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Affinity of Alpha-Synuclein and Lipid Vesicle Interaction

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Symbols and Abbreviations

- TIRFM: Total Internal Reflection Fluorescence Microscopy
- PD: Parkinson's Disease
- CD: Circular Dichroism
- DLS: Dynamic Light Scattering
- A-syn: Alpha-synuclein
- GUVs: giant unilamellar vesicles
- SUVs: Small unilamellar vesicles
- MUVs: Multilamellar vesicles
- POPS: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
- POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
- POPE: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine

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Abstract

Alpha-synuclein is a protein involved in several neurodegenerative diseases, such as Parkinson's, where it has been observed to form amyloid fibrils or Lewy bodies. However, how or to what extent alpha-synuclein is involved is not known. I have in this thesis been conducting experiments in order to design an experimental model system to study how alpha-synuclein interacts with a cell membrane. This system consists of giant unilamellar vesicles (GUVs) made up of various lipid species, including 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE). The vesicles are loaded with a fluorescent dye (fluorescein) which starts to leak out if the membrane becomes permeable. It was observed that GUVs with a diameter exceeding 20 µm could be made of different lipids and filled with fluorescein. The GUVs were photobleached and the movement of fluorescein in and out of them was measured. Upon addition of alpha-synuclein it was observed it caused no detectable structural damage of the GUV membrane during 20 minutes. In summary, I have investigated the steps in setting up a general method of studying protein-lipid interactions, where the most ideal way to monitor GUV membrane permeability was found, with specific parameters regarding lipid concentrations to ensure membrane stability.

Introduction

Neurodegenerative diseases are some of the most severe illnesses that plague human society [1-2]. They are largely incurable, with current medical efforts only being capable of slowing down the progress of these illnesses, but it doesn't stop the progressive degeneration and neural death that they cause. Some of these including genetic mutations - such as mitochondrial DNA mutations that contribute to faster aging - as well as protein misfolding and the accumulation of toxic intracellular proteins in neurons [2]. Due to their severe detrimental effects and uncertainty surrounding their causes and treatment, research into this group of diseases is steadily growing. There are a variety of risk factors that are known to contribute to the cause and progression of neurodegenerative diseases. Current medication for neurodegenerative diseases have a combined failure rate of 99.8%, well above that for any other illness. While some of the causes that can lead to neurodegenerative illnesses are understood, there is a lack of understanding regarding how these malfunctions in the human body happen on a cellular level [2-3].

Several neurodegenerative diseases are characterized by the aggregation of misfolded proteins. Alpha-synuclein is an intrinsically disordered protein in its natural state, but it can aggregate under certain circumstances such as a change in pH or salt concentration. The aggregation can result in either fibrils or formation insoluble Lewy bodies together with other proteins, a hallmark of Parkinson's disease (PD), Lewy body dementia, and Alzheimer's disease [4-6]. Alpha-synuclein has various properties, including its capabilities of binding to acidic phospholipids such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), which are a part of the cellular membrane and vesicles in the human body. This implies that alpha-synuclein has the capability to influence vesicles which contain neurotransmitters in synapses, and therefore culpable of the de-homeostasis of calcium ions by making neural membranes permeable to it. This is one of the main characteristics of PD, and a strong indicator that alpha-synuclein aggregates play a role in the development of the disease [1,2,6].

There has been a number of previous research efforts into alpha-synuclein and its effects on neurodegenerative diseases, such as various trials that show its influence on neuron degradation and cell death, especially in cases associated with PD [1]. This includes the studying of calcium signaling in neurons in a sufferer of PD, and how the involvement of PD related proteins can have an effect [1], due to alpha-synuclein's formation of insoluble fibrils.

In addition, two mutations in alpha-synuclein, Ala53Thr (A53T) and Ala30Pro (A30P), were identified in families with early onset PD [7], and these mutations can induce accelerated fibril formation [8]. Research in circular dichroism showed that these mutations can be indistinguishable from alpha-synuclein [8].

Other research has found that many aspects of neurodegeneration are a result of compromised mitochondrial functions, which is linked to specific genetic mutations, as well as toxins in the mitochondrial respiratory chain. While this is present in all cell types, a main characteristic of PD is the extreme selectivity of cell loss, which is restricted to dopaminergic neurons. It has been shown that the autonomous activity of these neurons is responsible for metabolic stress that is compensated by mitochondrial buffering. However, when mitochondrial function becomes compromised due to aging or mutations, then the metabolic stress overwhelms the protective mechanisms and neurodegeneration starts [1-2].

Further research has also attempted to characterize the toxic protein aggregates that are associated with neurodegenerative diseases, which is shown through measuring the extent of aggregate induced Ca ions and their entry into lipid vesicles [1-2].

The aim of this project is to setup a general method of studying how alpha-synuclein interacts with different lipid membranes. This can be used to study the effects alpha-synuclein has on the membrane and how this depends on parameters such as concentrations of lipids, proteins and calcium ions. The basis of this method is to form giant unilamellar vesicles (GUVs) of different lipid material, with POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) in the case of this project. The GUVs are subsequently filled with a fluorescent dye that only starts to leak out from the vesicles when alpha-synuclein has made the membrane more permeable. This process can be studied using fluorescence microscopy. Different steps in reaching this as well as characterization of the vesicles and alpha-synuclein will be presented.

Circular Dichroism

Circular dichroism (CD), is a method that measures the difference in absorption between right and left circularly polarised light [9-10]. Unlike linearly polarized light, where the electric and magnetic fields are both oscillating in perpendicular planes, circularly polarized light has the electric field vector rotating around the propagation axis with a constant amplitude. Should the left and right-handed circularly polarized light have equal amplitudes, they will superimpose on each other and the result is linear polarization. This is shown in figure (1) below, where the circularly polarized vector forms a helix along the direction of propagation, k. For left polarized light, the electric vector rotates counterclockwise, and clockwise for right polarized light, with propagation being towards the observer [9-10].

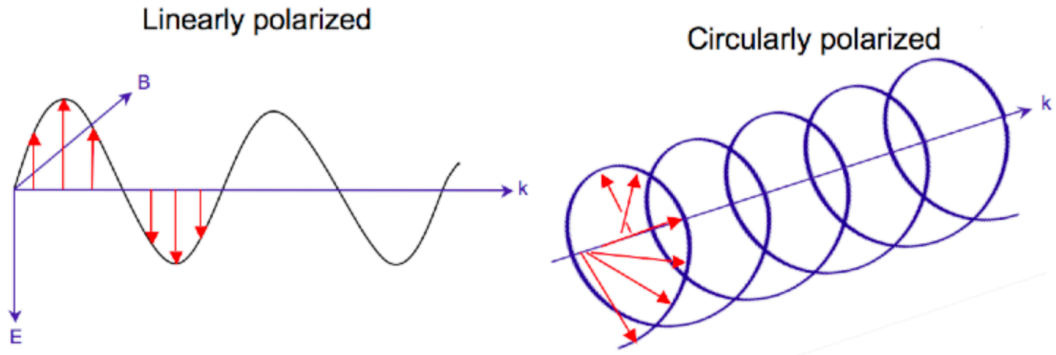

Figure (1). Schematic illustration showing the difference between linearly and circularly polarized light. The illustrations are taken from Castro AB, Stockert JC. Fluorescence Microscopy In Life Sciences. Bentham Science Publishers; 2017.

Circular dichroism is detected when a molecule contains chiral chromophores [10], which are pairs of mirror image isomers, that are not super-imposable and have identical physical and chemical properties. They are shown to have circular birefringence [10], so left and right circularly polarized light propagates through them at different speeds. Molar circular dichroism is the difference in molar absorptivity between the left and right polarized light.

CD is measured as ellipticity, given by:

$$\theta = \frac{(E_R - E_L)}{(E_R + E_L)} \quad (1)$$

Where E is the electric field vector's magnitude. Since the intensity of light, I, is proportional to the square of the electric field vector, then equation 1 can be written as the following:

$$\theta = \frac{I_R^{0.5} - I_L^{0.5}}{I_R^{0.5} + I_L^{0.5}} \quad (2)$$

By substituting for I using Beer's law, which relates the absorption of light with the properties of the material the light is traveling through, where

$$I = I_0 e^{-Aln10} \quad (3)$$

The ellipticity can then be written as:

$$\theta = \frac{e^{-0.5A_R ln10} - e^{-0.5A_L ln10}}{e^{-0.5A_R ln10} + e^{-0.5A_L ln10}} \quad (4)$$

where A_L is the absorbance of left-handed polarized light, and A_R is the absorbance of right-handed polarized light.

CD spectroscopy has various applications with regards to biological molecules. It can be used to detect protein unfolding, since there are clear changes in CD spectrum when the protein changes secondary structure. An unfolded protein has a random coil conformation, which gives a signal with a single peak around 195 nm. When the protein starts folding it changes into a alpha-helix or a beta-sheet. An alpha-helix is characterized by a double minimum in the signal at 208 and 222 nm and a beta-sheet by a peak at 195 and one at 217 nm with opposite sign as the one at 195 nm [9]. This is shown in figure (2) below.

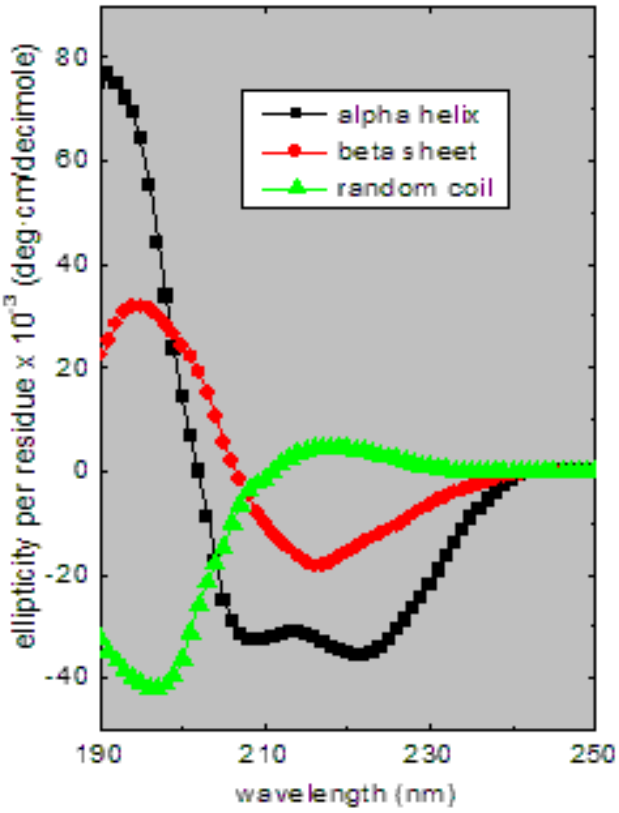

Figure (2). Shows CD signal of different protein secondary structures, and their observed minimums. The first sample shows the CD signal of the alpha-helix structure, while the second shows that of a beta sheet structure. The third shows a random coil formation. Image taken from Alliance Protein Laboratories (n.d) Circular Dichroism. From https://www.ap-lab.com/circular-dichroism

CD is also important in studying how outside stimuli can affect the formation of a secondary structure. For example, changes in temperature, or the addition of specific concentrations of molecules that the sample interacts with can have large effects on its structure. Thus, it can also be used to verify whether a protein is in its native conformation or not before proceeding with experiments.

In addition, CD is a rather cost-effective and quick method. While it gives less structural information than different methods, such as protein NMR spectroscopy, it is often faster in giving results, does not require a high concentration of protein samples, nor does it require an extensive amount of data processing [9,10,14].

Fluorescence Microscopy

Fluorescence is a phenomenon where a substance emits light after having absorbed some form of electromagnetic radiation. It has numerous applications in a variety of fields, from medicine to mineralogy. It's important in creating chemical sensors due to fluorescence spectroscopy, as well as fluorescent labeling [11]. It occurs when an excited orbital electron of a molecule relaxes back to its ground state by emitting a photon. This is best described by the following equations [11].

$$1.\text{Excitation: } S_g + hv_{ex} \rightarrow S_1 \qquad (5)$$

$$2.\text{Fluorescence: } S_1 \rightarrow S_g + hv_{em} + heat \qquad (6)$$

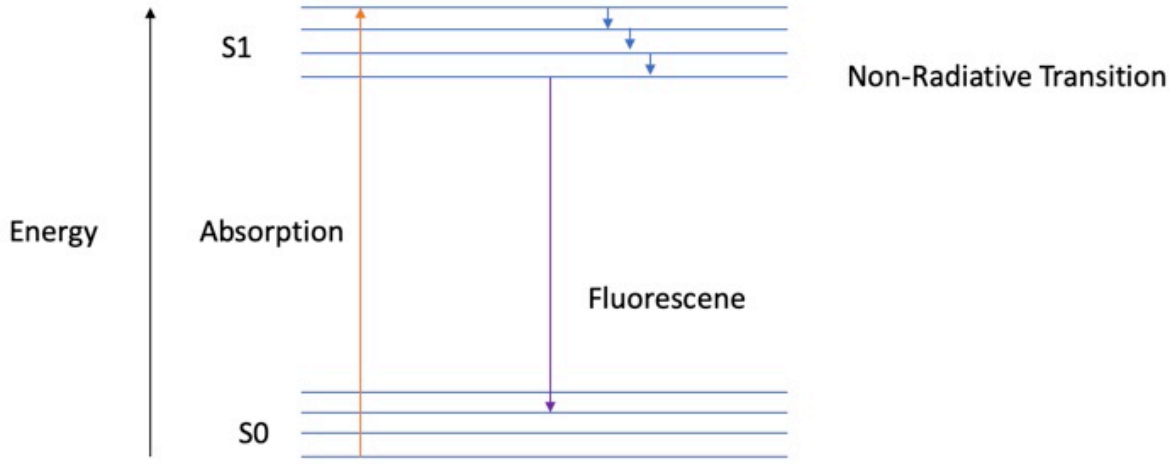

Figure (3). A Jablonski diagram is one that illustrates the electronic states and the transitions between these states for a molecule. It shows the absorption of a photon, which excited the molecule and transitions it to a higher energy state. Non-radiative transition occurs, before the molecule fluorescents and goes down to a ground state.

Here, hv describes the energy of the photon, with h being Planck's constant, and v being the frequency of light. The subscript ex stands for excitation light and em for emission light, where the frequency of the emitted light is less than that of the excitation light. S_1 is the excited state and S_g

the ground energy state of the fluorescent molecule and the excited fluorophore relaxes within the excited energy state giving off heat.

The fluorescence, in most cases, has a longer wavelength and thus lower energy than the absorbed light, which occurs due to non-radiative decay when the electron relaxes to the lowest vibrational level before de-excitation [11].

The fluorescence quantum yield gives information regarding the efficiency of process. It is the ratio of photons emitted to the ones absorbed, where the maximum possible result is 100% - each photon absorbed resulted in another one being emitted. The quantum yield is independent of the wavelength, due to excited photons decaying to the lowest available vibrational level before emission takes place [11].

Fluorescent materials have a lifetime, which is the time – on average – that a molecule stays in an excited state before photon emission occurs. It usually follows first order kinetics and is an example of exponential decay. This is shown by the following [11]:

$$[S_1] = [S_1]_0 e^{-t\Gamma} (7)$$

Here, $[S_1]$ Is the concentration of excited molecules at a time t, $[S_1]_0$ is the initial concentration, and Γ is the rate of decay. Typically, the lifetimes for fluorescent states are very short, and the decay times are around 5 to 20 nanoseconds. Excited molecules first undergo non-radiative decay into a relaxed state, before fluorescence occurs. This can be visualized using a Jablonski diagram, as seen in figure (3) above.

Using a fluorescent molecule as a marker for cellular structures of interest in microscopy is convenient since the emitted light can be filtered from the excitation light, thus avoiding unwanted signals from reflected light. The sample is illuminated with a specific wavelength of light, which is then absorbed by fluorophores – fluorescent chemical compounds that are capable of remitting light when they are excited – causing then to emit light at a longer wavelength. The weaker emitted light is separated from the illuminated source by a spectral emission filter. Typical components, as shown in figure (4), include a light source – such as high-powered LEDs and lasers – as well as the excitation filter, emission filter, and a dichroic mirror. The mirror and filters are specifically chosen

with the excitation and emission characteristics of the fluorophores that are labeling the sample in mind [13].

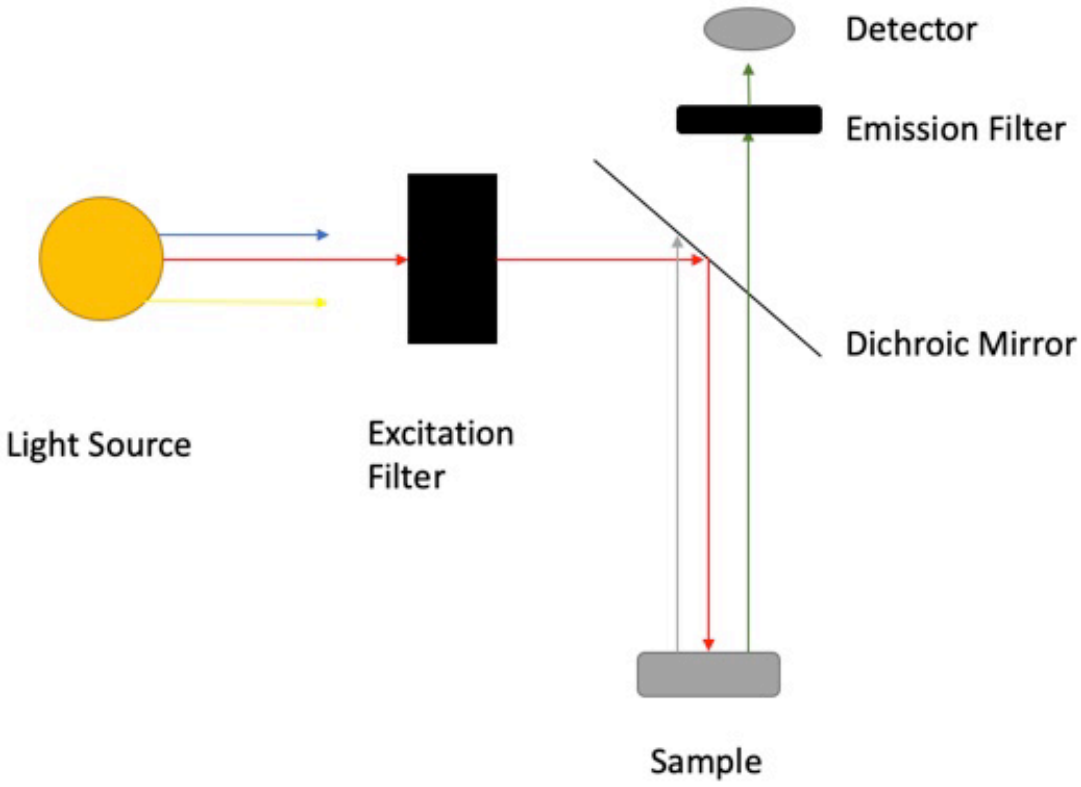

Figure (4). An illustration of a basic setup for a fluorescent microscope, with the various components used in the setup labeled.

Most setups are based on three filters [13]:

1. Excitation filter, which only passes light from the source at wavelengths absorbed by the fluorophore. This reduces the excitation of other sources of fluorescence in the sample, and allows a clearer image.
2. Dichroic mirror, typically used at a 45° angle, is used to reflect light in the excitation band, and transmit light in the emission band.
3. Emission filter, which passes the wavelengths emitted by the fluorophore, and blocks other sources, especially from the excitation light.

While important in various biophysical experiments, fluorescence microscopy has limitations. Fluorophores will eventually lose the ability to fluoresce as they are illuminated. Photobleaching occurs when there is a transition of an excited electron from a singlet state to a triplet state via intersystem crossing. Due to the long lifetime of the triplet state the excited molecule will have time to react with neighboring molecules, which might cleave covalent bonds and hence change the photochemical properties of the molecule. This phenomenon causes a fluorophore molecule to become permanently incapable of fluorescing. Photobleaching poses a problem, as it can greatly limit the time frame over which a sample can be observed, as the fluorescent sample will be eventually destroyed by light needed to stimulate them into fluorescing [13]. Photobleaching can be useful in studying the diffusion of molecules, such as fluorescence recovery after photobleaching (FRAP), where the movement of molecules can be found by observing the recovery of fluorescence at a site that was previously photobleached [13]. Minimizing illumination, or using more robust fluorophores, can help minimize the effects of photobleaching [13-14].

Dynamic Light Scattering

Dynamic light scattering (DLS) can be used to determine information about the size and concentration of particles or molecules in a solution. In DLS, monochromatic light is scattered by the sample, and the scattered light is collected by a photomultiplier. Only a small volume of the sample is illuminated and particles within this region gives a fluctuating signal that depends on how many particles there are in the volume and how fast they diffuse.

The scattered light is detected over a specific time period, and its intensity will fluctuate over time. The initial intensity is used to generate a correlation function, which describes how long a particle is located in the same spot within the sample. At the beginning of the measurement, this correlation function is linear and constant – which indicates that the particle is in the same location and no diffusion has taken place. The correlation function will exponentially decay, showing that the particle is moving. This decay shows a measure of the time that the particle needs to change position and gives information on the size dependent movement as well. Small particles move faster so the decay is faster, while larger particles are slower and thus the decay of the correlation function is delayed [12].

The diffusion coefficient, D, of a spherical particle can be obtained which according to the Stokes–Einstein equation is related to the radius of the particle according to the following expression:

$$D = k_B T / 6\pi\eta r \quad (8)$$

Where k_B is Boltzmann's constant, T is the absolute temperature, η is the dynamic viscosity of the solution and r is the radius of the particle. [12]

In addition to their use in determining the size and concentration of particles, DLS has other applications. Studies regarding the stability of particles can also be done using DLS. Periodical DLS measurements of a sample can highlight whether particles are aggregating over time or not. This is done by seeing if the hydrodynamic radius of the particles in the sample increases. A larger number of particles with larger radii implies that the particles have aggregated.

The setup of a DLS instrument can be rather simple. A single frequency laser is directed at a cuvette which contains the sample to be analyzed. The incident light is then scattered in all directions by the diffusing particles in the sample, which is then detected at a specific angle over time. This angle depends on how opaque the sample is, with side scattering (90°) or back scattering (15°) being the most often used, while an angle of 15° is more useful in detecting aggregation [12].

Alpha-Synculein and Ion Channels

Alpha-synuclein is a protein that is primarily found in neural tissues in humans, where it makes up around 1% of proteins in the cytosol [8]. While predominantly found in the brain, smaller amounts can be located in various other tissues, including the heart and muscles. It has been mainly found at the tips of neurons in presynapses, where it interacts with phospholipids and other proteins.

In order to better highlight the prominence of alpha-synculein's location in presynaptic terminals in neurons, one must understand the importance of chemical synapses. Synapses are biological junctions across which a neuron's signals can be sent either to other neurons, or to different types of cells, such as those in muscles or glands. These synapses allow neurons to form the equivalent of circuits in the nervous system, thus providing an irreplaceable functionality in living creatures

– they are not only crucial to the transmission of biological computations, but they give the nervous system the ability to connect with and control all other systems [1]. An illustration detailing this is shown in figure (5).

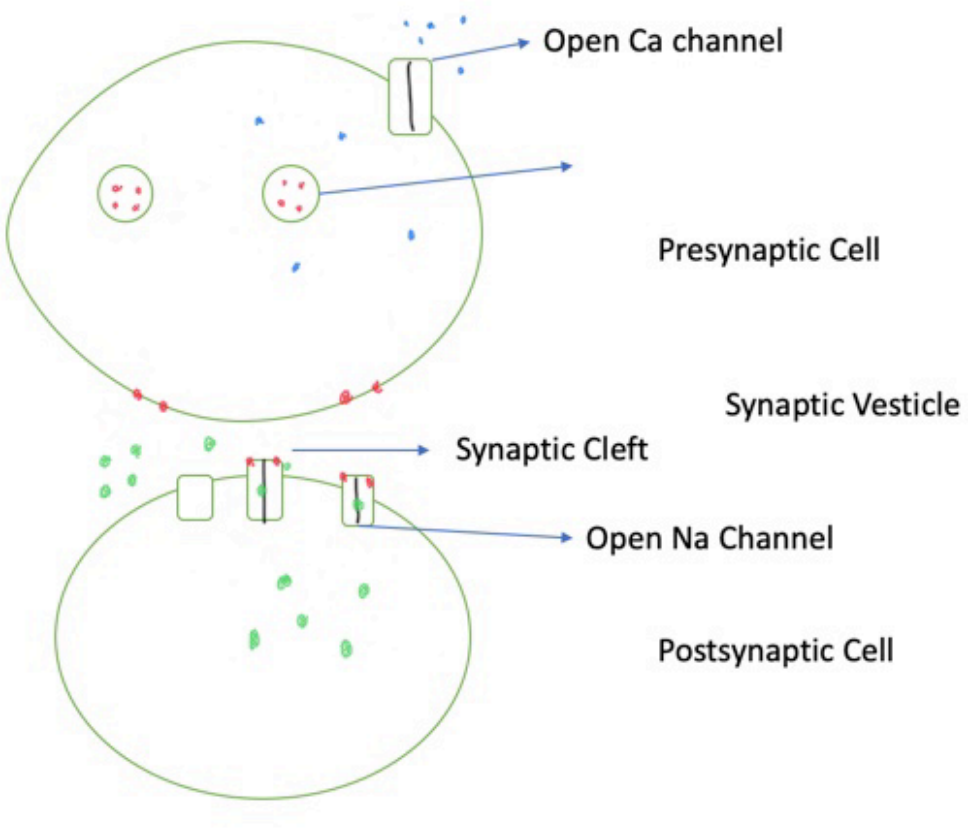

Figure (5). An illustration detailing a chemical synapse is shown, including the process of sending a signal via neurotransmitter from the presynaptic to postsynaptic cell. The neurotransmitters are stored in synaptic vesicles. The blue dots represent calcium Ions, the red neurotransmitter molecules, and green sodium.

The signaling process begins when an electrochemical excitation wave travels along the presynaptic cell, until it reaches the synapse. This causes an electrical depolarization, which leads to calcium ion permeable channels to open. The open channels result in calcium ions flowing through the presynaptic membrane, which will quickly increase in concentration. The high concentration of calcium activates calcium sensitive proteins that can contain a neurotransmitter chemical.

This process causes the proteins to change shape, which leads to vesicles containing neurotransmitter chemicals to fuse with the membrane of the presynaptic cell and release their contents into the synaptic cleft – a small gap, around 20nm wide - between the presynaptic and postsynaptic cells. This short distance is important, as it permits the rapid concentration changes for neurotransmitter materials [1,15]. Neurotransmitters diffuse within the cleft, until eventually an amount of them binds with chemical receptors on the membrane of the postsynaptic cell, which causes the receptors to activate. In usual cases, this happens by opening ligand-gated ion channels in the postsynaptic membrane, which causes ions to diffuse through the cell and changes the transmembrane potential. This is the key step in chemical synapses which allows a presynaptic cell to affect a postsynaptic one. The neurotransmitter is then removed in order to allow for the postsynaptic cell to continue the process. This can happen in various manners, usually by diffusion due to thermal vibrations, where it is either reabsorbed by the presynaptic cell or broken down [15].

While the functionality of alpha-synculein is poorly understood, a previous study has suggested that it is involved in the regulation and restriction of mobility for synaptic vesicles. [15] In addition, alpha-synuclein is known to bind to vesicles, specifically to negatively charged surfaces of phospholipids, such as the ones used in this project. This binding induces its alpha-helix structure.

Alpha-synuclein's binding has various effects on the lipid membranes, including changing the composition and forming small vesicles. It has also been shown to "bend" negatively charged phospholipid membranes to form tubules, in addition to having an effect that inhibits oxidation. The functions of alpha-synuclein require the natively unfolded structure of the protein [1,15].

As shown in figure (6), alpha-synuclein has three main sections in its structure:

- Residues 1-60 is an amphipathic N-terminal region. This region is comprised of four 11-amino acid imperfect repeats, and can shift to an alpha-helical conformation, which consists of two alpha-helixes that are separated by a short break. This is the part that gains a secondary structure, as the hydrophobic parts fold inwards while the hydrophilic fold outwards, making this section fold into an alpha-helix structure. The N-terminus has a net charge of +3 which means that it can interact with negatively charged lipids [15].

- Residues 61-95 is a central hydrophobic region. It is involved with protein aggregation and causes the formation of fibrils [15].
- Residues 96-140 is a highly acidic region with no specific inclination in its structure. This section has a charge of -12 [15].

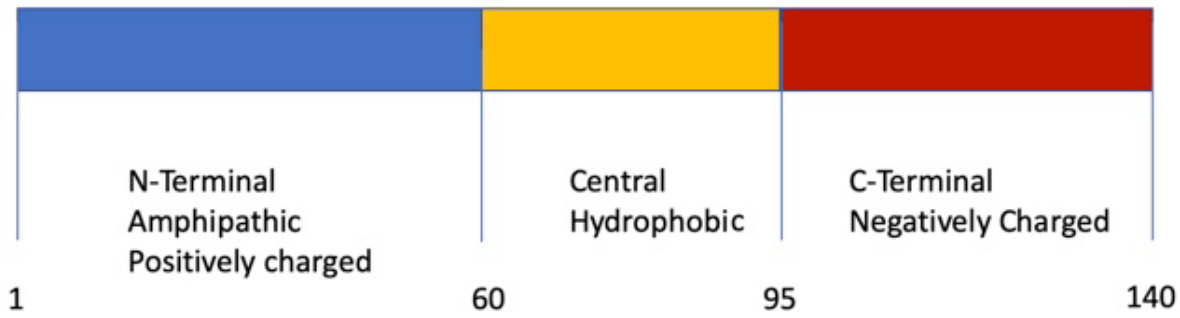

Figure (6). An illustration of alpha-synuclein's structure, with its three distinct domains shown.

Giant Unilamellar Vesicles

Giant unilamellar vesicles (GUVs) are cell sized model-membranes, used to study the functionality of biological membranes, how they interact with outside molecules such as proteins, and how such molecules can have an effect on the composition, shape, and chemical properties of the lipids that make them up. They are an important part of biomedical research, as they are useful in mimicking cell function, and thus in studying interactions with membranes, such as in membrane fusion, ion channels, and protein localization [16].

GUVs were researched under the motivation of developing a simplified way to study the complex cellular membranes that they are patterned. They are made from bilayer vesicles and are around 1-100 um in size. Their relatively large size compared to other vesicle complexes such as small

unilamellar vesicles (SUVs, with a diameter of around 50 nm), as well as their curved shape makes them ideal subjects to visualize using an optical microscope. GUVs have, therefore, found various applications where membrane interactions are to be studied, as well as other biophysical studies where membrane composition is manipulated using microscopy techniques, such as fluorescence imaging of lateral membrane organization [16].

There are several ways to produce GUVs. The original technique is to dissolve a mixture of lipids into a solvent, which is then spread on a glass. The solvent is then evaporated, and the lipids are then hydrated at a high enough temperature – higher than the transition temperature of the lipids in the mixture. Another similar method involves rehydrating SUVs instead of dried lipids, which minimizes the risk of denaturing the membrane proteins. However, these methods result in vesicles that are mostly multilamellar rather than unilamellar. Another method used is electroformation, where the lipid samples are placed on a conductive slide glass, and the rehydration of the dried lipids is expedited by an AC electric field, set at about 4 VPP and 10 Hz for 2 to 12 hours. The buffer used to rehydrate the lipids must contain as few ions as possible [16].

Materials and Methods

SUV preparation

Three types of phospholipids from Avanti Polar Lipids were used; 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE). The lipid stocks were diluted in chloroform (0.1-10 mg/mL). Lipids were measured from the stock solutions into samples of POPC/POPS (2 mg/ml of lipids, POPC:POPS 7:3), POPE/POPS (2 mg/ml of lipids, POPE:POPS 7:3), and pure POPC (2 mg/ml of lipids). After mixing, the sample was evaporated with nitrogen gas for ca 20 minutes to evaporate away the chloroform. The lipids were then resuspended in a 10 mM MES buffer, with a pH of 5.5.

When the lipids have been resuspended and formed multilamellar vesicles (MLVs) they are sonicated in order to form small unilamellar vesicles (SUVs) with a size of 20-100 nm. The sonication process uses the application of sound energy to the samples in order to agitate particles for various purposes, in this case breaking them down by disrupting the membrane, and thus decreasing their size. The samples were prepared using a CV18 tip sonicator from Chemical Instruments. Dynamic light scattering with a Malvern Zetasizer S was used to characterize the size of the SUVs.

Once the SUVs were formed, they were ready to test their interactions with alpha-synuclein.

GUV preparation

GUVs were prepared from the same lipid stocks as the SUVs into samples of of POPC/POPS (2.5 mg/ml of lipids, POPC:POPS 7:3), POPE/POPS (2.5 mg/ml of lipids, POPE:POPS 7:3), and pure POPC (2.5 mg/ml of lipids), diluted in chloroform. 1 mol% of fluorescently labelled lipids, 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine labeled with Atto 647N (ATTO647N- DPPE, AttoTec) was incorporated in the GUV samples to visualize the GUV membrane.

GUVs have a size of 1-200 µm and are increasingly used as a tool to study a variety of subjects, including membrane structure. [13] 5 microliters of the lipid solutions were spread on a conductive

indium tin oxide (ITO)-coated cover glass, which was then placed under vacuum for two hours, in order to eliminate any trace of chloroform, and then covered with buffer. A 10 mM HEPES,150 mM NaCl buffer with 200 mM sucrose and 10 nM- 10 uM fluorescein at a pH of 7.4 was then added to the dried lipids. Afterwards, electroformation - the process of running an electric current though the ITO slides - was carried out at a sinusoidal voltage of 10.00 Hz with an amplitude of 4.0 V peak to peak for an additional two hours. This results in vesicles with a diameter greater than 10 μm. Before imaging the GUVs they were diluted in a solution containing 200 mM glucose without any fluorescein. The larger molecular mass of sucrose compared to glucose makes the GUVs having an effectively higher density, sinking to the bottom of the sample due to gravity.

Fluorescence Microscopy

The microscope setup used in this project is a Nikon Apo TIRF with 60x magnification oil immersion objective, on a Nikon Eclipse TE2000-U microscope that is equipped with a Hamamatsu ORCA-Flash4.0 LT Digital CMOS camera. The pixel area of the camera used was 169 um^2, with a pixel array of 1024 x 1024, with 2x2 binning.

The lipid membrane of the GUV was visualised using a diode laser from Cobolt (06-01) operating at 638 nm, which excites the ATTO 647 DPPE lipids, which have an emission wavelength of 669 nm, whereas the incorporated fluorescein was excited using a diode laser from Cobolt (06-01) operating at 488 nm, with an emission wavelength of 512 nm. Suitable emission filters where used to separate the excitation light from the emitted light.

Circular Dichroism

CD was used to analyse alpha-synuclein's structure, both by itself and combined with the various SUVs used in this project. The structure of alpha-synuclein when interacting with lipid vesicles was analysed by a Jasco J-715 Spectropolarimeter.

Alpha-synuclein (wt), were expressed from E. coli bacteria and then purified using heat treatment, ion exchange and gel filtration chromatography as described previously [11]. The samples were prepared in a 10 uM MES to a final concentration of 2.5 uM, and left to incubate for 45 minutes

before being analysed. Three samples were prepared - alpha-synuclein by itself, as well as with POPS/POPE and POPS/POPC SUVs, at a 1:20 protein to lipid ratio.

Alpha-synuclein Interaction with SUVs

The main purpose behind the CD spectroscopy experiments was to analyze the protein structure of alpha-synuclein, both separately and accompanied by different SUVs. All the made SUVs samples had a similar mean diameter (between 55 nm and 63 nm) as measured by DLS. While alpha-synuclein as a monomer is intrinsically disordered, it has been shown to change into a secondary alpha-helical structure when interacting with phospholipids found in the prepared SUVs containing POPS. [6] This transition results in a shift in the CD spectrum producing a more pronounced dip around 220 nm as can be observed in figure (6) [3].

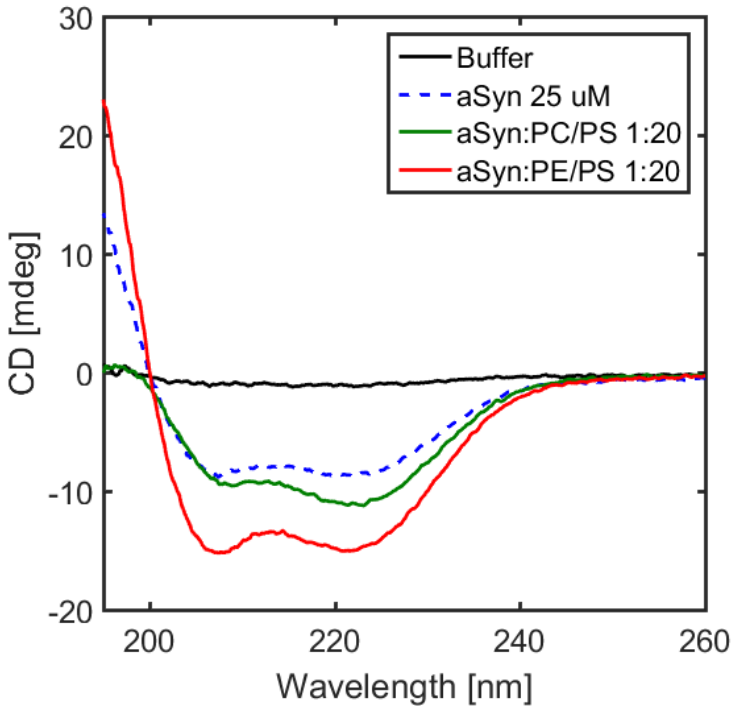

Figure (7). CD spectra for different alpha-synuclein samples.

As can be seen in figure (7), both samples containing lipid vesicles have a clear alpha-helical structure, with the sample containing PE having a more pronounced structure. That can be explained by the PE lipids having a smaller head-group that leads to tighter packing in the bilayer, which would eventually result in a hexagonal phase. Alpha-synuclein has been shown to be able to bend lipid membranes and help stabilising the vesicles, thereof the higher affinity for PE/PS vesicles than PC/PS vesicles. Due to this, the GUV samples containing POPE/POPS were used.

Studying the signal for the pure alpha-synuclein sample, it can be seen that the alpha-synuclein already seems to have an alpha-helical secondary structure, meaning that it has started to fold in absence of lipids. The reason for that could be that the freeze-dried alpha-synuclein was diluted in a MES buffer it was purified in. When freeze-drying, the salts and other solid material is remained in the sample together with the protein and therefore it is enough to dilute the freeze-dried sample in distilled water. Diluting it in buffer means that the pH will be lower than the expected value of 5.5, which would induce aggregation of the sample.

Originally, a buffer containing NaCl was used to dilute the alpha-synuclein, which was a mistake, as Cl ions have a high absorbance rate near the UV region, and because of this there was no signal to detect. In addition, cuvettes with a path length of 10 mm were originally used for the measurements, but were replaced with cuvettes with a path length of 2 mm. A shorter path length meant that there was less sample to absorb, which was needed as the sample was highly absorbing.

GUV Formation and Loading

The small size of SUVs makes them hard to visualize in fluorescence microscopy due to the diffraction of light preventing details smaller than a couple of 100 nm to be discerned. GUVs are, due to their size, a better option when the interaction should be visualized. [13] Various GUVs with diameters larger than 10 μm were therefore formed by electroformation, as seen in figure (8).

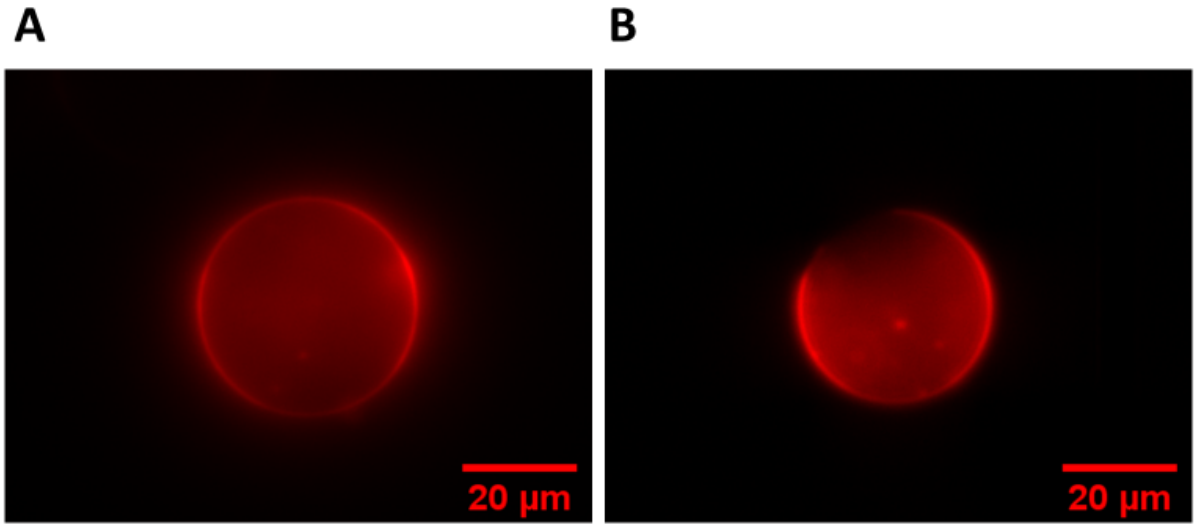

Figure (8). The lipid membrane of GUVs imaged with fluorescence microscopy. (A) GUVs consisting of POPC, (B) GUVs consisting of POPC and POPS. ATTO 647 DOPE was used as the fluorescently labelled lipid in both cases.

The vesicle consisting of POPC alone (figure 8A) had a homogenous edge profile with similar intensity at all positions around the GUV. For the sample containing both POPC and POPS (figure 8B) this was not the case and darker patches appeared on the GUV, such as the one on the upper left corner. This is likely due to domain formation of lipids, to which the ATTO 647 DPPE distributes differently. The lower brightness on the top and bottom corners of both GUVs, in comparison to left and right edges, can be due to an effect of the laser polarization being parallel or perpendicular with the oriented molecules of the bilayer. In addition, it can also be observed that there is a signal from within the GUVs, which becomes even more clear when looking at a cross section of the POPC GUV which can be seen in figure (9). The reason for this is that the fluorescence microscope also picks up emitted light from the top and bottom of the GUV.

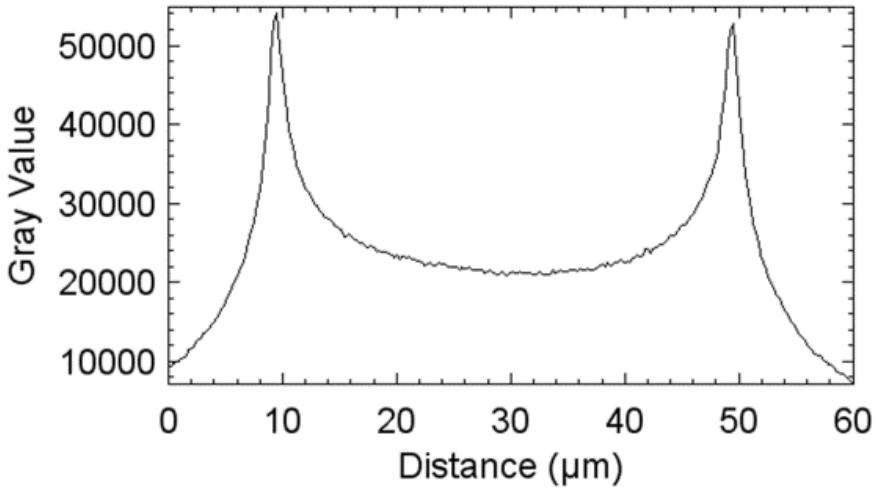

Figure (9). Line profile showing the intensity of the GUV in figure (8A).

The edges of the GUV also have a finite width, of approximately 2 um, as seen in figure (9). The reason for this is due to the diffraction limit of light which limits the resolution of optical microscopy to approximately the wavelength of the light. A more likely contribution to the membrane width (which is approximately 5-10 nm in living organisms) is that the pixel size of the camera is 13 um, which corresponds to a distance of 0.22 um with a 60x objective. The smallest width of the membrane would then appear to be 0.22 um, but since the light is diffracted, the photons will hit several pixels and the membrane will appear to be even wider. In addition, the signal inside the GUV, located at the top and bottom as seen in figure (8), might make the membrane appear to be a little wider than it is.

The GUVs were made in a solution with 10 nM fluorescein, which only slowly could pass the lipid membrane. Thus, when the GUVs where re-dispersed in a solution without fluorescein the interior of the GUV appeared brighter, as seen in figure (10).

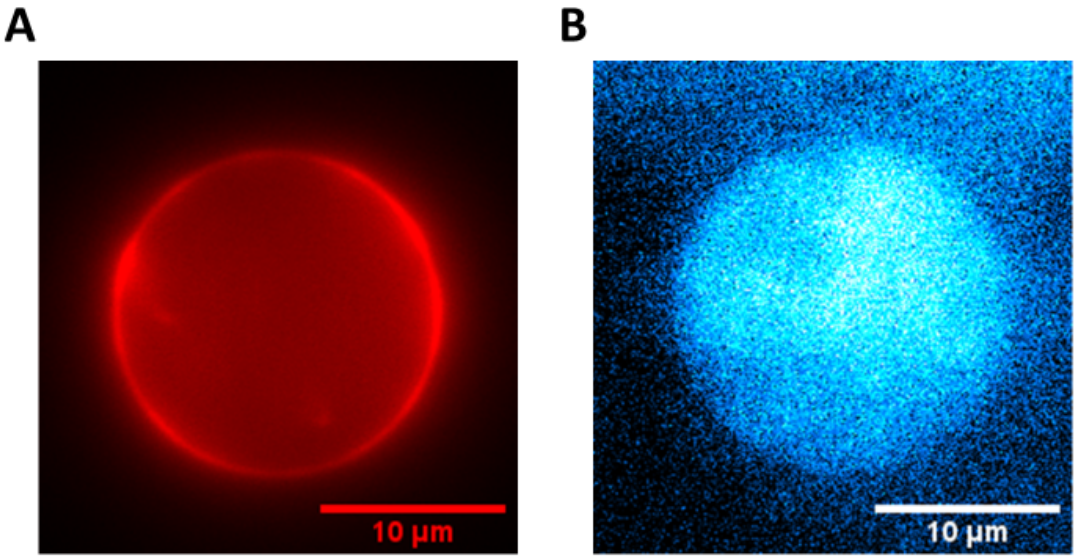

Figure (10). Fluorescence image of a GUV filled with fluorescein.

A lot of different tests had to be conducted in order to reach the GUVs presented in figure (8). It was found essential to use a vacuum pump for the initial period of GUV preparation. If instead of using a vacuum pump to incubate the vesicle sample after it was spread on ITO cover slides, in order to remove any trace amounts of excess chloroform, they were left to incubate outside, only very small GUVs were formed. A solution containing 200 mM sucrose was used, as the sucrose would increase their mass and ensure that the GUVs would stick to the surface of the glass slide. The samples were diluted in a buffer containing glucose, so that the contents of the GUV are heavier than the surrounding buffer, but the risk of obtaining a hypertonic solution inside the GUV that would lead to swelling and bursting of the GUV is minimized. A suitable amount of the fluorescent dye fluorescein was also investigated to produce stable GUVs, without too much or little fluorescent signal. It was found that 10 nM fluorescein was optimal.

GUV Permeability

The fluorescein inside the GUV was found to be bleached rather quickly, in the span of 5-10 seconds. When studying the flow of fluorescein across the vesicle membrane it was easier to fully bleach the fluorescein inside the GUV, and then observe if any fluorescein from the outside of the GUV could flow into the GUV, as seen in figure (11A).

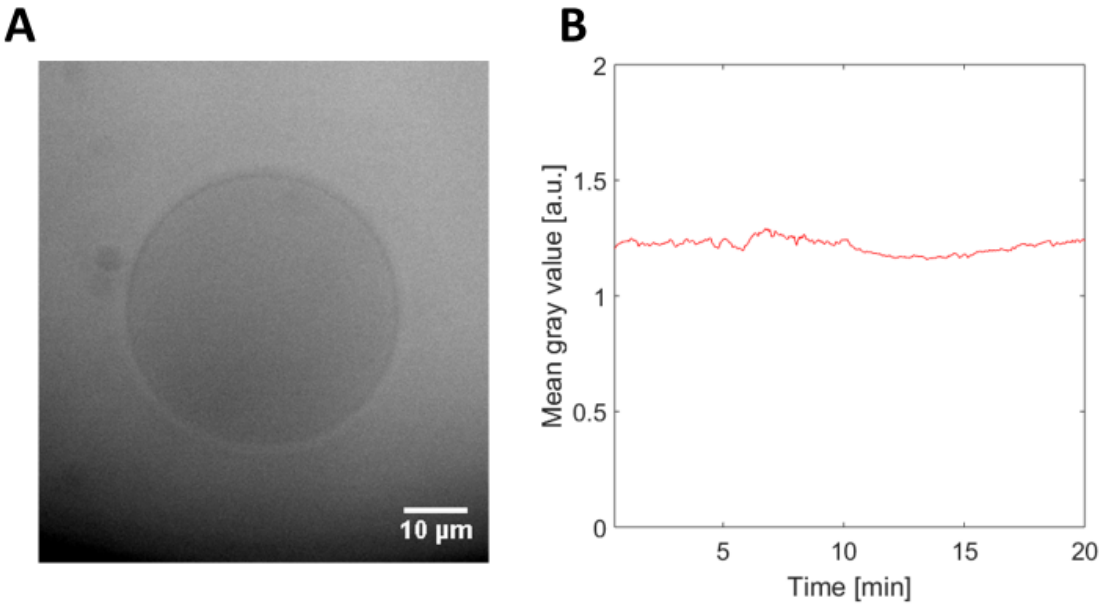

Figure (11). (A) Fluorescence image of a GUV filled with fluorescein where the fluorescein inside the GUV has been bleached. (B) The quotient between the intensity inside and outside the GUV as a function of time.

The quotient of the intensity inside and outside the GUV was followed at different times after the GUV had been bleached (Figure 11B). It was found that the quotient only changed marginally over the time course of 20 minutes, indicating that the fluorescein molecules did not pass significantly over the membrane in this time span. The microscope in this instance was taking 10 images per second to capture fluorescein before it was photobleached, in order to investigate if the GUV is permeable.

The GUVs tested were made from POPS/POPE lipids, which are the most permeable to alpha-synuclein due to the protein's preference of acidic phospholipids [3-4]. This is also why samples made from pure POPC were not made. However, before 50 uM of Alpha-synuclein could be added

to the GUVs to investigate its effect on state and permeability, the GUVs were found to burst. This is due to relative instability of POPS/POPE, as changes in temperature can disrupt them. The microscope area where they were observed was colder than the lab where they were formed, and this could have been avoided by heating them beforehand.

Additionally, a second run with POPS/POPC GUVs was conducted. While the GUVs did not burst when alpha-synuclein was added, there was no observable change in the GUVs themselves, as shown in figure (12). This is because of the lack of GUVs found that were stuck to the surface of the slide, which made observing them difficult, and thus observing them over time was impossible.

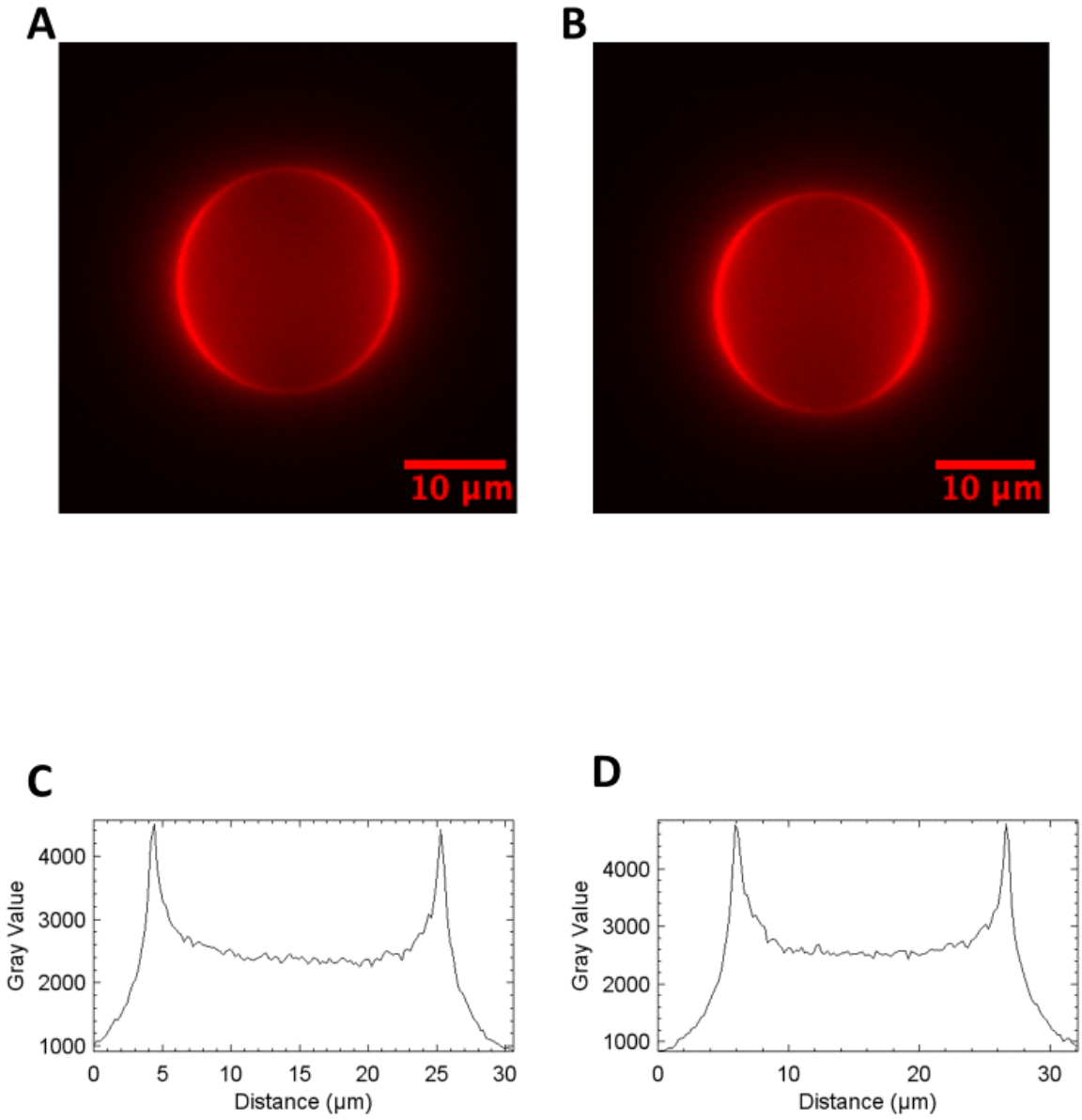

Figure (12). (A and B) The lipid membrane of GUVs imaged with fluorescence microscopy. (C and D) The line profiles of the two GUV samples (A and B).

Conclusions and Outlook

The preparation of GUVs proved to be a long and delicate process, with many different steps and aspects that can fail and produce unusable samples. Since the methods used to produce the GUVs in this project took around 5 hours, this essentially meant that producing poor GUVs would take up an entire day, as there would be no time to make more or run other experiments. It is thus important to carefully follow the protocols when making the GUVs. The GUVs could also be sensitive to temperature changes and some of the vesicles were expected to burst when transporting them from the laboratory where they were made to the colder microscope room. This could have been solved by maintaining the GUVs at room temperature for a while, before bringing them to the microscope. The metal holder of the microscope used could also have been heated up in advance in order to help maintain their temperature.

An inappropriate choice of voltage or electrode material during electroformation may also play a part in why the initial samples of GUVs were too small or too fragile. A wrong voltage can lead to lipid hydrolysis, as well as oxidation reaction. A study on the relationship between GUV formation and chemical impurities may be beneficial. Lowering the voltage applied to the GUVs during electroformation shortly before collecting them can help in producing more stable samples.
The conductive coating on the glass slides used (ITO), may also provide issues at the voltages that would be normally applied during electroformation, and can give rise to lipid breakdown products.

Additionally, the amount of fluorescence in GUVs took a while to get right. While it was not added until later in the experiment cycle, since the focus at first was preparing viable GUVs, the initial concentration of fluorescein was too much. Because of this, the beginning attempts to view GUVs and test their permeability, as seen in figure 9, proved to be difficult, as the brightness resulted in a severe amount of signal noise that made viewing the GUVs themselves difficult, and attempting to measure the permissibility of the membrane proved to be impossible under such circumstances.

The decision to fully bleach the fluorescence in the GUVs in order to track movement going inside it, as seen in figure 10, ended up being a superior method to test the permeability of the membrane, and the method that should be used in future experiments. This method provided an enhanced contrast between the inner and outer mediums of the GUVs, and made observing the transport in and out of them clearer.

In addition, the decision to use a sucrose-based buffer to make the GUVs ensured that they were heavier than their surroundings, and thus had a higher chance of getting stuck on the glass slide while observing them. Using glucose in the buffer around the GUVs made sure that molecules were lighter as well.

Due to lack of time it was not possible to perform more experiments of alpha-synuclein interacting with different GUVs. Future work would include observing the effects of different concentrations of alpha-synuclein on GUVs containing different lipid. Adding fluorescently labelled alpha-synuclein would also be interesting to observe how the alpha-synuclein interacts with the GUVs, is it for example binding to the surface or "punching a hole"?

Altogether, this project has provided guidelines in how to make and handle GUVs for interaction studies with proteins such as alpha-synuclein. The GUVs were filled with the fluorescent dye fluorescein to study vesicle permeability upon alpha-synuclein interaction, but could also be filled with other substances, for example calcium-sensitive dyes to study calcium ion transport across the lipid membrane.

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