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Cyclodextrins as lipid exchange mediators to synthesize artificial asymmetric model membranes

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Abstract of the Thesis

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The use of cyclodextrins have shown promise to exchange lipids of model membranes in various conditions. Several types of cyclodextrins have been shown to exchange lipids in different conditions. In this study, we used Methyl-alpha cyclodextrin (MαCD) to facilitate lipid exchange between donor multilamellar vesicles (MLVs) containing brain SM and large unilamellar vesicle acceptors (LUVs) composed of POPE, POPS and cholesterol. This technique is just one of several exploring the use of cyclodextrins to manipulate artificial membranes in order to obtain asymmetry. Overview of other studies involving other types of cyclodextrin are also discussed to explain what is known about cyclodextrin-lipid interactions. Mastery of the utilization of these types of molecules can have the potential to significantly impact the way we carry out future membrane studies. With more insight, cell membranes we find in nature can be replicated and tested on in laboratory settings to further our understanding of the membrane bilayer.

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List of Abbreviations

CD	Cyclodextrin
MαCD	Methyl-alpha-cyclodextrin
MβCD	Methyl-beta-cyclodextrin
HPαCD	Hydroxypropyl-alpha-cyclodextrin
HPβCD	Hydroxypropyl-beta-cyclodextrin
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
SM	Sphingomyelin
SUV	Small Unilamellar Vesicle
LUV	Large Unilamellar Vesicle
GUV	Giant Unilamellar Vesicle
MLV	Multi Lamellar Vesicle
PBS	Phosphate Buffered Saline
TLC	Thin Layer Chromatography

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1. General Introduction

All cells have a plasma membrane which encloses their cytoplasm, organelles, and other essential components which cells need to function properly. Lipid vesicles are spheres of lipid molecules arranged in bilayers which enclose an aqueous solution, equivalent to the plasma membrane. Replicating conditions of cellular membranes in vitro can give insight into understanding how cell membranes function in vivo. However, many of the cell membrane's functions are based upon their asymmetric lipid distribution (Pautot, et al., 2003).

1.1 Membrane bilayer

Cell membranes are quite complicated structures, consisting of a bilayer with an array of different structural lipids and membrane proteins which are essential to carry out a variety of processes. Eukaryotic cells invest a significant amount of energy and nearly 5% of the encoded genome to synthesize membrane lipids. The major structural lipids in eukaryotic membranes are glycerophospholipids, such as: phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS). The hydrophobic tail of lipids are made up of diacylglycerol which contain fatty acyl chains of varying lengths. Another major class of lipids, sphingolipids, have ceramide as their hydrophobic tail. Lipids have special geometries which depends on their polar head group and hydrophobic tail regions. Some may have conical shapes, while others are more cylindrical. The saturation of a hydrophobic tail refers to how many double bonds are present. More saturated lipids have fewer double, and more single bonds. Conversely, highly unsaturated lipids will contain more double bonds. The actual length of the molecule is also significant. The acyl chains of the hydrophobic tail can vary from

lipid to lipid. This depends on the amount of carbons involved in the chain. These features of lipids are important factors for how the membrane bilayer is arranged intracellularly (Van Meer, et al., 2008).

Inside a single cell different membranes display varying amounts of the various different lipids. The plasma membrane shows different distributions of lipids than that of the Golgi apparatus, which also differs from the membrane of a late endosome or mitochondria. This occurs due to the structure and distribution of lipids in a membrane, and is directly responsible for the functions of an organelle. One commonality is that none of these organelles display symmetry in their membranes (Van Meer, et al., 2008).

The unequal distribution of these lipids in the inner and outer leaflets of cellular membranes is called asymmetry. These lipids will group together in certain patterns based on their structure in order to execute different cellular functions. Their position on the inner and outer leaflet are vital for cellular survival. It has been shown apoptosis (Fadok, et al., 1992), metabolic inhibition (Post, et al., 1993), and aging of erythrocytes (Diaz, Schroit, 1996) all lead to loss of phospholipid asymmetry.

1.2 Asymmetry

In the 1970's it was shown that in red blood cells, or erythrocytes, the lipid distribution in the cell membrane was not symmetrical (Verkleij, et al., 1973). There was an uneven distribution of certain types of lipids on the outer leaflet, which differed substantially from the arrangement in the inner leaflet. In eukaryotic cells, SM and PC are often found in the outer leaflet, while PS, PI and PE are typically seen in the inner leaflet (Verkleij, et al., 1973). This difference causes the asymmetric distribution of these

molecules to have an effect on the behaviors of cell membranes including surface charge, membrane potential, shape, and permeability. The specific positions of these lipids lead insight onto their function. For example, It has been shown that when PS, normally found on the internal monolayer, is exposed in mammalian cells it is a marker for apoptosis and a signal for disposal by macrophages (Marquardt, et al., 2015).

Maintaining a membrane with asymmetry in living cells, it requires energy (ATP). Flipases and flopases are responsible for helping to maintain the correct distribution of membrane lipids (Van Meer, 2011). Not only does the difference of lipid molecules on the inner and outer leaflets contribute to the heterogeneity of membranes; the lateral distribution of lipids on the same leaflet contributes as well. Groups of lipids that can help carry out a signal, or allow membrane protein assembly are called lipid rafts. Lipid rafts contain high amounts of sphingomyelin (SM) and cholesterol (Chol) (Pike, 2006). On plasma membranes, lipids can take the form of several types of phases, including the liquid ordered (Lo), liquid disordered (Ld), and a gel state. In the liquid disordered state, the lipids are free to move around with the most freedom of the 3 states. The gel state is quite different; it is more rigid and laterally constrained. Interestingly, lipids with higher melting points, SM and Chol, are found to be in the Lo state (Heberle, Feigenson, 2011). For this reason, it has been proposed that lipid rafts are in the Lo state (Schroeder, London, Brown, 1994).

1.3 Cyclodextrins

Cyclodextrins have been used and tested in several membrane studies. Cyclodextrins (CDs) are unique compounds consisting of rings of glucose molecules

bound by glycosidic bonds which contain a hydrophilic exterior and a hydrophilic interior. These compounds increase the solubility of other molecules by trapping them inside the cavity. Since the interior cavity is both hydrophobic and lipophilic, it has the potential to interact with molecules like phospholipids and cholesterol (Huang, London, 2013).

There are several types of cyclodextrins including the α, β, and γ cyclodextrins. The different types vary based upon the number of glucose rings involved in the molecule, as shown in Figure 1. αCD has ring of 6 sugars, βCD has a ring of 7 and γ has a ring of 8. Because of their varying ring sizes, the cavities for these three CDs are different. αCD has a cavity of about 5Å, while βCDs have a 6.9Å diameter. γCD cavities are the largest with a diameter of 8.5Å (Cromwell, et al., 1985). The structure of the CD molecule has drawn great interest from pharmaceutical companies because of its ability to increase the solubility of a guest molecule. The hydroxyl groups found all over these molecules provide a hydrophilic environment. Inclusion complexes of cyclodextrins and a hydrophobic drug can greatly increase the bioavailability of that drug because it is able to penetrate body tissues due to the increased solubility (Becket, et al., 1999).

There have been studies to test the potency of how well each of the three types of CDs mentioned solubilize different components of an erythrocyte membrane (Somogyi, et al., 2006). αCD was the most efficient at making phospholipids more soluble, while βCD was found to be most efficient in taking up cholesterol. αCD is believed to be effective at extracting phospholipids while being inefficient with cholesterol due to its smaller cavity (Somogyi, et al., 2006). An αCD can fit one fatty acyl chain in its chamber, while cholesterols are too bulky. βCDs have a larger opening

which can fit these bulky cholesterols. γ was found to be the least efficient in solubilizing membrane components (Ohtani, et al., 1989). The efficient uptake of cholesterol by βCDs has profound effects for cells due to the fact that cholesterol is essential for many cell functions, including endocytosis (Rodal, et al., 1999). Cholesterol can be commonly found in lipid rafts, as much as 3-5 times the amount found in other areas of the membrane (Anchisi, 2012). These structures are a conglomeration of membrane proteins and lipids that carry out important cell functions, including endocytosis and signal transduction. The ability of αCDs and βCDs to be proficient in solubilizing and extracting lipids is important for our purposes because in order to manipulate a membrane to become asymmetric, we must extract certain lipids from artificial vesicles allowing the addition of other types of lipids.

Cyclodextrin structure can be altered by the addition of functional groups, for example adding methyl and hydroxypropyl. MβCD, HPαCD, and HPβCD have been investigated to create artificial asymmetric vesicles. These lipids carry out phospholipid exchange between small unilamellar vesicles (SUVs), MβCD being the most efficient lipid exchange mediator. HPαCD was found to be useful due to its ability to exchange phospholipids while being unable to interact with cholesterol (Lin, London, 2014).

1.4 Previous Asymmetric Membrane studies

Artificial asymmetric lipid vesicles can be utilized to give insight into the functions of the plasma membrane. However, the in vitro formation of these vesicles has been difficult. Most studies in the past have resorted to using symmetric vesicles because when lipids self-assemble they group together with the same lipids. However, it is

promising to consider that asymmetric vesicles can be synthesized in labs to help further our understandings of the inner working of the cell membrane. There have been several studies done to try to achieve this goal.

Pautot, Frisken, and Weitz devised a way to create asymmetric vesicles using two separate monolayers. Each leaflet of the bilayer is created independently. They used an inverted emulsion phase around water droplets dispersed in an oil, where the inner leaflet lipids assembled. This is then placed over an intermediate phase of the same oil but with the outer leaflet lipids. This is then placed in an aqueous phase where the two monolayers come together to form a bilayer. Because the water from the emulsion phase is heavier than the oil, the water droplet surrounded by the inner leaflet lipids drops down to the intermediate phase/aqueous phase interface where the outer leaflet can join the inner leaflet and become assembled in the final aqueous phase. Fluorescently-tagged lipids were used to confirm the asymmetry found in these unilamellar vesicles. By labeling specific lipids, they were able to see what the inner and outer leaflets were composed of due to the fact that the quencher can only reach the outer leaflet lipids (Pautot, et al., 2003). This is an example of achieving asymmetry without CD, but this method has its drawbacks. It is a short-lived asymmetry that this group achieved, so testing on the membranes in a lab would be restricted to only a few hours.

While the Pautot, Frisken, and Weitz method of achieving asymmetric vesicles lacked the use of CDs the following methods all utilize some form of CD. MβCD mediated lipid exchange has been used in many previous experiments.

One study carried out by Ilaria Visco, Salvatore Chiantia, and Petra Schwille achieved asymmetric membranes through the use of supported lipid bilayers on solid mica plates and vesicle fusion. Normally vesicle fusion does not allow asymmetric bilayers to form, however, this team was able to use MβCD to facilitate lipid exchange to ultimately get SM into the outer leaflet of the supported lipid bilayers. The resulting lipid bilayers showed asymmetry, but only for several hours. This was known to be true through measuring lipid mobility (diffusion times) of the inner leaflet relative to the outer leaflet. SM is a more saturated lipid than the others used in this experiment, therefore the outer leaflet displayed a slower diffusion time (measured by fluorescence spectroscopy). After several hours the diffusion times became almost equivalent, meaning asymmetry was lost (Visco, et al., 2014).

Another study carried out by London and Son showed that headgroup properties play a role in membrane asymmetry. Like the previous study, MβCD was used to carry out lipid exchange. But this time between small unilamellar vesicles (SUVs) instead of supported lipid bilayers on freshly cleaved mica. SM was introduced to outer leaflets of vesicles containing PG, PS, and PI in mixtures with PE, as well as PC and PA mixtures. It was shown that fully asymmetric membranes with SM on the outer leaflet were achieved when PS, or PE mixed with PG, PS, and PI was present. The asymmetry was evident for over a full day which suggests the asymmetry was stable. NMR further showed that SM was in fact found in the outer leaflet. In addition, melting temperatures were measured for symmetric and asymmetric vesicles containing SM, PE, and PG. The asymmetric vesicles had a higher thermal stability than symmetric vesicles containing the same lipid compositions. The exchanged asymmetric vesicles' melting

temperature was comparable to that of pure SM vesicles, which also suggests asymmetry in the exchanged vesicles. SM is shown to clearly be stable in the outer leaflet in the gel-like state as expected. It was concluded that PE and PS serve a function in keeping asymmetry stable (London, Son, 2013).

Asymmetric membranes similar to those found in nature were formed in another study by Cheng and London with SM and POPC in the outer membranes and DOPC, POPC, POPS, or POPS and POPE in the inner leaflet. MβCD was used to carry out the lipid exchange with SUVs to obtain the asymmetric vesicles. Cholesterol was also introduced into this system with a second MβCD lipid exchange. This study showed similar results to the previous one mentioned, where SM in the outer leaflet formed a solid state with high thermal stability comparable to pure SM vesicles. In addition, when cholesterol was exchanged into the vesicles it resulted in increased thermal stability suggesting cholesterol may play a role in stabilizing this ordered lipid phase (London, Cheng, 2009).

Cheng and London also did another experiment where large unilamellar vesicles (LUVs) were used in their MβCD lipid exchange method. SM and/or SM with POPC were exchanged into outer leaflets of vesicles containing DOPE and POPS with or without cholesterol. It was shown that when SM was exchanged into the outer leaflet, it increased the order of the inner leaflet. This finding shows that there are interleaflet interactions. The melting temperature of the SM-enriched outer leaflet was similar to that of vesicles containing solely SM and was higher than symmetric vesicles with equal SM content. This information suggests that higher temperatures weaken the interactions of the inner and outer leaflet. Asymmetric SUVs had similar properties to these

asymmetric LUVs, which suggests that curvature is not a factor in interleaflet interactions (Cheng, London, 2011).

Chiantia, Schwille, Klymchenko, and London were able to create asymmetric giant unilamellar vesicles (GUVs). This method also utilized MβCD-mediated lipid exchange to create asymmetric vesicles, like those done with SUVs and LUVs. A benefit to this experiment is the actual size of the vesicles created. GUVs are much larger and more closely resemble the size of cells. They are also large enough to use optical methods to confirm asymmetry. Fluorescence correlation Spectroscopy (FCS) was used to visualize lipid dynamics. Diffusion times were used to compare fluidity in the leaflets. As expected, when brain SM is exchanged into the outer leaflet diffusion time rises suggesting a more ordered, rigid state. On the DOPC inner leaflet, diffusion occurs more rapid, indicating a more disordered state. However, when brain SM was exchanged into vesicles with POPC vesicles, diffusion times increased in both leaflets. Another fact worth noting is that these asymmetric vesicles were stable for at least 4 hours, which is crucial for future studies (Chiantia, et al., 2011).

Once again, MβCD was used to facilitate lipid exchange for another experiment conducted by Son and London. This time they were looking at how PC acyl chain structure would affect asymmetry. During this study they used lipids with PC headgroups with varying acyl chain structures based on their length and saturation. It was shown that lipids that exhibit fast transverse diffusion are not suitable for stable asymmetric vesicle structures. The PCs with two short and/or unsaturated chains were more likely to diffuse across the membrane and disrupt the asymmetry. However, the

experiment did show that asymmetry can be achieved with a variety of different acyl structures (Son, London, 2013).

Another type of CD, HPα, was used in an experiment by London and Lin because the MβCD lipid exchange method had too high of an affinity for cholesterol. This resulted in difficulty making asymmetric lipid membranes that involved cholesterol. The smaller cavity of the αCDs reduced its affinity for cholesterol, which allowed them to exchange phospholipids without cholesterol hindering their efforts. Despite this, HPαCD may lack sufficient affinity for other lipids that may be desired in exchange experiments, like SM (Lin, London, 2014). Because different CDs have different physical properties they interact with lipids differently. It is important to take into consideration how these CD-lipid interactions work.

Huang and London carried out a study to examine CD-lipid interactions. In these sets of experiments they went through several types of CDs including MβCD, HPαCD, and HPβCD. MβCD was found to be the most proficient at exchanging lipids, however at concentrations that were too high the vesicles were solubilized as shown through light scattering and FRET experiments. Also, the lipid structure affected how much of the MβCD was needed for solubilization (Huang, London, 2013). The variety of different CDs and lipids used in this experiment are essential to figure out the best way to make specific types of asymmetric vesicles. More experiments like this will improve further efforts to make desired vesicles with controlled amounts of desired lipids.

Many studies have been carried out trying to create stable asymmetric vesicles in order to enhance our understanding of how membranes behave. Experiments have

varied using a wide variety of lipids, CDs, and vesicle sizes. However, there are still more questions to explore and many variables to modify in order to get a better sense of the optimal conditions for lipid exchange to create asymmetric membranes.

This thesis explores the ability of MαCD to carry out lipid exchange between donor MLVs of brain SM and acceptor LUVs composed of cholesterol, POPE, and POPS. The purpose of this study is to show that MαCD can be utilized to create artificial asymmetric vesicles. MαCD is believed to have potential to carry out phospholipid exchange due to the fact that αCDs can interact with phospholipids without affecting cholesterol due to the small diameter of the cavity. This study alone cannot determine whether MαCD is useful for our goal. However, examining this study along with several similar studies, and experimenting with a variety of lipids, we can hopefully gain insight on how cyclodextrins can act as lipid exchange mediators to create any desired asymmetric membranes. Creating these asymmetric membranes will have profound impacts on future studies involving model membranes.

2. Methods

2.1 Preparation of Cyclodextrin

The molecular weight of MαCD is 1225.32 g/mol. In order to get a desired concentration of 250 mM, we attempted to dissolve 306 mg/mL of MαCD in DI water. To then check the concentration we used the equation x= (y-1.33)/(1.49x10^-4) where y represents the index of refraction and x= the concentration of MαCD in mM. The equation was derived by Zhen Huang, a member in Dr. London's lab, using solutions of known MαCD concentration and recording their refractive indexes. These data points were then used in Excel to create a line of best fit, which the equation represents. The solution that we made was put on the refractometer and the number we recorded from this was plugged into the equation to get the exact concentration of the solution we made.

2.2 Preparation of Donor and Acceptor MLVs

Acceptor vesicles were made with 27.5% POPE, 27.5% POPS, and 45% Chol. The appropriate amounts of these lipids dissolved in organic solvent were dried in the same test tube under nitrogen gas, then vacuum dried for one hour. The dried lipids were then hydrated with 2000 µl of 25% (w/v) sucrose solution at 70°C. The test tube was then vortexed, shaken at 55°C for 15 minutes and left overnight covered with Teflon. These acceptors had a final concentration of 10 mM in the 2000 µl mixture.

Donor vesicles were made with brain SM. Two separate test tubes were used to make 16 mM and 20 mM lipid donors. Appropriate amounts of SM were syringed into the test tubes. They were then dried under nitrogen gas and vacuum dried for one hour.

Then in a 70°C water bath the SM was hydrated with the appropriate amounts of MαCD and PBS, shown in table 2. The volume of PBS and MαCD totalled 600 µl. After hydrating, they were vortexed and shaken for 2 hours at 55°C. 100 µl of material from each test tube was reserved for the L4A18 peptide assay.

2.3 Preparation of Acceptor LUVs

1000 µl of the acceptor MLV's stored overnight were freeze/thawed in acetone with dry ice 7 times then extruded through 0.1 um filter syringe 11 times. The sample was then layered over 3000 µl PBS and centrifuged at 37,500 rpm. After the centrifugation, we then removed the supernatant and resuspended the pellet at the bottom of the tube in 2000 µl PBS total.

2.4 Exchange process

We calculated acceptor dilutions and added appropriate amounts of PBS and acceptor LUVs to each of the donor MLV tubes to make the final sample with 16 mM donor lipid have an 8 mM acceptor lipid concentration and the 20 mM donor lipid sample have a 6 mM final acceptor lipid concentration. These values are shown in Table 2.

After adding the LUVs and the PBS samples were shaken in an oven at 55°C for 30 minutes and cooled to room temperature. We then layered the mixtures over 3 mL of 13% (w/v) sucrose and centrifuged at 37,500 rpm for 30 minutes. After the centrifugation was done, we carefully removed the top layer of the test tube with a Pasteur pipette and swabbed the sides with a Q-tip in order to remove any remaining

MLVs adhering to the sides. We left about 1 ml of the sucrose solution in the tube along with the LUV pellet at the bottom of the tube.

After adding another 3 mL of fresh PBS to the tube, we centrifuged for 30 minutes once more at 37,500 rpm. We removed the liquid after this step and resuspended the pellet at the bottom of the tube in 500 µl of fresh PBS, reserving 200 µl in each of the tubes for the TLC.

2.5 L4A18 Peptide Assay

We analyzed the remaining LUV sample with L4A18 peptides (acetyl-$K_2LA_9LWLA_9LK_2$-amide). These peptides help with identifying to what extent our lipids exchanged. The L4A18 peptide binds to PS, which has a negatively charged polar head, because the peptide contains positively charged lysines at each end. It also contains a tryptophan in the middle. We measured the Trp fluorescence on a SPeX2 fluorolog spectrophotometer after mixing vesicles and peptide with a vortex at room temperature. In each cuvette we started with 450 µl of PBS and 1 µl of the 0.5 mM peptide. We then took samples from the 16 mM donor tube, the 20 mM donor tube, the original acceptor LUV tube diluted to 1 mM lipid, and also a control tube where it was only 500 µl PBS plus 1 µl of the 0.5 mM peptide and put them in separate cuvettes to analyze with a spectrophotometer. We took readings after addition of 50 µl, 100 µl, 200 µl, and 300 µl of sample in each of the cuvettes for the 16 mM donor sample after MαCD-mediated lipid exchange, 20 mM sample after MαCD-mediated lipid exchange, and acceptor LUV tube.

2.6 Thin Layer Chromatography (TLC)

For the TLC, we utilized band intensities to give us a reading of the amount of lipid found in each sample. Because each lipid has different physical properties, they will travel up a silica TLC plate differently and therefore create separate bands. TLC plates were preheated at 100°C and cooled to room temperature ensure a dry surface before loading of the sample and standard lanes. For the mobile solvent phase, we used a mixture with the ratio 65:25:4:12 (v/v) of Chloroform:MeOH:H2O:Acetic Acid, respectively to separate the lipids. We used samples from 16 mM donor lipid MαCD exchange sample and 20 mM donor lipid MαCD exchange sample and prepared 2 sample lanes for each, one with 50 µl and one with 100 µl for each donor tube. We also had standard lanes containing 0.5 µg, 1 µg, 8 µg, 16 µg, and 32 µg of the lipids used in the experiment: SM, Chol, POPE, and POPS. The standard lanes were necessary in order to compare the sample bands to a known, controlled amount of lipid. The lipids were prepared by pipetting the desired amounts of each lipid and drying them under a nitrogen stream. They were then dissolved with 50 µl of a 1:1(v/v) mixture of chloroform and methanol. The mixture contained 2 µg/µl of lipid, so 0.25 µl, 0.5 µl, 4 µl, 8 µl, and 16 µl were used for the 5 lane, respectively. The dissolved lipids were loaded onto a preheated silica plate using a syringe 1 cm from the bottom of the plate and left to dry before placing them in a glass chamber with the mobile solvent phase mentioned before. When the mobile phase almost reached the top, the plate was taken out to dry for approximately 30 minutes. After drying, the plate was sprayed with 3% (w/v) cupric acetate and 8% (v/v) phosphoric acid solution and left to dry again for another 20-30 minutes. To visualize the lipids, we then placed the plate in a 180°C oven for approximately 5-10 minutes. The resulting plate showed the charred separated lipids.

Using a program called ImageJ, we were able to take band intensity readings of each band we see on the TLC plate. A blank reading was also taken as a reference point. By subtracting a blank reading from the band intensity reading, we were able to come up with the amount of each lipid in our samples, in micrograms.

From these data, we were able to find the total lipid quantity per sample, which will also gave us a percent yield for each lipid since we can figure out the final concentration relative to the starting concentration of lipids. Using these data we can see how well MαCD mediated exchange of SM into the LUVs.

3. Results

3.1 L4A18 peptide assay results

The results from this experiment are presented in Table 4. These readings are of maximum wavelength of the Trp fluorescence. The acceptor vesicles showed a stronger blue shift compared to the vesicles that underwent vesicle exchange. The two samples that contained vesicles that underwent the MαCD mediated lipid exchanged both exhibited blue shifts, but the sample with the 20 mM donor sample showed a slightly stronger shift.

3.2 Thin Layer Chromatography Results

Figures 3a, 3b, and 3c all show the amount of lipids found in the standard lanes for our TLC analysis. We have a positive linear regression on all of these graphs because each standard lane has progressively more of each type of lipid. Also shown in figure 2 is an actual sample TLC plate. The 4 lanes to the right are the 50 µl and 100 µl samples of the 16 mM mixture and 20 mM MαCD mixtures, respectively.

The lipid quantity was derived from the band intensities using ImageJ. Band intensity values were then input to an excel sheet shown in tables 3a, 3b and 3c. From these numbers we were able to estimate the number of µgs of each type of lipid in each of the sample lanes shown also in these tables. We were then able to deduce the percent yield of the experiment because we knew the starting amount of PE and PS. The percent yield analysis was done in moles, and the lipid quantities were converted to µmoles. By working with the numbers we were able to determine the percentage of lipid in each sample. Our final lipid percentages in each sample showed 92% SM, 5% POPS, and 3% POPE in the 16 mM samples, while there was 80% SM, 10% POPS, and 10% POPE in the 20 mM samples as shown in figure 3D.

4. Discussion

4.1 General Discussion

It is important to see how cyclodextrins can help facilitate lipid exchange to create asymmetric cell membranes in a laboratory environment. These are just a few experiments that have utilized these special molecules to manipulate lipid distribution in model membranes. Cyclodextrins are used widely for their unique structure and properties. It is an important goal to carry out studies with these molecules so we can fine tune the process of making artificial asymmetric membranes for future studies.

4.2 MαCD Discussion

For the L4A18 peptide assay, we should expect to see a stronger blue shift in the samples containing PS compared to those without it. From the data collected we see evidence to suggest that MαCD worked in exchanging SM into the outer leaflet of the

acceptor vesicles. The acceptor vesicles before the exchange should show the stronger blue shift compared to the vesicles after the exchange because prior to exchange, vesicles will have more anionic PS that can bind the positively charged lysine residues in the L4A18 peptide. Both the acceptor LUVs before the exchange, as well as our exchanged samples showed blue shifts, but the acceptor blue shift was a little stronger. This tells us that PS has been exchanged out of the exchanged acceptor vesicles and replaced by SM. The L4A18 peptide and PBS is there as a control and recorded the highest wavelength. The maximum wavelength of this control sample should be the highest of the wavelengths due to lack of binding to any vesicles. Increasing the amount of sample in each reading was done to make sure the vesicles were saturated with the peptide to give the strongest result. The weaker blue shifts after exchange suggests that MαCD carried out successful lipid exchange

From the TLC plate and ImageJ we were able to calculate a significant amount of SM in the vesicles. According to the calculations we saw the there was a significant amount of SM in our exchanged LUVs. The 20 mM donor exchanged samples had a slightly higher percentage of SM found in their vesicles compared to the 16mM donor samples, which suggests increasing the concentration will increase the exchange efficiency. However, the amount of SM is above the 50% value expected for exchange. This suggests come donor vesicles contaminated the preparation. This experiment needs to be repeated several times to be confident in these results and conditions changed to minimize contamination.

The results of the MαCD experiment are not conclusive at this point in time, but according to our protocol we should be able to predict what the results should look like if

MαCD can act as a mediator for lipid exchange. The data we have does not prove or disprove the feasibility of using MαCD as an efficient lipid exchange mediator. More conclusive data need to be collected using MαCD, along with other types of lipids to see if it can be utilized as a sufficient mediator for lipid exchange to create asymmetric model membranes.

Figures:

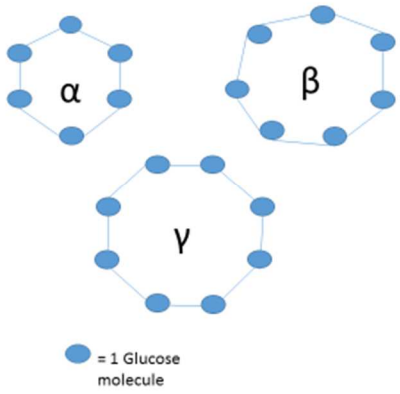

Figure 1. General Structure of α, β, and γ cyclodextrins. The structure of these molecules have a hydrophilic outside with a more hydrophobic interior which makes it excellent for interacting with lipids. The difference between these types of CDs is the size of the ring. They have 6, 7, and 8 glucose rings, respectively.

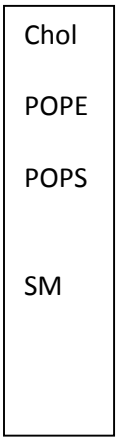

Figure 2. TLC Plate. The five left lanes are the standard lanes each containing 0.5 µg, 1 µg, 8 µg, 16 µg, and 32 µg of each type of lipid used. Lanes 5, 6, 7, and 8 on the right are the sample lanes containing 50 µl and 100 µl of each of the MαCD mixtures, with 16 mM and 20 mM donor lipid, respectively. The abbreviations on the left side indicate the type of lipid in each row. The SM quantity is too high, evident by the high intensity of the SM bands in the sample lanes, which can be attributed to donor vesicle contamination.

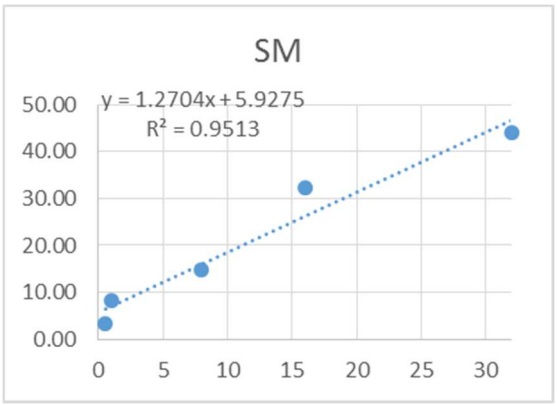

Figure 3a. Standard Curve for SM quantity. Data points were derived using ImageJ based on band intensities from the TLC plate shown in Figure 2. Final band intensity values were determined by subtracting a blank intensity value, which were measured using a blank area of the TLC plate.

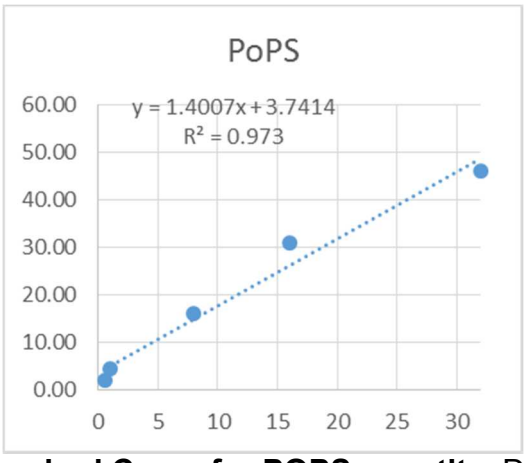

Figure 3b. Standard Curve for POPS quantity. Data points were determined the same way as mentioned in Figure 3a.

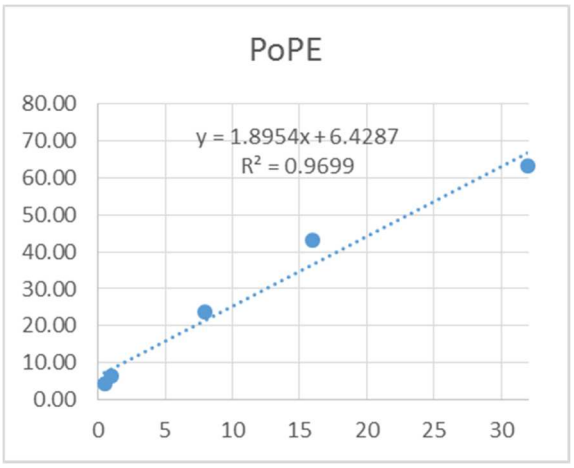

Figure 3c. Standard Curve for POPE. Data points were determined the same way as mentioned in Figure 3a.

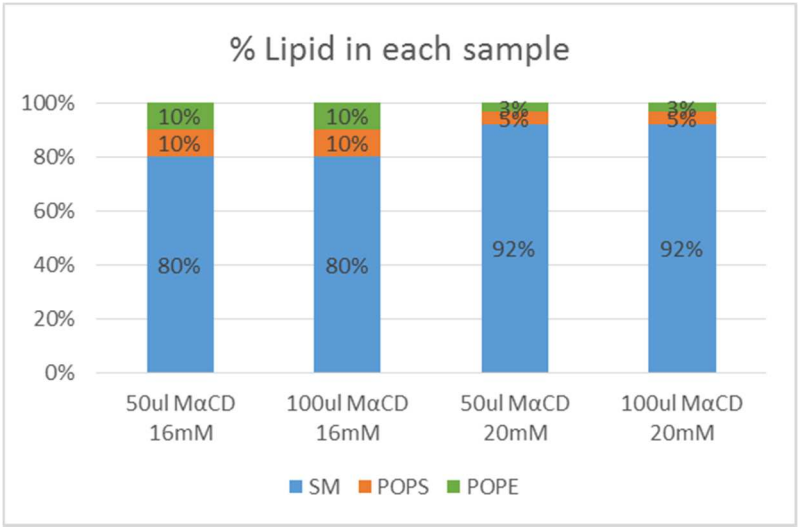

Figure 3d. Final Lipid percentage in exchanged LUVs. This graph is a representation of the final quantity of lipids found in our exchanged vesicles. µMoles were calculated by dividing µg of each lipid by their respective molecular weights. Then the µMoles were converted to a percentage for each of the samples.

Tables:

Table 1. Donor Tube contents. These numbers were derived by using 600µl as the total volume and by deriving our CD concentration using a refraction index vs. [MαCD] curve, x=(y-1.33)/(1.49x10^-4), where x= [MαCD] and y= refraction index.

MαCD conc.	SM conc.	PBS added	CD added	Total Volume
30 mM	16 mM	436 µl	164 µl	600 µl
30 mM	20 mM	436 µl	164 µl	600 µl

Table 2. Acceptor Tube Contents. The table shows the final acceptor dilution that was added to the donor tubes containing the MαCD and SM. The starting acceptor concentration was 10 mM. Appropriate amounts of PBS were added to end up with correct final acceptor concentrations.

Final Donor Conc.	PBS	10mM Acceptor	Final Acceptor Conc.
16 mM	100 µl	400 µl	8 mM
20 mM	200 µl	300 µl	6 mM

Table 3a- Band Intensity Analysis for SM. Band intensities were derived from the TLC plate using ImageJ. Blanks were taken using the empty space on the TLC image. Final µg calculations in each sample were done in excel based on the standard curve.

Lane	Sample	Band Intensity	Blank	Band Intensity - Blank	Calculation (µg) by Excel
1	std 0.5	8.95	5.655	3.29	
2	std 1	14.03	5.655	8.37	
3	std 8	20.54	5.655	14.88	
4	std 16	37.89	5.655	32.24	
5	std 32	49.56	5.655	43.91	
6	50µl MαCD 16mM	52.70	5.655	47.04	18.83
7	100µl MαCD 16mM	58.35	5.655	52.70	21.62
8	50µl MαCD 20mM	52.943	5.655	47.29	18.95
9	100µl MαCD 20mM	64.661	5.655	59.01	24.73

Std number gives the amount of lipid standards loaded in µg
For lanes 6-7: number gives the volume of asymmetric vesicle preparation and 16mM gives the concentration of donor vesicles used
For lanes 8-9: number gives the volume of asymmetric vesicle preparation and 20mM gives the concentration of donor vesicles used

Table 3b- Band intensity analysis for POPS. Band intensities were derived from the TLC plate using ImageJ. Blanks were taken using the empty space on the TLC image. Final µg calculations in each sample were done in excel based on the standard curve.

Lane	Sample	Band Intensity	Blank	Band Intensity - Blank	Calculation (µg) by Excel
1	std 0.5	8.201	6.236	1.97	
2	std 1	10.607	6.236	4.37	
3	std 8	22.252	6.236	16.02	
4	std 16	37.154	6.236	30.92	
5	std 32	52.211	6.236	45.98	
6	50µl MαCD 16mM	19.837	6.236	13.60	2.93
7	100µl MαCD 16mM	24.532	6.236	18.30	5.10
8	50µl MαCD 20mM	18.966	6.236	12.73	2.53
9	100µl MαCD 20mM	18.177	6.236	11.94	2.16

Std number gives the amount of lipid standards loaded in µg
For lanes 6-7: number gives the volume of asymmetric vesicle preparation and 16mM gives the concentration of donor vesicles used
For lanes 8-9: number gives the volume of asymmetric vesicle preparation and 20mM gives the concentration of donor vesicles used

Table 3c- Band Intensity Analysis for POPE. Band intensities were derived from the TLC plate using ImageJ. Blanks were taken using the empty space on the TLC image. Final µg calculations in each sample were done in excel based on the standard curve.

Lane	Sample	Band Intensity	Blank	Band Intensity - Blank	Calculation (µg) by Excel
1	std 0.5	13.033	8.801	4.23	
2	std 1	15.161	8.801	6.36	
3	std 8	32.667	8.801	23.87	
4	std 16	52.141	8.801	43.34	
5	std 32	72.132	8.801	63.33	
6	50µl MαCD 16mM	26.019	8.801	17.22	3.04
7	100µl MαCD 16mM	31.049	8.801	22.25	4.76
8	50µl MαCD 20mM	17.877	8.801	9.08	0.25
9	100µl MαCD 20mM	21.284	8.801	12.48	1.42

Std number gives the amount of lipid standards loaded in µg
For lanes 6-7: number gives the volume of asymmetric vesicle preparation and 16mM gives the concentration of donor vesicles used
For lanes 8-9: number gives the volume of asymmetric vesicle preparation and 20mM gives the concentration of donor vesicles used

Table 4. Analysis of L4A18 peptide assay. These numbers are the maximum wavelengths of the tryptophan fluorescence recorded with our spectrophotometer. A control reading was taken of 1 µl of 0.5 mM L4A18 peptide in PBS. The Acceptor sample contained the LUVs prior to lipid exchange. The 1st row contains the contents of the exchanged vesicles made with 16 mM donor lipid. The 2nd row contains the contents of the exchanged vesicles made with 20 mM donor lipid.

Sample added	+50 µl of sample	+100 µl of sample	+200 µl of sample	+300 µl of sample
16 mM MαCD exchanged sample	359	358	356	354
20 mM MαCD exchanged sample	358	357	354	352
Acceptor	356	354	349	347
PBS+Peptide	360			

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