Fluorescent Tagging of Liposomes

Objective: This lab examines the liposome as a carrier of fluorescent tags used in cancer identification. The liposomes will be created from raw materials, tagged, extruded to a proper size, filtered, and characterized.

BACKGROUND MANUFACTURING AND TAGGING

A liposome is an artificially-prepared vesicle composed of a lipid bilayer and an aqueous core. As shown in Figure I, the liposome has an aqueous core, a fatty acid boundary, and an outer boundary that is attracted to water. As shown in Figure I, the liposome can be used in vivo to deliver drugs, fluorescent tags, DNA/RNA, or nutrients. Hydrophilic drugs/nutrients/tags can be trapped in the central aqueous core of the liposomes, lipophilic drugs can be solubilized within the lipid bilayer, and material can be attached to outer core of the liposome. This experimental section of this lab will be used to verify where fluorescent tags reside in these boundaries.

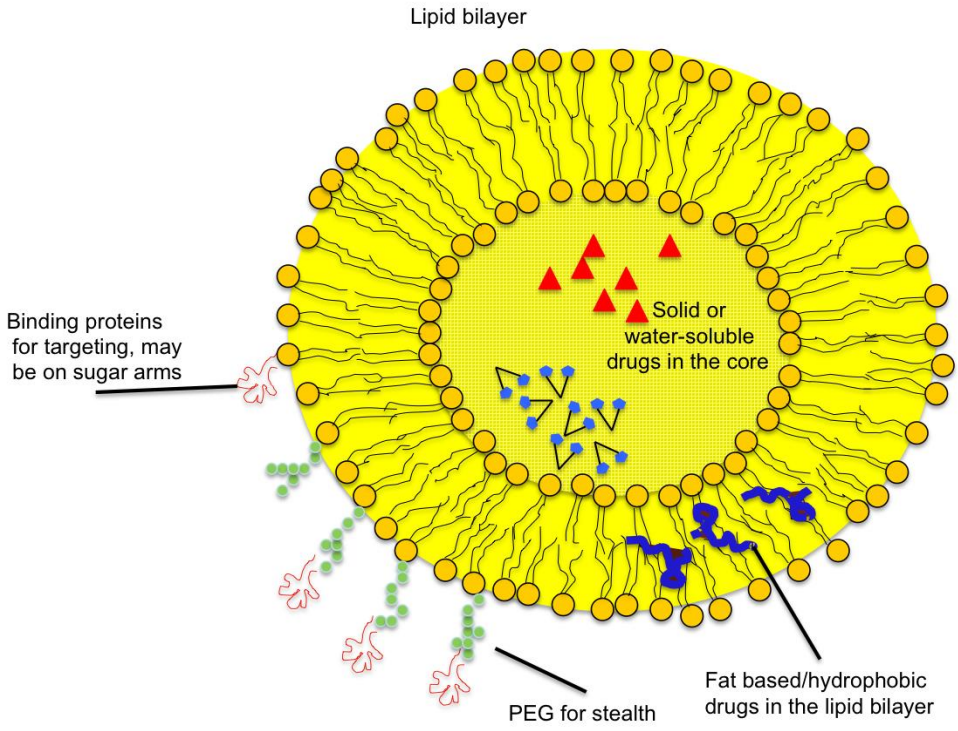

Figure I: Liposome with boundaries

Liposomes have many desirable attributes such as, they are biodegradable, they can incorporate both water and fat soluble drugs or nutrients, and liposomes can be used to deliver tagging molecules. This tagging can reside inside the liposome and/or in the

* Further information can be found in the Appendix section

lipid shell of the bilayer, or on polymers bonded to the liposome shell. Liposomes are in clinical use because they can preferentially deliver these tags and drugs to tumor sites.

In non-cancerous tissue healthy human blood vessels are encapsulated by endothelial cells that are bound together by tight junctions. These tight junctions stop any nanoscale particles in the blood from leaking out of the vessel. So, healthy vasculature prevents small nanoparticles such as liposomes from leaking into healthy tissue. Tumor vessels do not contain the same level of seal between cells and are diagnostically "leaky". This deficiency is known as the Enhanced Permeability and Retention (EPR) effect. Tumors are porous and small particles can permeate into these defects. So liposomes under 100 nm in diameter are a popular option to passively target neoplasms. Therefore healthy tissue will keep out small particles such as fluorescently tagged liposomes, and tumors will allow passive inclusion of these tagged liposomes. Due to this scale, we will use chromatography to determine where the florescent tag resides in the liposome.

The basic method to create tagged liposomes is to start with commercially available lipid powder. The powder is mixed with an organic solvent, and then this organic solvent is evaporated under low temperature. This forms a lipid cake in the bottom of the flask. An aqueous solution containing a tag or drug material is added to the lipid cake along with water to hydrate the lipids and form a wide distribution of liposome sizes and types as shown in Figure II.

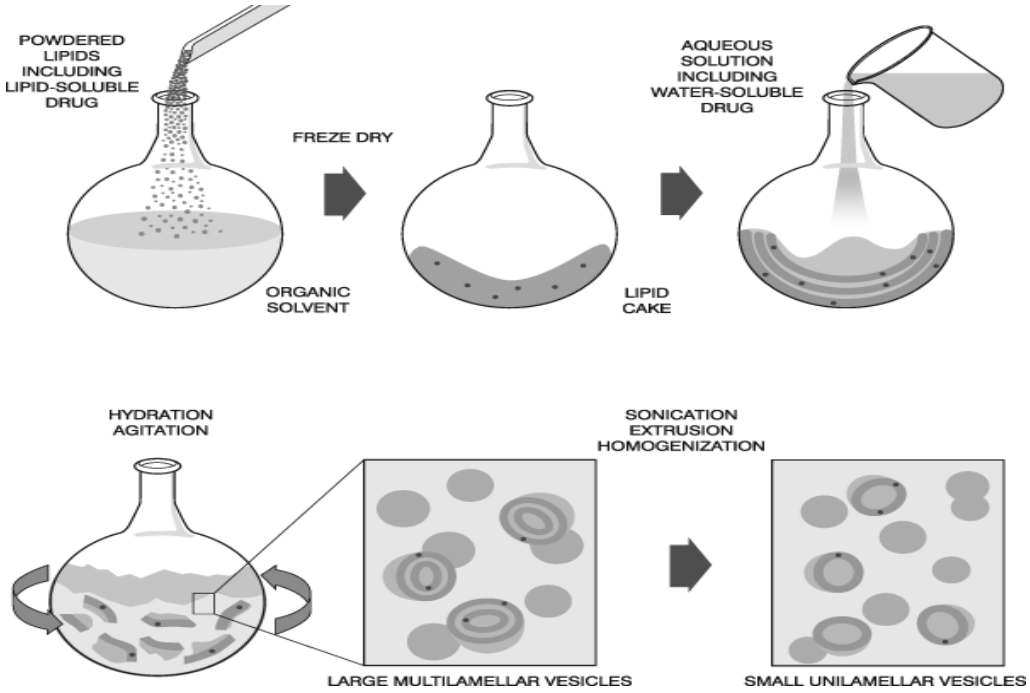

Figure II. Liposome preparation and hydration procedure

* Further information can be found in the Appendix section

As shown in Figure III, when the dry lipid film is hydrated, the lipid cake layers will begin to swell. Upon agitation of the hydrated cake layers, multilamellar vesicles (MLV's), large unilamellar vesicle (LUV's), and small unilamellar vesicles (SUV's)can form. The various sizes and types of liposomes can be extruded down to the desired nm sizes through a commercially available extrusion unit

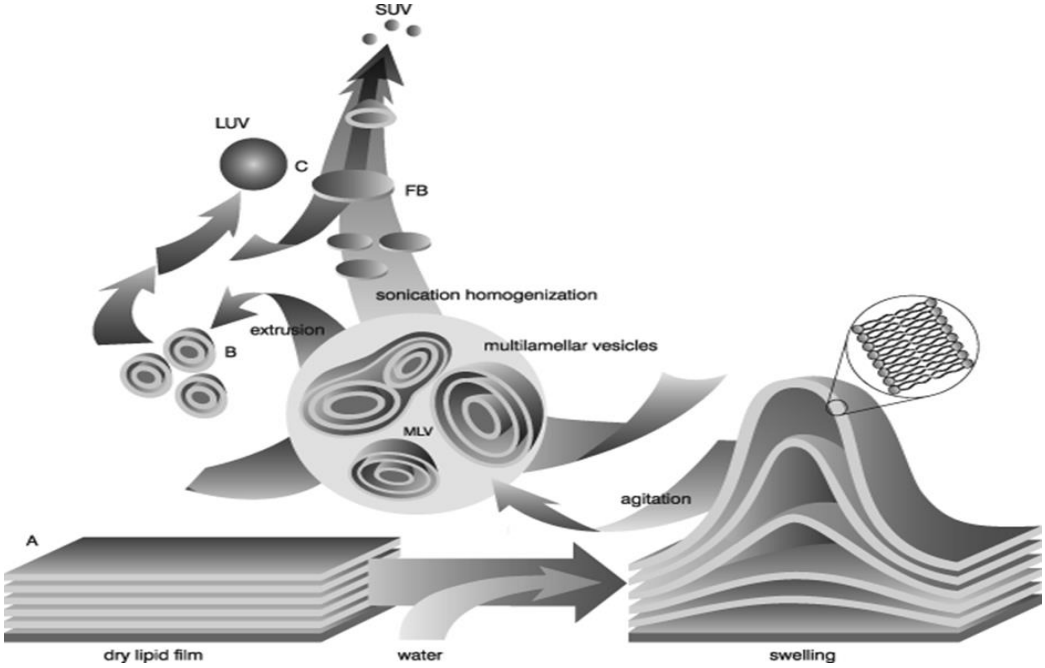

Figure III. Production of lmv's through hydration and agitation of lipid solution

EXTRUSION

Lipid extrusion is a technique in which a lipid solution is pushed through a polycarbonate filter that has a certain pore size to give the experimenter a particle size that is desired. The Avanti mini-extruder is a tool that performs lipid extrusions to produce a distribution of sizes that will be used to resize the liposomes into small unilamellar vesicles (suv). This tool shown in Figure IV can either use 1 mL or 250 µl syringes that push the lipid solution back and forth through a porous polycarbonate membrane filter that will resize the liposome. Avanti offers the PC membranes in 30, 50, 80, 100, 200, 400, 800, and 1000 nm sizes. This experiment will be using a 100 nm PC membrane to give 100 nm suv's. Another key feature of this tool is the heating block that allows the extrusion to be kept at elevated temperatures to match the transition temperature of the liposome. The figure below shows the internal component of the Avanti mini-extruder and where the filter supports are placed relative to the polycarbonate membrane that will be used.

* Further information can be found in the Appendix section

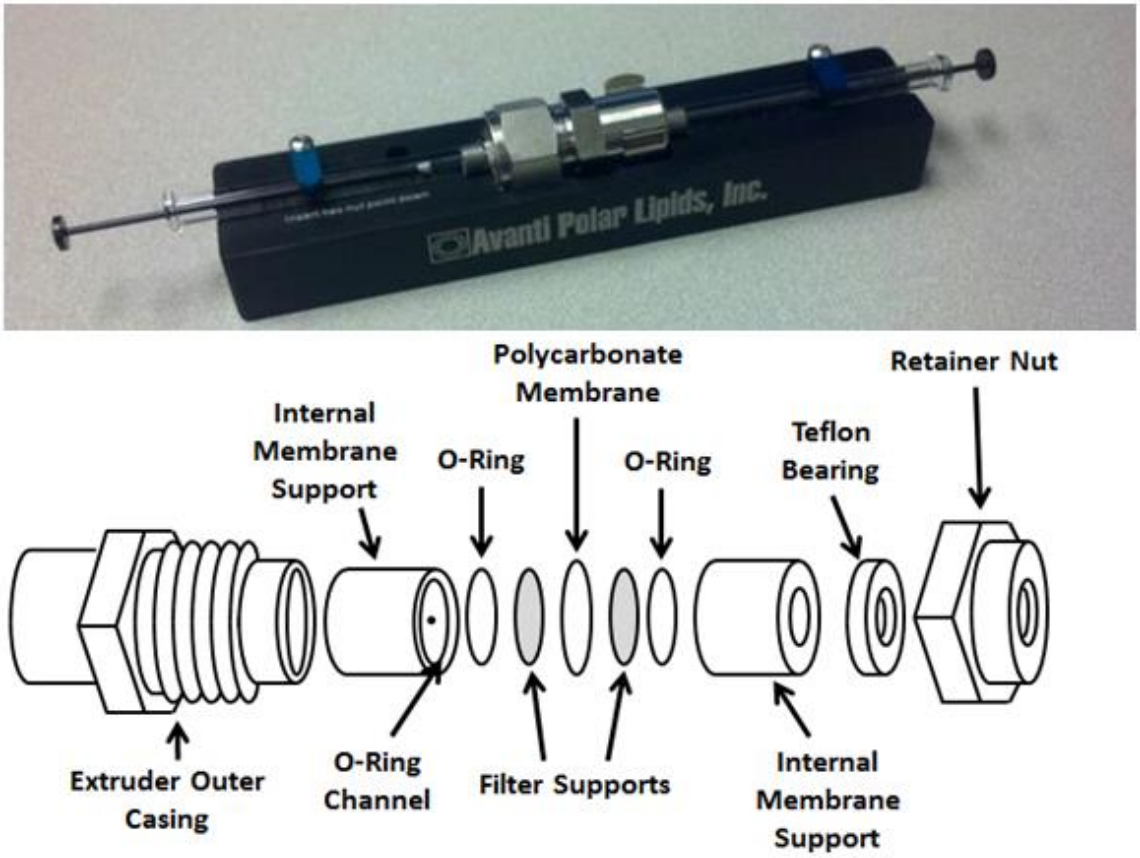

Figure IV. Avanti Mini-Extruder with internal component part setup.

LIPOSOME FILTERING

Chromatography is defined as a technique used to separate mixtures. The various constituents of the mixture travel at different speeds through some type of "filter" depending on the type of chromatography, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation. There are many different types of chromatography that can be performed on a mixture. Several main types of chromatography used can be seen in Table I, which include gas*, affinity*, ion exchange* and gel chromatography*. Gel chromatography is appropriate for the liposome separation since it allows different sized particles to elute down the column at different speeds based on size. The reason for filtering the mixtures of liposomes through this method will show whether the liposome dye is membrane bound or is encapsulated inside the aqueous core. This can be answered by filtering particle sizes through the column. The liposomes filter down through the column first because they have a larger molecular weight than the dye. So if the dye is membrane bound than the experimenter

* Further information can be found in the Appendix section

should see a single band filtering through the column. On the other hand, if the dye is encapsulated in the aqueous core of the liposome then two bands should be present. This is because the dye that is not encapsulated will filter slower than the liposomes with the dye in the aqueous core. Their color should be similar but the molecular size will be different.

Types of Chromatography	
Gas Chromatography	Mixture is vaporized and carried by an inert carrier gas (usually helium or nitrogen) through a gas chromatograph.
Affinity Chromatography	Process in separating biochemical mixtures based on a specific interaction between a receptor and ligand, antigen and antibody, or a enzyme and substrate.
Ion Exchange Chromatography	Process that allows the separation of polar molecules and ions to be separated by their charge.
Gel (Size Exclusion) Chromatography	Column is packed with a permeable gel that will separate a liquid by sieving or molecular filtration action.

Table I. Four different types of chromatography.

Gel chromatography, otherwise known as size exclusion chromatography, separates based solely on the molecules sizes when they travel through the column packed with polymer beads. The larger molecules filter through the column faster than molecules of a smaller size. The reason this occurs is because the beads themselves are made of a cross-linked structure with different pore sizes. When the solution travel down through the beads, the smaller molecules will enter the pores and travel through the bead structure as shown in Figure V. Since the larger molecules don't enter the pores, they are able to travel down the column a lot quicker than the smaller molecules. Also if there are molecules of intermediate size, those molecules will enter the beads to some extent but will still travel down the column faster than the smaller molecules since they don't travel as long through the beads. This method of column chromatography is primarily used to show distribution of molecular weights as well as the purification of the unknown molecules or proteins. Since we are seeking to separate liposomes with the fluorescent tag from the dye, this method will give us the separation that is desired. Because the liposomes with the encapsulated dye will have a higher molecular weight than the unencapsulated carboxyfluorescein, therefore allowing the separation of the mixture that is desired.

* Further information can be found in the Appendix section

Figure V below shows the gel chromatography process with the glass column packed with a small ball of glass wool and the gel (Sephadex). The glass wool serves two purposes for this filtration process. The first is that it allows the gel to pack in the column, where it will be used for separation. The second reason is to keep the gel beads from being filtered out the column, since the swollen gel beads are too large to escape through the wool. Figure V also shows how the column is packed, where the solvent is placed, and the clarification of the mobile and stationary phase.

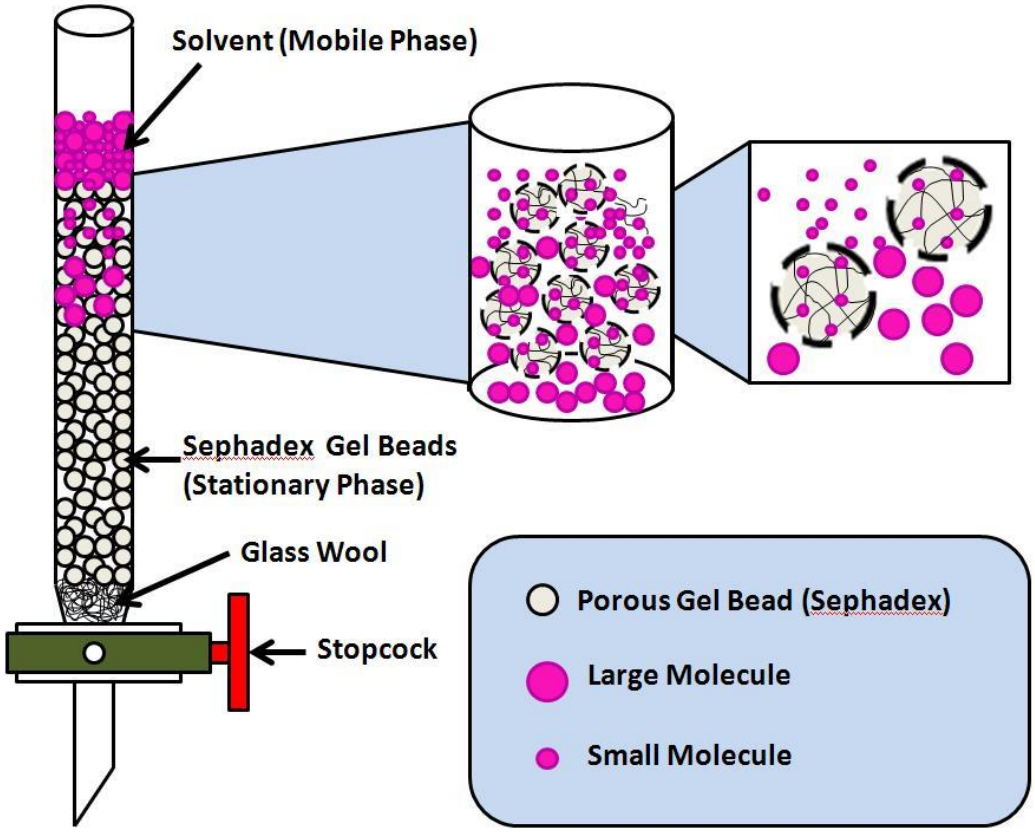

Figure V. Gel chromatography experimental column assembly

The Sephadex* gel beads used for this experiment are polymer beads created by cross linking dextran (complex, branched glucan) with epichlorohydrin (an organochlorine compound and an epoxide). These gel beads have pore sizes that allow small particles to pass through them while not allowing larger particles. This causes smaller molecules to take longer to filter through the column than larger molecules, thus separating the larger and smaller molecules through filtration.

* Further information can be found in the Appendix section

CHARACTERIZATION

Once the liposome fractions are collected from each of the column filtrations, the vials with the most liposomes will be determined with a laser light deflection to show which vials had the strongest concentration of liposomes containing the dye. Those vials will then be characterized with a Malvern Zetasizer Nano to determine the size distribution that our extrusions had on the liposomes.

The Malvern Zetasizer Nano* that can be seen in Figure VI, has the capabilities to measure molecular weight, electrophoretic mobility, zeta potential, size, and microrheology by using various types of light scattering methods. This characterization tool can measure particles and molecules from less than a nanometer in size to several microns. Since the liposomes for this experiment are desired to be 100 nm in size, this tool will give a size distribution of the variation of liposomes in the sample solution and thus why it is used in this experiment.

Figure VI. Malvern Zetasizer for characterizing the size of the liposome

The Zetasizer Nano uses a laser for dynamic light scatting detection in order to determine particle size by measuring the speed at which those particles are diffusing due to Brownian movement in the solution. Through measuring the intensity rate change through a solution, the zetasizer can send that information through a detector and into a correlator. This correlator, will then use various algorithms based on the light detected from the detector to determine the size distribution based on the relative intensity of scattered light of various sized particles. The laser path can be seen in Figure VII. Depending on the model of the Zetasizer, it could either have a detector positioned at an angle of 90˚ or 173˚.

* Further information can be found in the Appendix section

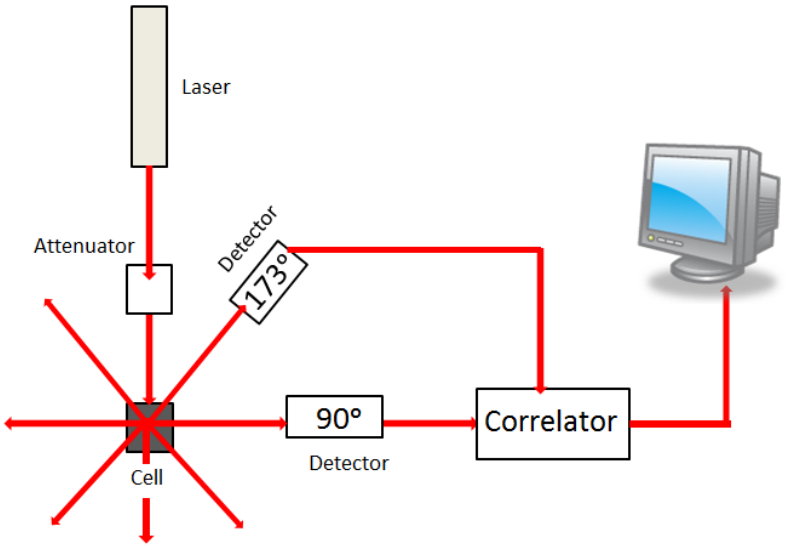

Figure VII. Malvern Zetasizer laser path for dynamic light scattering detection

The zetasizer will be used to examine 3 samples that were passed through the Avanti mini-extruder with different number of passes. The zetasizer will use dynamic light scattering and zeta potential to determine size of the liposomes as well as show a size distribution of the liposomes in the vial. An example of a size distribution chart can be seen in Figure VIII below.

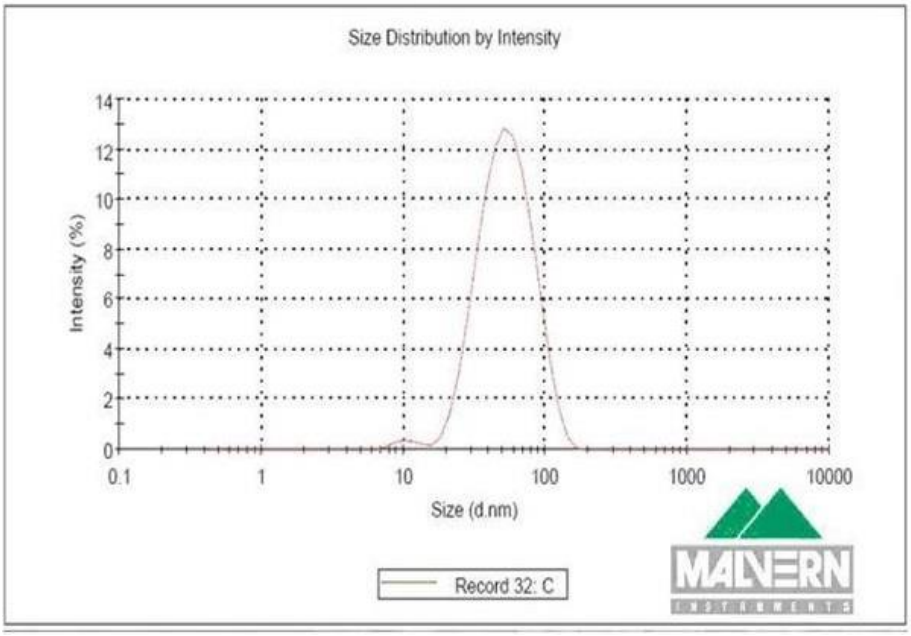

Figure VIII. Example of a particle size distribution

* Further information can be found in the Appendix section

Experiment: This lab will examine tags in the aqueous core and the fatty wall.

Liposome Gel Filtration Procedure

Equipment:

Avanti mini-extruder (Avanti Polar Lipids), Glass column, Sephadex G-50, glass wool, 5mg DOPC, 5mg DOPC/Nile Red, Carboxyfluorescein solution, water, support stand with clamp

Procedure: This experiment will contrast two sets of liposomes with tags in the aqueous core or tags in tags in the lipid bilayer of the liposome. Sample A will have the dye inside the liposome bilayer while sample B will have the tag in the aqueous core. Below in Figures IX and X, shows the general block diagram of the procedures that need to be done for sample A and B.

STEP	PROCEDURE
1	Obtain a scale and measure out ~3g of Sephadex G-50 in a beaker or flask with water. Use excess water to completely cover the Sephadex because the beads will swell. Sephadex can be hydrated overnight in a refrigerator, but 1-2 hours of hydration at ambient temperature should be sufficient. 3g of Sephadex is enough to fill the 10"x1/2" column
2	For sample A, add 1ml water (DI, sterile, distilled, or purified) to one vial of 5mg DOPC/Nile Red, and allow it to hydrate at ambient temperature for about 1hour.
3	For sample B, add 1ml carboxyfluorescein to one vial of 5mg DOPC, and allow it to hydrate at ambient temperature for about 1 hour.

(VIDEO OF WEIGHING OUT AND HYDRATING THE SEPHADEX and LIPOSOMES

http://www.engr.psu.edu/mediaportal/flvplayer.aspx?FileID=b04b651a-71b0-460e-a90a-8

STEP	PROCEDURE
4	Assemble the Avanti mini-extruder with a 0.1um membrane according to the provided instruction. This membrane should provide 100 nm liposomes using the appropriate number of extrusion passes.

(VIDEO OF MINI EXTRUDER ASSEMBLY)

* Further information can be found in the Appendix section

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STEP	PROCEDURE
5	Once sample A has hydrated, use a 250µl syringe to extrude the sample 11 passes through the mini-extruder. (Note that 11 passes will leave the sample on the "clean side' of the extruder.) Repeat step one more time to obtain a total of 0.5ml of sample A.

(VIDEO OF EXTRUSION SAMPLE A)

http://www.engr.psu.edu/mediaportal/flvplayer.aspx?FileID=8d9e54f3-795e-43d6-a935-a

STEP	PROCEDURE
6	Disassemble the mini-extruder and rinse the parts and syringes with clean water and dry extruder parts.

(VIDEO OF SYRINGE AND EXTRUDER CLEANING)

http://www.engr.psu.edu/mediaportal/flvplayer.aspx?FileID=5e5e3f18-1388-44c7-b0b3-1

STEP	PROCEDURE
7	Once sample B has hydrated, use a 250µl syringe to extrude the sample 11 passes through the mini-extruder. (Note that 11 passes will leave the sample on the "clean side' of the extruder.) Repeat step one more time to obtain a total of 0.5ml of sample A.

SAMPLE B WAS EXTRUDED THE SAME WAY AS SAMPLE A

* Further information can be found in the Appendix section

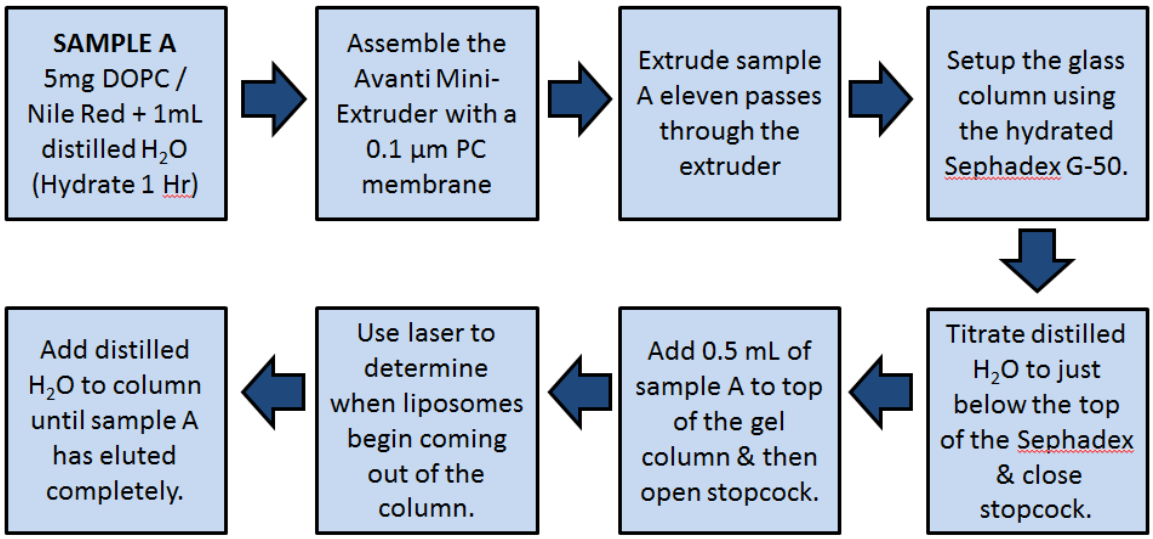

Figure IX. Block diagram of sample A procedure

8	Add a small piece of glass wool to the glass column, and push it down into the column near the point where the column narrows just above the stop cock. About a ½" diameter ball (loosely balled) of glass wool is sufficient.
9	Attach the column vertically to a support stand with a clamp.
10	Pour the hydrated Sephadex gel into the column being careful to minimize air bubbles in the column. Open the stop cock and allow some of the water to run out which will help the gel to settle. Add more gel as needed until the column is almost fully packed with Sephadex. Do not completely fill the column; leave a 1/2" of space from the gel to the top of the column. When the column is packed, turn off the stop cock to stop the flow. **Note: Do not allow the column to run dry. This will create air pockets which will decrease the effectiveness of the gel filtration. The Sephadex needs to be kept wet until the experiment is complete. If needed, add water to the top of the column.**
11	Once all of the resin has settled, open the stop cock and allow the water level to decrease until just below the top of the resin. Close the stop cock.

(VIDEO OF GEL CHROMATOGRAPHY COLUMN SETUP)

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STEP	PROCEDURE – SAMPLE A
12	Slowly add the 0.5ml of sample A to the top of the gel column. Add the volume and all following volumes carefully to try not to disrupt the gel.
13	Open the stop cock and allow the sample to elute down the column. Add water to the top of the column as needed.

* Further information can be found in the Appendix section

14	This sample has a membrane bound dye, so the sample should elute down the column as a single band.
15	A laser can be shined through the drops coming out of column to determine when the liposomes begin coming off the column. The laser will shine through fractions containing only water, and the laser will be diffracted through samples that contain liposomes.
16	Collect the fractions into test tubes and allow the entire sample to elute off of the column to prepare for the next sample. Add water to the top of the column as needed.

(VIDEO OF CHROMATOGRAPHY OF SAMPLE A)

http://www.engr.psu.edu/mediaportal/flvplayer.aspx?FileID=77554e4e-df9c-416a-9577-8

17	Once the entire DOPC/Nile Red sample has eluted from the column, open the stop cock and allow the water level to decrease until just below the top of the resin. Close the stop cock.

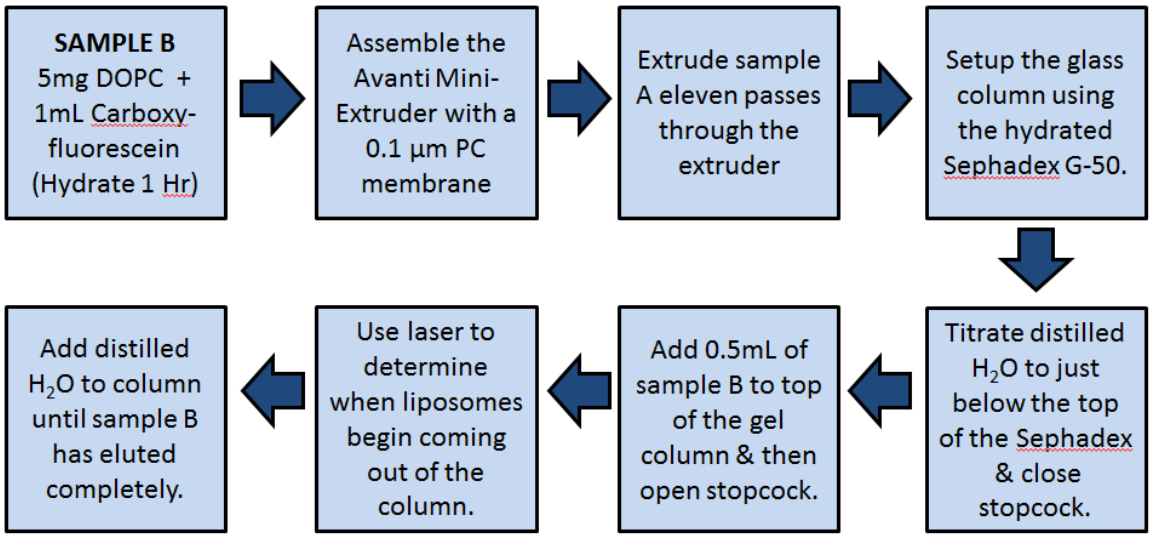

Figure X. Block diagram of sample B procedure

* Further information can be found in the Appendix section

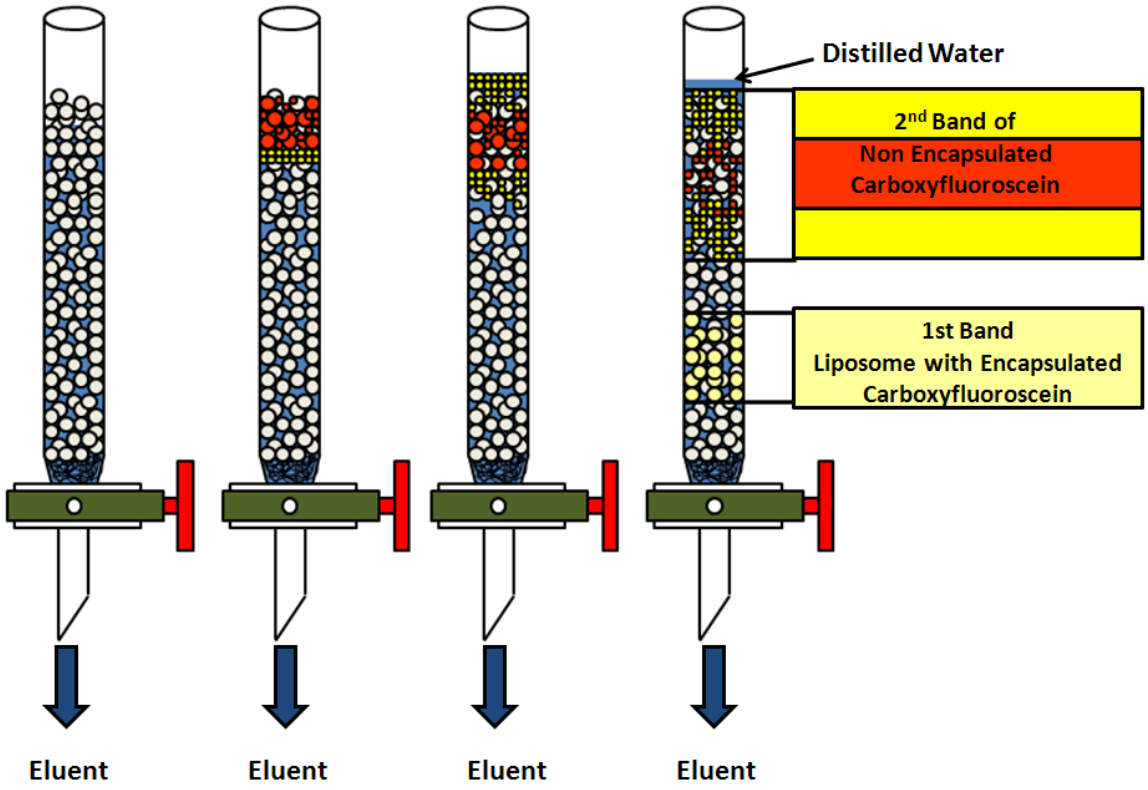

Figure XI. Sample B eluting down the gel column.

STEP	PROCEDURE – SAMPLE B
18	Once sample A has fully eluted off of the column and the distilled water is just below the top of the resin, add 0.5ml of sample B to the column. Once again make sure not to disrupt the gel when sample B is poured into the column.
19	Open the stop cock and allow the sample to elute down the column. Add water to the top of the column as needed.
20	This sample has is self-quenching and will encapsulate the carboxyfluorescein inside the liposomes aqueous core. This sample should elute down the column in two bands. The first band will be the liposomes with the carboxyfluorescein (amber color) and the second band will only be carboxyfluorescein that was not encapsulated (Darker amber color). Both of the bands should be separated by a thin clear layer of water.
21	A laser can be shined through the drops coming out of column to determine when the liposomes begin coming off the column. The laser will shine through fractions containing only water, and the laser will be diffracted through samples that contain liposomes.
22	Collect the fractions into test tubes and allow the entire sample to elute off the column.

* Further information can be found in the Appendix section

(VIDEO OF CHROMATOGRAPHY OF SAMPLE B)

http://www.engr.psu.edu/mediaportal/flvplayer.aspx?FileID=3032c274-73b1-4771-99a4-1

STEP	PROCEDURE
23	Take the test tubes of sample A and B that that contain liposomes and add dawn dish soap.
24	Clean the column, all parts used during the experiment and place back into correct locations.

Results

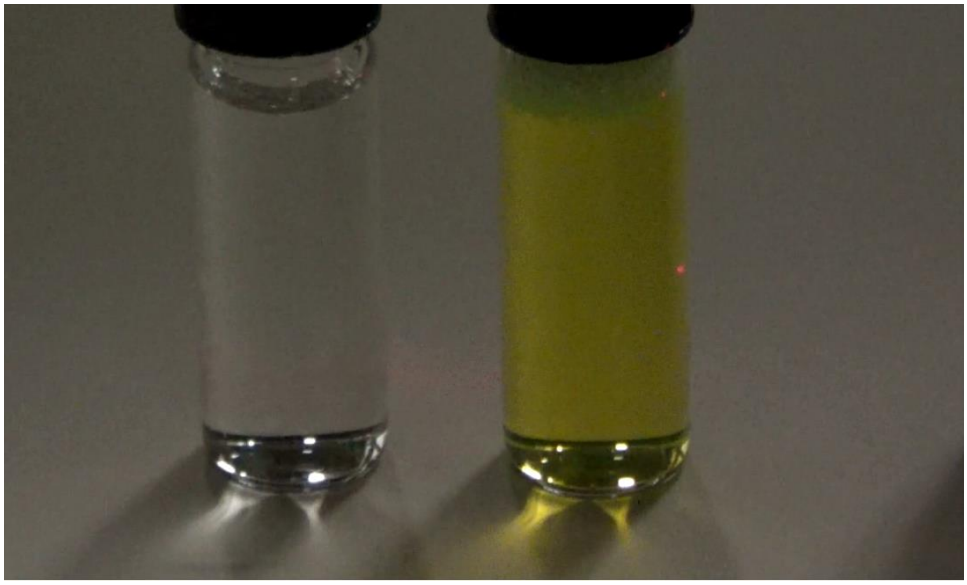

Figure XII. Sample A and sample B after adding the Dawn dish detergent

Once the Dawn dish detergent was added the liposomes were broken down in both sample A on the left and sample B on the right. The laser was currently being directed through the samples but neither had any deflection of light as they did before adding the detergent. When looking at sample A the color of the solution still stayed a light pink color because the dye was membrane bound and did not contain any other fluorescents. Therefore when the liposomes were destroyed the dye was still in the solution so the pink color of the dye remained in solution. Sample B on the other hand changed from a yellow to a more yellowish green color that resembled more of the color saw on the fringe of the carboxyfluorescein in the column chromatography. The reason for this is because once the liposome was broke apart the carboxyfluorescein was further

diluted that caused it to change color. This showed us that the carboxyfluorescein was inside the aqueous core. Since the change in color came once the liposome was broke down.

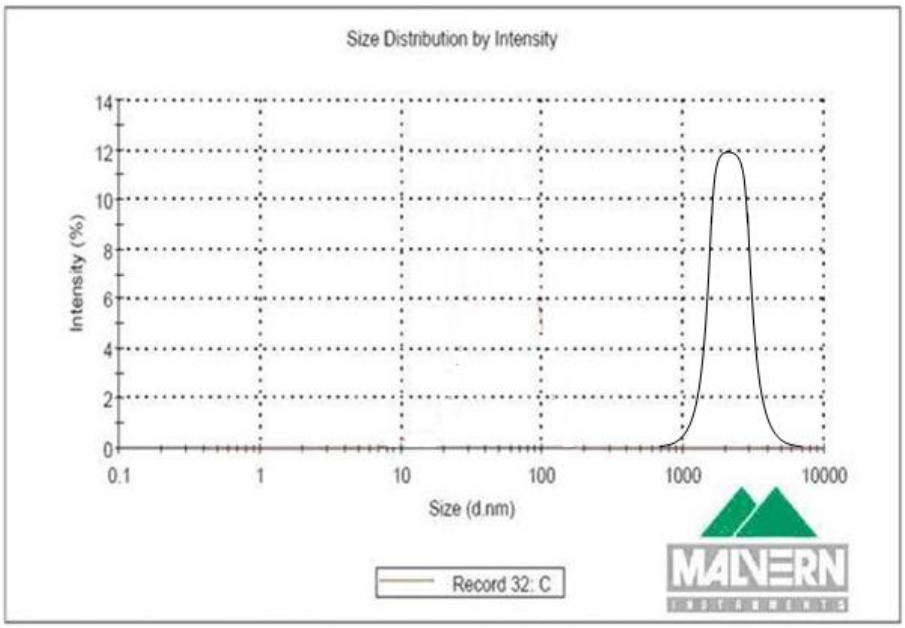

Figure XIII. Malvern zetasizer distribution graph of liposome solution that was not extruded using an Avanti mini-extruder

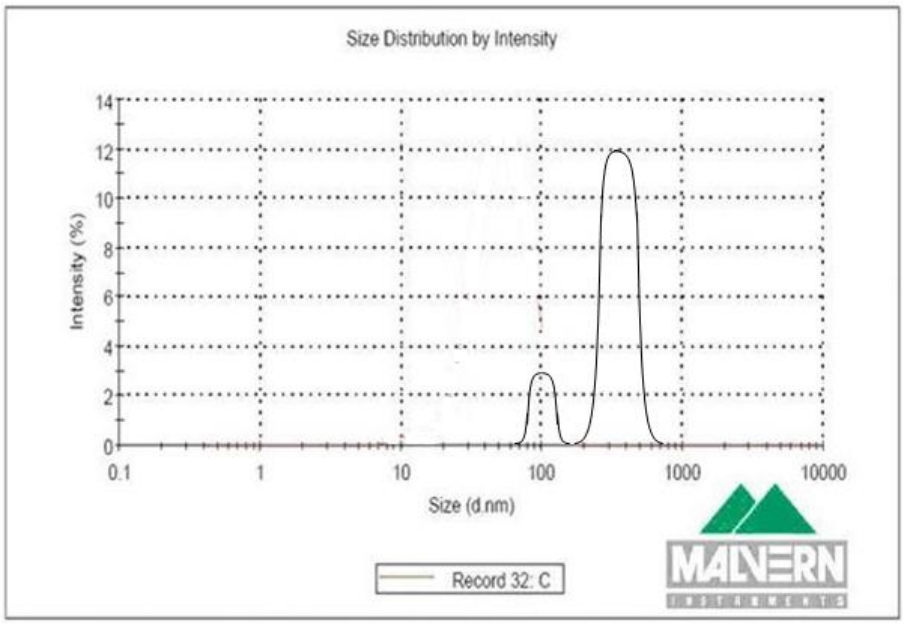

Figure XIV. Malvern zetasizer distribution graph of liposome solution that was extruded with 3 passes through an Avanti mini-extruder with 100nm PC membrane

* Further information can be found in the Appendix section

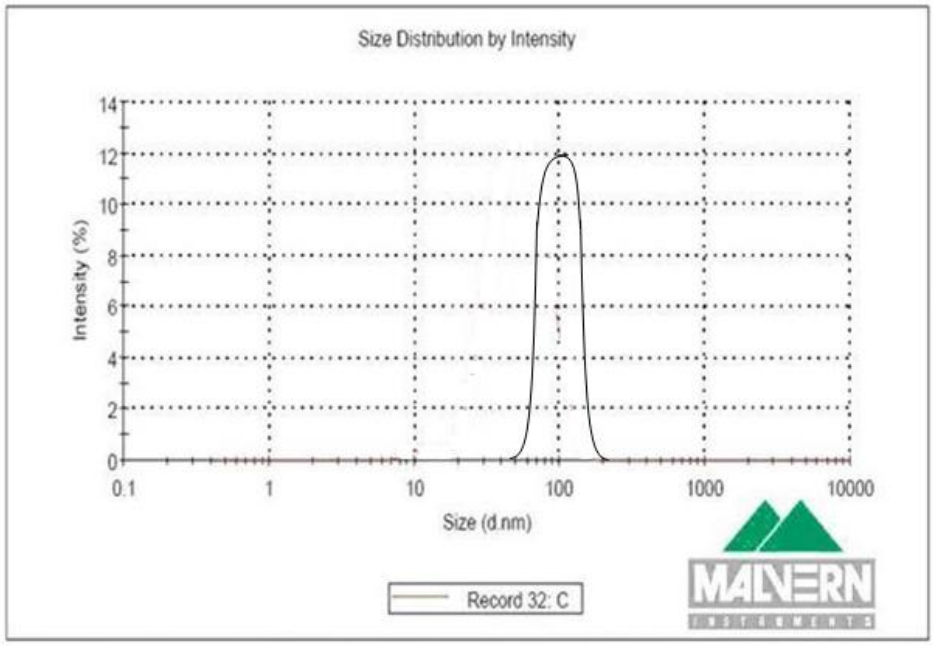

Figure XV. Malvern zetasizer distribution graph of liposome solution that was extruded with 11 passes through an Avanti mini-extruder with 100nm PC membrane

The reason gel chromatography was chosen for this experiment is because we want to be able to separate the liposomes from the mixture. Sample B that contains the 100 nm liposomes with carboxyfluorescein should separate into 2 distinct bands. The first band should be the liposomes that have encapsulated the carboxyfluorescein. After this layer, should be a thin clear layer of distilled water followed by another distinct band that is only carboxyfluorescein. The reason there are two layers is because, not all of the carboxyfluorescein is quenched into the liposomes aqueous core. Therefore the liposome with the carboxyfluorescein will filter down first since it has a higher molecular weight than just the carboxyfluorescein. The carboxyfluorescein that was not encapsulated is a smaller molecule and will be filtered down through the pores of the beads which will slow it the molecules as it elutes down the column. This creates two separate bands, which allows the experimenter to collect fractions that contain the liposomes that have encapsulated carboxyfluorescein

Questions:

1. Liposomes can be used in the body to deliver which of the following?

2. What interface of the liposome can a tag reside?

3. What classification of material makes a liposome stealthy?

* Further information can be found in the Appendix section

4. When a lipid solution is being hydrated, make sure that the solution always stays above what?

5. After the lipid film hydrated, the lipid cake layers can form into what shapes upon agitation?

6. How many times can a polycarbonate membrane be used before a new one needs to be put in the extruder?

7. How long was Sample A and Sample B hydrated before extruding?

8. What type of chromatography was used during this lab?

9. Which type of silica gel beads did the experiment use?

10. What is the desired liposome size for this experiment?

11. Smaller particles travel through the gel column faster than larger particles?

12. The Malvern zetasizer has the capabilities to measure which of the following?

13. The extruded liposome solution should always be removed from the "clean" side?

14. The Malvern Zetasizer uses what to determine particle size?

15. Based on the Malvern Zetasizer data, what number of extrusions produced liposomes with the best size distribution to the desired size?

16. What was used to clean the gas tight syringes and Avanti mini-extruder?

17. What was the result when dawn soap was added to the DOPC/Nile Red Liposome sample?

18. What was the result when dawn soap was added to the DOPC/Carboxyfluoroscein Liposome sample?

APPENDIX

* Further information can be found in the Appendix section

The **basic method to create tagged liposomes** is to start with a commercially available lipid powder. This powder is then mixed with an organic solvent to assure a homogenous mixture of lipids is obtained. This step is usually done with chloroform or a chloroform:methanol mixture to get a clear lipid solution. Once the lipids are mixed well, the organic solvent is removed by evaporation and a lipid film will be left in the round bottom flask. Depending on the volume that is being prepared either rotary evaporation or a dry nitrogen or argon stream may be used under a fume hood. Then the lipid solution is frozen, then placed on a vacuum pump and lyophilized until dry. Lyophilization is simply drying a stable preparation by freezing in a high vacuum. Then the dry lipid cake can be stored in a freezer until it is needed for hydration.

In **gas chromatography**, a mixture is vaporized and carried by an inert carrier gas (usually helium or nitrogen) through a gas chromatograph. The most commonly used detectors in gas chromatography are flame ionization and thermal conductivity detectors. There are three types of gas chromatography that include gas adsorption, gas-liquid, and capillary-gas chromatography. These methods are mainly used to separate gas mixtures, therefore this type of chromatography is not a method that is desired for this experiment.

In **ion exchange chromatography**, molecules or proteins are separated based on their charge with the use of resin or beads with a fixed charge. The two different types of ion exchange chromatography are cation-exchange and anion-exchange. The difference is that cation-exchange uses negatively charged beads or resins, while anion-exchange uses positively charged ones. This type of chromatography works by introducing a solution into the column that is packed with either positive or negatively charged beads. The molecule/protein with an opposite charge of the bead will bind while a molecule/protein with the same charge will not bind and flow down the column. Then a solution of high ionic strength is added and which will separate the protein from the bead, which then allows the molecule/protein to elute down the column.

In **affinity chromatography** there is still two phases which are involved, that being the stationary and mobile phase. The stationary phase though is usually a gel and it is typically agarose gel. This method starts with a liquid solution such as blood and targets molecules that can be found by defined properties that are exploited during the purification process. One common way this is used is to put antibodies on the stationary phase, such the agarose gel and flow a solution with a mixture of proteins over the gel. Then later in the process they can be extracted by changing the pH or ionic strength to separate the proteins to collect them. Since we are not working with proteins and instead looking for allocation of fluorescent die in the liposome we will not be using this method either.

* Further information can be found in the Appendix section

The **Sephadex** gel used for this experiment are polymer beads created by cross linking dextran (complex, branched glucan) with epichlorohydrin (an organochlorine compound and an epoxide). It was launched by the company Pharmacia in 1959 where it name originates from the words separation Pharmacia dextran or Sephadex. These beads are commonly used in gel filtration column chromatography and come in a dry form that swells when introduced to aqueous solution. There are 6 different G-type Sephadex gels from Sigma-Aldrich that vary in their degree of cross-linking as well as degree of swelling and molecular fractionation range. They are Sephadex G-10, G-15, G-25, G-50, G-75, and G-100. When selecting beads for an experiment, the two factors that need to be taken into account are particle bead size and the pore size. Some types of Sephadex such as G-50, like the one that will be used in this lab, comes in variations of coarse, medium, fine, and superfine. Table III, shows the different sizes of the beads for each of these variations. This is a factor because the particle bead size or otherwise known as the mesh size has to do with resolution. When a smaller bead size such the superfine is used, the experimenter will get higher resolution but will end up having a slower flow rate and therefore a slower separation.

Sephadex Variation	Bead size
G-50 Coarse	100-300 micrometers
G-50 Medium	50-150 micrometers
G-50 Fine	20-80 micrometers
G-50 Superfine	20-50 micrometers

Table III. Sephadex variations with corresponding bead sizes.

The other factor previously mentioned is the pore size of the gel bead. The image below in Figure XVI, shows the exclusion limits of each type of Sephadex. The range includes an upper and lower value over which they can be used to separate macromolecules. This upper value, also known as the exclusion limit, indicates the size limit for molecules that cannot penetrate the pores of the gel beads. In other words if the molecular mass of the molecule is above this value than it will be excluded which means it will not go through the pores and will travel down the column first. Molecules that have a smaller molecular mass will than this value will therefore enter the pores for various amounts of time depending on the size of the molecule. An example would be Sephadex G-10 that has an exclusion limit of 700 Dalton's. This means that the any molecule that has a molecular weight over 700 Daltons would not be able to enter the bead pores and will elute down the column.

* Further information can be found in the Appendix section

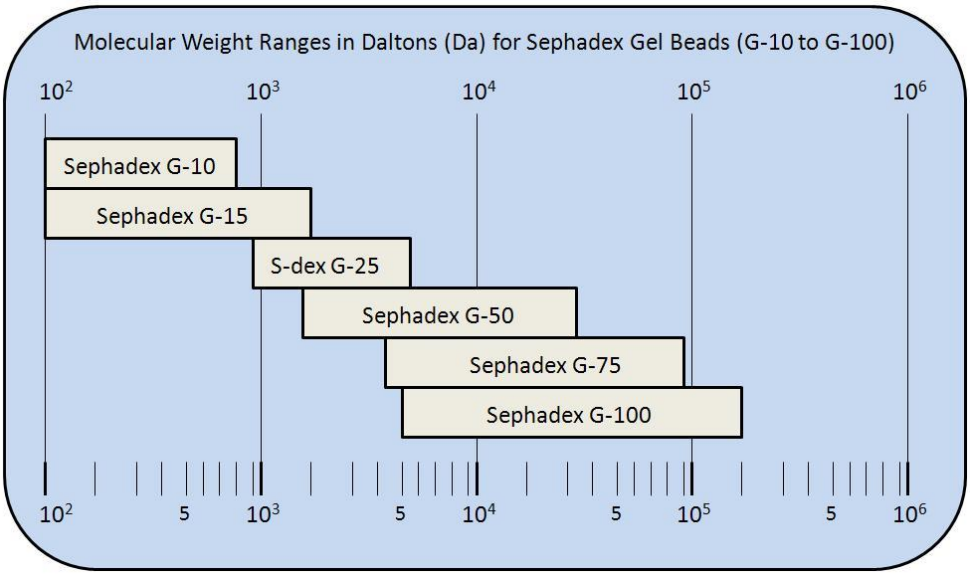

Figure XVI. Comparison of Sephadex variations.

Video of Malvern Zetasizer being used to determine size of particles

http://www.engr.psu.edu/mediaportal/flvplayer.aspx?FileID=e2f8e124-b573-4bbe-a940-d

http://www.engr.psu.edu/mediaportal/flvplayer.aspx?FileID=9bf235a1-aea3-434e-b75e-9

* Further information can be found in the Appendix section