Incorporation of Tyrosine Kinase Receptor into a Hybrid Membrane for High Throughput Screening

A Major Qualifying Project Report

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Abstract

This project focused on the development of a hybrid membrane for high throughput screening for testing type II diabetes drugs. Self assembled monolayer (SAM) of dodecane thiol (DDT), octadecane thiol (ODT), and hexadecane thiol (HDT) were created on a gold surface and characterized by contact angle goniometry, ellipsometry, and impedance spectroscopy. Liposomes were created from injection of the lipid, dimyristoylphosphatidylcholine (DMPC) and TEM images of the vesicles were taken. A cloned tyrosine kinase receptor, with no extracellular portion, was incorporated into these liposomes. A hybrid membrane of DDT and liposomes was formed and characterized by impedance spectroscopy and quartz crystal microbalance (QCM). Activity of the protein was detected with a kinase-glo assay. This hybrid membrane system can be used for high throughput screening to test type II diabetes drugs.

Introduction

Diabetes is a disease where the body cannot regulate glucose levels in the blood. Insulin is a hormone that controls the level of glucose in the blood. Type I diabetes is caused by a lack of insulin production. Type II diabetes is caused by the insulin receptor not responding in the presence of insulin.

There are currently six main types of type II diabetes drugs, biguanide, thiazolidinedione, sulfonylurea, meglitinides, DPP-4 inhibitors, and alpha-glucosidase inhibitors. Biguanides decrease the amount of glucose that the liver produces in an attempt to decrease the amount of glucose in the blood. Thiazolidinedione also decreases the amount of glucose produced in the liver, but it also increases the efficiency of insulin in fat and muscle cells. Sulfonylurea and meglitinides both affect the pancreas, making it produce more insulin, to be released into the blood. DPP-4 inhibitors prevent glucagon-like peptide-1 (GLP-1) from being broken down in the body. GLP-1 breaks down glucose in the blood, but breaks down quickly in the body without the DPP-4 inhibitor. Alpha-glucosidase inhibitors work by preventing the breakdown of starches, into glucose. Also, they can slow the breakdown of some sugars, such as sucrose. All of these drugs either work by decreasing the amount of glucose, or increasing the amount of insulin. Of these drugs, only thiazolidinedione affects the insulin receptor, increasing its affinity to bind insulin.

The insulin receptor is a tyrosine kinase receptor, found in the lipid bilayer cell membrane of skeletal muscle cells. The tyrosine kinase receptor has three sections, an extracellular portion that binds insulin, and a transmembrane portion and an intracellular portion that use that insulin to be come active. (Alberts et al., 1994)

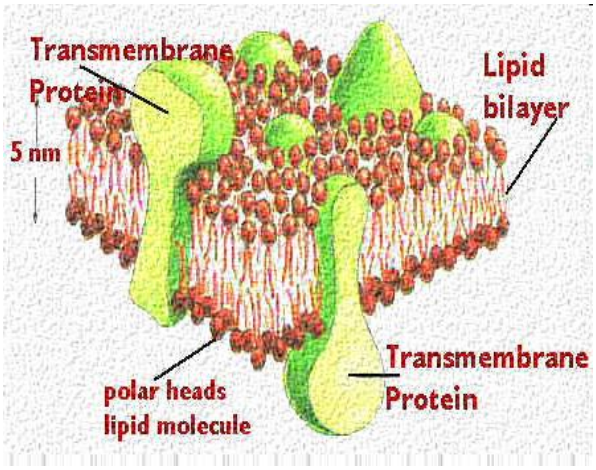

Figure 1: diagram of transmembrane proteins in a lipid bilayer
Image from "Molecular Biology of the Cell" Third Edition, by Bruce Alberts, Dennis Bray, Julian Lewis, Keith Roberts, and James D. Watson. Garland Publishing, Inc. New York & London, 1994. page 477

Insulin binds to the receptor on the outside of the cell, and when two bound receptors are close they combine together to form a dimer. Once they dimerize, they can cross-phosphoralate the tyrosine groups on the intracellular portion of the enzyme in the presence of ATP. This phosphorylated tyrosine then sends a signal to relay proteins. This signal reaches the glucose channel, activating it. Once it's active, it opens to allow glucose to enter the channel from the blood, and then closes that end and opens to allow the glucose to enter the cell. To incorporate this protein into a hybrid membrane created on a gold slide, one end of the protein must be removed. The transmembrane portion goes through the hybrid membrane. One end sticks out the top, but the other end cannot pass through the gold surface. The intracellular portion is the section that is needed to test for activity with type II diabetes drugs. It is for these reasons that the protein was cloned, by Bluesky Biotech (Worcester, MA), with no extracellular portion.

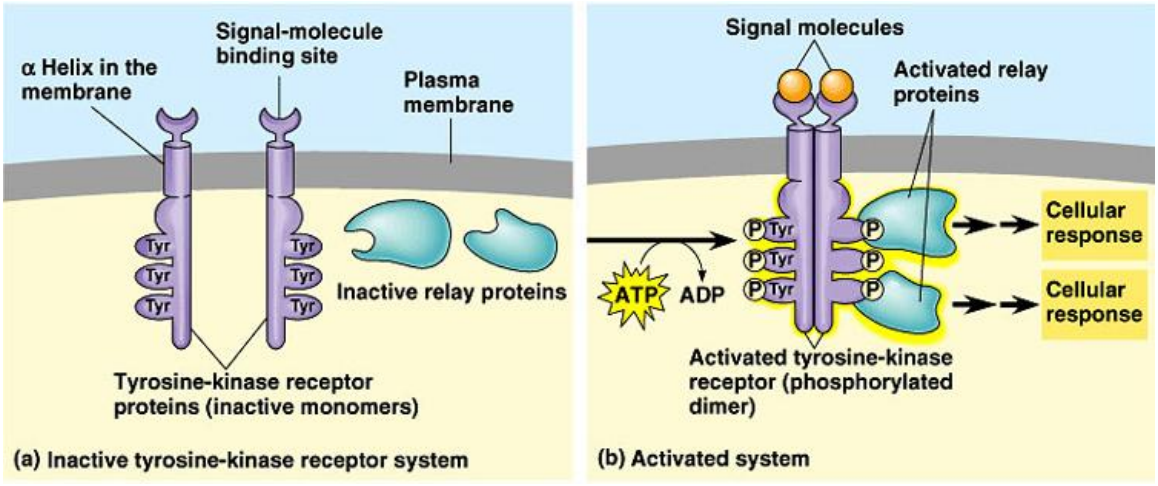

Figure 2: diagram of tyrosine kinase receptor forming its dimer
http://www.bio.miami.edu/~cmallery/255/255prot/255proteins.htm

The tyrosine kinase receptor is only active in a membrane because it cannot dimerize unless it is in a membrane. Lipid bilayer membranes act like a two dimensional fluid. The proteins can move through the membrane but they cannot leave the membrane. This forces the protein in the same direction so the dimer can form.

The tyrosine kinase receptor with the extra cellular portion removed can be incorporated in a hybrid membrane so it can be used for high throughput screening. A hybrid membrane has two layers. The first is a self assembled monolayer on a gold surface. The second layer is liposomes added on the SAMs.

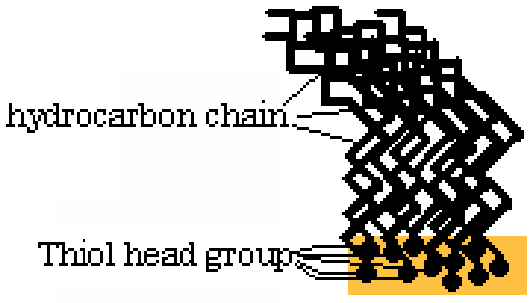

Figure 3: diagram of a SAM on a gold slide

Self assembling monolayers, or SAMs form due to their strong gold-thiol covalent bond, giving them stability, and the Van der Waal forces of their nonpolar hydrocarbon chains attracting to each other. These Van der Waal forces can cause the SAM to angle slightly, but more importantly they keep the SAM organized and well packed on the gold surface.

Phospholipids have two main components: a hydrophilic phosphate polar head, and a hydrophobic nonpolar hydrocarbon chain for a double tail. In a polar solution, like water, these lipids form lipid vesicles, or liposomes. These vesicles have a bilayer membrane in order to minimize the surface area of the nonpolar sections of the lips and maximize the surface area of the polar hydrophilic heads. The polar heads face both inward to the center of the vesicle and outward to the rest of the solution. This leaves the hydrophobic tails in between the polar heads, away from the polar solution.

Figure 4: Diagram of a phospholipid

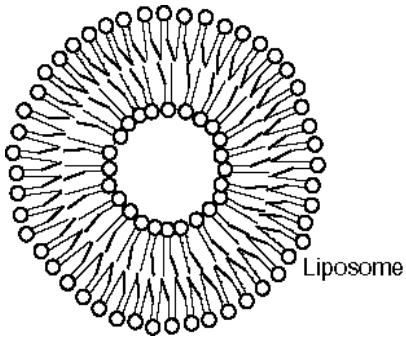

Figure 5: diagram of a liposome

A hybrid membrane is a combination of a SAM and a lipid vesicle. The SAM acts as one half of the lipid bilayer, and the liposome attaches to the SAM to form the second half. In the image below, the SAM is the bottom layer, which is formed on the gold surface first. The top lipid layer is added after the SAM is formed, to complete the bilayer hybrid membrane.

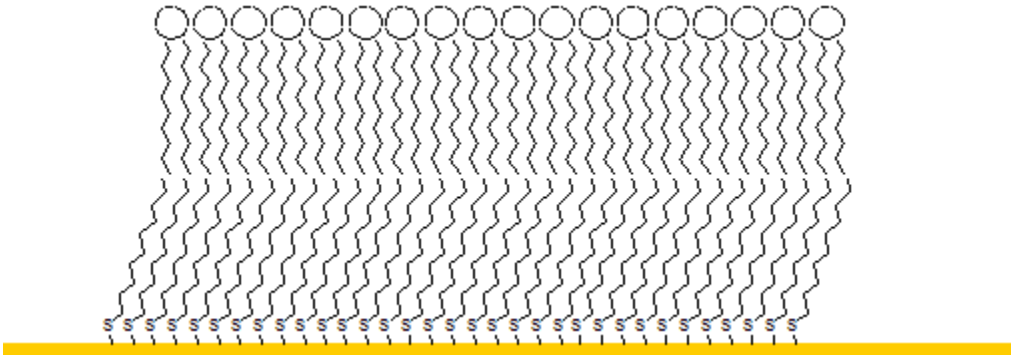

Figure 6: diagram of a hybrid membrane of a SAM and lipid on a gold

In the body, the tyrosine kinase receptor is in a lipid bilayer cell membrane. A lipid vesicle is similar to a cell membrane in that the protein can also be incorporated into the bilayer membrane of a lipid vesicle. The vesicle, with the protein still attached, can then be added to a self assembling monolayer on a gold surface, to stabilize it. Once the protein is stabilized in the hybrid membrane of the lipid vesicle and self assembling monolayer, it can undergo a high throughput screening process and help with type II diabetes drugs research.

A high-throughput screening system is an assay method that can test hundreds of thousands of compounds. A single protein is chosen, and tested against all of these compounds. Any compounds where the protein shows activity can be tested further, either again by machine or this time by hand. These compounds are tested in 96-, 384-, 1536-, or 3456-well plates. Usually, a machine can be used to conduct the assay and check which wells, if any, show signs of activity. Some of these entire processes can be run by robots, while others still have to be done more manually. Once initial tests are done on every compound that was on the list to be tested, follow up tests can be done just on the compounds that showed activity or appeared to be of interest.

1. I + RTK ↔I-RTK

2. I-RTK+I-RTK↔ dRTK

3. dRTK + ATP → activated dRTK + ADP

4. Activated dRTK + signal proteins → Activates glucose channel

Figure 7: diagram of tyrosine kinase activity
I=insulin
RTK=tyrosine kinase receptor
dRTK= dimmer of tyrosine kinase receptor
ATP= adenosine triphosphate
ADP= adenosine diphosphate

High throughput screening can be used to test the tyrosine kinase receptor in the hybrid membrane against thousands of different compounds that could potentially be used to make drugs that will activate the tyrosine kinase without binding insulin to it. The goal of these drugs is to be able to skip equations 1 and 2 in the diagram above. These drugs will transition from the RTK to dRTK.

RTK + new drugs → dRTK

This HTS will greatly reduce the number of potential drug candidates. Once the HTS finds compounds that activate the tyrosine kinase, research can be done to try and turn the compounds into actual drugs that can work in the body.

3. Experimental: Materials and Methods

Materials

The tyrosine kinase receptor was cloned and obtained from BlueSky Biotech (Worcester, MA). The kinase-glo plus luminescence assay kit was purchased from Promega (Madison, WI). The dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma (St. Louis, MO). Dodecane thiol was purchased from Aldrich (St. Louis, MO). Decane thiol, pentane thiol, octadecane thiol, hexadecane thiol, 11-mercaptoundecanoic acid, and adenosine triphosphate were purchased from Alfa Aesar (Ward Hill, MA). Gold slides were purchases from Evaporated Metal Films (Ithaca, NY).

3.1 Formation and Characterization of SAMs

Gold Slides, 100 nm gold on 5 nm chromium on top of glass, were cut into 2 cm by 1 cm slides. A piranha solution of 70 mL concentrated H_2SO_4 and 30 mL 30% H_2O_2 were poured carefully into a crystallization dish. After 10 min the gold slides were placed in the solution. This piranha solution cleans the slide by dissolving any random particles on the gold surface. After 15 minutes, they were removed from the piranha solution and put into distilled water. They were then rinsed with ethanol and dried with nitrogen gas. After drying they were plasma-cleaned with oxygen radicals using a Plasma Prep II plasma cleaner by SPI Supplies. This was done by putting the gold slides in a vacuum sealed chamber and flowing oxygen into it. A Voltage was sent through the oxygen until a purple color was visible. This color indicates that there are oxygen radicals. After 45 seconds, the voltage was stopped and the slides were removed.

Once the slides were clean, they could be placed in each thiol solution and left overnight to form the self assembling monolayer (SAM). After overnight incubation the slides were removed, rinsed with ethanol, and dried with nitrogen. Each thiol

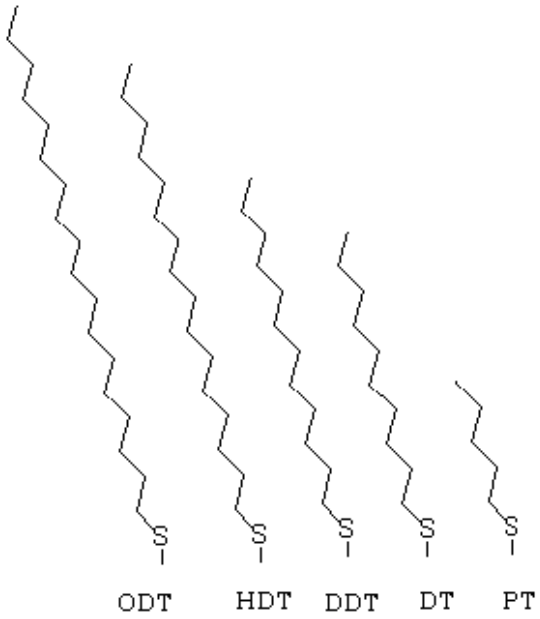

Figure 8: Different length SAMs
ODT- octadecane thiol
HDT- hexadecane thiol
DDT- dodecane thiol
DT- decane thiol
PT- pentane thiol

solution was composed of 50 mL ethanol and 0.75 mmol of the thiol, for a concentration of 0.015 M. Solutions were made for octadecane thiol, hexadecane thiol, dodecane thiol, decane thiol, and pentane thiol. They have carbon chain lengths of 18, 16, 12, 10, and 5 respectively.

Once the SAMs were created, a goniometer was used to measure contact angle by putting a drop of water in the surface of the SAM and measuring the angle from the surface to the bottom of the drop of water. Ellipsometry was used to determine the

thickness of the SAM. The ellipsometer was used to measure the angles of polarized light that reflect off the surface. These angles can be used in an ellipsometry program to calculate the thickness of the SAM on the gold surface. Electrochemical impedance spectroscopy was used to determine capacitance and resistance. Impedance spectroscopy sends an AC voltage through the sample, and measures the impedance over a range of frequencies. The SAM on the surface is the capacitor in the circuit, which makes the calculated capacitance value of the system, the capacitance of the SAM. The electrochemical impedance was run at both room temperature, and in a water bath at 37 ^{o}C. The DC voltage was set to 5 mV and AV voltage at -0.3 V. (Plant 1993)

3.2 Formation and imaging of Vesicles

Lipid vesicles were formed by lipid injection of DMPC into a buffer solution. A buffer solution of 10 mL phosphate buffered saline (PBS) was heated in a water bath to a constant 37 ^{o}C. Both the buffer solution and the water bath were stirred vigorously with a magnetic stirrer at high. A solution of 12.5 mg of DMPC in 0.5 mL of ethanol was injected with a 22-gauge syringe needle into the PBS buffer solution at a rate of 3 mL/hr. If the lipid vesicle solution was not used immediately after its creation, it was kept at 4^{o}C for storage. (Kremer et al. 1977)

Once the liposomes were created, transmission electron microscopy (TEM) images of the vesicles were taken with an electron microscope. First, the vesicles had to be prepared for imaging. The vesicle solution (1.0 mL) was centrifuged in a 1.5 mL centrifuge tube at 14,000 rpm for five minutes. In order to cross link the vesicles and make them easier to see, osmium tetroxide (0.4 mL of a 4% solution) was added to the vesicle solution and after 30 minutes of occasional shaking the solution was centrifuged again at 14,000 RPMs for 30 minutes (as is all centrifuging done for vesicle preparation). The supernatant was removed again and 1 mL of Ethanol was added. The solution was mixed by a vortex for 30 seconds and then centrifuged again. This removal of supernatant, replacing it with a new solution, vortexing, and centrifuging again was repeated again for ethanol, then twice for propylene oxide. Both the ethanol and propylene oxide are added then removed to dehydrate the solution, removing any water with ethanol, and then removing any propylene oxide with water. Once the second propylene oxide supernatant was removed, a mixture of propylene oxide to resin in a 2:1 ratio was added and let sit for 1 hour. It was then centrifuged and the supernatant was

removed and replaced with a propylene oxide/resin mixture in a 1:2 ratio this time and allowed to sit for an hour. The solution was again centrifuged and the supernatant was removed. These mixtures were used to try and remove any leftover propylene oxide from the vesicles. Resin was added and the vesicles were cured overnight at 70 ^{o}C to allow the resin to harden. Once cured, the resin was cut with a diamond blade into 50-60 nm slices, and placed on formvar coated carbon grids. The grids were then placed in the electron microscope and TEM images were taken.

3.3 Formation and Characterization of Hybrid Membrane

The hybrid membrane was characterized by impedance spectroscopy and quartz crystal microbalance (QCM). The hybrid membrane was formed separately for each characterizing test. For impedance spectroscopy, a water bath was prepared at 37 C. A beaker with PBS buffer solution was placed in the water bath and the electrochemical probes were placed in it. The 1 cm by 2 cm gold slide with the SAM on it was placed halfway into the buffer solution giving the slide a surface area of 1 cm^2 in solution. The impedance spectroscopy was taken. Then, lipid vesicle solution (10 mL) was added to the buffer solution. The impedance spectrum was taken as soon as it was added, and then again every five minutes until the capacitance leveled off and stopped decreasing.

For the QCM, a water bath was heated to 37 ^oC, and a beaker of PBS buffer solution was equilibrated to 37 ^oC in the water bath. Gold slides were not used for the QCM measurements. The quartz crystals, coated in gold were used instead. The SAM was added the same way as described in section 3.1, by overnight incubation in the same ethanol and thiol solution used for the gold slides. The quartz crystal is cut to a specific resonating frequency. Adding anything to the surface of this crystal changes that resonating frequency. When the liposomes are added to the SAM, the crystal changes frequency, which is measured and recorded by the QCM. The mass is calculated from the frequency. This allows the QCM to measure the change in mass from before the liposomes have been added, to after the hybrid membrane as formed.

The quartz crystal with the SAM on it was placed in the crystal holder and connected to the QCM with the connecting cable. The holder was placed in the PBS solution. The mass and frequency was measured until the temperature equilibrated and a

baseline was established. Then, lipid vesicle solution (10 mL) was added to the PBS solution. This addition caused a sudden spike in the mass. The QCM measurements were taken until the mass and frequency levels stopped changing.

3.4 Kinase Assay for Tyrosine Kinase Receptor

Tyrosine kinase receptor cloned by BlueSky biotech was incorporated into the lipid vesicles, by mixing the liposomes and protein in a 1:8 ratio and heating in a water bath at 37 ^{o}C for two hours. Kinase-glo luminescence kit from Promega was used to test the activity of the tyrosine kinase receptor. The assay has a luciferase that luminescence in the presence of ATP. The luminescence decays over time, and if the kinase is active it uses ATP as well, making the luminescence decrease at a faster rate. The kinase-glo substrate was mixed with the kinase-glo buffer solution and heated at 37 ^{o}C for one hour to form the kinase-glo reagent. A 96-well plate was used for the kinase assay. First, the vesicle protein solution was added to the wells, 22.5 μL in the first well, 12.25 μL in the second, 6.12 μL in the third, 3.06 μL in the fourth, 1.53 μL in the fifth, and 0.76 μL in the sixth. ATP (1 μL) was added to each of these wells, and one additional empty well. The kinase-glo reagent was added to all of these wells to fill them up to a total volume of 45 μL. One additional well of just 45 μL kinase-glo reagent was added as a control. Each of these eight wells was done in triplicate. The well plate was shaken linearly for ten minutes by the 96-well plate reader on normal speed, before the luminescence was measured. The luminescence was measured again every five minutes for three hours.

3.5 Incorporation of Protein into Hybrid Membrane

The protein was incorporated into the liposomes by mixing the liposomes and protein in a 1:8 ratio and heating in a water bath at 37 ^{o}C for two hours. A new hybrid membrane was created from these liposomes with incorporated protein. The same impedance spectroscopy and QCM experiments that were used in section 3.3 to form the original hybrid membrane were repeated with the protein vesicle solution, replacing the original vesicle solution.

The kinase assay was modified to include the hybrid membrane and repeated again. Gold sides were cut to about 0.7 by 0.7 cm instead of the usual 1 cm by 2 cm and a SAM of DDT was formed on them in the same method described in section 3.1. The assay was done in a 24-well plate so the gold slides could fit. PBS buffer solution (0.8 mL) was added to four wells. Gold slides were in the first two wells. The vesicle protein solution (100 µL) was added only to one of the wells with a gold slide and to one with out. ATP (5 µL) and kinase-glo reagent (100 µL) was added to each well. All four wells were done in triplicate. The plate was shaken in the plate reader for ten minutes before the first reading. Luminescence readings were taken every five minutes for two hours.

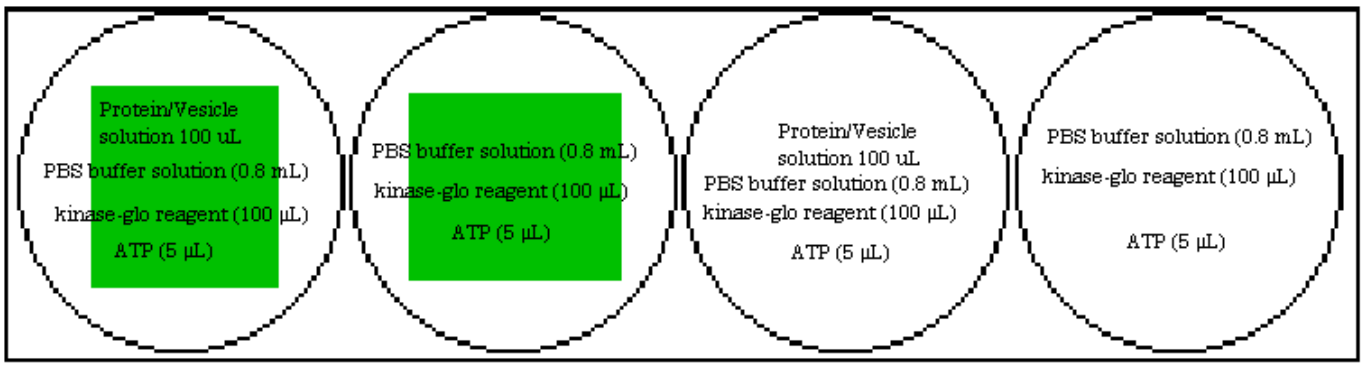

Figure 9: Contents of each Plate Well

4. Results

4.1 Formation of SAMs

The three SAMs that were initially formed were octadecane thiol, hexadecane thiol, and dodecane thiol. They were characterized by ellipsometry, contact angle, electrochemistry, impedance spectroscopy, and QCM.

Contact angle can be measured to show how hydrophobic a surface is. The greater the angle is, the more hydrophobic the surface. This is the simplest way to determine if a hydrophobic SAM has been successfully attached to the gold surface. However, it only works on a macroscopic scale, and does not necessarily determine how organized the SAM is or how well packed. This is because the hydrocarbon chains will still repel the water, even if there are scratches or impurities in the SAM. 11-mercaptoundecanoic acid (MUA) is known to be hydrophilic and was used as a control against the hydrophobic SAMs. The data shown below is the average of three measurements on each of three different slides for nine total values per SAM.

Table 1: Contact Angles

	HDT	DDT	ODT	MUA	Gold
AVG angle	100.1±1.41	99.8±2.02	99.8±2.41	15.8±3.18	60.7±1.02

Ellipsometry can be used to calculate the thickness of the SAM. The manual ellipsometer was used to measure the angles of polarized light as they refracted or reflected off the gold surface. These angles were entered in to the ellipsometry analysis

program (Rudolf Ellipsometry), along with the known refractive index for gold (0.47). From these angles, the height or thickness of the SAM was determined. The data shown below is the average of three measurements on each of three different slides for nine total values per SAM.

Table 2: Thickness of SAMs from Ellipsometry

	Average Height (nm)
DDT	1.57±0.4
HDT	1.61±0.6
ODT	2.79±0.4

Impedance spectroscopy measures the resistance of the SAM, the resistance of the solvent, and the capacitance of the SAM. If the SAM is well organized and not very permeable, it will have a higher resistance and a lower capacitance than a SAM that is not as well packed and more permeable. Impedance spectroscopy was taken for ODT, HDT and DDT, and DDT was later chosen as the best choice to proceed to make the hybrid membrane on. Since this was the smallest of the three chosen, impedance spectroscopy was taken again for PT, DT and DDT. This tested two smaller SAMs than DDT to see if they might be better choices to work with. (Plant, 1994)

Table 3: Resistance and Capacitance from Impedance Spectroscopy

	DDT	37°C DDT	DT	37°C DT	PT	37°C PT
Surface resistance (Ohms)	8580±989	1710±308	2870±782	2610±452	3550±452	2580±993
Solvent resistance (Ohms)	20.3±1.81	17.5±2.01	21.0±5.09	18.2±3.38	19.0±3.38	14.7±1.72
Capacitance (μF)	6.6±0.35	31.1±5.03	19.1±1.85	63.3±8.57	13.0±0.76	68.5±8.24

Again, all impedance data is the average of three measurements on each of three different slides for nine total values per SAM.

4.2 Imaging of Vesicles

Images of the vesicles were taken through TEM imaging by an electron microscope. Vesicles were measured against the scale and the average of 30 vesicles measured came out to 76.8 nm, with a range of 63 nm to 101 nm. These vesicle sizes should be consistent with the needle gauge (22) and injection rate (3 mL/hr) that is used.

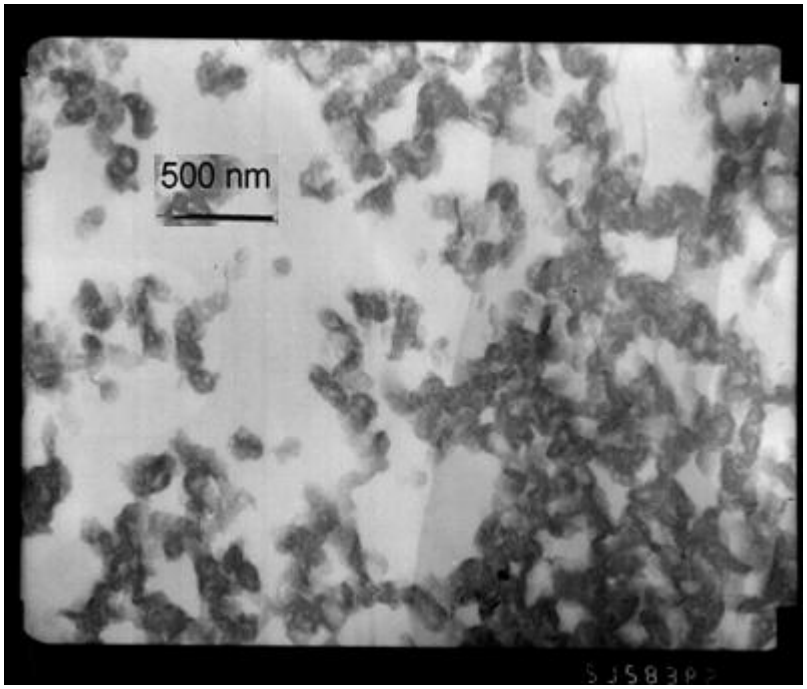

Figure 10: TEM image of Lipid Vesicles

4.3 Characterization of Hybrid Membrane

Impedance spectroscopy of the hybrid membrane was taken and the capacitance was determined by applying the Randles model using the Simplex Method. The Randles is a model circuit containing two resistors and a capacitor, to which the data is fit. There is a capacitance for the SAM and a resistance for the SAM and the solution in the model. The capacitance was taken every five minutes until it leveled off. The difference between the initial and final capacitance, 45.3 μF, shows the formation of a hybrid membrane on the SAM. Also, the time it takes to form, can be approximated to about 20-25 minutes based on where the capacitance levels off and stops decreasing.

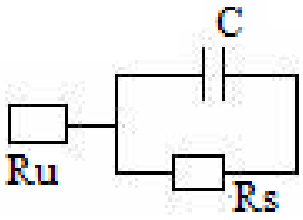

Figure 11: Randles circuit model
Ru-surface resistance
C- capacitance of SAM
Rs- charge transfer resistance of SAM

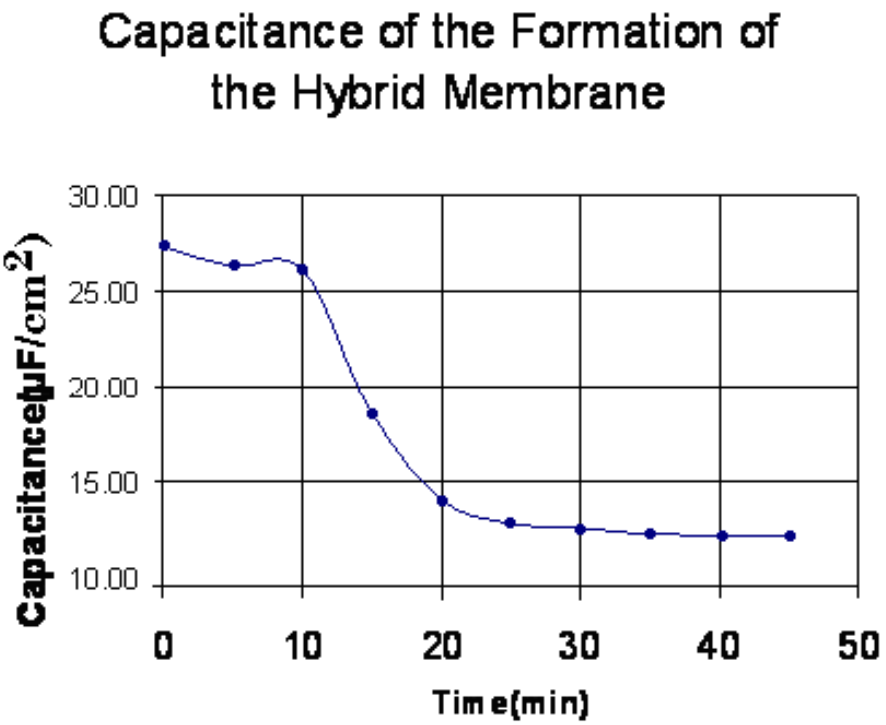

Figure 12: : Capacitance from Impedance spectra of the hybrid membrane

The QCM measures the change in frequency of the quartz crystal over time. The change in mass is calculated from this frequency. The important data gained from QCM is the change in mass from the initial baseline of the mass to the final, after the lipid solution has been added. This change in mass was calculated as 0.243 $\mu g/cm^2$- 0.008 $\mu g/cm^2$= 0.235 $\mu g/cm^2$.

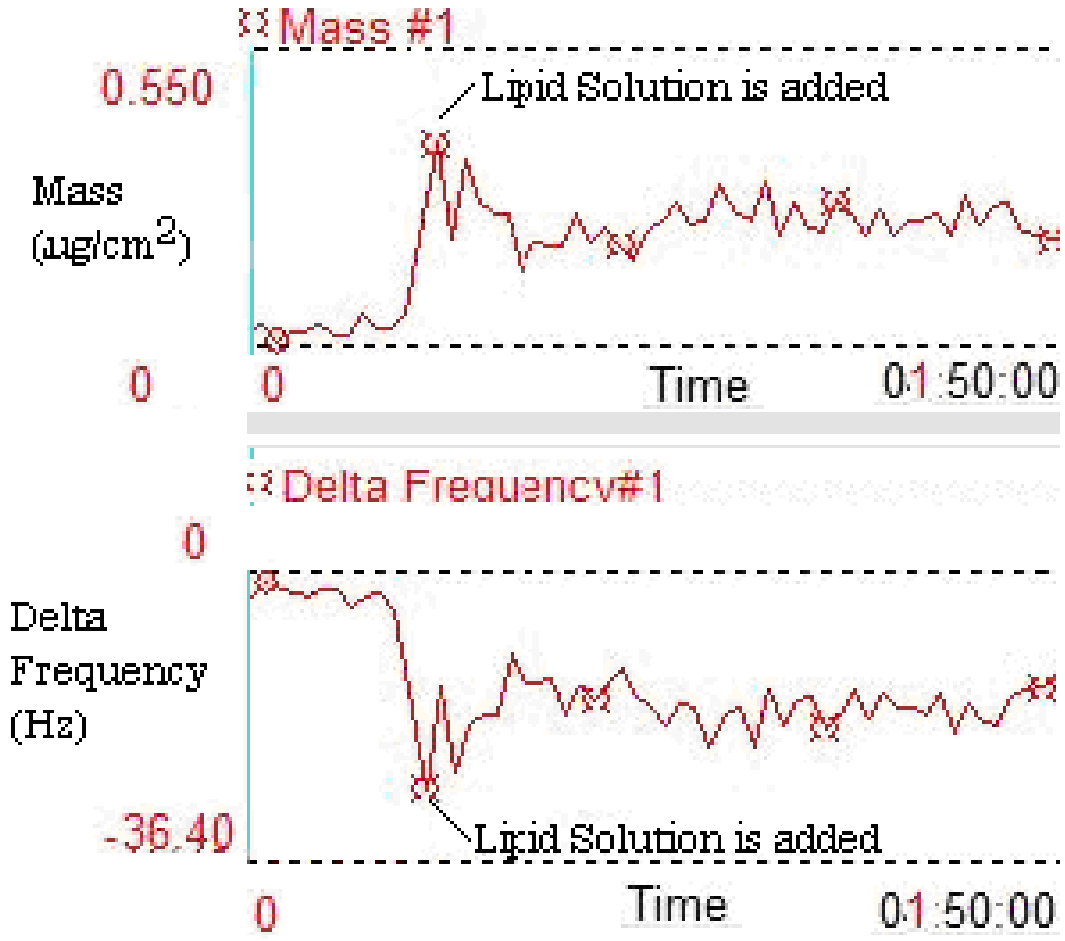

Figure 13: QCM graph of mass and frequency of the formation of the hybrid membrane

4.4 Kinase Assay for Tyrosine Kinase Receptor

The tyrosine kinase receptor's activity was measured both with the kinase in a buffer solution, and, and in a solution with liposomes. The luminescence decay was measured over time, and the slopes of the decays were used to determine the rate of ATP usage.

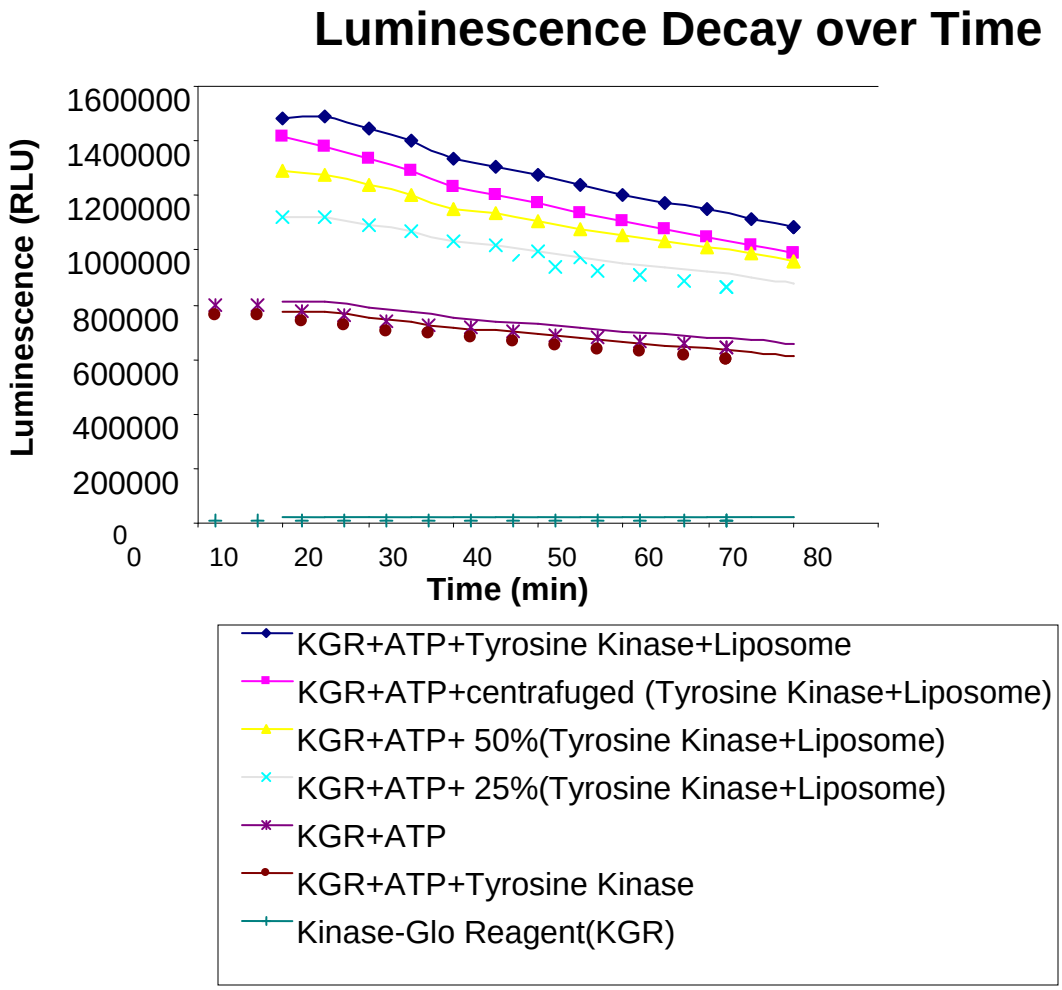

Figure 14: Luminescence decay of kinase assay

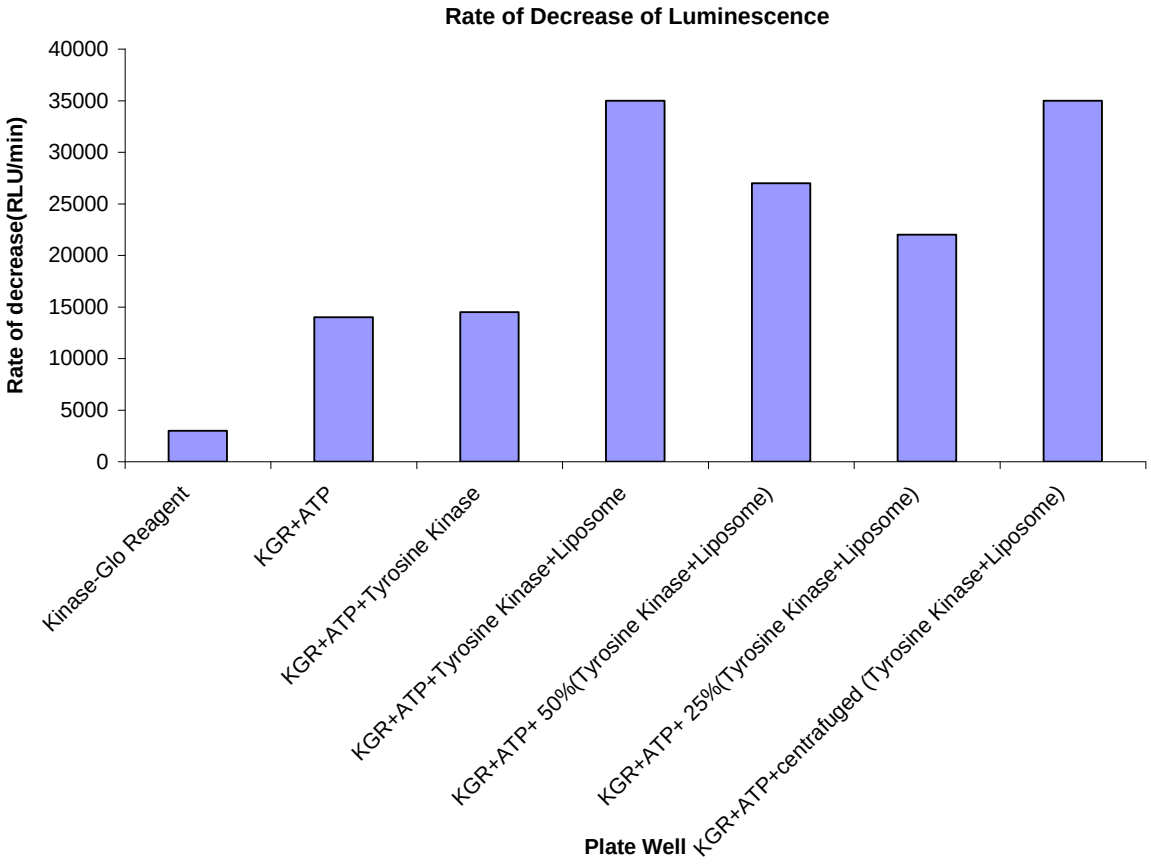

Figure 15: Rate of luminescence decay of kinase assay

The luminescence assay kit uses a plate reader to measure the decrease in luminescence over time. The luminescence decreases because there is less ATP available for the luciferase to use. This assay effectively measures the amount of ATP that the system is using. The luciferase will use a constant amount of ATP each time, which is measured by the control of kinase-glo reagent and ATP. Any further decrease in ATP is cause by another enzyme being active. In this experiment the tyrosine kinase receptor is actively using ATP in a membrane, which causes the ATP concentration, and the luminescence, to decrease faster than the control of kinase-glo reagent and ATP.

4.5 Analysis of Incorporation of Protein into Hybrid Membrane.

Once the protein was successfully incorporated into the liposomes, new hybrid membranes were formed with those liposomes. The same electrochemical and QCM experiments were performed again to ensure that a hybrid membrane was still being formed. The total change in mass was 0.302 μg/cm^2. With a known change in mass, and known mass of each liposome, the average number of liposomes on the surface can be calculated to 2.69*10^14 liposomes per cm^2.

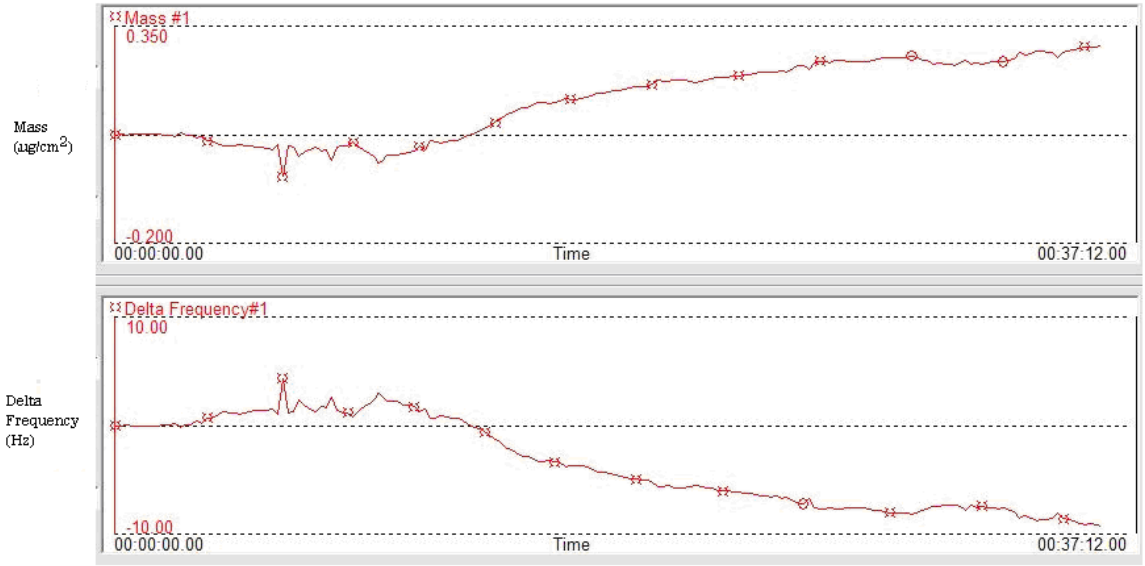

Figure 16: QCM graph of mass and frequency of the formation oh hybrid membrane with incorprated protein

The kinase assay was also run again with the hybrid membrane in the well plates. These tests had to be done in a 24-well plate, in order for the gold slides to fit.

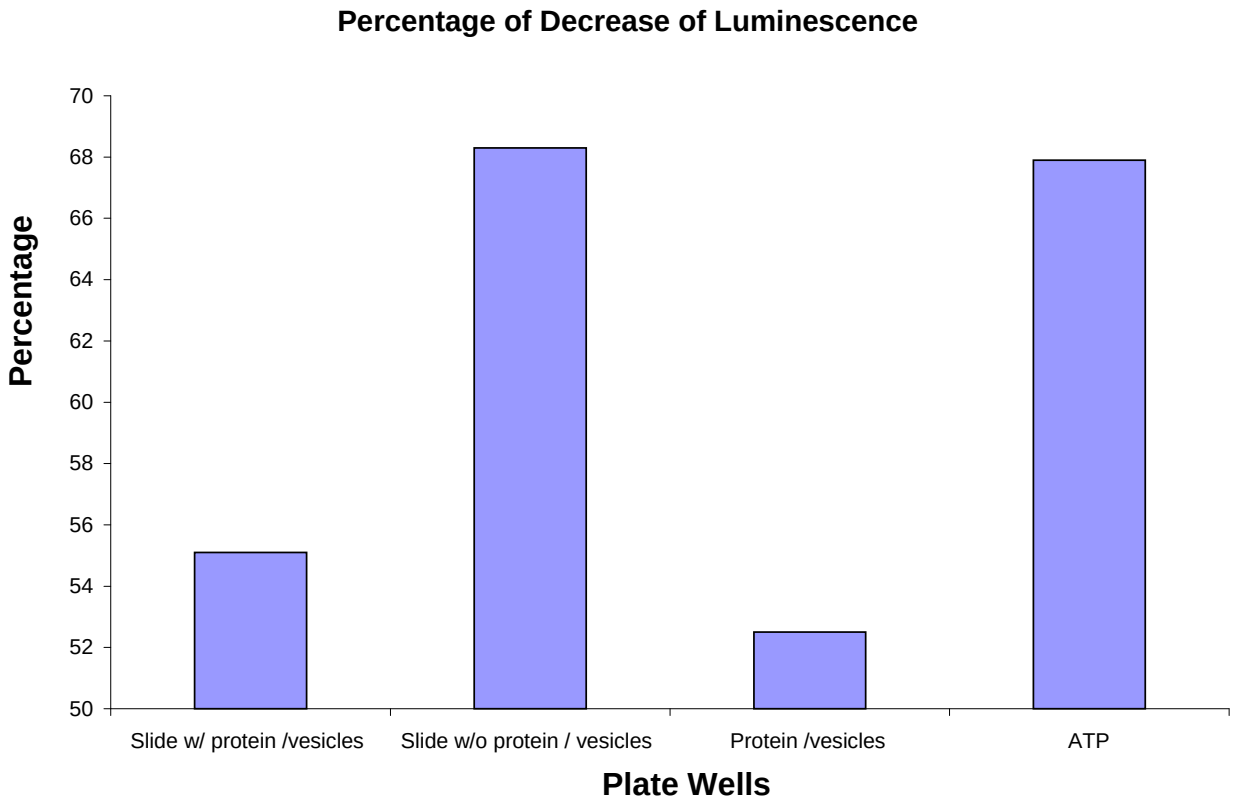

Figure 17: Percentage of luminescence decay of kinase assay

5. Discussion

5.1 Formation of SAMs

The SAMs form with a strong covalent gold-thiol bond. This strong bond makes them very durable, and can be used for months, potentially years, after their creation date. The extending hydrocarbon chains coming out from the SAMs interact with each other through Van der Waal's forces. This interaction allows them to form the well packed, self assembling monolayer. Also, the Van der Waal's forces can cause them to not be completely vertical 90 degrees from the gold surface, but at an angle instead. Three SAMs were chosen to start with, DDT, HDT, and ODT. All three SAMs were well formed, and appeared to be able to form hybrid membranes. However, with the larger chains, HDT and ODT, it is harder to determine by electrochemistry if a hybrid membrane has formed. The capacitance is already quite high due to the length of the chain and how well packed and organized it is, that when adding a new layer on top it may be harder to detect the change. For the small chain of DDT, it is still able to form a hybrid membrane, but has a higher capacitance, and is easier to see a new layer added on top, by measuring the change in capacitance. Since the smallest of the three original chains was chosen it made sense to try some even smaller chains of DT and PT. While the two additional SAM choices, DT and PT, may have worked for creating a hybrid membrane, DDT already worked at least as well and it was decided to continue working with DDT. The most important factor in choosing a SAM was to have it organized enough to form a hybrid membrane on, but not too organized, that we would be unable to see a change in resistance or capacitance when forming the bilayer. It is important to

form the bilayer successfully, but it is also important to be able to determine that we have formed it.

5.2 Imaging of Vesicles

Once the vesicles were formed by lipid injection, an initial unofficial test was done by shining a laser through it to look for the particles, or vesicles in the solution. If a laser is shined through pure distilled water, or even PBS buffer solution without vesicles, the water's refractive index will scatter the beam. However, if there are vesicles in the solution the beam will bounce off each particle. This contact with the particles allows the beam to be seen clearly in the solution. This was not very informative, and simply was a test to see if any vesicles might be there or if the injection failed and needed to be tried again.

The TEM imaging was done to definitively check for vesicles, as well as determine the average size. The preparation was very important, because just putting vesicles under the microscope would not allow pictures of to be taken. The liposomes are composed of mostly light elements like carbon, oxygen and hydrogen.

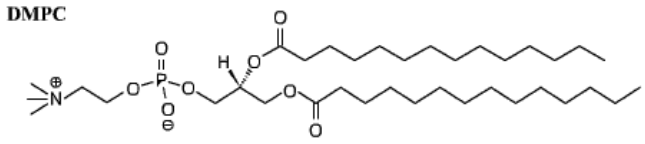

Figure 18: diagram of a DMPC molecule

These lighter elements do not refract the electron beam from the TEM imaging enough to leave an imprint on the negative of the film. For that reason, osmium tetroxide was used to crosslink the liposomes. The DMPC lipids used are not completely saturated with double bonds. This allowed the osmium tetroxide's oxygen to bond to a single bond on the carbon chain of a lipid. Each osmium tetroxide molecule can bond to four different lipids, either on the same vesicle or different vesicle. Each liposome could potentially

bond to a large number of osmium molecules. By simple statistics, some osmium molecules will bond multiple vesicles, and those vesicles will be bonded to other vesicles. This creates cross linking between multiple liposomes and concentrates them, making them easier to find under the electron microscope. Also, the high density of the osmium can diffract the electron beam that the TEM imaging uses, leaving a more defined mark on the negative of the film.

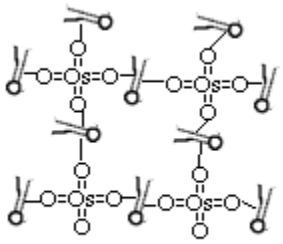

Figure 19: diagram of lipids cross linked by osmium tetroxide

5.3 Formation and Characterization of Hybrid Membrane

The hybrid membrane is formed by adding the liposomes to the SAM to complete the simulated lipid bilayer. The formation of this bilayer, in solution is stabilized by the decrease in entropy when it forms. The PBS solution is polar, so the hydrophilic heads of the liposomes can exist anywhere in the solution. The polar thiol group in the end of the SAM is bonded to the gold, leaving the nonpolar hydrocarbon chain extending out into the solution. The only way these hydrocarbon chains can get protection from the polar solution is by forming the bilayer membrane with the liposomes. This also keeps the hydrophobic tails of the lipid vesicles out of solution, by being in between the SAM and the polar head group.

The QCM measurements were the most accurate way to determine if the hybrid membrane had been formed, being able to measure the mass with an error of ±0.02 $\mu g/cm^2$. The lipid vesicles, forming on the surface of the SAM increased the mass on the gold surface. This mass changed the frequency at which the quartz crystal was vibrating.

From the frequency, the mass was calculated. This overall change in mass shows how much lipid was added on the surface of the SAM.

5.4 Kinase Assay for Tyrosine Kinase Receptor

The kinase assay uses the kinase-glo luminescence assay kit to measure the decrease in luminescence overtime. The luminescence is caused by the ATP activating the luciferase enzyme in the kinase-glo reagent. This luminescence decreases over time as ATP is used by the reaction. If there is a secondary enzyme that is also using ATP, then the luminescence will decrease faster, due to less ATP being available for the luciferase enzyme. The tyrosine kinase receptor uses the ATP when it is active in a membrane. It is only active inside the membrane, because it is only active when two tyrosine kinase receptors dimerize together. Once they dimerize, the intramembrane amino acid groups can cross-phosphoralate the tyrosine in the presence of ATP. Usually this cross-phosphoralation triggers other kinase to open a glucose channel in the cell, but without the tyrosine kinase being in an actual cell, none of these other steps can occur. The phosphoralation of the tyrosine still occurs though if it does dimerize, and it still uses ATP. This ATP usage is measured by the assay to check for activity of the tyrosine kinase receptor.

5.5 Analysis of Incorporation of Protein into Hybrid Membrane.

The percentage of luminescence decreased more for the wells that had the hybrid membrane in it that for the slide without protein or vesicles. The decrease was not quite as great as it was for the vesicles and protein solution. This was expected, because even if the hybrid membrane is saturated with protein there will still be some excess that will be removed, so there is not as much protein in the hybrid membrane as there is in the

pure solution. The same electrochemical impedance spectroscopy and QCM experiments that were performed on the hybrid membrane before protein was added were performed again after the protein had been incorporated into the hybrid membrane. The impedance spectroscopy yielded the same results. The QCM also yielded very similar results, but had a slightly higher increase in mass. This increase in mass could be due to the proteins being added into the hybrid membrane. However, the change in mass was not very great (0.058 $\mu g/cm^2$) and at least some of that change could be due to error. More QCM experiments would need to conducted to be able to tell with certainty, but it does appear that adding the protein into the hybrid membrane increases its mass.

6. Conclusions

The goal of this project was to create a hybrid membrane and insert a cloned tyrosine kinase receptor with no extracellular portion into the hybrid membrane while still keeping the tyrosine kinase receptor active. A dodecane thiol SAM was successfully formed on a gold surface. Liposomes were successfully formed and tyrosine kinase receptors were incorporated into the liposomes. These liposomes were added to the SAM to form a hybrid membrane, and the tyrosine kinase retained activity (at room temperature) in the membrane. Future experiments could be conducted using different SAMs or different sized vesicles to see if these affect the amount of protein incorporated in the hybrid membrane. A better combination of SAM length and vesicles size could increase amount of liposomes that form on the SAM or increase number of proteins that are incorporated into the liposome. Either of these would increase the activity of the protein in the hybrid membrane. Also, activity of the tyrosine kinase receptor in the hybrid membrane can be checked at body temperature (37 ^{o}C) to see if the activity increases.

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