Extrusion and suspension of phospholipid liposomes from lipid fims

James R. Collins, Krista Longnecker, Helen F. Fredricks, Benjamin A. S. Van Mooy

Abstract

The protocol can be used to create and extrude phospholipid liposomes. This extrusion protocol roughly follows some guidelines published by the manufacturer. However, we found that the manufacturer's directions were insufficient in several areas. In particular, the manufacturer guidance on initial preparation of lipids was incomplete; 'good' liposomes cannot be prepared directly from dry lipid mixtures. In addition, our protocol provides supplementary guidance for extrusion and suspension of different classes of polar lipids; efficacy of the various matrixes we recommend was largely determined through trial and error. The manufacturer directions (downloaded March 4, 2017) are attached to this protocol as a .pdf document.

This protocol was original created by Krista Longnecker and Jamie Collins for creating lipsomes to be used in lipid photo-oxidation experiments. The results of these experiments are detailed in Chapter 4 of:

Collins, J. R. 2017. The remineralization of marine organic matter by diverse biological and abiotic processes. Ph.D. thesis. Cambridge, Massachusetts: Massachusetts Institute of Technology, 300 pp; doi:10.1575/1912/8721

A manuscript of the chapter is forthcoming.

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Before start

Note: Plan to use the liposomes you are making within a day or two because they are not stable for longer periods of time. If necessary, store the extuded liposome solutions at 4ºC.

This protocol assumes you have an Avanti mini-extruder with heating block, available at https://avantilipids.com/product/610000/

Protocol

Obtain lipid films

Step 1.

Obtain lipid films for the desired liposomes. The companion lipid film preparation protocol contains instructions for producing the necessary films.

PROTOCOL

. Preparation of lipid films for phospholipid liposomes

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Obtain desired lipids from supplier

Step 1.1.

For this work, we used a variety of phosopholipids obtained from Avanti Polar Lipids, Inc. Where available, we obtained lipids as a powder. When the desied lipid was not available from the supplier as a powder (e.g., 22:6, 22:6 PC), we ordered the product dissolved in chloroform. The lipids given in the reagents list for this step were those used for the lipid photo-oxidation experiments, but this protocol can be followed for preparation of films of any phospholipid.

REAGENTS

1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1, 18:1 PC) 850375P by Avanti Polar Lipids, Inc.

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0, 16:0 PC) 850355P by Avanti Polar Lipids, Inc.

1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine (16:1, 16:1 PC) 850358P by Avanti Polar Lipids, Inc.

1,2-distearoyl-sn-glycero-3-phosphocholine (18:0, 18:0 PC) 850365P by Avanti Polar Lipids, Inc.

1,2-dilinoleoyl-sn-glycero-3-phosphocholine (18:2, 18:2 PC) 850385P by Avanti Polar Lipids, Inc.

1,2-dibehenoyl-sn-glycero-3-phosphocholine (22:0, 22:0 PC) 850371P by Avanti Polar Lipids, Inc.

1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine (22:6, 22:6 PC) 850400C by Avanti Polar Lipids, Inc.

Weigh out desired quantity of lipid(s) into a glass test tube

Step 1.2.

Weigh out desired quantity of lipid(s) into a glass test tube. Use a round-bottom tube, such a disposable culture tube or small (i.e., 20 mL) round-bottom vial from an HPLC fraction collector. In applying this protocol, we aimed for approx. 1.65 mg of each lipid per tube. If making up multiple

tubes of lipid fims for each lipid, weigh out the total quantity of lipid needed (1.65 × no. of tubes) into a single tube; you can then aliquot it into separate tubes using a solvent-rinsed glass syringe.

Dissolve lipids in chloroform

Step 1.3.

In a fume hood, add 200 µL of chloroform to each glass test tube to dissolve the lipids. Just add enough chloroform that the lipids dissolve. The actual volume of chloroform does not matter because it will be removed in a later step.

Note that this step **does not apply** if lipid was obtained from vendor already in chloroform.

If making multiple films of each lipid: Add more chloroform to this first tube — 200 µL × no. of desired films — and then aliquot the solution into separate tubes in volumes of 200 µL.

REAGENTS

Chloroform by Contributed by users

Disposable borosilicate glass culture tubes, with rounded bottom 14-961-26 by Fisher Scientific

SAFETY INFORMATION

Chloroform is a hazardous solvent. Conduct all work in a fume hood, while wearing proper PPE.

Optional: If precise concentration of lipid in initial chloroform solution must be known

Step 1.4.

If you want to know the exact concentration of lipids in any of these initial chloroform solutions, remove an aliquot of the lipid/chloroform solution at this point, and keep track of the volume. The lipid solution will need to be diluted further with chloroform before it can be analyzed. Helen Fredricks (HPLC-MS guru in the Van Mooy Lab) has suggested a final concentration of 0.01 mg mL^{-1} for analysis.

Optional: Dispense ~ 200 µL aliquots into different tubes

Step 1.5.

If you made up more than 200 µL of lipid solution in your initial tube, now is the time to dispense 200 µL aliquots into separate round-bottom tubes. A volumetric, solvent-washed glass syringe can be used for this purpose.

Blow down the lipid/chloroform solution in each tube using a nitrogen gas blow-down system

Step 1.6.

The object here is to create a lipid 'film' that evenly coats the inside of the lower side walls and bottom of each tube once the solvent has been evaporated. This requires some concentration and attention when evaporating the solvent.

You cannot just stick the vial under a needle with N_2 flowing out of it and walk away, because that will not form the lipid film needed for this method to work.

Best practices:

1. Connect a disposable glass pasteur pipette (use one with a longer length) to the nitrogen supply by way of some flexible non-reactive (e.g., Tygon) tubing. Use some lab tape or other means to secure the pipet at about eye height inside the fume hood. You will want the end of the pipette to point slightly downward. The precise orientation of the pipette doesn't matter all that much; you want it oriented in such a way to make the next few steps convenient and easy!
2. Hold a tube containing 200 µL of the lipid solution so that the end of the pipette protrudes just into the mouth of the tube.
3. While contantly rolling the tube in your fingers, gradually let a weak stream of the N_2 blow down the chloroform. Remember: You don't want to concentrate all the lipid at the very bottom of the tube. The goal is to create a film that evenly and continuously coats the lower third of the side wall and bottom of the tube.
4. At the end, you should have an even film of the lipid coating the lower side walls and bottom of the tube. If you end up with any chunks or visible crystals, add chloroform and repeat the film-coating and blowdown process again.

REAGENTS

- Glass pasteur pipettes by Contributed by users
- Nitrogen gas, UHP by Contributed by users

Remove any residual chloroform

Step 1.7.

Once the vial appears dry, remove any residual chloroform by putting the tubes in a Speed Vac (or Vacufuge) for one hour.

SAFETY INFORMATION

Note: Make sure you use a vacuum evaporator and tubing suitable for work with organic solvents.

Top fims with argon and store until needed

Step 1.8.

Top the dry lipid film with argon (as a stabilizer), cap, and then and store at −20ºC until needed.

We have stored the films for up to 4 months when argonized and capped.

REAGENTS

- Argon gas, UHP by Contributed by users

Prepare matrixes for lipid suspension

Step 2.

In this protocol, you will resuspend lipids from lipid films in a buffered solution prior to beginning the extruding process. By trial and error, we determined that the following matrixes were appropriate for various classes of polar lipid.

Class(es) of lipid	Solution(s) appropriate for suspending lipid films prior to extrusion
PC	Milli-Q water 100 mM NaCl 100 mM NaCl / 50 mM Tris 10 mM Na_2SO_4 260 nM NaCl / 50 mM Tris[†]
PC:SQDG	100 mM NaCl / 50 mM Tris
PC:MGDG	Milli-Q water
PC:DGDG	Milli-Q water
PC:PG	100 mM NaCl
PC:Soy CRB	10 mM Na_2SO_4
PC:SPG	Milli-Q water
PC:PCE	Milli-Q water
PC:Di-O-P	Milli-Q water
PC:Di-O-H	Milli-Q water

[†] This was the chosen solution for preparation of PC liposomes for our photo-oxidation experiments.

REAGENTS

- MilliQ water by Contributed by users
- Sodium Chloride PubChem CID: 5234 by Contributed by users
- Tris-buffered saline (TBS), 1x solution BP24721 by Fisher Scientific
- Sodium sulfate PubChem CID: 24436 by Contributed by users

Warm ThermoMixer

Step 3.

Power on ThermoMixer and warm to 60°C. Use the ThermoMixer tube block appropriate to the size of tube in which you prepared the lipid films (and will be resuspending them). An incubator/shaker warmed to the same temperature could be used in lieu of a ThermoMixer.

Warm liposome mini-extruder block

Step 4.

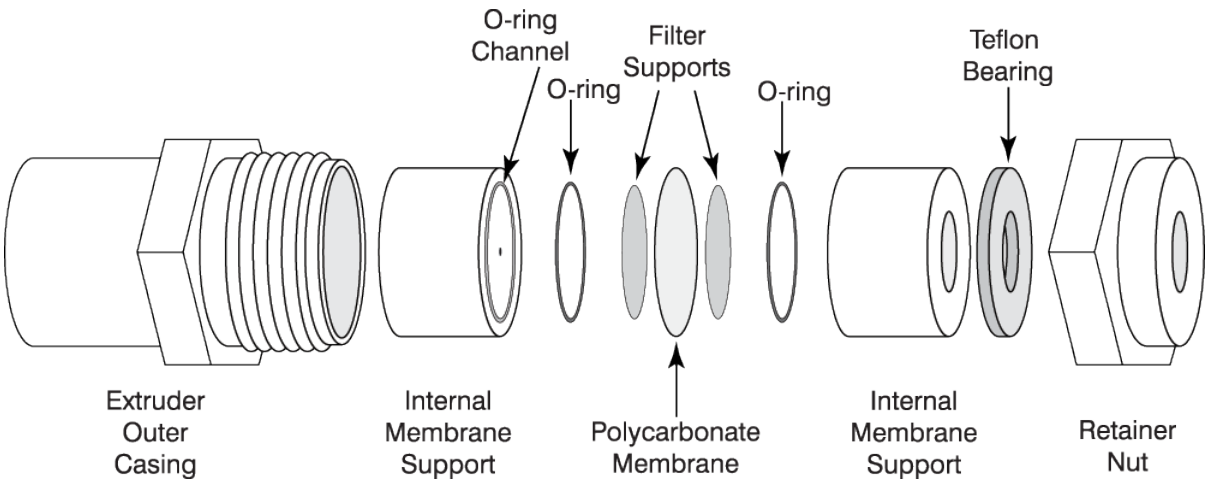
Place the Avanti mini-extruder heating block (heavy black metal component) on a heating block or warming plate. Set temperature to 60°C. Monitor temperature of the extruder block using a

thermometer inserted into the provided hole. *Do not let the extruder block get hotter than 70ºC*, even if you increased the set temperature of the ThermoMixer to a higher point during the suspension process. Temperatures above 80°C can damage the syringes.

Assemble the mini-extruder

Step 5.

Assemble the mini-extruder according to the following figure, linked from the Avanti web site:



1. Place the two Internal Membrane Supports o-ring side up on the counter.
2. Pre-wet two of the small (5 mm) filter supports with Milli-Q water, and place over the holes in each of the Internal Membrane Supports.
3. Insert one of the Internal Membrane Supports with small filter support into the Extruder Outer Casing with the o-ring side facing up.
4. Place a 0.2 µm polycarbonate membrane[‡] over filter support and o-ring.
5. Carefully place the second Internal Membrane Support (o-ring facing down) over the polycarbonate membrane. Do not twist the Membrane Supports.
6. Place the Teflon bearing into the Retainer Nut.
7. Put the Retainer Nut onto the Extruder Outer Casing
8. Tighten the nut enough to allow it to fit into the spot on the extruder heating block (do not use a wrench, but this will need to be quite tight).
9. Wet the extruder parts by passing a syringe filled with the chosen buffer solution through the extruder. Discard this volume of buffer.

[‡] A different size membrane could be used. We found the 0.2 µm pore size suitable for our needs.

REAGENTS

0.2 µm polycarbonate membranes 610006 by Avanti Polar Lipids, Inc.

10 mm filter supports for Avanti mini-extruder 610014 by Avanti Polar Lipids, Inc.

Hydrate lipid films

Step 6.

Hydrate the lipid film(s) by adding some volume of the appropriate buffer/suspension matrix to the glass test tube(s). 200-500 µL was suitable for our purposes, but the volume will depend largely on the capacity of your glass syringes.

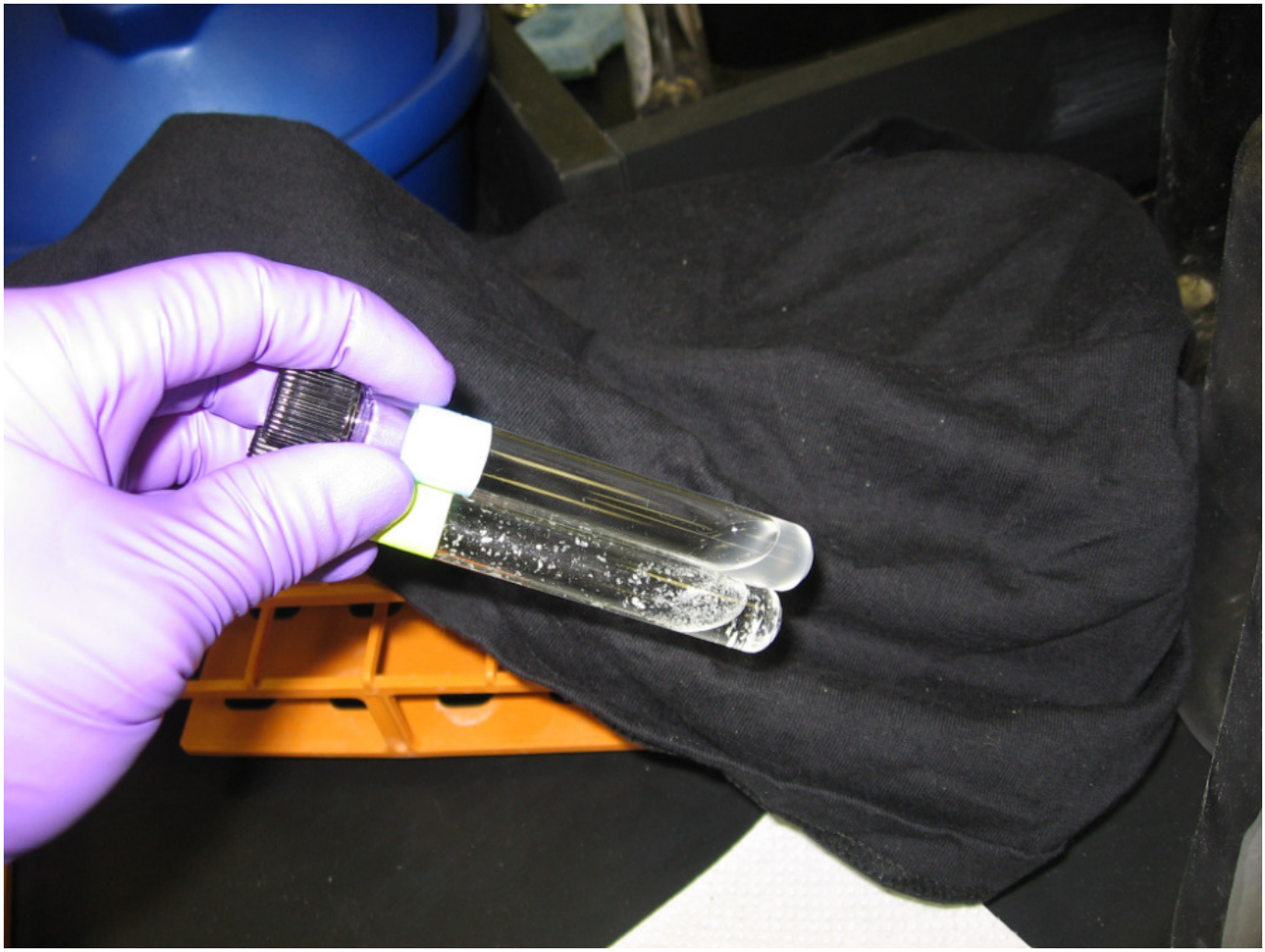
Suspend lipids

Step 7.

Insert the tube(s) containing the lipid films and suspension solution(s) into the ThermoMixer. Mix for 30 minutes at 60ºC.

After 30 minutes, take the tube out of the ThermoMixer and shake/swirl the solution. At this stage, you should see a milky/cloudy solution. If you see chunky bits of lipids floating around in the solution, something is wrong. Try again, using a new film, new buffer, and a higher or lower suspension temperature.

In this image, the top vial contains a liposome suspension which has been properly hydrated. The bottom vial has been improperly hydrated and should be discarded.



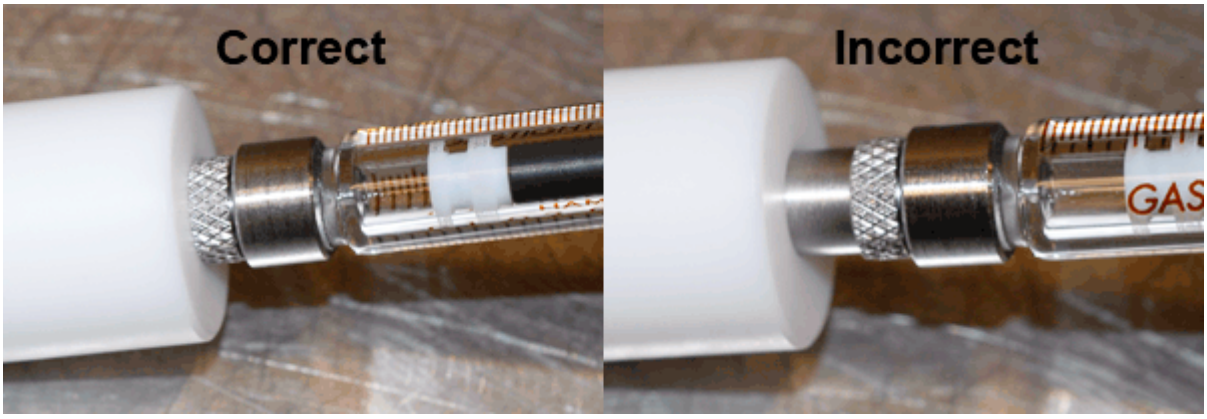
Note: The 60ºC temperature should be sufficient for most IP-DAG composed of short- and medium-chain fatty acids with at least some degree of unsaturation. However, you may need to increase the temperature for certain lipids containing saturated, long-chain fatty acids; these can have very high phase transition (i.e., melting) temperatures. A useful table of phase transition temperatures is provided by the manufacturer at
https://avantilipids.com/tech-support/physical-properties/phase-transition-temps/

DURATION
00:30:00

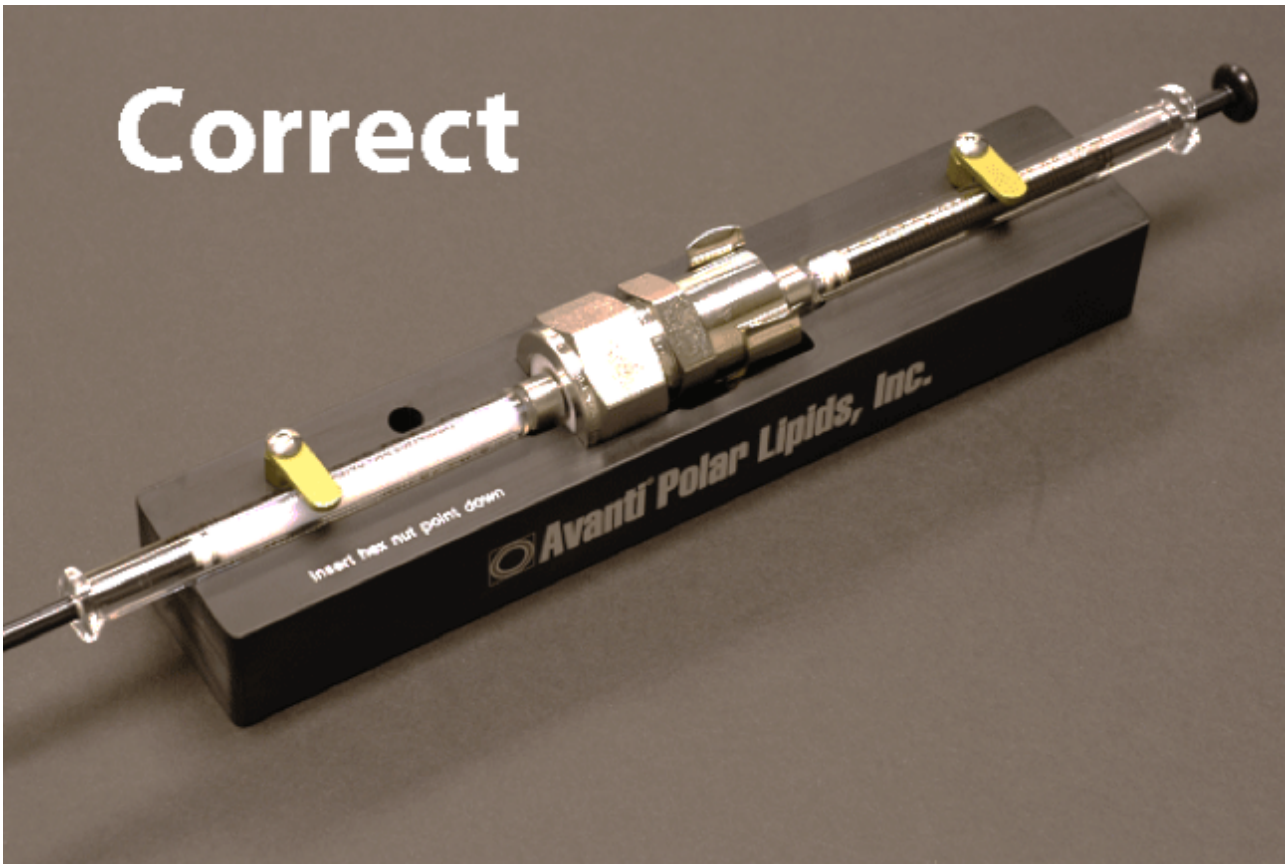
Transfer suspension to syringe and allow temperature to equilibrate

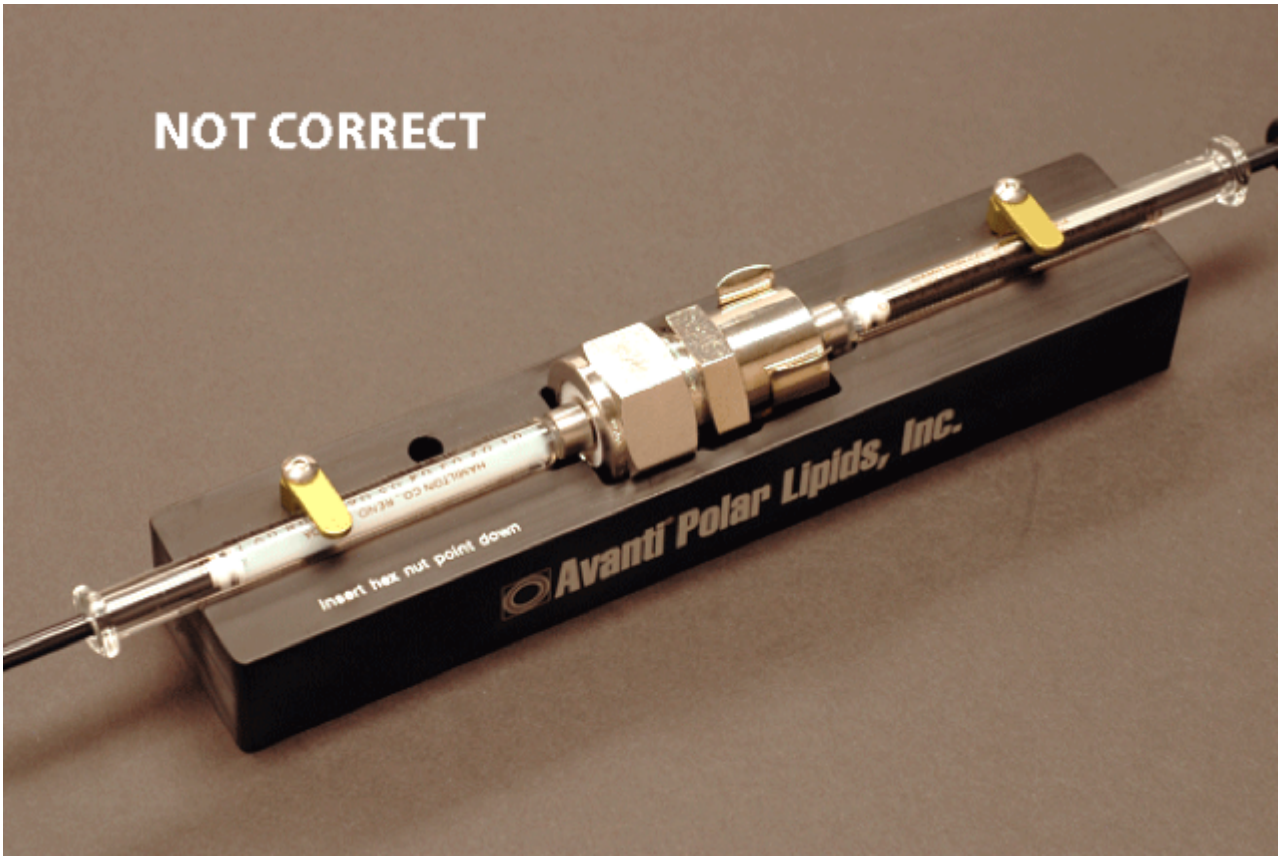
Step 8.

1. Immediately transfer the solution to a glass syringe; ensure this syringe has been solvent-rinsed and is compatible with the extruder. Insert the syringe into the Teflon pieces of the mini-extruder as shown in the figure below (from manufacturer site):



2. Then put the assembled extruder (with syringes on both ends) into the extruder block. Use the yellow clips to hold each syringe in place. Insert the hex nut so that the apex of the hex nut points straight up:





3. Allow the temperature of the lipid suspension to equilibrate with the temperature of the extruder block (5 minutes).

Further directions and guidance on proper extruder assembly are provided on the manufacturer web site: https://avantilipids.com/divisions/equipment/mini-extruder-extrusion-technique/

DURATION
00:05:00

Extrude lipsomes from the lipid suspension(s)

Step 9.

1. Gently push the plunger of the filled syringe until the lipid solution is completely transferred to the alternate syringe.
2. Then, push the plunger of the alternate syringe to transfer the solution back to the original syringe.
3. Repeat the above steps multiple times in order to have at least ten passes through the membrane inside the extruder. The final pass through the extruder should fill the alternate syringe.
4. Remove the extruder from the block, and then remove the syringe from the extruder by pulling the syringe straight out of the extruder.
5. Inject the lipid solution into a clean vial.

Other directions and guidance are provided on the manufacturer web site: https://avantilipids.com/divisions/equipment/mini-extruder-extrusion-technique/

Clean up

Step 10.

Clean the extruder apparatus with Milli-Q water and thoroughly dry it before storing. Solvent-rinse syringes before storing. If extruding additional liposomes, disassemble and clean all parts of extruder and replace the membrane and filter supports.