

Project 8B: Liposome Permeability and Phase Transition

Part 1: Liposome Permeability Probed by HeNe Laser Light Scattering

Part 2: Lipid Phase Transition Probed by Diode Laser Light Scattering

LABORATORY REPORT: Informal Report

PRE-LAB ASSIGNMENT

- Read the entire laboratory project described in the following pages. Also read the source papers by Bittman and Blau¹ and Craig *et al.*²
- Prepare, on a typed sheet of paper, the Project Objectives of this lab; on the same sheet, complete the assignment below:
 - 1) Concerning the measuring of liposome permeability via light scattering,
 - a. when liposomes swell, transmitted light intensity (I_{tr}) goes _____.
 - b. In the presence of hyperosmotic impermeant solutes, liposome volume goes _____.
 - c. In the presence of hyperosmotic permeant solutes, liposome volume goes _____.
 - d. Which permeates liposomes faster, methanol or butanol? _____.
 - e. Which permeates liposomes faster, methanol or water? _____.
 - 2) Draw molecular structures of DSPC and DCP at pH 7; include their MWs.
 - 3) For the permeability lab (Part 1), you are instructed to make an *aqueous lipid stock solution* that is 10.0 mM in total lipid, 96.0 mol% DSPC, 4.0 mol% DCP, and 0.80 mL in total volume.
 - a. Calculate the moles and masses of DSPC and DCP necessary to make this *aqueous lipid stock solution*.
 - b. Calculate the volumes of chloroform stock solutions of DSPC (10. mM) and DCP (3.0 mM) necessary to deliver the required amounts of each lipid to make the 0.80 mL *aqueous lipid stock solution*.

¹ Bittman, R. and Blau, L. "Kinetics of Solute Permeability in Phospholipid Vesicles" *J. Chem. Educ.* **1976**, *53*, 259-261.

² Craig, N.C.; Fuchsman, W.H.; Lacuesta, N.N. "Investigation of Model Cell Membranes with Raman Spectroscopy: A Biochemistry Laboratory Experiment" *J. Chem. Educ.* **2003**, *80*, 1282-1288.

- c. You will begin the permeability assay with a volume of the permeant osmolyte solution (V_{osm}) in a stirred cuvet. To this osmolyte solution you will add an aliquot of the *aqueous liposome stock solution* described above. If the final cuvet solution is 1.50 mL and 0.167 mM total lipid, calculate the aliquot volume of the *aqueous liposome stock solution* (V_{lip}), and also V_{osm} .
- d. Why are lipids delivered from chloroform stock solutions, instead of just weighing them out?
- 4) For the phase transition lab, Part 2, we consider two common pure phospholipid phases.
- Which phase is favored at low temperature? _____.
 - Which phase features tighter packing and lower permeability? _____.
 - Which is more motionally free and is a better biological membrane model?
 - As T rises, will liposomes shrink or swell? _____.
 - As T rises, transmitted light, I_{tr} , should ____crease.
- 5) Rank these four phospholipids from lowest to highest transition temperature:
 di-14:0 phosphatidyl glycerol (DMPG); di-14:0 phosphatidyl ethanolamine (DMPE);
 di-14:2 phosphatidyl ethanolamine (D2 π PE); di-16:0 phosphatidyl ethanolamine (DPPE)
- lowest T_{tr} : _____ < _____ < _____ < _____ ; highest T_{tr}

Part 1: Liposome Permeability Probed by HeNe Laser Light Scattering

INTRODUCTION

Phospholipids self-assemble in aqueous solution to form lipid bilayers, with the non-polar fatty acid chains from each monolayer sequestered on the inside, away from water, and the polar head groups on each outer surface, in contact with water. Under certain special conditions, aqueous lipids can be induced to form a single lipid bilayer surrounding an aqueous core; this is known as a *bilayer vesicle*. Under most conditions, concentric shells of bilayers form around a central

aqueous core, in a particle called a *multilamellar liposome*. Here each bilayer is separated from adjacent bilayers by an aqueous shell.

Lipid bilayers encasing interior aqueous phases make effective osmometers: The bilayer serves as a semi-permeable membrane across which solutes and solvent diffuse in response to differences in chemical potential (i.e., concentration gradients). Liposomes suspended in a hypo-osmotic (low external solute concentration) medium will slowly gain water from their exterior and swell. Due to the size and multilamellar structure of the liposomes, this swelling will cause a decrease in total light scattering, and an increase in the intensity of light transmitted (I_{tr}) through the suspension. By monitoring I_{tr} increase with time, we can determine the first order rate constant for liposome swelling, which should be identical to the rate of water permeation (influx) across the membrane. Please read Bittman and Blau¹ for further details and discussion.

If liposomes are suspended in a hyperosmotic (high external solute concentration) medium, in principle two things can occur: (a) water will leave the liposome interior and cause the particle to shrink; (b) at the same time, solute can also permeate into the liposome interior, and this latter process increases the interior solute concentration, causing water to move back into the liposome, which then swells. To summarize, in hyperosmotic media, water efflux (a) causes liposome shrinkage and a decrease in I_{tr} , whereas solute influx (b) causes liposome swelling and an increase in I_{tr} . If the solute is impermeant (e.g., salts), then water efflux (a) is the only process observed. On the other hand, if the solute permeates the bilayer (e.g. long chain alcohols), liposome volume changes are almost entirely due to solute influx (rather than water efflux); because differences in solute concentration (i.e., activity) alter the activity of water very little, water efflux tends to be a minor process in this case.

The main determinant of transmembrane permeability is polarity. Non-polar molecules tend to partition into the membrane interior more readily, and thus permeate faster. Polar molecules permeate the membrane more slowly, and very polar solutes (e.g., salts, glucose) essentially do not permeate at all on the time scale of several minutes. Molecular size matters, but not nearly as much as polarity.

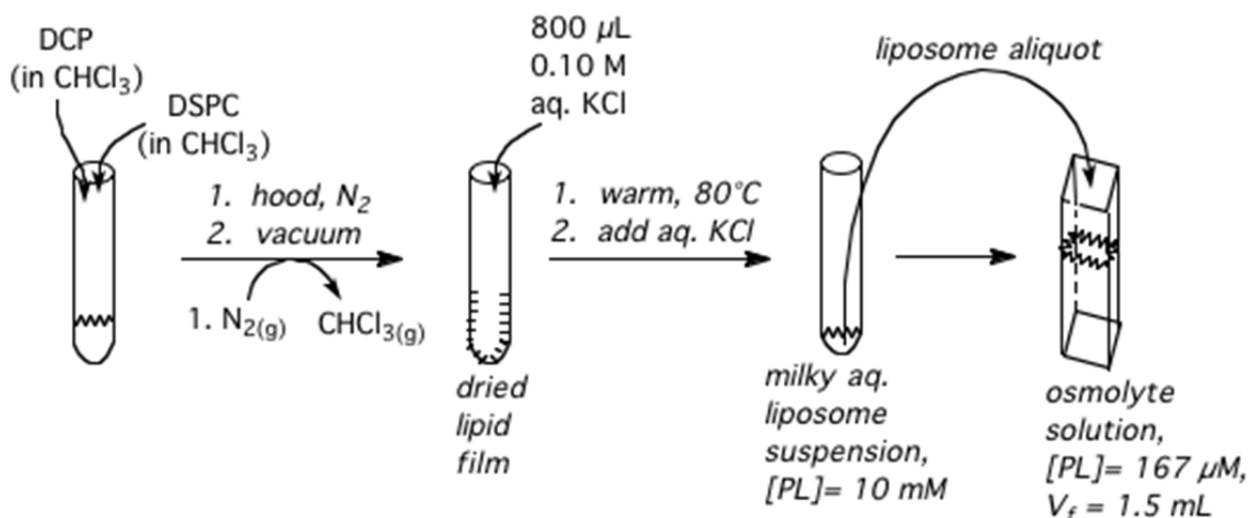
In this experiment you will make multilamellar liposomes comprising two mixed lipids: distearoyl phosphatidyl choline (di-C_{18:0} PC = DSPC) and dicetyl phosphate (di-C_{16:0} PO₄H = DCP). You will use these liposomes as osmometers to probe the permeability of a series of solutes in so-called “osmolyte” solutions.

EXPERIMENTAL: LIPOSOME PREPARATION (PART 1)

Start by setting up an 80° C water bath: Place a 250 mL beaker with water on a hot plate. **Allow at least 30 min.** for the water temperature to equilibrate. For this part of the lab, you will make up an aqueous liposome stock suspension, using three pre-made stock solutions:

1. DSPC in chloroform, about 10 mM (record actual concentration)
2. DCP in chloroform, about 3 mM (record actual concentration)
3. aqueous KCl, 0.10 M

For this permeability experiment, liposome preparation occurs in two stages (see Scheme 1 below): First, aliquots of the *chloroform* stock solutions 1 and 2 will be mixed together, and then the chloroform will be evaporated, leaving a dried film of mixed lipids on the test tube walls. Next, an aliquot of solution 3 will be added, to make an *aqueous* stock solution in the test tube that is 0.10 M KCl, and 10 mM in total suspended lipid. Later, when you make your permeability measurement, you will add an aliquot of this liposome stock suspension to an osmolyte/permeant solution in a cuvet; then you will mix the solution in the cuvet and measure light scattering/trans-mission changes as the liposomes swell or shrink.



Scheme 1: Making an aqueous liposome stock suspension from lipid-chloroform stock solutions.

While one student makes up the liposome aqueous stock suspension, the second student on the team will obtain (or prepare) four different osmolyte solutions to add to the cuvet; you will need 5 or 10 mL of each osmolyte solution. Student #1 will make liposomes from a mixture of lipids that is 96 mol% distearoyl phosphatidyl choline (di-C_{18:0} PC = DSPC) and 4 mol % dicetyl phosphate (di-C_{16:0} PO₄H = DCP). The final aqueous liposome stock suspension will have a total lipid concentration of 10 mM, and a final volume of 800 μL (using 0.10 M KCl as solvent).

- Calculate the number of moles of DSPC and DCP that you will need in order to make the final aqueous liposome stock suspension.
- Obtain the actual concentration of the chloroform stock solutions of DSPC and DCP.
- Calculate the volumes that you will need of the chloroform stock solutions of DSPC and DCP.
- Check these calculations with your laboratory instructor.

To deliver calculated volumes of the lipid-chloroform solutions, student #1 must use glass Hamilton syringes³, as pipet tips dissolve in chloroform. Add each calculated lipid-chloroform stock solution to a 13 × 100 mm test tube. After mixing the DSPC and DPC chloroform aliquots, use a controlled stream of N_{2(g)} to carefully evaporate the chloroform *in a fume hood*

³ Graduated Hamilton syringes of various volumes may be obtained from the lab instructor. Soon after you're done with the syringe, be sure to rinse it several times with ethanol or acetone; then, return the syringe to the instructor.

(chloroform is a carcinogen!); take care to tilt and rotate the test tube so that an even thin film of lipid is deposited on the tube wall. Place the tube in a stoppered side arm vessel and pump off any remaining chloroform under house vacuum for 20-30 minutes. After the lipid mixture is pumped dry, warm the dried lipid tube in your pre-equilibrated 80 °C bath for > 30 s, then add 800 μ L of 0.10 M KCl to the lipid and warm for > 30 s. While holding firmly to the top of the test tube, vortex the warm aqueous liposome suspension vigorously(!) for several seconds. Return the tube to the water bath to re-warm, then vortex again. Repeat until all of the lipid is dispersed into a slightly cloudy/opalescent suspension, and the solid lipid film is no longer visible on the side of the test tube. Store the aqueous liposome dispersion on ice.

EXPERIMENTAL: OSMOLYTE SOLUTIONS

Each team will be testing a number of different osmolyte/permeant solutions, as specified in Table 1 below. You will add an aliquot of the aqueous liposome stock suspension to osmolyte solution in a cuvet, and use light scattering to measure the liposome swelling rate. The final solution volume in the cuvet will be 1.5 mL, and the final concentration of suspended total lipid will be 167 μ M. Recall that the aqueous lipid stock suspension is 10 mM in total lipid.

Table 1: Osmolyte permeants studied by student teams.

<u>Teams</u>	<u>osmolytes</u>
A, E	urea: 0, 0.2, 2, and 6 M
B, F	2 M alcohols: MeOH, EtOH, PrOH, Et-glycol, glycerol
C, G	2 M salts: KCl, KOAc, NH ₄ Cl, NH ₄ OAc, and HOAc (1 M)
D, H	KCl: 0, 0.2, 1.5, 3.5 M

- Calculate the volume of the aqueous liposome stock suspension that you will add to the cuvet.
- Calculate the volume of osmolyte that you will start with in the stirred cuvet.
- Check these calculations with your laboratory instructor.

Add the calculated volume of osmolyte solution to a stirred 4 mL glass cuvet, and place the cuvet in the holder. At the appropriate time, you will add an aliquot of the aqueous liposome stock solution to the stirred cuvette that already contains the osmotic solution that you wish to test.

EXPERIMENTAL: LIGHT SCATTERING MEASUREMENTS

On the optics table in the laser lab find the cuvet holder with stirring unit. You will use a standard 4 mL glass cuvet with a tiny spinbar. The HeNe laser (black cylindrical tube) emits 632.8 nm light. The light path on the optics table includes an attenuating filter to decrease incident light intensity, two mirrors to bend the light toward the cuvet, a chopper wheel for lock-in amplification, and a computer-controlled electronic shutter to control when light hits the cuvet. Beyond the cuvet, an iris blocks scattered light, transmitting only light travelling at the same angle as the incident light beam. Finally, a photodiode detects the transmitted light, I_{tr} , as an electrical signal (units = millivolts). The photodiode detector toggle switch must be up (on).

Below the optics table, the laser power supply, labeled “Red 632.8”, must be turned on with its key. It may take a minute or so to warm up before lasing starts, and another several minutes for the laser to reach steady intensity. The electronic stirrer control unit on the table, next to the cuvet holder, must be turned on with the switch on the rear right of the unit. Adjust the initial stirring speed to 1200 rpm. The shutter controller unit, next to the stirrer control, must be turned on with the toggle switch on the left. The toggle switch on the right manually opens the shutter; try this, in order to test the unit. Leave the shutter closed, so that the computer can control its operation. The chopper wheel controller, to the left of the shutter controller and under the black breadboard, must be turned on with the switch on the rear right of the unit.

The SR830 Lock-In Amplifier, at the bottom of the electronics rack, must be turned on; its power switch is on the left back side of the instrument, requiring a walk around to the rear of the electronics rack. Above the lock-in amplifier is a multi-use bin containing the SR245 Computer Interface. Turn on the electronics bin power with the toggle switch on the far right side.

The computer folder you will use is entitled “Exp. Biochem”. Find (or make) the subfolder of your year (e.g., S’13); finally, make a subfolder named with your initials, and make sure that at least one document in this folder lists the full names of your team members. The program you will use is entitled “LIA Collect Liposome Transient.” Double click on this icon, and select your collection time in minutes. Start with 10 minutes; you can adjust downward if I_{tr} changes seem complete much earlier. Place the cuvet containing only the osmotic solution (no liposomes) in

the cuvet holder. Make sure that the stirrer is working. If at any point the stir bar becomes “frozen,” press the “capture” pad on the stirrer control keypad to reset.

Start the computer data collection process by clicking on the white forward arrow in the upper left corner. This initiates the collection of two background signals: a ten-second “dark” signal with the shutter closed, and a twenty-second “reference” signal with the shutter open. When these are completed, the program will prompt for the addition of the liposome suspension aliquot. Do this by inserting the pipet tip containing liposome suspension near the bottom of the cuvet to achieve efficient stirring. Press down on the delivery shaft on the pipettor to expel the aliquot, and withdraw the tip from the cuvet with the shaft still depressed: **Avoid sucking up solution** from the cuvet! Once you observe complete mixing in the cuvet (about 5-10 s for most solutions, but longer for osmolytes of high density), quickly lower the stirring speed to 400 rpm.

If it looks like your signal is still changing dramatically as you approach the end of your initially selected data collection period, you can increase the data collection time by clicking on the “+1 Minute” button. On the other hand, if the signal reaches equilibrium well before the end of your initially selected data collection period, you can terminate the run by clicking the “STOP” button. The left-hand window on the computer screen contains all of the data in your run, but you can omit the initialization signals (0 and 100%) and any early mixing artifacts by selecting a delay time offset for the right-hand zoom window. You can adjust the delay time for this zoom window in 10 s intervals, by clicking the up or down arrow in the time delay box. Note that this feature is only available while data are being collected. At the conclusion of the data collection, save your data as an appropriately named Excel file (e.g., KCl_3M_Chuck.xls) in your team’s data folder inside of the Exp. Biochem folder.

DATA ANALYSIS:

During the first 10-20 sec after liposome addition, mixing artifacts may produce an anomalous increase in I_{tr} . Ignoring these I_{tr} changes within the first 10-20 sec, most of your transients should show first order exponential changes: declines for hyperosmotic impermeant solutes, or rises for hyperosmotic permeant solutes and hypoosmotic solutions. Below we summarize the highlights of the curve-fitting process for your I_{tr} vs. t curves. For a complete description of the data analysis process, please refer to Appendix 1.

The equation for first order exponential decline from an initial voltage V_0 to zero is $V_t = V_0 * e^{-kt}$, but your I_{tr} transients do not decay to zero. The equation for exponential decay to a finite final voltage at time infinity = V_f is:

$$V_t = V_f + \Delta V_{\max} * e^{-kt} \quad (1)$$

where ΔV_{\max} is the difference between V_0 and V_f . You may fit such a first order exponential decline using Kaleidagraph:

$$(Y=) m3 + (m2 * \exp(-m1 * m0)); m3=100; m2=1; m100=0.005 \quad (1K)$$

where $m3 = V_f$, $m2 = \Delta V_{\max} = V_0 - V_f$, and $m1 = k$, the first order rate constant (units = sec^{-1}).

If your curve features a first order exponential **rise** in V , from V_0 to V_f , you may still use equation (1K) to fit your data.⁴ The only difference is that your value of $m2 = \Delta V_{\max}$ will be negative.

After converting all I_{tr} values from V to mV, use equation (1K) to fit your I_{tr} (mV) vs. t (s) data sets, and determine the first order rate constants for liposome shrinking/swelling due to solute or water permeation. Please consult Appendix 1 for a more detailed explanation of the data analysis procedure.

⁴ Alternatively, the equation $V_t = V_0 + \Delta V_{\max} * (1 - e^{-kt})$ is sometimes used to fit rising exponentials.

POST-LABORATORY: RESULTS TABLE

Post your fitting results for each curve in the class Google doc spreadsheet, before the deadline listed on the course schedule. Include all Kaleidagraph-fitted plots, plus a table that lists:

- permeant, including concentration; this will be either water, or the osmolyte
- whether I_{tr} rose or fell;
- $k \pm$ uncertainty, in min^{-1} ; your x -values are in units of s , so you must convert your fitted k values from s^{-1} to min^{-1}
- $\Delta V_{\text{max}} \pm$ uncertainty, in mV
- R^2

You will characterize results from the whole class, comparing the permeation processes by their direction (rise/fall), their magnitude (ΔV_{max}), and their rate constant (k).

POST-LABORATORY: INTERPRETATION

Membrane permeation depends mainly on the ability of a molecule to diffuse through the membrane, which in turn should depend on polarity: Nonpolar molecules should be highly membrane permeable, and very polar molecules should be membrane-impermeant. There are several ways to characterize the polarity of a compound. For the purposes of permeation, the octanol/water partition coefficient (P) is probably the most relevant.⁵ P is determined by exposing the solute to a two-phase mixture of water and octanol, and measuring the solute concentration in both phases at equilibrium:

$$P = [\text{solute}]_{\text{octanol @eqm}} / [\text{solute}]_{\text{water @eqm}} \quad (2)$$

In general, high values of P (positive $\log P$) occur for non-polar, membrane-permeant molecules; low values of P (negative $\log P$) occur for polar, membrane-impermeant molecules. Intermediate

⁵ Besides partition coefficient, other measures of compound polarity include the dielectric constant (ϵ , unitless), and the dipole moment (μ , debyes).

values of P may indicate a slowly permeating solute. $\text{Log}P$ values can be obtained at the SRC website, <http://www.syrres.com/what-we-do/databaseforms.aspx?id=386> by inserting the CAS# of the compound of interest. This website also gives water solubilities (in mg/L or ppm), which, when converted to mol/L, also relate to membrane permeability. In general, polar compounds will have high water solubility and low membrane permeability, whereas nonpolar compounds will have low water solubility and high membrane permeability.

POST-LABORATORY: QUESTIONS

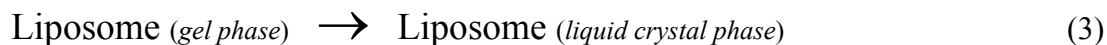
1. Describe or explain briefly (one paragraph or less)
 - a. the biochemical system that you worked with,
 - b. the chemistry of osmosis, and
 - c. the physics of light scattering/transmission.
2. In your Results section, include
 - a. exemplary plots showing your I_{tr} transients, and
 - b. a table giving fitted permeation rate constants (in min^{-1}) and ΔV_{max} (in mV) for all systems tested by the whole class.
3. If your I_{tr} vs. time trends are more complicated than a single first order exponential change, give a possible explanation.
4. Discuss your results, as well as those from the whole class. This is the heart and soul of your report for part 1, so please answer this question carefully and thoroughly. Note that your curve-fitting results give you information about both the kinetics (k) and thermodynamics (ΔV_{max}) of the permeation process. Using both prose and plots, explore how permeation kinetics and/or thermodynamics are influenced by
 - a. solute concentration,
 - b. solute (and water) polarity,
 - c. and perhaps even solute acidityYou may need to distinguish between
 - d. the permeability of water vs. that of osmolyte,
 - e. influx vs. efflux of permeant molecule, and
 - f. swelling vs. shrinkage of the liposomes.

Part 2: Lipid Phase Transition Probed by Diode Laser Light Scattering

INTRODUCTION

Phospholipid molecules have a glycerol backbone, two of whose OH groups are esterified to fatty acids, with the third esterified to a phosphate anion. The phosphate is also esterified to an R group which can be neutral or charged (mostly cationic). The fatty acids differ in their chain length (number of C atoms) and unsaturation (number of pi bonds). Other fairly common membrane lipids besides phospholipids include cholesterol and sphingolipids. As we discussed above, phospholipids self-assemble to form bilayers comprising hydrophilic polar head groups (glycerol plus phosphoester) on the two outer surfaces facing water, and hydrophobic fatty acid chains on the inside, protected from water. Aqueous suspensions of pure phospholipids can exist in many different structural phases; two of the most commonly observed phases are a pseudo-solid “gel” phase at low temperature and a pseudo-liquid “liquid crystalline” phase at high temperature. The *gel* phase features a well-organized, condensed, and somewhat rigid arrangement of hydrocarbon chains with trans-type configurations throughout. At higher temperatures, the *liquid crystal* phase loses this order: The hydrocarbon chains gain motional freedom, bend out of the trans-type configuration and move apart.

Conversion between these two states can be represented as a “melting” reaction:



This “melting” reaction is endothermic, as energy is required to break the London forces holding the well-organized hydrocarbon chains together. The change in entropy of the reaction is also positive, as fatty acid hydrocarbon chains in the liquid-like phase have much more freedom of motion than those in the semi-solid phase. As a result, just like true melting, the conversion from gel to liquid crystal becomes spontaneous at “high” temperatures, and the temperature at which the two phases exist in a stable 50/50 equilibrium is the transition temperature (T_{tr} or T_{melt}).

The gel to liquid-crystal phase transition temperature can be measured in a number of different ways, including differential scanning calorimetry (see Ohline *et al.*⁶) and Raman scattering.² In part 2 of this lab project we will use a novel method, laser light scattering, to identify the phase transition temperature of multilamellar liposomes. As you learned in the previous liposome experiment, an increase in liposome size causes an increase in the amount of light transmitted through a suspension, while a decrease in liposome size causes a decrease in transmitted light. You will use this information to determine T_{tr} and also to decide whether the gel phase or the liquid-crystal phase of a liposome is more expanded.

Finally, prior to the gel-to-liquid-crystal “melting” transition, a rippling of the liposome surface occurs, often a few degrees below the temperature at which the liposome undergoes the complete phase transition. The surface-rippling process is referred to as a pre-transition (see Ohline *et al.*⁶, Figs. 2 and 5), and is readily detected by laser light scattering because rippling of the liposome surface alters the liposome’s light scattering characteristics.

EXPERIMENTAL: LIPOSOME PREPARATION (PART 2)

This process will take about 1.5 hr, so plan your lab time accordingly.

Each team will make a single 3 mL aqueous liposome sample (167 μ M in total lipid) and incubate it at temperatures controlled by the Neslab temperature controller, monitoring changes in light scattering due to liposome swelling or shrinking. Although the total lipid concentration will be 167 μ M for each team’s liposome sample, the lipid composition will be different for each team (see Table 2 below). Your final liposome suspension will be 3.0 mLs of 167 μ M total lipid. In addition to the DSPC lipid-chloroform stock solution you used last week, others will be provided; be sure to confirm the concentrations of these solutions when you come in.

As you did in part 1,

- *calculate how many moles of each lipid you’ll need to make your liposome suspension.*
- *From moles needed and stock concentrations, calculate the volume of lipid-chloroform solution(s) you’ll need to make your liposome suspension.*

⁶ Ohline, S.M.; Campbell, M.L.; Turnbull, M.T.; Kohler, S.J. “Differential Scanning Calorimetric Study of Bilayer Membrane Phase Transitions: A Biophysical Chemistry Experiment” *J. Chem. Educ.* **2001**, 78, 1251-1256.

- *Check these calculations with your instructor.*

Table 2: Liposome lipid compositions and solvent for each student team.

<u>team</u>	<u>% DSPC</u>	<u>% chol.</u>
A	100	0
B	95	5
C	90	10
D	80	20
E	70	30
F	60	40
G	100% DPPC	0
H	100% DMPC	0
I	100% DMPE	0

Your procedure for making liposomes will be slightly different from that of part 1. Set up an 80 °C water bath next to the vortexer, allowing ≥ 30 min. for equilibration. Obtain from your instructor appropriate graduated glass Hamilton syringes³ to measure out the aliquot(s) of lipid-chloroform solution. Blow off the chloroform with a stream of nitrogen *in the hood* and pump off any remaining chloroform using the house vacuum for 20-30 minutes.

Set up an ice bath adjacent to the 80 °C hot plate. Measure out a 3.0 mL aliquot of 0.1 M KCl (by pipet or graduated cylinder). Warm the dried lipids in the 80 °C bath for a minute, then add ≈ 0.5 mL of the 0.1 M KCl taken from the 3.0 mL aliquot, and warm; next, vortex *vigorously* the warm dispersion. Add the remaining volume of the 3 mL aliquot of 0.1 M KCl to the lipid dispersion, vortex again, then return the lipid tube to the 80 °C bath and incubate for 3 minutes. Cool the lipid tube in the ice bath for 3 minutes, vortexing once or twice during the incubation. Repeat this cycle twice more: hot/cold, hot/cold, vortexing periodically. Your liposome suspension should be opalescent: slightly cloudy but not opaque. Unlike your liposome preparation for the permeability experiment (part 1), this week your liposome prep ends here – you will NOT be diluting this 3.0 mL liposome sample.

Instead, place this liposome suspension and a small magnetic spin bar in a crimp top vial. Put an aluminum cap with a white septum (shiny side down) on the vial, and then crimp the vial shut. Install the vial in the laser light scattering dewar with the assistance of a faculty member. Confirm that the magnetic spin bar is spinning stably and mixing the liposome solution well. The speed control knob for the stirring unit is a light blue dial on the lower right side of the dewar base.

EXPERIMENTAL: TEMPERATURE-CONTROLLED LIGHT SCATTERING MEASUREMENTS

You will run a LabView program called “V7 Liposome Phase Behavior.” This program allows you to change the Neslab temperature, open and close the electronic shutter in front of the diode laser, read the detector signals, and set up an automated data collection run. Confirm that the laser light transmitted through the vial reflects off of the corner of the square mirror and makes it through the iris in front of the photodetector. If the light instead strikes the iris, then adjust the tilt controls on the mount for the square mirror until the laser makes it through the iris and the transmitted intensity displayed on the computer is maximized.

Using the software, prepare an automated data collection run to go from about 8°C below the literature value⁷ of the phase transition temperature of your lipid mixture, to about 5°C above, in 0.1 °C steps. Set the delay equilibration time to be 240 s. If you are running overnight, see if there will be sufficient time to collect data by first increasing and then decreasing the temperature (↑↓) rather than just increasing the temperature (↑). Enter an appropriate file name for your data, and then start the data collection. When the run is complete, remove your crimp top vial from the instrument with the assistance of a faculty member. Uncap the vial by breaking the aluminum ring; clean and return the stir bar, and discard the glass vial and cap.

⁷ Lipid phase transition temperature literature values can be found: (a) in the papers cited in the lab manual; (b) in Prof. Silverstein’s “CHEM 351 Handouts” booklet under “Lipid Phase Transitions”; and (c) at the Avanti Polar Lipids website:

http://avantilipids.com/index.php?option=com_content&view=article&id=1700&Itemid=419

If your lipid contains **cholesterol**, keep in mind that it can lower T_{tr} by up to 4 °C for compositions up to 20-25 mol% cholesterol. Above 25-30 mol% cholesterol, the gel/liquid crystal phase distinction disappears, and lipid packing increases as a smooth function of temperature. {Chong and Choate, *Biophys. J.* **55**, 551-556.}

DATA ANALYSIS:

The data file will have three columns – the temperature reported by the thermistor probe, the transmitted light intensity, and the scattered light intensity collected at a small angle relative to the transmitted light beam. Convert all temperature values to Kelvin, and generate graphs of transmitted light (V or mV) versus $T(K)$ and small-angle scattering versus $T(K)$. Be sure to label the plots clearly, and distinguish between the warming and the cooling curve (different point symbols, colors, labels, etc.).

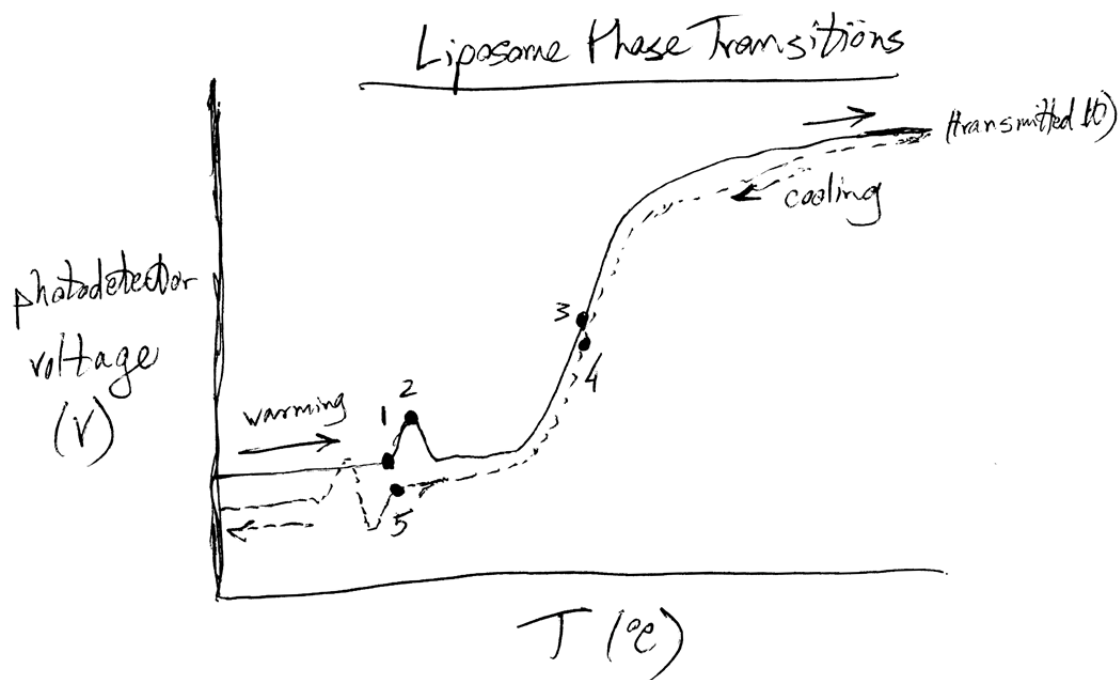
From each curve you will determine five characteristic temperatures, as outlined below. The most well-defined temperature will be that of the main gel/liquid crystal phase transition (T_{tr} , also called T_m or T_{melt}). This is the temperature at which the two phases are in a stable 50/50 equilibrium. T_{tr} can be determined by eye from the curve (see Figure below), but a more informative method is to fit the data to the equation (see Appendix 2 for a derivation):

$$y = \frac{y_{\min} + y_{\max} \cdot \exp\left[\frac{\Delta H_{tr}^{\circ}}{R} \left(\frac{1}{T_{tr}} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H_{tr}^{\circ}}{R} \left(\frac{1}{T_{tr}} - \frac{1}{T}\right)\right]} \quad (4)$$

Using Kaleidagraph, you will have four fittable parameters: $m1 = y_{\min}$; $m2 = y_{\max}$; $m3 = \Delta H_{tr}^{\circ}$; and $m4 = T_{tr}$ (in K); also, $m0 = T(K)$.⁸ The fitted value of ΔH_{tr}° is in units of kcal (or kJ) per mole of cooperative thermal phase transition units.⁹ Each cooperative melting unit comprises n lipid molecules, where n can vary up to 100, depending on the lipid and the experimental conditions. The value of ΔH_{tr}° in units of kcal/mol lipid thus = $\Delta H_{tr}^{\circ}(\text{fit})/n$. Some literature values of ΔH_{tr}° in units of kcal/mol lipid can be found in Gennis's *Biomembranes*⁹ and elsewhere. Knowing the literature value of ΔH_{tr}° per mole of lipid (from Gennis⁹) and ΔH_{tr}° per mole of cooperative phase transition units, you can calculate n , the number of phospholipid molecules per cooperative melting units.

⁸ Temperature must be in Kelvin because of the units of R , the universal gas law constant.

⁹ Gennis, R.B. *Biomembranes: Structure and Function* Springer-Verlag, NY, 1989; p. 66-68.



From each of your curves, estimate the following temperatures (see accompanying figure above), with associated uncertainties and correct significant figures:

1. T_{initial} for the pre-transition in both the warming and cooling curves; in the accompanying figure, T_1 and T_5 , respectively;
2. T_{max} for the pre-transition in the warming curve (T_2), and
3. T_{trans} for the main phase transition in both the warming and the cooling curve (T_3 and T_4 , respectively).
4. Calculate average \pm std. dev. values for
 - a. $T_{\text{pre-transition, initial}}$ and for
 - b. $T_{\text{main transition}}$.
5. Calculate $\Delta T = T_{\text{main transition}} - T_{\text{pre-transition, initial}}$.
6. Report $\Delta H_{\text{tr}}^{\circ}(\text{fit})$ and n (value \pm uncertainty) for both the warming and cooling curves. Provide a reference to the exact pages in your laboratory notebook where sample calculations can be found for your reported value of $n \pm$ uncertainty.
7. Post your results in the class Google doc spreadsheet, before the deadline stipulated on your class schedule sheet.

POST-LAB QUESTIONS:

8. *Do liposomes swell or shrink during the gel to liquid-crystal “melting” phase transition? Which phase is more condensed? Does this match your expectation? If so, how; if not, why not?*
9. *Discuss trends discerned from results of the whole class. How does T_{tr} vary with head group? Fatty acid chains? Cholesterol content? Does this make sense?*
10. *Provide a Summary paragraph as described in the introductory section of the lab manual, “Writing in the Chemistry Laboratory: The Informal Report.”*

FURTHER READING:

“Kinetics of Solute Permeability in Phospholipid Vesicles”

Bittman, R. and Blau, L. (1976) *J. Chem. Educ.* **1976**, 53, 259-261.

“Investigation of Model Cell Membranes with Raman Spectroscopy: A Biochemistry Laboratory Experiment”

Craig, N.C.; Fuchsman, W.H.; Lacuesta, N.N. (2003) *J. Chem. Educ.* **80**, 1282-1288.

“Differential Scanning Calorimetric Study of Bilayer Membrane Phase Transitions: A Biophysical Chemistry Experiment”

S. M. Ohline, M. L. Campbell, M. T. Turnbull, and S. J. Kohler (2001) *J. Chem. Educ.* **78**, 1251-1256.

“Calorimetric studies of the effects of cholesterol on the phase transition of C(18):C(10) phosphatidylcholine”

P. L.-G. Chong and D. Choate (1989) *Biophys. J.* **55**, 551-556.

“Phase Transitions of Lipid Membranes” Moscow State University

Appendix 1: Liposome Solute Permeability Data Analysis/Curve Fitting

Load your Excel data files onto your H: drive. Open each file and resave it in format: “Excel Workbook” (i.e., .xls) instead of “Text (Tab delimited)”. Make a third data column to convert all I_{tr} readings from V to mV.

Select all data and make a scatter plot of I_{tr} (mV) vs. t (s). You may wish to clean up this plot by: (a) double-clicking on the “Plot Area” and changing “Fill: Color” to “no fill”; (b) double-clicking on a single data point and deselecting “Marker: shadow” – uncheck the box; and (c) double-clicking on an x-axis numerical value and selecting “Number” then under “Category:” select “Number” and decrease the number of decimal places to zero.

You’ll note that there are four phases in your plot: (1) the initial values of your plot are zero (shutter closed); followed by (2) a series of constant values around 700 mV (shutter open, buffer only in cuvet). (3) After this, I_{tr} falls precipitously as the pipet tip is inserted into the cuvet, blocking the light beam, and liposomes are added, then stirred. (4) The data series that is of experimental interest follows the precipitous decline in I_{tr} ; this smooth curve represents changes due to permeation. To examine data in this fourth phase more carefully, double-click on the y-axis, select “scale”, and choose y (min) and y (max) such that only the points in this smooth curve comprising the fourth phase are shown on the plot. Note whether the I_{tr} values: (a) increase exponentially (light scattering declined, hence liposomes swelled); (b) decrease exponentially (light scattering rose, hence liposomes shrank); or (c) show a more complex change, e.g. double exponential (fast then slow), or exponential followed by linear.

Move the cursor over the first point in the fourth phase, and note its x,y values. Now in the spreadsheet, select the two columns [t (s) and I_{tr} (mV)], from the first point of the fourth phase to the last, and copy these values. Open a Kaleidagraph data file, and paste these values into the Kaleidagraph spreadsheet. Label the columns “ t , s” and “ I (tr) name, mV”. The “name” here will describe the buffer in the cuvet, e.g., KCl 1M, EtOH 2M, water, etc.

In the Kaleidagraph menu at the top, select “Gallery/Linear/Scatter”, and select the “t, s” values for the x -axis, and the “ $I(t_r)$, mV” values for the y -axis. Adjust the resulting Kaleidagraph plot if you need to: remove grid lines, add an informative title, alter axis legends if necessary, etc.

Now fit these values to the proper equation for first order kinetics, or if necessary, something more complex.¹⁰ Recall that the equation for first order exponential **decline** from an initial voltage V_0 to zero is: $V_t = V_0 \cdot e^{-kt}$. Your I_{tr} transients do not decay to zero, but rather to a finite final voltage at time infinity = V_f . The equation for such a decline is:

$$V_t = V_f + \Delta V_{\max} \cdot e^{-kt} \quad (5)$$

Where ΔV_{\max} is the difference between V_0 and V_f . You may fit such a first order exponential decline using Kaleidagraph. Under “Curve Fit/General/Edit General”, “add” a new fit, click on that new fit in the left hand box, and rename it in the bottom left hand box, e.g., “1st order fall to Yf”. Click “Edit”, and type in the fit equation:

$$(Y =) \quad m3 + (m2 * \exp(-m1 * m0)); m3=100; m2=100; m1=0.005$$

Where $m3 = V_f$, $m2 = \Delta V_{\max} = V_0 - V_f$, and $m1 = k$, the first order rate constant (units = s^{-1}). The initial fit values used here would be for a curve with $m3 = V_f \approx 100$ mV, $m2 = \Delta V_{\max} \approx 100$ mV (i.e., $V_0 \approx 200$ mV), and $m1 = k \approx 0.005$ s^{-1} . If Kaleidagraph cannot fit your data using these initial values, alter them based on a careful examination of your I_{tr} vs. t curve. Recall from first order kinetics that $k = \ln(2)/\tau_{1/2}$, where $\tau_{1/2}$ = the half-time for the exponential change in I_{tr} . You must examine your curve to estimate $\tau_{1/2}$, then calculate from this an initial fit value for k .

Once you type in the equation, click “OK” and “OK”, then select this curve fit under “Curve Fit/General”, click the column name with your y -values, and “OK”. You should get a red line fit, and a red fit box with best fit values (plus uncertainties) for $m3$ (V_f), $m2$ ($\Delta V_{\max} = V_0 - V_f$), and $m1$ (k). Please convert the value of k , as well as its uncertainty/error, from sec^{-1} to min^{-1} units; the value of ΔV_{\max} , as well as its uncertainty/error, should already be in mV units. Note

¹⁰ For example, if you observe a first order change followed by a linear region, the full equation for the fit would be the first order equation + $m4 * x$.

that if R^2 is less than about 0.5, you're not using the correct fit equation for your data. See the instructor and look more closely at your curve.

If your curve depicts a first order exponential **rise** in I_{tr} , from V_0 to V_f , you may use the same equation to fit your data. In this case, ΔV_{max} will be negative.

Appendix 2: Phase Transition Temperature (T_{tr}) Data Analysis

From equation (2), the equilibrium constant for the gel to liquid crystal phase transition is given by

$$K_{tr} = \frac{[PL(\text{liquid crystal})]_{eq}}{[PL(\text{gel})]_{eq}} \tag{6}$$

The phase transition heating curve features a change in some measured y value (e.g., photodetector voltage) that tracks the change in lipid phase with temperature. The curve is sigmoidal: y_{min} at low T is characteristic of the pure gel phase; as T rises, y rises sigmoidally, asymptotically approaching y_{max} , which is characteristic of the pure liquid crystal phase. Given y at any particular T , $[PL(\text{liquid crystal})]_{eq}$ is proportional to $y - y_{min}$, and $[PL(\text{gel})]_{eq}$ is proportional to $y_{max} - y$. Thus the phase transition equilibrium constant can be written as

$$K_{tr} = \frac{[PL(\text{liquid crystal})]_{eq}}{[PL(\text{gel})]_{eq}} = \frac{(y - y_{min})}{(y_{max} - y)} \tag{7}$$

which can be rearranged to

$$y = \frac{(y_{min} + y_{max}K_{tr})}{(1 + K_{tr})} \tag{8}$$

At $T = T_{tr}$, $\Delta G^\circ_{tr} = \Delta H^\circ_{tr} - T_{tr}\Delta S^\circ_{tr} = 0$ (9)

Rearranging, $\Delta S^\circ_{tr} = \Delta H^\circ_{tr}/T_{tr}$ (10)

So the Gibbs Free Energy Law becomes

$$\Delta G^\circ_{tr} = \Delta H^\circ_{tr} - (T/T_{tr})\Delta H^\circ_{tr} = \Delta H^\circ_{tr}(1 - T/T_{tr}) \tag{11}$$

Also, $K_{tr} = \exp(-\Delta G^\circ_{tr}/RT) = \exp[(\Delta H^\circ_{tr}/RT)(T/T_{tr} - 1)] = \exp[(\Delta H^\circ_{tr}/R)(1/T_{tr} - 1/T)]$ (12)

Combining equations (8) and (12), we get,

$$y = \frac{y_{min} + y_{max} \cdot \exp\left[\frac{\Delta H^\circ_{tr}}{R} \left(\frac{1}{T_{tr}} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H^\circ_{tr}}{R} \left(\frac{1}{T_{tr}} - \frac{1}{T}\right)\right]} \tag{13}$$