disc, 25 mm	Whatman	230600
Zetasizer ZS90	Malvern Panalytical	

Zetasizer 7.01 software	Malvern Panalytical

Comments/Description

Size for mini extruder

for PAMPA studies

Size for mini extruder
Size for large extruder

Dilute to 1x

Size for large extruder

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