Laboratory 4 – Liposomes

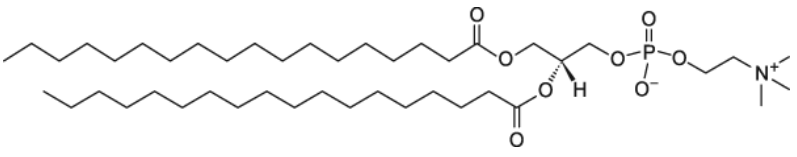
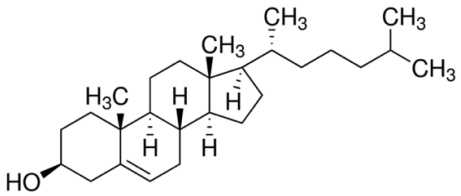
Liposomes are one of, if not the, most common nanoscale vehicles for drug delivery. They allow for increased uptake and distribution of compounds by increasing stability and solubility of compounds. Liposomes are phospholipid vesicles consisting of one (unilamellar) or more (multilamellar) concentric lipid bilayers enclosing discrete aqueous spaces. They are able to trap both lipophilic and hydrophilic compounds, allowing a wide range of molecules to be encapsulated. Hydrophilic molecules can be loaded into the aqueous core, and hydrophobic compounds integrated into the lipid bilayer. Research using liposomes is expanding rapidly, and liposome-based drug formulations are becoming increasingly common in clinical trials as vehicles for targeted and non-targeted drug delivery. Liposomes have been used for applications ranging from the delivery of small molecule cancer therapeutics, mRNA vaccines, such as the Pfizer/BioNTech and Moderna COVID vaccines, and various diagnostic imaging applications. Liposomes offer several advantages over other drug delivery systems in that they are more biocompatible, can self-assemble, carry large payloads, and have easily modifiable physiochemical properties that allow easy alteration of particle size, lipid composition, charge, surface groups, and number of lamellae.

There are a number of different methods that are commonly used to make liposomes including extrusion, sonication, reversed phase evaporation, and freeze-dried rehydration. Extrusion has been preferred historically due to the method allowing the quick and easy production of unilamellar liposomes with a consistent size distribution (low polydispersity). However, other methods such as those based on microfluidics have taken off in recent years, and have had great commercial success (this being the method used to make the liposomes encapsulating both the Pfizer/BioNTech and Moderna COVID vaccines).

1. Preparation of liposomes

a) Background

In this lab we will be using extrusion to produce liposomes that are a 55:45 molar ratio of the lipid DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine, a type of phosphatidylcholine), and cholesterol that contain the fluorescent dye Nile Blue A.

	
DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) MW: 790.145 g/mol Vendor: Avanti Polar lipids (850365P-1G)	**Cholesterol** MW: 386.65 g/mol Vendor: Sigma-Aldrich (C8667)

Drugs can be loaded into extruded liposomes by both passive and active methods. Active methods generally use buffers at different pHs and the principles of ion trapping to achieve a high loading efficiency of drug into the liposomes. The downside of this method is that it typically requires a fair bit of time, several dialysis steps, each taking 12-24 hours, to exchange buffers and purify the final liposomes being required. Passive loading has the advantage of being much simpler – just mix your drug and lipids together before production of the

liposomes., however has the downside that your drug is able to easily diffuse out of your liposome, leading to relatively low loading efficiencies.

The pore size of membranes used during extrusion will dictate both the size of our particles as well as how uniform they are in size. To produce consistently sized unilamellar liposomes, membranes with a pore size less than 200 nm must be used. Using larger pore sizes will result in the production of multilamellar liposomes with high polydispersity.

b) Creation of the lipid solution and thin film

NB: Due to time and equipment constraints, this section will have been done for you, and the dry thin films produced stored at -20°C until needed for the lab.

1. Using glass tubes, weigh out powdered DSPC and cholesterol to give a 55:45 molar ratio of DSPC to cholesterol.
   - This is equivalent to a weight ratio of 71.41% DSPC to 28.59% cholesterol

2. Prepare 50 mg/mL stock solutions of each lipid in a 5:1 mixture of chloroform:methanol in glass vials
   - Solvent: 20 mL chloroform + 4 mL methanol
   - ____ mg DSPC / 50 mg/mL = ___ µL solvent
   - ____ mg cholesterol / 50 mg/mL = ___ µL solvent

3. Measure the volume of each lipid needed into a clean round bottom flask so that you have the desired 55:45 molar ratio.
   - V = (mg * % needed)/concentration
     1. DSPC: V = (40 mg * 0.7141)/50 mg/mL, V = 571 µL
     2. Cholesterol: V = (40 mg * 0.2859)/50 mg/mL, V = 229 µL

4. Mix the solution (800 µL total volume) thoroughly via vortex.

5. Set up the rotary evaporator
   - Attach the round bottom flask to the apparatus
   - Heat the water bath to 40°C
   - Fill the condenser with dry ice and slowly add acetone until the ice is covered

6. Slowly degas the lipid solution, ensuring that there is no splashing

7. Allow to incubate while rotating for ~5-15 minutes until lipid is dry.

8. Remove flask and turn off the rotary condenser.

9. This is your lipid thin film. The flask can be sealed with parafilm and stored at -20°C until needed.

c) Hydration and drug loading of the thin film

1. Add 2 mL of a 100 µg/mL solution of Nile Blue A (MW: 732.85 g/mol) in 300 mM $(NH_4)_2SO_4$ (ammonium sulfate), ~pH 5.5, that has been passed through a 0.45 µm filter, to the flask containing the thin film.

2. Seal flask using parafilm to prevent any spills

3. Place in a flotation rack

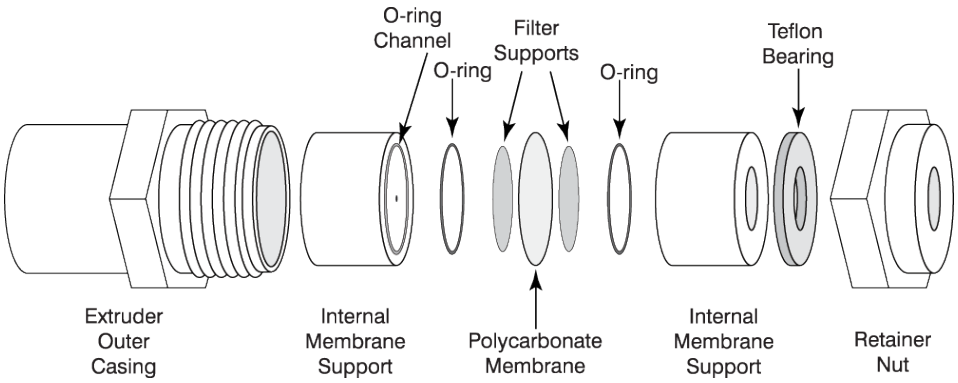
4. Sonicate in a 60°C water bath for a minimum of 10 minutes, vigorously shaking every ~2 minutes. The longer this sonication period, the better the emulsion of lipid and aqueous drug.

d) Creation of liposomes via extrusion

1. Rinse the two extrusion syringes and teflon inserts in mQH_2O.

2. Place the extrusion device on a hot plate set to 70°C and allow to warm
   - A thermometer placed in the extruder base should indicate that the extrusion device is at ~60°C.

3. Wet the 2 filter supports and the 400 nm polycarbonate membrane in mQH_2O and assemble the extrusion apparatus as instructed by your TAs:
   - Do **not** over-tighten (stripped metal is very difficult to repair)
   - Use mQH_2O to ensure there are no bubbles between the membrane and the filter supports.



4. Test the assembled apparatus for leaks
   - Carefully insert a clean, rinsed syringe into the side of the apparatus opposite the retainer nut.
     - Do **NOT** put it at an angle as this may break the needle and/or extrusion apparatus!
     - Carefully place syringe and membrane apparatus into the pre-heated extrusion block on the hotplate.
     - Ensure the blunt needle tip is securely attached to the syringe!

   - Fill the second syringe with 1000 µL of water, and insert into the other side of extrusion apparatus
   - Ensure that the syringe retaining arms are set.
   - Slowly depress the plunger of the water-filled syringe to transfer the water from one syringe to the other.

    - There should be a small amount of resistance when pressing the syringe plunger
- Examine the receiving syringe to confirm that there is approximately the same amount of liquid in the receiving syringe as was in the original syringe.
- Repeat the transfer a few times, examining the syringes each time for any loss. Up to ~10% loss is acceptable, but confirm with your TA before proceeding.
    - If there is >10% loss, inform your TA, and the apparatus will be taken apart and reassembled to fix the leak before re-testing and continuing with the protocol.

5. Transfer all the water into the left-hand syringe (on the side of the apparatus with the retainer nut).

6. Gently remove the syringe from the apparatus. Discard the water into the liquids discard beaker.

7. Remove your flask of sonicated and hydrated lipid from the sonicating bath and fill a syringe with 1 mL of the blue, hydrated lipid solution

8. Re-insert the syringe into the extrusion device as shown by the TAs.

9. Allow the syringes to equilibrate to temperature (~60°C) for 3 minutes.

10. You will now form your nanoparticles by extrusion. One person should hold the apparatus in place, using a heat resistant glove, and two other people will operate the syringe plungers. Rotate through everyone in your group to ensure everyone gets a chance to try the procedure.

Potential Issues:
Leaking: O-ring forgotten, syringe not correctly inserted, bolt is not tight enough. Don't panic, you can suck up the lost solution with the syringe

Common causes of pressure:
Aggregates, or air bubbles.
STOP pressing the plunger, wait for a couple seconds, and try again with gradual pressure. If this doesn't work, inspect the filter and/or supports. DO NOT PULL BACK THE PLUNGER.

Pass through feels too easy:
The membrane is likely displaced. STOP, disassemble the apparatus and inspect the filter.

11. Carefully press the plunger of one syringe to force the liquid through the membrane and into the other syringe. All the liquid should pass through the filter
    - If there is substantial resistance, stop pressing, then try again. If there is still a lot of resistance, ask your TA for assistance

12. Repeat this using the other syringe to force the lipid back to the starting side.

13. Repeat this process of forcing your lipid through the membrane an additional 20 – 30 times.
    - The more times you pass your lipids through the filter, the more uniform in size your final particles will be.

14. After the final extrusion, collect all the liquid into the left side syringe, remove syringe from the apparatus and dispense your lipids into a labelled 13 mm diameter glass tube.

15. Carefully disassemble and clean the extrusion apparatus as directed by your TAs. Discard the membrane and filter supports, and ensure that the internal membrane supports and extruder outer casing are clean and free of lipid.

16. We will now repeat steps 3-15 twice more. First using a 200 nm filter, then a 100 nm filter.

17. Remove the extrusion apparatus from the heating block and allow it to cool to room temperature.

18. Pipette 200 µL of your extruded liposomes into a microfuge tube labelled with "unpurified liposomes" and your group number. These will be used to measure the size of your extruded liposomes.

e) Purification of Liposomes

In the lab, typically liposomes are purified by dialysing them against water or buffer overnight. However, due to time constraints, we will perform a quick purification using a cold extrusion apparatus (per the protocol outlined by Alves et al., 2013) to separate our liposomes away from the ammonium sulfate buffer containing unincorporated free dye.

1. Reassemble your extrusion apparatus with a 50 nm filter disk, and test it to confirm there are no leaks.

2. Draw your remaining 100 nm liposomes into a syringe and insert into the right-hand side of the extrusion apparatus, and an empty syringe into the left-hand side.

3. Push through the majority of your liposome mixture into the empty syringe, leaving ~100 µL in the right-hand side syringe.

4. Remove the left-hand syringe and discard the buffer into the waste beaker. Rinse the syringe in mQH_2O, then fill it with ~900 µL of fresh 300 mM ammonium sulfate, pH 5.5.

5. Re-insert the syringe into the extrusion apparatus, and press the buffer through the filter and into your left-hand syringe.

6. Repeat steps 3-5 twice more.

7. Remove the left-hand syringe containing your filtered liposomes and dispense into a labelled 1.7 mL microfuge tube. This tube now contains your purified liposomes in 300 mM ammonium sulfate, pH 5.5.

8. Carefully disassemble and clean the extrusion apparatus as directed by your TAs. Discard the membrane and filter supports, and rinse the components in 95% ethanol to remove residual lipids and dye, then in mQH_2O.

2. Quantification of Liposome Loading efficacy

In order to determine the loading efficiency of our liposomes, we must break them open to release the Nile Blue A dye trapped inside. This can be done by the addition of Triton X-100 to lyse the liposomes. Nile blue is very sensitive to the presence of other compounds, and its fluorescence will change depending on the presence or absence of the Triton X-100. In order to examine both lysed and unlysed liposomes, we'll need to make two standard curves, one with Triton X-100, and one without. We will then compare our samples to the correct curves.

a) Preparation of Nile blue standard curves

NB: The technician or your TAs will generate the standard curves for you, and run them alongside your samples.

1. Prepare serial dilutions of Nile Blue A in 300 mM ammonium sulfate, pH 5.5. or in 1% Triton X-100 made in 300 mM ammonium sulfate, pH 5.5. Each curve will consist of eight points: 500 ng/mL, 400 ng/mL, 300 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, and 0 ng/mL of Nile Blue A.

2. Load 100 µL of each point of the standard curve onto the plate in triplicate.

b) Measure the amount of fluorescence of Nile blue in your liposomes

1. In a tube labelled unlysed liposomes, combine 50 µL of your purified liposomes with 450 µL of 300 mM ammonium sulfate, pH 5.5.

2. In a second tube, labelled, "lysed liposomes", combine 50 µl of purified liposomes with 450 µl of 1% Triton X-100 made in 300 mM ammonium sulfate, pH 5.5.

3. We will measure our samples in triplicate:
   - Pipette 100 µL of your diluted, unlysed liposomes into 3 separate wells of a black 96-well plate
   - Pipette 100 µL of your diluted, lysed liposomes into 3 separate wells of a black 96-well plate
   - Record your loading order!

4. Measure the absorbance of each sample using an endpoint fluorescence read:
   - Excitation: 635 nm
   - Emission: 680 nm

c) Calculate the entrapment and loading efficiencies of your particles

1. Calculate the amount of dye present in each sample using the standard curve
   - Use the equation of the line to calculate the concentration of dye present in your sample
   - Adjust for the fact that we did a 1/10 dilution of our sample when preparing it for measurement
     1. This will give us the concentration of dye present in our sample

2. Calculate the mass of dye that would be present if we had measured our entire sample:
   - Total amount = concentration measured * total volume made (2 mL)

3. Calculate the mass of dye we added initially:
   - 2 mL of 100 µg/mL Nile blue = ____ µg Nile blue

4. Calculate the entrapment efficiency of our particles:
   - $Entrapment\ Efficiency\% = \frac{\text{mass of dye in nanoparticles}}{\text{initial mass of dye}} \times 100$

5. Calculate the loading efficiency of our particles:
   - Each of our thin films contained 40 mg of lipid
   - $Loading\ Efficiency\% = \frac{\text{mass of dye in nanoparticles}}{\text{mass of nanoparticles}} \times 100$

3. Liposome sizing

Background

Nanoparticle size can be determined using a spectrophotographic technique known as dynamic light scattering (DLS). The motion of particles in a solution causes light from a monochromatic laser shot into the solution to be scattered at varying intensities. Analysis of the fluctuations in intensity yields the velocity of the particles' Brownian motion, and allows the calculation of particle size using the Stokes-Einstein relationship.

Fortunately, machines and software exist that perform the measurements and math for us so that we do not need to manually interpret the raw data. We will be using a Malvern Instruments Zetasizer Nano to determine the size of our liposomes.

a) Dynamic light scattering with the zetasizer

1. We will measure two samples and compare particle size, uniformity, and quality.

2. Open the Malvern Instrument software and ensure that it is set to the correct settings for measuring our particles
   - Material: lipid polymer
   - Dispersant: water
   - Cell: 4 ml polystyrene cuvette
   - Measurement: 173° backscatter, manual duration: 11 runs, 10 sec, 3 measurements

3. Prepare your unpurified liposomes sample for measurement
   - To a 4 ml cuvette, add 980 µL of mQH_2O and 20 µL of your unpurified liposomes. Mix well by pipetting.

4. Place the cuvette into the machine, ensuring that the triangular shape embossed in the plastic is facing forwards.

5. Place the cuvette cover on the cuvette, and close the lid of the machine.

6. Start the measurement process. This will take approximately 5 minutes, and the machine will display intermediate results on the screen as it progresses.

7. Record the results on your lab worksheet

8. Repeat this process using a new cuvette and your purified liposomes. Record the results.

4. References

Alves NJ, Cusick W, Stefanick JF, Ashley JD, Handlogten MW, and Bilgicer B (2013) Functionalized liposome purification via Liposome Extruder Purification (LEP). *Analyst* **138**:4746–4751.

Sercombe L, Veerati T, Moheimani F, Wu SY, Sood AK, and Hua S (2015) Advances and Challenges of Liposome Assisted Drug Delivery. *Frontiers in Pharmacology* **6**.

Laboratory 4 – Liposomes Worksheet [22 points total]
Due: In lecture, the day after lab.

Liposome production:

1. Describe the appearance of your thin film and the lipid-dye mixture before and after sonication. [2]

2. After performing the buffer exchange to remove unincorporated dye at room temperature, what did your liposome solution look like compared to before the exchange? [1]

Liposome loading efficiency:
Use the data uploaded to Canvas to answer the following questions.

3. What were the equations of the line and the r^2 values for the standard curves of Nile blue in 300 mM ammonium sulfate, pH 5.5 and 1% Triton X-100? [2]

4. What was the average absorbance and standard deviation of your unlysed and lysed liposomes? [2]

5. What was the average concentration, in µg/mL, of Nile Blue A dye in the liposomes you diluted in 300 mM ammonium sulfate, pH 5.5, and in the 1% Triton X-100? [2]

6. Using the amounts of Nile blue that you calculated to be present in your lysed and unlysed samples, what was your liposome entrapment efficiency? [1]

7. Using the amounts of Nile blue that you calculated to be present in your lysed and unlysed samples, what was your liposome loading efficiency? [1]

8. Was there any difference in the amount of absorbance (and calculated dye amounts) between your lysed and unlysed liposomes? [1]

9. Nile blue is a weak base with a pKa of 9.7. Explain how we are able to improve retention of drug within the liposomes by preparing our drug in 300 mM ammonium sulfate, pH 5.5. Use the Henderson-Hasselbalch equation to support your answer. [2]

Liposome sizing:

10. What was the average size and PDI of your group's unpurified liposomes? [2]

11. What was the average size and PDI of your group's purified liposomes? [2]

12. Was there any difference between the average size and PDI of your purified vs the unpurified liposomes? Why could there be a difference between these samples? [2]

13. Describe how you could produce higher quality liposomes containing a weakly basic drug of interest if you needed to manufacture a liposomal formulation of your drug for an actual research lab. [2]