



Microcavity Array Supported Lipid Bilayers Biomimetic Platform for Study of Membrane Associating Proteins and Membrane-Protein Aggregation

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Thesis Submitted in Fulfilment of Examination Requirements Leading to the Award of PhD

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Declaration

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Glossary of Abbreviations

Aβ	Amyloid Beta
AC	Alternating Current
ACF	Auto Correlation Function
AFM	Atomic Force Microscopy
AnV	Annexin-V
Aβ	Amyloid Beta
BLM	Black Lipid Membrane
C	Capacitance
CPE	Constant Phase Element
CV	Cyclic Voltammetry
DC	Direct Current
D_L	Lateral Diffusion
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
DOPS	1,2-dioleoyl- <i>sn</i> -glycero-3-phospho-L-serine
EIS	Electrochemical Impedance Spectroscopy
EggPC	L- α -phosphatidylcholine [Egg, Chicken]
EggPA	L- α -phosphatidic acid [Egg, Chicken]
FCS	Fluorescence Correlation Spectroscopy
FLCS	Fluorescent Lifetime Correlation Spectroscopy
GUVs	Giant Unilamellar Vesicles
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LB	Langmuir-Blodgett
LUV	Large Unilamellar Vesicle
MSLB	Microcavity Suspended Lipid Bilayer
PBS	Phosphate buffered saline
PDMS	Polydimethoxysilane

PS	Polystyrene Spheres
R	Resistance
RT	Room Temperature
SAM	Self-Assembled monolayer
SEM	Scanning Electron Microscopy
SLB	Suspended Lipid Bilayer
THF	Tetrahydrofuran
Z	Total Impedance
WTGal3	Wild Type Galectin 3
Gal3 Δ Nter	Truncated Galectin 3
Ω	Ohm
Δ	Delta

Research Outputs

Poster Presentation

- Investigation of Transmembrane Proteins on Suspended Lipid Bilayers on Gold Microcavity Arrays using Electrochemical Impedance Spectroscopy, 69th Annual ISE Meeting, Bologna, Italy, September 2018

Oral Presentation

- Investigation of Membrane Associating Proteins on Suspended Lipid Bilayers on Gold Microcavity Arrays using Electrochemical Impedance Spectroscopy, Second ISE Satellite Student Regional Symposium on Electrochemistry in Ireland, Limerick, November 2018

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- Chemistry Research Day, Dublin City University, May 2018 – Organiser
- 35th European Peptide Symposium, Dublin City University, August 2018 – Volunteer

Peer Reviewed Publications

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- Robinson J, Sarangi NK, Keyes TE “Role of Phosphatidyl Serine in Beta Amyloid Oligomerization at Asymmetric Phospholipid Bilayers.” Manuscript submitted to ACS Chemical Neuroscience.
- Sarangi NK, Shafaq-Zadah M, Berselli GB, Robinson J, Dransart E, DiCicco A, Levy D, Ludger J, Keyes TE, “A biophysical study of Galectin-3 binding to $\alpha 5\beta 1$ integrin reconstituted in pore suspended biomembranes”, manuscript submitted to Biophysical Journal.
- Robinson J, Sarangi NK, Shafaq-Zadah M, Berselli GB, Dransart E, DiCicco A, Levy D, Ludger J, Keyes TE, “Glycoswitch of $\alpha 5\beta 1$ integrin reconstituted in pore suspended biomembranes on the effects of binding of Galectin 3.” Manuscript in preparation

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Abstract

Microcavity Array Supported Lipid Bilayers Biomimetic Platform for Study of Membrane Associating Proteins and Membrane-Protein Aggregation

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The plasma membrane is a semi-permeable barrier system. It separates the extracellular and intracellular environments, supports cell-signalling, cell adhesion and membrane transport. Proteins can be embedded in or bound peripherally to the membrane. The living cell membrane's profound complexity has proven a difficult environment for investigating protein binding mechanisms. This has led to the development of biomimetic model membranes to allow for the study of dynamics and functions of individual cell membrane processes isolated from its complex nature. Chapter 1 describes the background to this work and overviews the current state of the art model membranes. Chapter 2 describes the experimental studies for MSLB preparation and analysis.

Annexins are soluble membrane-binding proteins that associate in a calcium dependent manner with anionic phospholipids. They play roles in membrane organization, signalling and vesicle transport and in disease states including thrombosis and inflammation. Annexin V is believed to be involved in membrane repair. Through binding to phosphatidylserine exposed at damaged plasma membrane the protein forms crystalline networks that stabilises small membrane tears. Chapter 3 models this mechanism to simulate membrane healing at microcavity array supported, asymmetric, lipid bilayers. Fluorescence spectroscopy confirmed that when DOPS was made available at the external leaflet annexin assembled rapidly at the membrane where it formed a layer. From electrochemical impedance, the formed layer decreased membrane capacitance whilst reducing resistance.

Amyloid peptide aggregation, particularly A β (1-42), triggers neurotoxicity and is believed to play a central role in progression of Alzheimer's disease. An exact link between Alzheimer's disease and A β has not been determined but hypothesized that its self-aggregation of monomeric forms of A β to oligomers or fibrils has an important role in the progression of the disease. Chapter 4 investigates the effects of DOPS on A β oligomer formation by varying the DOPS & A β concentrations, and membrane orientation. It was seen that when more DOPS is present at the external leaflet, less concentration of A β is needed to form oligomers before the bilayer is destroyed.

Integrins are transmembrane, conformationally switching receptors involved in cell-cell and cell extracellular matrix adhesion. Integrins are believed to exist in conformational states that regulate their affinity to their ligands, proteins, or the extracellular matrix. Normally present in their bent resting state or can be activated to an extended state. Galectins are known to cluster with Integrin. Chapter 5 investigates Galectin-3 clustering on the different states, resting vs activated, of Integrin, $\alpha 5 \beta 1$. It was seen that in the resting form, galectin 3 was seen to form oligomers. However, on activation the oligomers of galectin 3 were not observed.

Chapter 1: Literature Review (Introduction)

1.1 Cell Membranes

Cell membranes, Figure 1.1, are dynamic and highly complex matrices composed of phospholipids, sterols and proteins that surround all living cells. The eukaryotic plasma membrane is a selectively permeable barrier of 4 – 10 nm thick that shields and protects the inner workings of a cell. ^{1,2} Danielli et al. proposed one of the earliest membrane models in 1935, stating that lipids organise into bilayers and a protein layer is associated closely with the polar head groups of the lipids.³ In 1972, the Fluid Mosaic Model was further developed by Singer and Nicolson, who suggested that the cell membrane was composed of a homogenous mixture of macromolecules such as lipids, proteins, glycoproteins, etc.⁴ Over the years the Fluid Mosaic Model has evolved and it is no longer thought that the membrane is homogenous but rather compartmentalised into dynamic, restless domains that constantly disband and reform within the membrane at different rates.⁵ The plasma membrane lies between two aqueous environments, the intracellular area (cytoplasm) and the extracellular area. Phospholipids spontaneously arrange into a bilayer with the hydrophilic heads towards each aqueous environment to form the exterior of the membrane protecting the hydrophobic tails. The hydrophobic tails align towards each other, while the polar head groups face the aqueous environments.⁶ Proteins constitute up to 50% by weight of the membrane composition and can be bound to the membrane on the exterior or the interior, or embedded partially or fully within the phospholipid bilayer, and are responsible for a number of processes. ⁷ Material transport (e.g., ions, waste, amino acids, etc.) from the cell's interior to exterior (and vice versa), cell adhesion and cell signalling are examples of processes for which these highly selective barriers are responsible for, many of which are mediated by proteins.

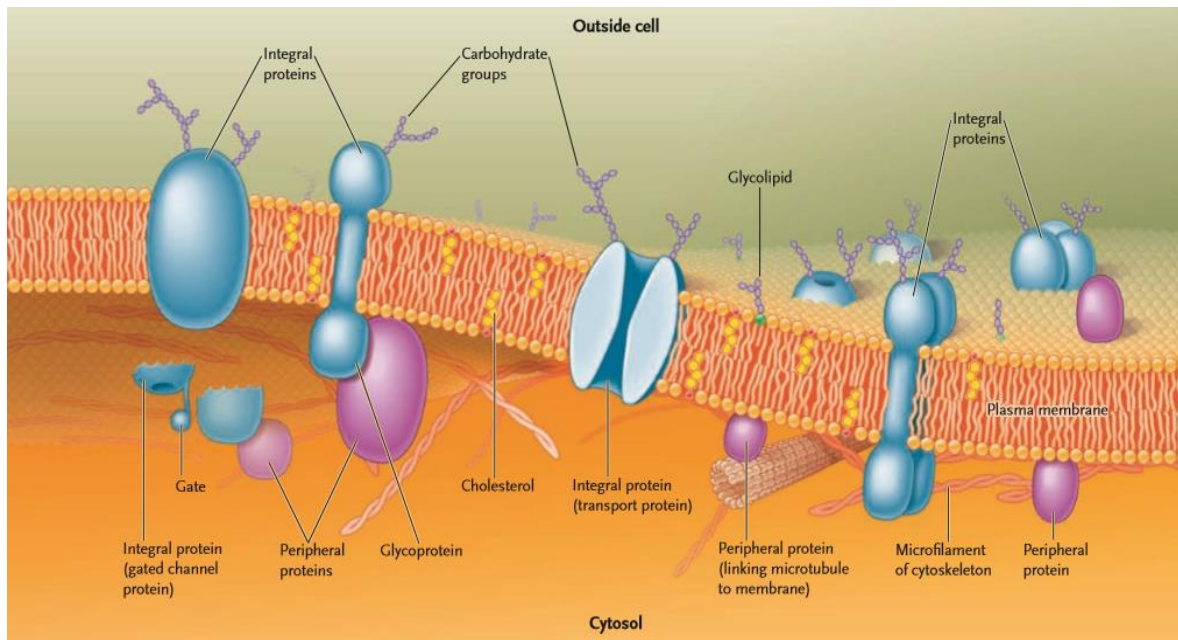


Figure 1.1: Illustration depicting the multiple components of the cell membrane. Reproduced from reference ⁷.

1.1.2 Lipid compositions

Amphiphilic lipids are one of the main constituents of the plasma membrane. There are thousands of naturally occurring lipids, forming numerous subclasses including phospholipids, glycolipids, and sphingolipids. Phospholipid molecules are amphiphilic; meaning they consist of a hydrophobic tail composed of two fatty acid chains, usually 14 to 24 carbons long, and a hydrophilic polar head. Molecules such as choline, serine, glycerol, or ethanolamine attach to a phosphate group to form the polar head group. The two fatty acid chains are covalently linked to the same phosphate group with a glycerol backbone to form the non-polar tail which may be saturated or unsaturated. Phospholipids have an important role in the membrane structure due to this amphipathic nature. When exposed to an aqueous environment they readily assemble into bilayers with the head groups orientated towards the aqueous environment.⁸ At low concentrations phospholipids exist in monomeric forms, however as concentrations rise the polar head groups start to exert repulsive forces to neighbour units. Hydrophobic-hydrophobic interactions of the tails prevent the self-organisation of the bilayer.^{8,9} The packing and mobility of the membrane is also influenced by length of the hydrophobic tails. Shorter chains allow lipids to easily diffuse within the bilayer. Unsaturated lipids are more fluid in the bilayer but cause a more rigid area with their neighbour. Phosphocholine (PC) is one of the most abundant phospholipids within animal cells and can be naturally found as egg-PC or soy-PC, or

synthetically as 1,2,-dioleoyl-sn-glycero-3-phosphocholine (DOPC). Other phospholipids which are present within the membrane are phosphatidylethanolamine (synthetically DOPE) and phosphatidylserine (synthetically DOPS). At physiological pH, DOPC and DOPE are zwitterionic while DOPS is anionic.¹

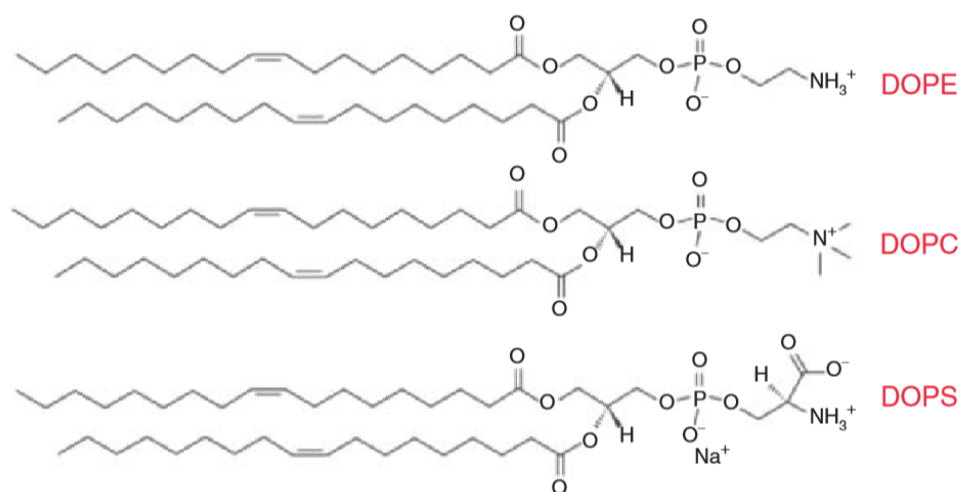


Figure 1.2: Structures of dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine (DOPS) Reproduced from reference¹.

Sphingolipids, another type of lipid found within the cell membrane are aliphatic amino alcohols composed of a polar head group and a non-polar tail, similar to phospholipids. They comprise a long aliphatic chain, generally 18 unsaturated carbons in length. More complex sphingolipids can form an amide-link to a long chain fatty acid.¹⁰ The presence of the amide bridge and hydroxyl groups facilitate the formation of H-bonds with other lipids and proteins. Sphingomyelin, an important example of a sphingolipid, is composed of a phosphocholine or phosphatidylethanolamine head group and is packed tightly into the bilayer due to their long, saturated acyl chains. This allows little movement laterally in the outer leaflet where sphingomyelin is predominantly found. Sphingolipids play a role in the stability of the bilayer by helping with the formation of rafts.¹⁰ A high concentration of sphingomyelin is found in the myelin sheath surrounding nerve cell axons and acts as an electrical insulator.¹¹ Membrane lipid composition varies significantly between organisms, cell type and depends on the stage of the cell cycle. Environmental factors can also play a role on the composition of the membrane.¹²

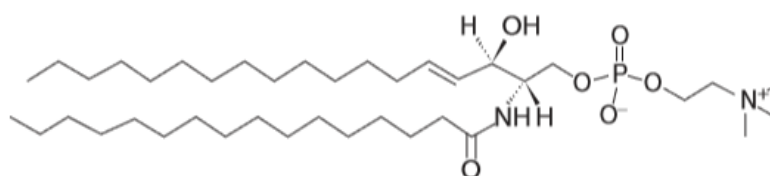


Figure 1.3: Structure of a sphingolipid, sphingomyelin. Reproduced from reference ¹.

Sterols are a non-phospholipid class of lipids that can be found naturally within the membrane of the cell. Cholesterol is the most well-known water insoluble sterol in mammalian cells and has a hydrophobic rigid ring structure with a short “floppy” hydrocarbon tail. Cholesterol also has a polar hydroxyl group meaning it is amphipathic in nature. The hydrophobic ring in the sterols is adjacent to the fatty acid tails of phospholipids and the polar head forms a hydrogen bond with the polar area of the phospholipid. The packing of cholesterol interferes with the fatty acid tails of lipids and causes the fluidity of the bilayer to decrease but mechanically strengthens the membrane. Cholesterol is thought to cluster with sphingomyelin and assists in raft formation which may have a role in membrane trafficking, signalling and endocytosis. Cholesterol and sphingomyelin form hydrogen bonds which condense the membrane structure believed to aid in stability of the bilayer, whilst also decreasing the permeability.^{10,13,14} Cholesterol often makes up 50% of the plasma membrane in human cells.¹⁵ Ergosterol is typically the main sterol found within plant cells.¹

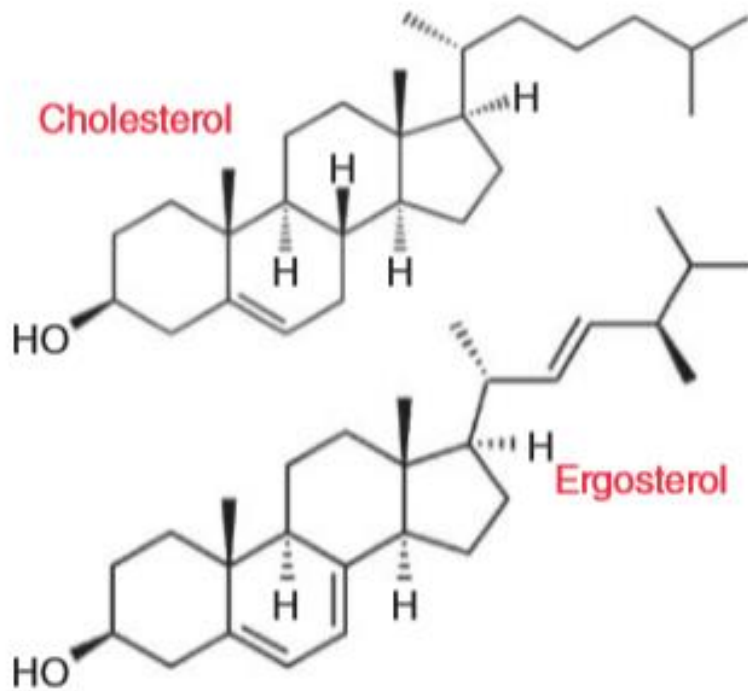


Figure 1.4: Structures of the predominant sterols in mammalian cells, cholesterol, and plant cells, ergosterol. Reproduced from reference ¹.

1.1.3 Lipid Structure in the Plasma Membrane

As stated, the bilayer is composed of two different leaflets, however the composition of exterior and interior leaflets can differ hugely, leading Bretscher to propose asymmetrical organization in 1972. ¹⁶ It has since been seen that certain lipids are only found on either the interior or the exterior. The outer leaflet of the membrane contains mainly phosphatidylcholine and sphingomyelin, whereas predominantly phosphatidylethanolamine and phosphatidylserine, and other negatively charged phospholipids such as phosphatidylinositol, are found on the inner leaflet. ^{17,18}

The regulation of lipid asymmetry is carefully controlled in living cell membranes. The functional roles of structure and regulation have been linked to asymmetry in the phospholipid distribution. ¹⁹ For instant, Annexin, protein kinase C and spectrin interact with PS, which is found on the cytoplasmic leaflet. ^{17,20,21} The disruption of asymmetry in the membrane can expose the PS to the extracellular environment which can generate an interaction of the membrane with the extracellular proteins. This translocation of PS for example can lead to a procoagulant surface on platelets which triggers macrophage recognition of apoptotic cells. ¹⁹

Transmembrane lipid asymmetry therefore contributes to the cells complexity and needs to be considered for a more realistic biomimetic membrane model.

Transversal diffusion or “Flip Flop” is the diffusion of lipids from one membrane leaflet to another. Although much slower than lateral diffusion, flip flop can be affected by the membrane composition or temperature. Cholesterol is expected to diffuse freely between the membrane leaflets due to its small molecular size.²² Kiessling et al showed using single molecular tracking that in a polymer tethered supported lipid bilayer biomimetic model, asymmetry was lost over 15 hours in a uniform bilayer consisting of brain PC, PE, PS and cholesterol.²³

The permeability and structure, in particular, phase and lateral order, of cell membranes can be affected by molecular events occurring at the interface or within the bilayer.¹ Due to variation in melting temperatures depending on lipid chain length, degree of saturation and the presence of sterols then different physical states between lipid classes can be seen at different temperatures.²⁴ The domains formed within the membrane can be separated into many phases but simplified into two domains: lipid ordered or lipid disordered phases.²⁵ As suggested by the name, lipid ordered domains are rigid and well organised phases, forming rafts believed to act as an anchor for protein binding. The nanoscale domains are believed to be consist of saturated lipids and sterols within the membrane and are termed “rafts”.²⁶ Due to the greater van der Waals forces along the length of lipid tail, saturated lipids pack tightly together in membranes leading to more rigid domains or even gel phases. Lipid disordered domains are less rigid.²⁷ Unsaturated lipid tails reduce the lateral packing of the membrane due to a higher hydrophobic bulk volume which results in a more flexible and fluidic domain. The degree of unsaturation of lipids is related to the poorly packing of membranes and it is strongly indicated that the packing effects the membranes diffusivity.²⁸ These lipid domains may alter the dynamic process of transversal diffusion, with more loosely packed phases being more

enabling.²⁹

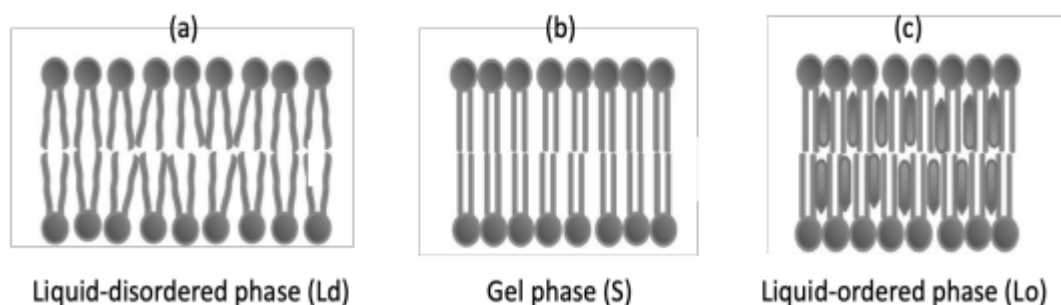


Figure 1.5: Representation of lipid phases: (a) unsaturated lipid molecules in a disordered domain, (b) Gel phases formed by saturated lipids, and (c) lipid ordered phase comprised of saturated lipids and cholesterol. Figure adapted from reference ³⁰.

Lipid models composed of lipids with a mixture of low and high melting points, cholesterol segregates into ordered domains, preferentially with saturated phospholipids. Ternary lipid compositions, typically containing sphingolipids and cholesterol have been observed to form lipid rafts. Occasionally rafts have been observed to not contain sphingolipids, however cholesterol must be present.²⁴ Rafts were originally thought to be driven by membrane proteins. Sevcsik et al completed a study of single molecule tracking in live cell membranes, utilising immobilised raft proteins and saw that no substantial effect on the membrane environment, suggesting the formation of rafts is driving by the lipids themselves.^{24,31}

The impact of lipid phases has been studied for the diffusivity effects with different membrane models. Ariola et al integrated a monophasic, biphasic, and ternary compositions of Giant unilamellar vesicle with a fluorescently tagged BODIPY cholesterol. They observed that cholesterol's diffusion coefficient in monophasic lipid bilayers with DOPC was $7.4 \pm 0.3 \mu\text{m}^2/\text{s}$ using fluorescence correlation spectroscopy. For the gel phased sphingomyelin membrane, the BODIPY cholesterol was observed to diffuse at $0.35 \mu\text{m}^2/\text{s}$. This diffusion decrease was attributed to the more viscous ordered phase of the membrane.³²

Other lipid models have also been used to investigate phases in the lipid membrane. Woodward and Kelly again used fluorescence correlation spectroscopy to monitor the diffusion coefficient of a lipid in both lipid ordered and disordered phases utilising a glass supported lipid bilayer. The diffusion coefficient reported for the lipid ordered phases was lower at $2.4 \mu\text{m}^2/\text{s}$ compared to $4.7 \mu\text{m}^2/\text{s}$ whilst in a pure DPPC membrane.³³ The enhanced lateral packing of lipids in the

ordered domains indicates that lipid diffusion coefficients are significantly lower than when the membrane is in the lipid disordered phase.

The interaction between lipid leaflets is also believed to be important for asymmetric leaflets and can lead to interdigitation.³⁴ Interleaflet coupling has been observed in experiments with supported lipid bilayers. Wan et al showed using Epifluorescence Microscopy Imaging that in a tethered supported lipid bilayer more ordered domains were apparent in asymmetric membranes containing PC/PE/PS/Cholesterol in the outer leaflet and PC/SM/Cholesterol in the inner leaflet, compared to a symmetric PC/SM/Cholesterol bilayer.³⁵ This further suggests that asymmetry alongside the phases of the membrane have a vital impact on the membrane structure.

Overall, lipid asymmetry and phases of the membrane are important physical properties that are responsible for maintaining biological activities in cells. The understanding of lateral and transversal diffusion amongst the asymmetric lipid membrane is vital for unravelling the functionality of lipid and proteins in lipid membranes. The inclusion of membrane proteins into lipid bilayers may affect the properties of the cell membranes. Frick et al showed using Fluorescence Recovery After Photobleaching (FRAP) that diffusion properties in cell plasma membranes were reduced when the total protein content was increased.³⁶

1.1.4 Membrane Proteins

While the basic structure of the cell membrane is provided by the lipid's constitutions mentioned above, lipids do not compose the entire plasma membrane. Proteins make up 50% of the membrane by weight with a ratio of one protein molecule for every fifty lipid molecules.³⁷ Membrane proteins perform the majority of the specific functions of membranes. Some membrane proteins are embedded within the membrane for support and structure, named the structural proteins. Functional proteins partake in processes such as the flow of matter, energy, or information. Membrane proteins can be classified by the nature of their assembly at the membrane whereby they may be fully inserted, also known as integral proteins, or partially inserted such as peripheral proteins, indicated in Figure 1.6.

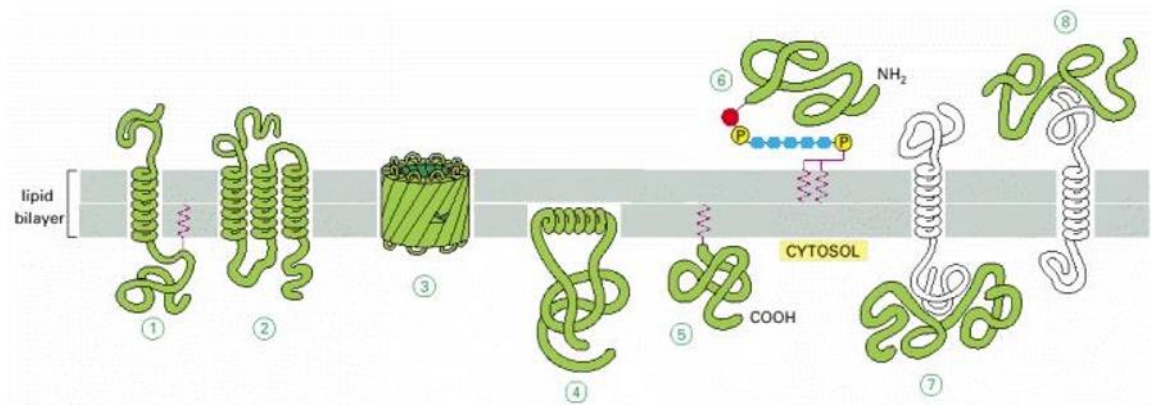


Figure 1.6 : Different types of membrane proteins. Transmembrane proteins may extend across the lipid bilayer as single a single alpha helix (1), multiple alpha helices (2), or as rolled beta-sheets (3). Peripheral proteins can be anchored to the cytosolic surface by an amphipathic alpha helix that partitions into the cytosolic monolayer of the lipid bilayer through the hydrophobic face of the helix (4). Others are membrane attached via a covalently attached lipid chain in the cytosolic monolayer (5). Some may be membrane attached via an oligosaccharide linker, to phosphatidylinositol in the non-cytosolic monolayer (6). Finally, peripheral membrane proteins interact with the lipid membrane via non-covalent interactions with other membrane proteins intracellularly (7), or extracellularly (8). Reproduced from reference ⁹.

Amphipathic proteins have a hydrophobic domain which will interact with the hydrophobic tails of the lipid components of the bilayer. This causes the protein to integrate into the membrane in a way that the hydrophobic amino acids of their polypeptide chains are within the membrane forming an alpha helix and the hydrophilic regions are arranged into one or both of the aqueous environments.¹ Thus, these proteins are referred to as transmembrane proteins, and can traverse the membrane partially or fully. Integrins are an example of single-pass transmembrane proteins that will thread the bilayer only once with its alpha helix (Figure 1.6 pt. 1), whereas bacteriorhodopsin, a multi-pass transmembrane protein, threads through of the membrane 7 times, (Figure 1.6 pt. 2), and acts as a proton pump for the cell.^{38,39} Transmembrane proteins may be found both intracellular or extracellular of the membrane.⁹ It has been postulated that protein-lipid interactions are crucial for proper insertion, folding and topology of membrane proteins.⁴⁰ Studies have indicated that positively charged amino acids will interact with negatively charged phospholipids which may help in the insertion of the

membrane protein.⁴¹ Pe is required for LacY to maintain active transport of lactose due to the orientation required.⁴² Similarly MscL requires anionic phospholipids to stabilise it in the open state. In the presence of zwitterionic or cationic lipids, the open state is disrupted.^{43,44} Peripheral proteins do not integrate into the lipid bilayer but rather associate with either the extracellular or intercellular region of the membrane. Peripheral proteins bind weakly to one leaflet typically through electrostatic interaction or hydrogen bonds through the polar head groups of the lipids or associate with integral proteins embedded in the membrane. Similar to transmembrane proteins, peripheral proteins may require optimum lipid environments to function. Binding is often mediated through ions such as calcium. They elicit their function in a number of different ways, such as in network formation during apoptosis & thrombosis (e.g. annexin V), cell signalling, metabolic pathways and stability.^{45–48} Membrane fluidity and tension is vital for protein activity. For example, Amyloid beta is thought to self-assemble within the membrane to form oligomers and fibrils, which are thought to be the cause of toxicity in Alzheimer's disease.⁴⁹

1.1.5 Aggregation of Proteins and Network Formation.

This thesis focusses on application of microcavity supported lipid bilayers to study membrane associated proteins and their interactions with the lipid membrane. Specifically we are concerned with proteins that aggregate or network at the membrane. Such interactions are difficult to study in cells due to their complexity and in conventional biophysical models because of their poor addressability and stability, but particularly because the most versatile models, the SLBS show low fluidity. These issues are addressed in MSLBs and studies of protein aggregation, which is facilitated by lateral diffusion are enabled. The lipid membrane is particularly important to multivalent proteins that can bind to more than one receptor, lipid, or protein.

Chapter 3 focuses on Annexin V. Annexins, comprise a large family of soluble proteins, that bind peripherally to negatively charged phospholipids of lipid membranes in a manner dependant on calcium.^{46,50–54} Annexin V (AnV), is thought to be critical in plasma membrane repair, as well as linking of biological structures.⁵⁴ AnV is thought to form a two dimensional network of crystal structures over the lipid membrane in response to membrane damage.^{17,55–57} Annexin V is also used biomedically as a marker for apoptosis. Apoptosis occurs during

development and aging as a way to maintain cell homeostasis. Apoptosis also occurs as a defence mechanism also when a normal healthy cells cannot repair damages such as oxidative stress or ruptures which can be caused by cell damage by disease or toxic agents.^{58,59} Upon cell damage, phosphatidylserine translocates from the interior leaflet to the exterior leaflet allowing for AnV to bind in the presence of calcium.^{60,61,62} Annexins are thought to link or hold together biological samples, such as gametes and cell membranes, in processes such as membrane organization, signaling and vesicle transport and are implicated in a number of disease states and physiological processes including thrombosis and inflammation.^{54,63–65} According to Huber et al, Annexins usually form a 4 fold repeat of 70 amino acid domains with variable N-terminal domains thought to link to the family's functionality, seen in Figure 1.7.⁶⁶

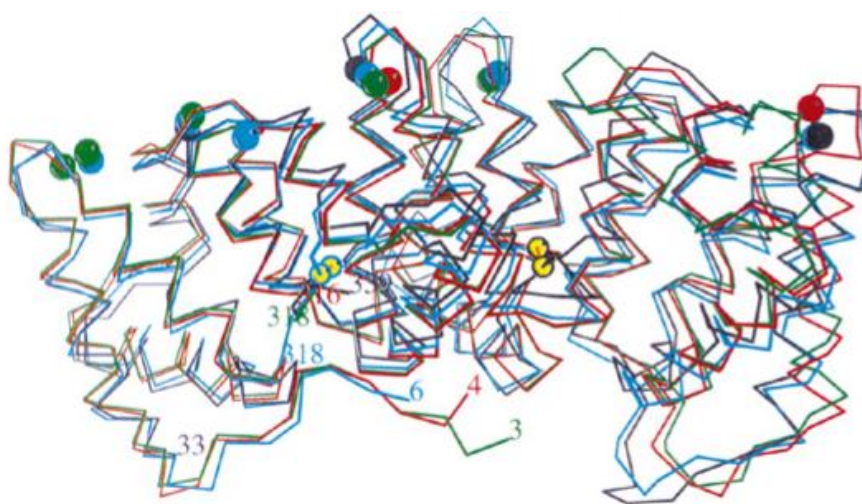


Figure 1.7: Overlay structures of Annexin I (blue), Annexin II (black), and Annexin V (green). Molecule view from the side. Calcium ions are represented as spheres in the same associated protein colour. Reproduced from reference ⁶⁷.

Another membrane protein that is seen to aggregate and form a 2D crystal layer is Streptavidin.⁶⁸ Streptavidin interacts with the biotin within bacterial membranes and aggregates to form a 2D layer. Tetrameric in nature, streptavidin is thought to have multiple biotin binding sites.^{69,70} The interaction of streptavidin and biotin within a bacterial membrane is thought to be a natural defensive mechanism of the bacterial cell. By binding with biotinylated enzymes associated with CO₂ transfer, the process is inactivated and therefore inhibits bacterial growth relying on these enzymes.⁷¹ Darst et al formed two dimensional crystal structures of Streptavidin when bound to monolayers of biotinylated lipids. They found that with fluorescence microscopy that the interaction of biotin and streptavidin on monolayers lead to

the formation of large protein domains with up to 200 μm diameters.⁷² Both annexin V and Streptavidin are peripheral proteins that interact with components of the cellular membrane. Transmembrane proteins are also expected to aggregate within the membrane and the development of fluidic membrane models that can incorporate the protein are vital.

Beta amyloid ($\text{A}\beta$), the focus of Chapter 4, is a peptide whose self-aggregation is a primary step in Alzheimer's disease. Alzheimer's is the most common form of dementia and is an irreversible age-related, progressive brain disorder that causes memory loss and impairs thinking skills. Amyloid precursor protein (APP) is a membrane glycoprotein within the brain that plays a role in a range of biological activities that includes neuronal development, signalling, transport, and neuronal homeostasis. APP is proteolytically cleaved by β - and γ -secretases to form beta-amyloids.⁴⁹ $\text{A}\beta$ is mainly composed of 36 - 43 amino acids that spontaneously self-aggregate through hydrophobic-hydrophobic interactions.⁷³⁻⁷⁵ Initially, $\text{A}\beta$ monomers are disordered but self-assemble to form β sheets, structured oligomers, and fibrils. The fibrils are then thought to block synaptic pathways in the brain which leads to memory loss of the patient.⁷⁶ Such oligomers are expected to be the toxic agent in Alzheimer's disease. Much of their toxic action is membrane based.

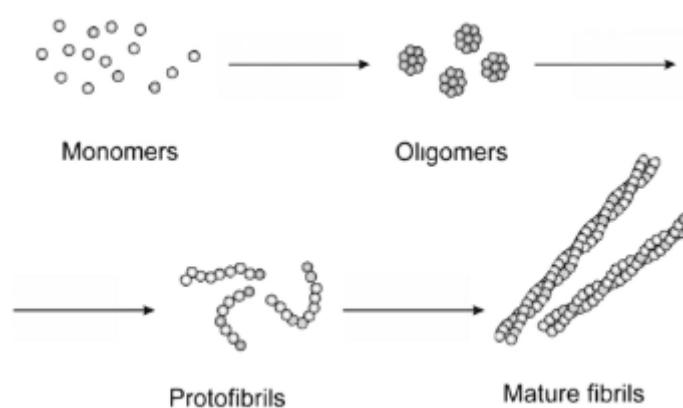


Figure 1.8: Simplified assumed path way for $\text{A}\beta$ fibril formation. Reproduced from reference ⁷⁶.

$\text{A}\beta$ is suspected to disturb the membrane and cause oxidative stress and inflammation, damage metabolism and upset calcium homeostasis.⁷⁷⁻⁷⁹ To date the models suspected to explain $\text{A}\beta$ oligomerisation toxicity in the membrane include the poration hypostasis, the carpet model and the membrane dissolution model. The poration hypothesis proposes that transmembrane pores

form in the neuronal membrane which disrupts intracellular homeostasis.⁷⁹ The carpet model proposes that the monomers peripherally adsorb to the interface which destabilised the structure of the membrane.⁷⁷ The dissolution model indicated that upon aggregation of A β , the plaques strip off and pull the lipids damaging the membrane in the process.⁷⁸ By understanding the mechanisms of this amyloid aggregation an early detection method and possible therapeutic treatment of Alzheimer's may be developed and given the role the membrane plays in this accurate membrane models are essential. Glycosphingolipids, cholesterol and anionic lipids present within the membrane are suspected to enhance fibril formation of A β .^{80,81} The interaction of transmembrane proteins and peripheral proteins can also influence membrane protein aggregation.

Another group of proteins, of growing biomedical importance, and believed to be involved in aggregation and network formation at the membrane are the Galectins. Galectins are a family of 15 lectins that contain a highly conserved carbohydrate-recognition domain (CRD) that recognise β -galactosides. Galectins recognise a range of glycosylated proteins and lipids in the extracellular cell membrane and modulate cell adhesion and migration. Galectin 3, the lectin of focus in chapter 5, oligomerization capacity has been linked to binding of surface glyco-receptors..⁸²⁻⁸⁷ The aggregation of Galectin3 with integrins has been linked to the spreading of cancer cell lines and has been associated also with diverse diseases including diabetes and depression.^{88,89} The term "integrin" was first published in 1987 to describe a family of heterodimeric transmembrane receptors.⁹⁰ There are 24 known glycoproteins in the integrin family in vertebrates and all are of significant biomedical importance. All integrins are comprised of alpha and beta subunits that integrate with the cell membrane and mediate signals between extra- and intracellular spaces, Figure 1.9A.⁹¹ Integrins are regarded as two faced receptors: one ligand receptor orientating to the extracellular space and the other orientating to the cell interior. A hallmark of the integrin family is the ability of individual family members to recognise multiple ligands.

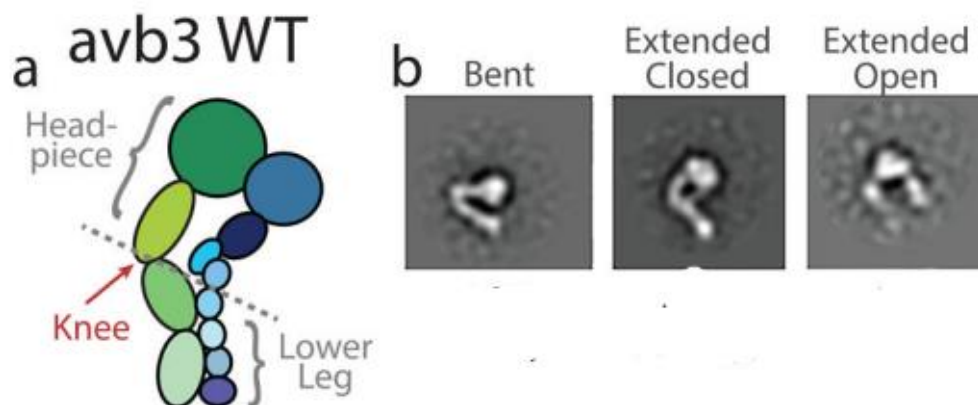


Figure 1.9: A) Schematic of structure of integrin $\alpha_v\beta_3$. B) Cryo Electron microscopy of integrin in the bent (resting), the extended closed (partially activated) and the extended open (fully activated) conformation states.. Modified from reference ⁹².

Integrins are normally integrated in the cell membrane in their resting state which has been characterized across many studies (by electron microscopy) as a bent conformation, Figure 1.9B. ^{92,93} Activation of integrins results in a gross conformation change from bent to an extended arrangement and as such can have an effect on the affinity of the integrin. ⁹⁴ Activation can be stimulated by the integrin binding to its ligand (such as fibronectin, vitronectin, collagen and laminin) or by binding to an activator protein or indeed through chemical activation by reduction of the disulphides, removal of the metals from the MIDAS region or through treatment with Mn(II), the latter leads to partial activation. ^{94–96} The activation state of integrin and its effect on aggregation of galectin is investigated in Chapter 5. The activation of integrin at the surface of blood platelets is a key step in the initiation of thrombosis. ⁹⁷ Gaul et al reported that platelet integrin $\alpha_{IIb}\beta_3$ can be reconstituted into GUVs of varying lipid compositions, a diffusion coefficient of $2.52 \mu\text{m}^2/\text{s}$ using FCS. Activation of this platelet integrin with fibrinogen decreased the diffusion value and also caused sub diffusion to occur. ⁹⁸ The decrease of diffusion could indicate that upon activation clustering of the integrin occurs and could lead to a platelet build up in the veins and arteries. This ability of integrin clustering has also been linked to the survival of human melanoma and suggests that integrin clustering has a big impact on cancer research. ⁹⁵ Galectin 3 has been shown to have altered levels amongst various types of cancers and the clustering of Galectin 3 with integrins could be the cause of chemotherapeutic resistance. ^{89,99}

Other multivalent proteins that require aggregation in the cell membrane include Cholera Toxin and Shiga Toxin.^{100,101} Cholera Toxin cross links with glycosphingolipids with promote stabilization to allow for protein cellular uptake.¹⁰² This allows for the protein to freely diffuse amongst the membrane to aggregate with other receptors to initiate cell infection.¹⁰³ Berselli et al monitored Cholera Toxin clusters using FCS on different asymmetric lipid membranes (utilising microcavity arrays) revealing two populations of the protein, one fast diffusing component and one slow diffusion component linked to the Cholera toxin aggregates. Increasing concentrations of Cholera toxin lead to an increased percentage of the slow moving component.¹⁰⁴ Overall, proteins are an active part of the cellular membrane, in which the lipid membrane is required to perform certain activities such as aggregation, material transport, signalling and trafficking. The cell membrane as stated is a complex, dynamic structure and can be complicated and difficult to study. Biophysical membrane models can be a useful way to study biochemical processes as they can include or exclude constituents of the membrane that may confound study. The next section covers the main models used to mimic the cellular environment to explore the properties of the lipid membrane with the assembly of peripheral and integral proteins.

1.2 Biomimetic Membrane Models

As evident in the above examples, the cell membrane is a complex and dynamic structure comprising a mixture of lipid and proteins that interact extensively with each other. Due to this complexity, specific interactions, and mechanisms behind biochemical processes at the cell membrane can be difficult to study. As such, artificial membranes can be used as models for cellular membranes for studying protein interaction with lipid bilayers under variable experimental conditions while still remaining well defined.^{50,105} Specifically, understanding the role of lipid-protein interactions and protein aggregation has an important value for disease, both in understanding unique mechanisms in the disease progression and also in therapy design. To accurately investigate protein-membrane interactions and aggregation in artificial models, the need for a fluidic lipid bilayer is vital to ensure the natural mobility of both proteins and lipids. The stability of artificial membrane models is crucial for the investigation of protein aggregation and network formations and prevention of denaturing, both the protein and the membrane, is vital. Within the literature, a range of biomimetic systems have been used to mimic the cell membrane. Lipid vesicles, Black lipid membranes and planar lipid bilayers are

some of the most studied types of membrane models. Recently, pore suspended lipid membranes have been introduced, which expands the possibilities of protein aggregation capabilities. Despite natural cell membranes asymmetry, majority of research concerning biomimicking models relies on pure symmetric membranes models. However, more stable asymmetric lipid bilayers have been observed in the literature for both lipid vesicles^{106,107} and supported lipid bilayers^{108,109}. Chiantia et al investigated interleaflet coupling, using FCS on asymmetric GUVs containing DOPC and sphingomyelin.¹⁰⁶ Cheng et al prepared asymmetric small unilamellar vesicles consisting of DOPC, sphingomyelin, POPC, cholesterol and POPS and used fluorescent based assays to confirm the asymmetry of the vesicle.¹⁰⁷ Reich et al used Synchrotron X-Ray Reflectivity to investigate the asymmetry of SLBs containing cholesterol and GM1.¹⁰⁸ Visco et al investigated asymmetric DOPC and sphingomyelin SLBs with stability up to at least 4 hours using FCS.¹⁰⁹

1.2.1 Vesicles

Lipid vesicles, or liposomes, are enclosed lipid bilayers that are free standing, quasi-spherical structures. They are one of the earliest membrane models and were discovered in 1964 by Dr Alec Bangham by imaging phospholipids with an electron microscope.¹¹⁰ Vesicles can be prepared in a number of ways. The most typical method is by drying lipids and rehydrating in a polar solvent such as water or buffer and upon rehydration vesicles form spontaneously.^{111,112} Vesicles formed in this way can vary widely in size and are classified as such (small, large or giant). The structure of liposomes are termed uni-, oligo- or multilamellar.¹¹³ The term “unilamellar” refers to a vesicle formed with only one lipid bilayer. The size of liposomes are controlled to form the liposome of choice: Small Unilamellar Vesicles (SUVs), Large Unilamellar Vesicles (LUVs), Giant Unilamellar Vesicles (GUVs) or Multilamellar Large Vesicles (MLVs) shown in Figure 1.10.

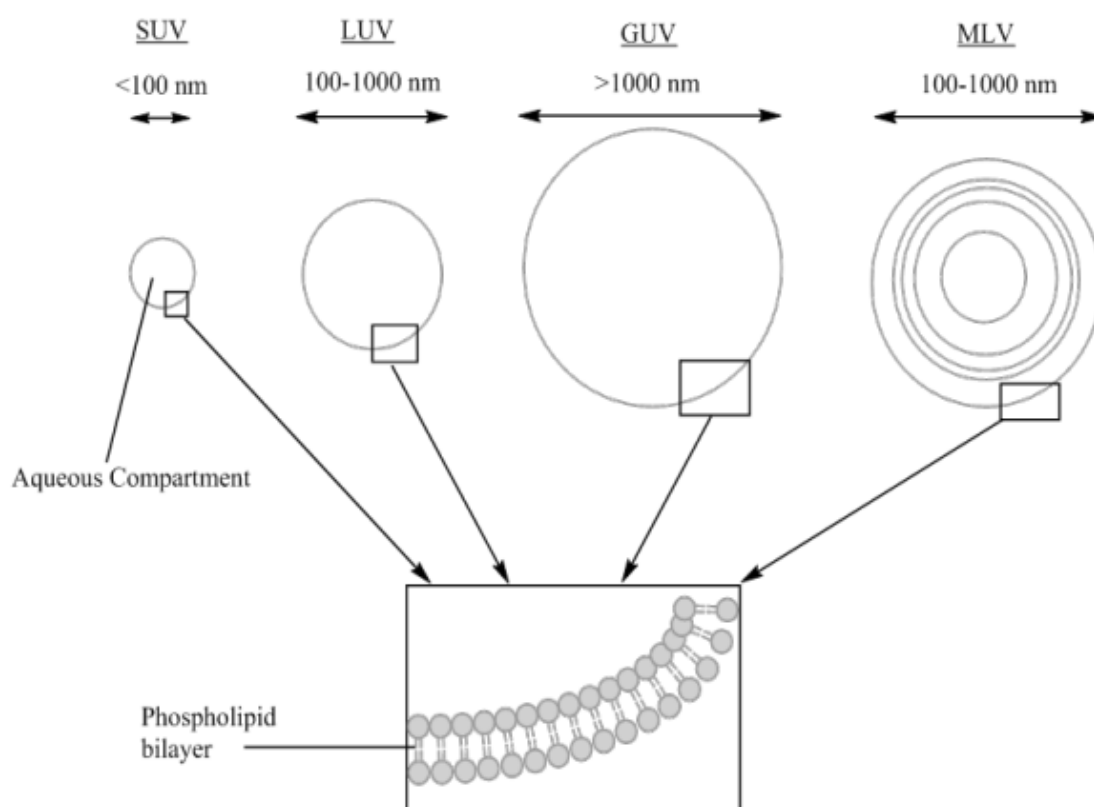


Figure 1.10: Representation of Size distribution for SUVs, LUVs, GUVs and MLVs.

As mentioned, vesicles are usually made by rehydration of dried lipid films. This method is usually done by dissolving the phospholipids molecules of choice in an organic solvent such as chloroform and then evaporating the solvent. In doing so the phospholipid molecules self-assemble into a stack of bilayers on the substrate. Upon rehydration in an aqueous buffer, the phospholipids are poorly soluble unless they self-assemble into bilayers to exclude the bulk water phase using their hydrophobic tails. The bilayers separate from the surface and form spherical liposomes.^{112,114} The rate at which rehydration occurs affects the lamellarity of the liposomes and can lead to multilamellar vesicles. For certain applications such as drug delivery or formation of supported lipid bilayers, multilamellar vesicles are not ideal and more homogeneity is desirable. In order to make more uniform vesicles, extrusion through a polycarbonate membrane, sonication, or freeze-thaw cycles are performed which results in more controlled liposome distributions. In this work liposomes were prepared by extrusion of multilamellar vesicles through a polycarbonate membrane with specific pore dimensions.¹¹⁵

Drug delivery systems in therapeutics and physiochemical lipid-protein properties have been utilized by liposomes due to their simple spherical structures and easy preparation. Liposomes have also been used for medicine carriers due to having good biocompatibility and biodegradability.¹¹⁶ To enhance liposomes biological activity, they can be functionalised with artificial molecules such as PEG or biological molecules such as antibodies or peptides.¹¹⁷ Liposomes also have been reported as effective delivery systems for various genetic material (mRNA, DNA, peptides) to cells.¹¹⁸ This delivery system with liposomes and genetic material has been utilised for the development of new vaccines for virus such as Zika, Chikungunya and SARS-Cov-2.^{119–121}

Membrane proteins require a lipid environment to retain structure and activity. Proteoliposomes are liposomes which contain membrane proteins and they have gained importance as biophysical tools for investigation into lipid protein interactions as they preserve the structure and functions of proteins as if in their native state. Different models for protein reconstitution of purified membrane proteins into lipid models have been reproduced. However the success of reconstitution is dependent on many parameters including nature of the protein, type of lipids used, solvent used and detergents.^{122,123} Chemical reconstitution using organic solvents, direct incorporation into preformed liposomes by a mechanical method or detergent-mediated reconstitution are three protocols for the formation of proteoliposomes.¹²⁴

The most studied method of membrane protein incorporation is the detergent method. Organic solvents and mechanical agitation have both been used for the incorporation of proteins.^{125,126} However both methods have a high risk of denaturing protein.¹²⁴ Using the detergent method the risk of denaturing is minimised. Lipid and proteins can be solubilized with a detergent, or the membrane proteins can be mixed with premade liposomes.¹²⁷ Detergent is then removed with dialysis, filtration or incubation with hydrophobic resins.¹²² The removal of detergent leads to lipid molecules rearranging into bilayer structures so as to isolate the hydrophobic cores from water molecules. The membrane proteins stay associated with the lipid bilayer. The choice of detergent for this process is important for successful protein incorporation, liposome size and homogeneity and should be chosen carefully. Rigaud et al reported different surfactants such as Triton X-100, Octyl Glucoside and sodium cholate had different implications for the incorporation of bacteriorhodopsin into purified egg yolk phosphatidylcholine (EggPC). Triton X-100 and Octyl Glucoside successfully inserted bacteriorhodopsin when partially and totally solubilized. In contrast sodium cholate needed full

solubilization of the liposomes for incorporation into the liposome.^{124,128} The surfactant must be completely removed to allow the formation of proteoliposomes and is traditionally removed using dialysis or detergent absorbent beads, such as BioBeads SM2.

Giant unilamellar vesicles (GUVs) are an important class of lipid vesicles that are of diameter greater than 1 μm and are typically produced by the gentle hydration of a dried lipid film.¹²⁹ Applying an alternating current (AC) electric field to the rehydrated lipids results in a great rate of bilayer separation leading to unilamellar vesicles varying from 10 to 100 μm .¹³⁰ As GUVs are large in size they are useful as a membrane model for optical imaging and are also closer to the dimensions of cells.^{115,131} Lipid dynamics, raft formation, membrane fusion and protein behaviour have all been studied using GUVs.^{98,132–134,134–137} Due to GUVs free standing properties and membrane fluidity they are one of the most common biomimetic models applied to proteins, however analysis is largely limited to fluorescence and imaging experiments and addressing them with interfacial methods is difficult.

Whilst vesicles have been useful model membranes, they have limitations. Liposomes are not easily electrochemically addressable and liposome movement during imaging or fluorescence experiments can affect focus during measurements. Boxer et al tethered fluorescently labelled liposomes using oligonucleotides to a planar supported lipid bilayer, which allowed for individual liposome visualisation by fluorescence video microscopy.¹³⁸ Asymmetry is complicated to build in lipid vesicles, which causes difficulty in studies that require true biomimicry. Steinkuhler et al reported asymmetric distribution of negatively charged PS in GUVs investigated with fluorescence quenching, however the asymmetry was nearly lost above room temperature which is usually required for lipids with higher melting points such as sphingomyelin.^{139,140} Other issues such as long term stability and liposome geometry are important drawbacks for use as a biomimetic model. However the use of liposomes as a precursor to preparations of other biomimetic models including Droplet interface bilayers, Supported Lipid Bilayers or for use of protein incorporation are a useful tool.^{141,142} In this work liposomes were used for building the MSLBs.

1.2.2 Droplet Interface Bilayers

Droplet interface bilayers (DIB) are an interesting approach to obtain lipid membranes. Aqueous microdroplets (usually 10 to 100 micrometre diameter) are immersed in an immiscible phase surrounded by a lipid monolayer. A lipid bilayer is formed at the interface

between droplets when the droplets are brought together.¹⁴³ DIB are mainly used to analyse the effects of membrane lipid structure for transport due by direct observation of the interfaced membrane.¹⁴⁴

DIBs can be prepared in two ways: by solubilizing lipid molecules in an oil (known as Lipid-Out) or by utilising liposomes as the droplets (known as Lipid-In).^{145,146} The first method utilizes water and oils immiscibility, in that when a drop of water is added to the lipid oil mixture, the phospholipids rearrange forming a monolayer at the interface of the aqueous drop. Asymmetric bilayers are easily obtainable for DIBs prepared in oil, however it is typically not suited to the incorporation of membrane proteins.¹⁴⁶ The second method of forming DIBs is by introducing an aqueous solution of liposomes to an oil phase. The lipid vesicles will in time fuse at the interface of the aqueous drop. This method allows for the use of a micropipette to manipulate the droplets in the oil phase together, leading to lipid bilayers at the intersections of two or more droplets. By preparing the liposomes to incorporate membrane proteins, the inclusion of proteins in the DIB is achievable and has been reported for proteins such as OmpG¹⁴⁶ and MscL.¹⁴⁷

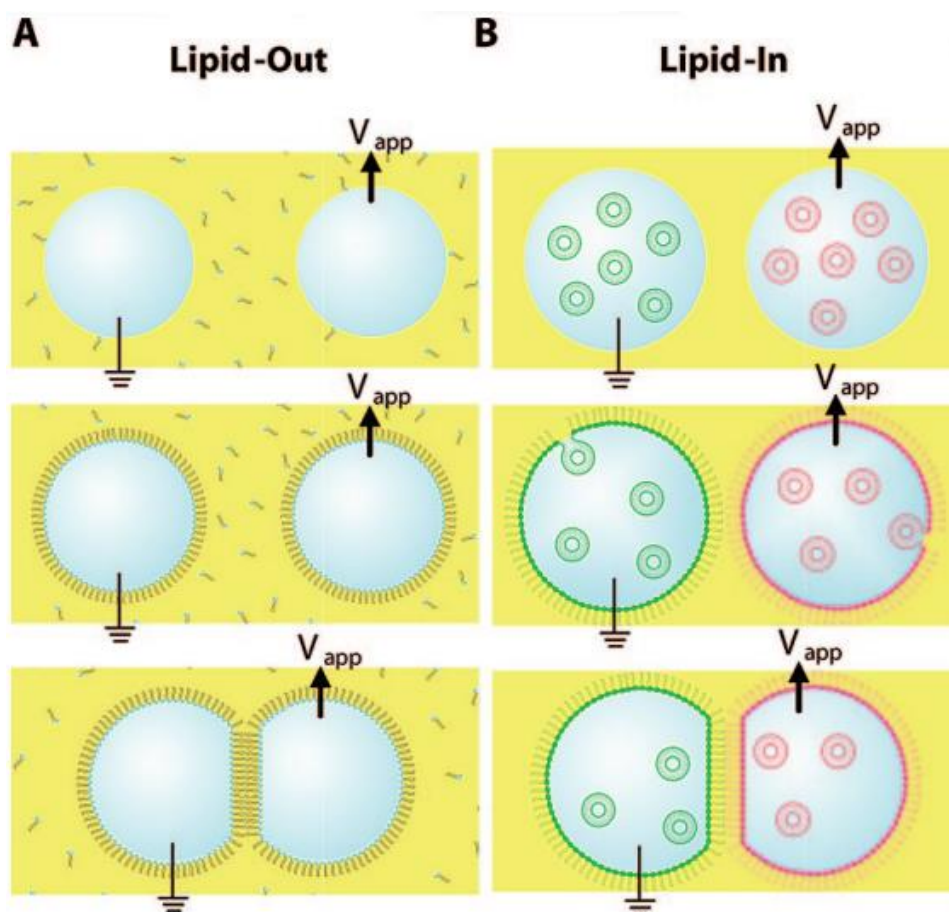


Figure 1.11: Assembly of DIBs. A) Lipid-Out formation of DIBs in which lipids have been solubilized into oil and aqueous drops have been added to form the lipid monolayer (<5 minutes). When brought into contact the DIB formed. B) Lipid-In formation of DIBs, in which aqueous drops of two different liposomes have been placed onto an oil layer. The liposomes diffuse out to the interface and disrupt to form a monolayer (<30 minutes), in which when drops are manipulated together form a bilayer. Reproduced from reference ¹⁴⁶.

DIBs are amenable to electrochemical interrogation and have been applied to assess the area, thickness, and surface potential of membranes as membranes behave as biological parallel plate capacitors. ¹⁴⁸ Liposomes themselves are not amenable to electrochemical studies, however electrodes can be inserted in the DIBs to enable study of the membranes electrochemical properties. Pore forming peptides can therefore be studied using DIBs by monitoring the ionic current flowing through the membrane.¹⁴⁵ The membrane protein, MscL, has been well characterized by electrochemical methods using DIBs. ^{147,149,150} Haylock et al determined the electrochemical properties of MscL by monitoring the pore network activity using

chronoamperometry. The reconstituted MscL was activated with MTSET and by monitoring the electrochemical signal of a series of DIBs, the pore channel activity could be followed.¹⁴⁷

DIBs are a versatile way of preparing asymmetric lipid membrane, which can include membrane proteins. However they rely on contact between two monolayer droplets to form the bilayer, reducing their appeal for more relevant studies over larger surfaces. The effect of the higher viscosity surrounding media of the oil bath can impact the lipid diffusivity directly. In addition, pH or salt concentrations cannot easily be adjusted of DIBs and limits the biomimicry and applications.

1.2.3 Black lipid membranes

In the 1960's, Mueller et al. reported a planar model membrane composed of extracted brain lipids in a methanol/chloroform solution that were painted on an underwater hole of 1 mm diameter on a polyethylene surface.¹⁵¹ Bilayers under these conditions appear black when studied with reflected light and as such have been termed black lipid membranes (BLMs). BLMs are composed of lipid layers painted over a small aperture on a hydrophobic material using organic solutions in an aqueous environment as seen in Figure 1.12.¹⁵²

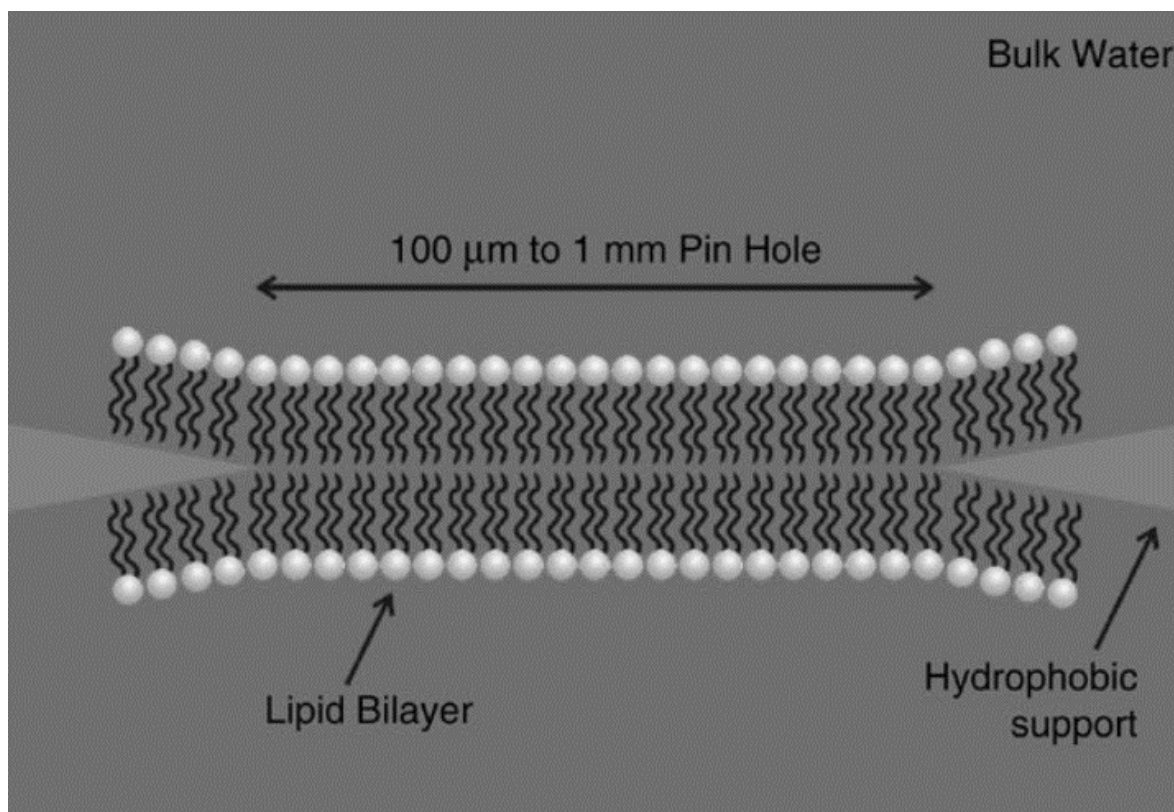


Figure 1.121: Schematic of a Black Lipid Membrane over a 100 μm to 1 mm aperture of a hydrophobic substrate. Modified from reference ¹⁵².

As either side of the bilayer is in contact with water, BLMs have been used extensively for investigating electrical properties of the membrane, and for transport of biomolecules. Cholesterol within the bilayer has been examined by utilising BLMs. ¹⁵³ The use of organic solvents in the preparation of BLMs leads to problems such as solvent retention in the lipid bilayer, making it very difficult to reconstitute protein into such structures and limiting the biomimicry of the environment. Another key disadvantage of BLMs is that asymmetry and controlled variations in compositions are difficult to achieve. The painting over the aperture method also leads to issues with reliable bilayer formation and usually resulting in the formation of multilayers and stability can also be problematic and limits precision preparation e.g. of asymmetric bilayers or protein reconstitution, though in electrochemical measurements they can often give giga ohm resistance as they seal the electrode so well but this is often a consequence of their multilayer . ^{1,136,154}

1.2.4 Supported Lipid Bilayers

Years after the formation of BLMs Tamm et al formed a stable lipid bilayer on oxidized silicon wafers, and thus reported the first solid supported lipid bilayer.¹⁵⁵ Supported lipid bilayers (SLBs) are highly versatile models of cellular membranes and can be used to study different aspects of membrane biophysics in well-defined experimental conditions.^{53,156} SLBs have been since shown to form on a variety of materials, such as mica, gold, PDMS, etc, provided they are smooth, clean and hydrophilic.^{155,157,158} A variety of lipids can be used to study different compositions and asymmetry of the membrane.⁵⁰ SLB formation is done through one of three methods. Vesicle disruption is the process in which SUVs are prepared and left to disrupt onto a flat surface to form a monolayer and upon contact with more SUVs a formation of a bilayer occurs.¹¹⁵ The second method, namely the Langmuir Blodgett method, is where a monolayer of lipid is transferred to a substrate from an air/water interface.¹⁵⁹ Lastly, the third method is a combination of the vesicle disruption and Langmuir Blodgett techniques. As variation in composition, asymmetry and protein incorporation are possible, all methods are discussed further in the Langmuir Blodgett section 1.3.1.¹ A major advantage of SLBs is that they are considerably more stable over time than either BLMs or liposomes. Additionally, SLBs also are far more addressable, and can be interrogated with a variety of techniques such as atomic force microscopy, quartz crystal microbalance and photo-physical and spectroscopic methods leading to a better and deeper understanding of membrane properties.^{160–163}

Substrate selection for preparation of SLBs is important as the resulting bilayer can be affected, and certain substrates are required for different methods of analysis. For example, hydrophobicity can influence the water layer thickness between the membrane and the substrate surface, which can affect the fluidity of the lipid within the bilayer.¹⁶⁴ Glass has commonly been chosen as the substrate of choice for SLBs. Naturally, glass is hydrophilic and usually only requires cleaning before bilayer formation.¹⁶⁵ Glass however has a rough surface and flatter smoother surfaces are required such as mica, silica, or flat gold for techniques such as atomic force microscopy. Key drawbacks of tradition SLB are that fluidity is low and biomimicry is limited. Frictional interaction between the substrate and membrane can lead to poor diffusivity, usually limited within the range of $0.3 - 4 \mu\text{m}^2/\text{s}$.^{166,167} Przybylo et al reported using FCS that under identical conditions a DOPC GUV exhibited 2 times higher diffusion at $7.8 \mu\text{m}^2/\text{s}$ compared to the DOPC SLB on mica, $3.1 \mu\text{m}^2/\text{s}$.¹⁶⁸ A ternary GUV composition of DOPC/Cholesterol/sphingomyelin was reported to have a diffusion of the lipid probe in the

liquid ordered region of $2.5 \mu\text{m}^2/\text{s}$ and a diffusion coefficient of $6.1 \mu\text{m}^2/\text{s}$ for the probe in the lipid disordered region. In contrast when the same membrane composition was investigated using an SLB on mica the lipid ordered and disordered diffusion coefficients were reduced, 3.4 and $0.11 \mu\text{m}^2/\text{s}$ respectively by FCS.^{169,170} Interleaflet coupling of the membrane can be important in the case of SLBs. A strong interleaflet couple exist between lipids in SLB and causes both leaflets to diffuse at the same velocity. In the absence of interleaflet coupling the exterior leaflet would diffuse similarly to GUVs, since the diffusional decrease in SLBs is seen to be 2 fold less than GUVs it was concluded that a strong interleaflet coupling is occurring.^{163,168} The lack a reservoir to mimic the cytoplasm can cause the reconstituted protein to insert improperly. Frictional or electrostatic coupling of the SLB to the substrate may affect the morphology of the phase separation in comparison to free standing membranes.^{111,171} Polymer materials are another common substrate used for SLBs, for example PDMS.¹⁷² PDMS is attractive as a material as it is low cost, biocompatible and compatible with light methods due to its optical transparency, has a low reflectivity and low fluorescent background. Modification is required for PDMS, however as it is naturally hydrophobic, plasma treatment has been shown to be effective for the formation of bilayers on PDMS substrates.¹⁷³ Gold has also been shown to be an attractive substrate choice for SLBs, although hydrophobic in nature, modification is easily achieved from self-assembly monolayers which can render the surface hydrophilic.^{174,175}

1.2.5 Modified Supported Lipid Bilayers

Modifications to traditional SLBs systems have been employed to further enhance the capabilities of SLBs. Versatility and stability are key qualities of traditional SLBs, however a major drawback is the membrane substrate interaction which can hinder natural diffusion of the lipids and membrane proteins. Frictional interactions of the substrate can cause incorrect reconstitution of protein to the membrane and can also lead to nonspecific binding of the protein to the substrate which can cause the protein to become inactive or denatured.¹⁷⁶ With this in mind, a focus has been put on attempting to increase the distance between the bilayer and the substrate. Polymers have been added onto the substrate to act as spacers or cushions to minimise interactions of lipids with the substrate. Many materials have been used to as these cushions, for example Elender et al used Dextran, branched poly-glucosides, to functionalize a hydrophobic surface to support a dimyristoylphosphatidylcholine bilayer and was proven to be stable for at least 7 days.¹⁶⁵ Györvary et al reported with fluorescence recovery after photobleaching that a SOPC membrane exhibited a diffusion coefficient of $2 \mu\text{m}^2/\text{s}$ however

ATP synthase shown no sign of lateral diffusion.¹⁷⁷ This was attributed to the fact the polymer cushion increased the lipid diffusion, but the hydrophilic links were too short for membrane protein accommodation. Other issues reported for polymer cushioned SLBs were that proteins lost functionality which hinders their application for biomimetic studies.¹⁷⁸

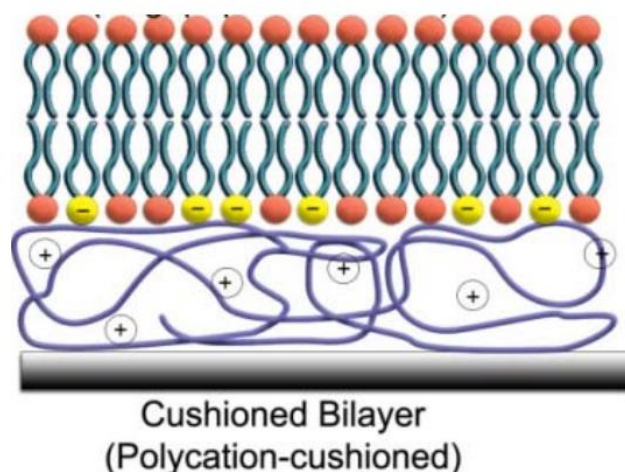


Figure 1.13: Polymer cushioned supported lipid bilayer. Reproduced from reference ¹⁷⁹.

Although polymer cushions have been successful in increasing the distance between the substrate and the bilayer, strong interactions between the cushion and bilayer have been shown to cause similar problems and diffusion rates were seen to be similar to an unmodified supported lipid bilayer array.¹⁶⁷ Another approach has been explored, whereby a tether is created between some lower leaflet lipids and the substrate. By modifying lipid groups with thiols, it allows for self-assembly of the lipids on gold surfaces, Figure 1.14, or by modification with silane groups, self-assembly on silicon is possible.^{180–182} This in turn increases the distance between the bilayer and the substrate and limits the amount of frictional interactions with the cushions used. However, this limits the number of substrates that can be used for the formation of the SLB. And, even with these modifications, studies have consistently shown that membrane proteins do not diffuse properly – anomalously slow diffusion of both lipid and protein, compared to liposome and low mobile fraction are widely reported in tSLBs and cushioned platforms. Budvytyte et al proposed that longer chain lengths for the tether causes a greater separation of substrate and lipid bilayer leading to greater fluidity. For HC18 a diffusivity value of $4.0 \mu\text{m}^2/\text{s}$ was obtained by FCS in comparison to a shorter tether resulting in membrane diffusion coefficients of $2 \mu\text{m}^2/\text{s}$.¹⁸³ Wagner and Tamm investigated the

peripheral proteins annexin V and cytochrome b and obtained diffusion coefficients of 0.3 and 1 $\mu\text{m}^2/\text{s}$ respectively, by utilising FRAP.¹⁷⁸

Polymer and tethered SLBs improve the diffusion coefficients of lipids when compared to SLBs on unmodified supports. However in comparison to free standing models, frictional interactions are still present with these models.

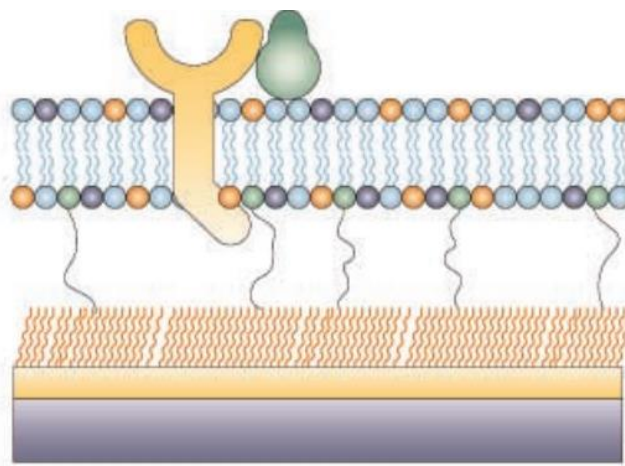


Figure 1.14: Supported lipid bilayer tethered to a gold substrate with different length thiol groups. Modified from reference¹⁷⁹.

In recent years, pore supported lipid bilayers have been introduced. The advantage is that they offer areas the freestanding bilayer where the membrane would not be in contact with the substrate and allow for unhindered diffusion and protein interactions to occur, Figure 1.15.^{15,184} Pore supported bilayers, otherwise known as free standing membranes, have been proposed to bridge the gap between supported lipid bilayers and BLMs.¹⁸⁵

Bilayers can be spanned directly over hydrophilic porous substrates due to the membrane support achieved by the interfacial water layer at the solid substrate point of contact. A range of different materials can be used to assemble such pore supported substrates for example porous silicon, alumina, silicon nitride or gold coated silicon nitride.¹⁸⁶ Some substrates can be functionalised with molecules to increase the hydrophilicity at the pore edges which improves the affinity to the membrane. Gold-thiol chemistry or silane chemistry have been shown in supporting the membrane of pore supported lipid bilayers.^{15,142,187,188} Steinem et al produced 50 – 60 nm porous surfaces by tempered aluminium foil at 500 °C for 3 hours, followed then by electropolishing in sulfuric acid. The polished foils then underwent

electrochemical etching and chemical etching to achieve the desired pore sizes. The porous substrate was then modified with gold and 3-mercaptopropionic acid to support the lipid bilayer.¹⁸⁹

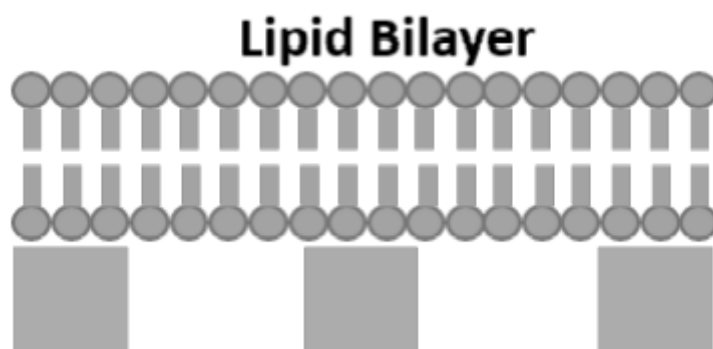


Figure 1.152: Representation of pore supported lipid bilayers.

Similarly to SLBs, pore suspended lipid bilayers can be obtained using different methods, including but not limited to, lipid transfer using Langmuir Blodgett, vesicle fusion or a combination of both methods. For the vesicle fusion, ensuring the liposome has a greater diameter than the aperture of the pore is required. This therefore limits the use of SUVs and LUVs as they might disrupt inside the pores rather than spanning the array. Different bilayers on different substrates spanned over cavities in the nano-dimensions by vesicle disruption and Langmuir-Blodgett techniques have been reported. Han et al has shown stability of up to 4 days on silicon nitride containing nanopores of a palmitoyl-oleoyl-phosphatidylcholine bilayer.^{185,190} However, in a lot of the cases reported, the bilayer formed is not continuous but rather bilayer patches are formed. Nussio et al reported using atomic force microscopy that lipid islands of DMPC and DPPC were formed over silicon nanopore substrates rather than a continuous bilayer.^{191,192} Jönsson et al have reported bilayers to form over 80 nm pores, however, the cavities were dry which limits the biological relevance of the system.¹⁹³ The pore sizes that have been reported in the literature are usually on the nanometre scale and due to diffraction limits of microscopy there are in reliably measuring such pores, further more difficulties in measuring diffusion and binding of proteins over the pore alone have been noted. Micron pore dimensions are more attractive as they enable single pore interrogation in microscopy.^{15,104,189,194} Heinemann and Schwille prepared a free standing membrane using fusion of GUVs

to a support with 2.5 μm pores. Using FCS they determined a lateral lipid diffusivity of around $10 \mu\text{m}^2/\text{s}$.¹⁴² Kocun et al used a similar method however using solvent free pores of a diameter of 1.2 μm and reported lipid diffusion measured by FRAP of $8 \mu\text{m}^2/\text{s}$. Similarly to Nussio's report, the GUV fusion membranes did not cover the full substrate but created patches of lipid bilayer.¹⁹⁵

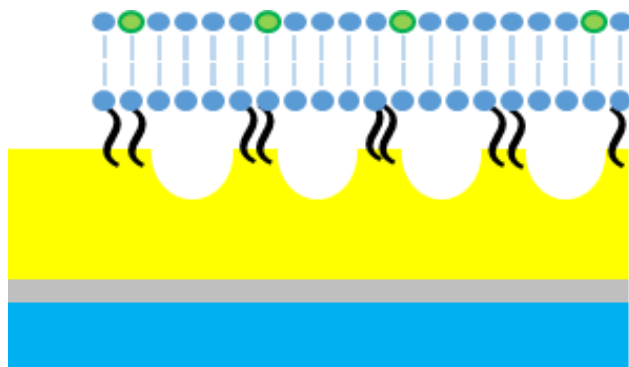


Figure 1.163: Representative Gold MSLB as pioneered by Keyes et al.

Keyes et al have pioneered microcavity supported lipid bilayer (MSLB) array platforms to study lipid membranes, and preparation is discussed in Chapter 2.^{104,194,196,197} MSLB have an advantage of eliminating the interaction of the bilayer with the underlying support whilst maintaining stability and robustness of the membrane. By utilising a combination of Langmuir Blodgett and vesicle fusion resulting in an aqueous interface for both leaflets, coverage of the porous substrate and incorporation of proteins have been achieved. Basit et al reported the incorporation of glycoporphin A and $\alpha_{II}\beta_3$ using the MSLBs. The reconstituted proteins had high mobility exhibiting diffusion coefficients of 4 and $3.2 \mu\text{m}^2/\text{s}$ respectively obtained by FLCS.¹⁹⁴ Furthermore, Keyes et al have developed the microcavity arrays utilising different materials such as PDMS and gold in which lipid membranes have been used to investigate protein activity, drug permeability and drug activity.^{196,198–200} The lipid diffusion values reported in the use of MSLBs is comparable to lipid molecules diffusivity in GUVs, $10 \mu\text{m}^2/\text{s}$.^{142,201} These values are 4 to 10 times higher than diffusion coefficients found in SLBs, showing the great advantage of the MSLB platform, and pore suspended membranes. Investigation into the interaction of antimicrobial vancomycin as a function of 5 different membrane compositions was completed. The change of resistance and capacitance values obtained using the MSLB

reflected the nature and extent of vancomycin interaction. A high affinity of vancomycin, $0.116/(\text{L}\mu\text{M}^{-1})^n$, was obtained for an E Coli membrane obtained from EIS.¹⁹⁸

In the work presented here, MSLBs have been utilised in order to study the effects of aggregation of proteins on the different membrane. The cavities are filled with aqueous solution prior to bilayer deposition, which in turn limits the issues of membrane-substrate interactions, increased lipid mobility and protein diffusion values.^{172,186,196,202} The lack of interactions between the membrane and substrate allows for a fluidic membrane. Asymmetry in the bilayer achieved through the layer by layer assembly method combined with the fluidity allows for a robust system to be studied, in the absence of harsh organic solvents.

Overall, artificial membranes represent a simplified matrix to biological membranes that can provide biophysical insight into lipid-lipid and lipid-protein interactions. Each system explained here has advantages and drawbacks.

1.3 Background to key techniques used in this thesis

1.3.1 Langmuir Blodgett

The Langmuir-Blodgett (LB) method uses the properties of both hydrophilic and hydrophobic components of the phospholipids, causing them to self-assemble as a monolayer at an air/water interface. In this work LB was used for monolayer formation throughout the MSLB preparation. In 1892 Agnes Pockles first proposed this mechanism, but it was further developed in 1917 by Irving Langmuir.²⁰³ Katherine Blodgett used the mechanism to transfer monolayers from a trough onto a surface.²⁰⁴ When the amphiphilic lipids are introduced to the surface of the water, the hydrophilic heads are spread across the interface while the hydrophobic tails are orientated away from the water surface. This monolayer formation is known as the Langmuir monolayer. As the monolayer accumulates, the surface tension of the water is lowered. Surface pressure (Π) is the measure used for surface tension, i.e. the difference between a monolayer present and no monolayer present. A Wilhelmy plate, filter paper or platinum, partially immersed into the subphase is used to measure the surface pressure and this is plotted against the surface area resulting in a pressure-area isothermic plot. The curve provides information such as stability and properties of the monolayer. Continuous cyclical compressions of the surface area covered by lipids at a constant temperature gives rise to the pressure-area curve. Initially uncompressed, the lipids deposited act as a two dimensional gas and the effect on the surface pressure is negligible. The number of lipids present remains unchanged throughout

compressions but the orientation of the hydrophobic tails changes causing a surface pressure change due to the Langmuir monolayer undergoing phase changes, gaseous (G), liquid-expanded (LE), liquid-condensed (LC), and solid (S) phases (Figure 1.17).

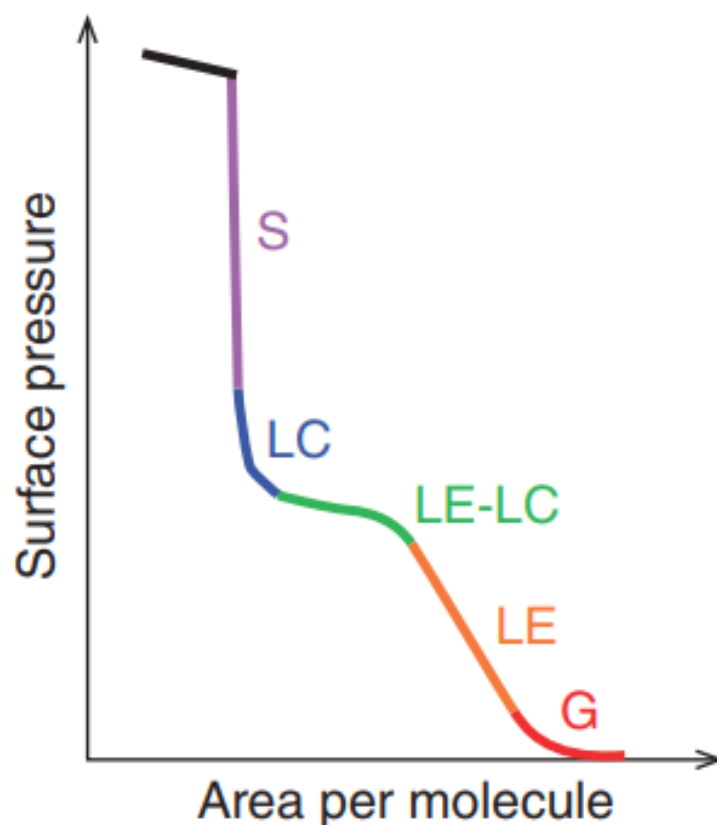


Figure 1.17: Representation of a Pressure area isotherm showing the different phases a Langmuir monolayer undergoes from gaseous to solid phases. Reproduced from reference ¹.

In the LE phase molecules begin to interact with one another causing a surface pressure increase. Upon further compressions, the LC phase is achieved as intramolecular interactions are stronger. Once the monolayer becomes compact and ordered it is said to be acting as a solid interface. If compressions are continued after reaching the solid phase, the surface pressure drops due to three dimensional structures forming on the trough. This is termed collapsing of the monolayer. ^{1,204}

Upon reaching the S phase, the floating lipid monolayer can be transferred to a solid support such as gold, PDMS, mica etc. The orientation of the monolayer depends on the wettability of

the substrate. In this work, the substrate is hydrophilic. The substrate is lowered to achieve the transfer with a dipping arm, controlled with computer software, at a perpendicular angle into the trough. The substrate is then pulled upwards out of the trough and in doing so the hydrophilic head groups of the lipids are transferred to the substrate, leaving a monolayer of lipids on the substrate of choice. The Langmuir-Blodgett technique is completed by transferring a second layer of lipids onto the monolayer by dipping the arm into the trough again to form a bilayer of lipids (Figure 1.18A). Vesicle Fusion (VF) is another way mono and bilayers on substrates are formed. Vesicle fusion is when a vesicle solution is put in contact with a substrate and the vesicles disrupt to form a monolayer. To form a bilayer a second vesicle solution can be put in contact with the monolayered substrate and upon disruption a second lipid layer is formed by spontaneous liposome disruption (Figure 1.18B). By combining the Langmuir-Blodgett technique and the vesicle fusion method a third variation of bilayer formation is achieved (Figure 1.18C). This process involves the transfer of the monolayer of lipids onto the substrate using the LB method. The monolayered substrate incubated with a vesicle solution forms a bilayer upon disruption of the liposomes.^{152,159,165} The LB-VF method is used throughout this work. This method allows for an accurate control of film thickness, composition, compactness and homogeneous deposition.¹

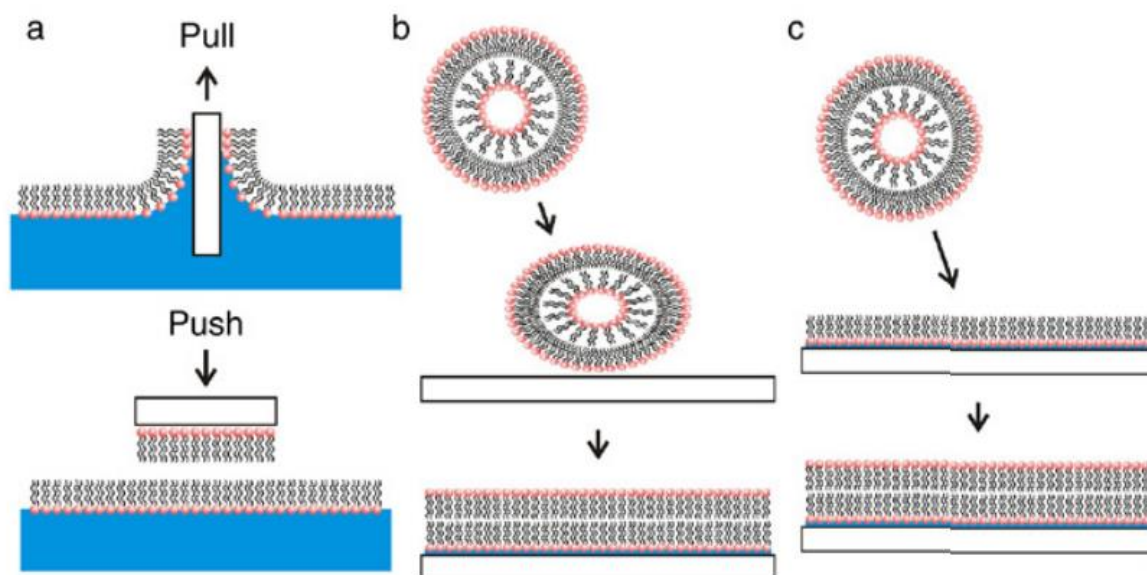


Figure 1.184: Schematics showing methods of forming supported lipid bilayers. a) The Langmuir-Blodgett/Langmuir- Schafer Method: deposition of an underlying lipid monolayer

on the substrate using the Langmuir-Blodgett method followed by the formation of a second layer by horizontally dipping the substrate. b) Vesicle fusion method: the addition of unilamellar vesicles to a hydrophilic substrate and their spontaneous formation of a lipid bilayer c) Langmuir-Blodgett/Vesicle Fusion method: deposition of an underlying lipid monolayer on the substrate using the Langmuir Blodgett method followed by the addition of unilamellar vesicles which fuse and form the upper leaflet. Reproduced from reference ¹⁵².

1.3.2 Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Lifetime Correlation Spectroscopy (FLCS) are two related techniques used to monitor dynamic processes and molecular interactions by measuring fluctuations of the emission intensity of a fluorophore in the interrogation (confocal) volume of the microscope. FCS is a single molecule method.^{48,158,205} The application of FCS to determine diffusion dynamics of molecules of interest by fluorescence fluctuations is of key interest in the context of this work. Fluorophores are excited by focusing an excitation laser onto a sample. A confocal pinhole blocks light emissions other than those originating from the thin focal plane, so that the emitted photons are filtered spatially before a suitable sensitive detector detects the readings. Suitable detectors for FCS can be SPAD or hybrid detectors (Figure 1.19A). By blocking around the pinhole, a well-defined femtolitre focal volume is obtained and only fluorophores within this are registered. A fluctuation time-intensity trace is obtained by carrying out this process over a time scale between 10 seconds and 1 minute. An auto-correlation curve can then be calculated using the fluorescence fluctuations and the following calculation (Figure 1.19B):

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F \rangle^2}$$

Where F is the fluorescence intensity, t is the time and τ is the lag time. The brackets “<” and “>” signify the averaged time over fluorescence signal, allowing for signal at time t to be compared with time t + τ , with the similarity averaged over all times. Short interval times mean that fluorophores within the detection volume have not moved far, and fluorescence intensity will not change hugely. For this reason, small lag times and a high value for the auto-correlation curve are obtained. Longer interval times mean the fluorophore has moved further leading to more significant changes in signal obtained and low value of auto-correlation curve as the decay tends to zero (Figure 1.19**Error! Reference source not found.**C). Slow diffusing

molecules will spend more time in the confocal volume and therefore the auto-correlation decay will be slower than with fast diffusing molecules.²⁰⁶

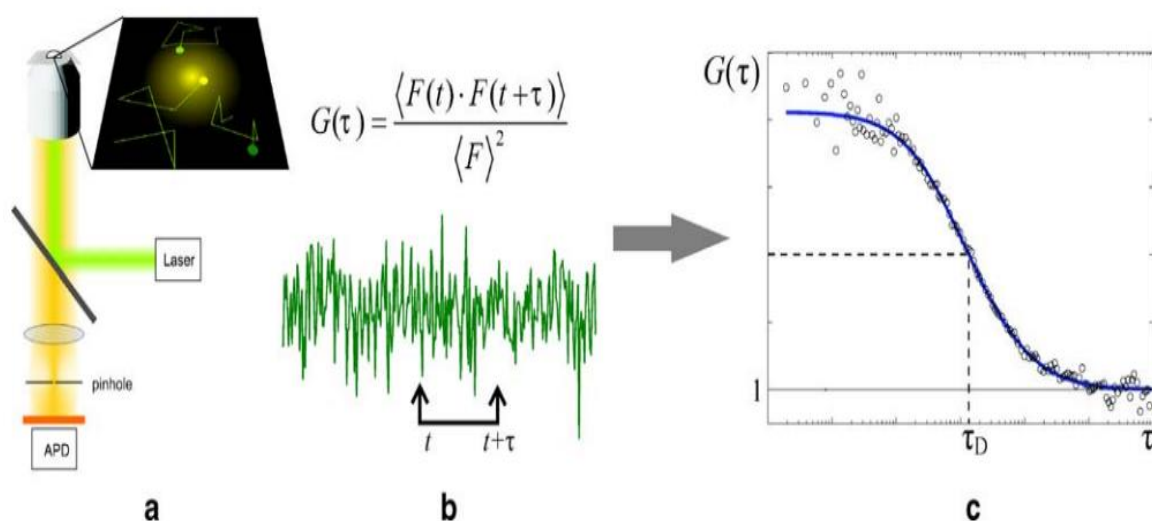


Figure 1.19 : The basic FCS set up. A) An excitation laser is focused onto a sample to excite fluorophores; a detector is then used to register emitted photons from the small focal area. B) Fluorescence fluctuations are measured over time and auto-correlation curve is formed. C) An auto-correlation curve. The data is fitted to a suitable model, i.e. 2D, in order to determine diffusion co-efficient of the fluorophore. Reproduced from reference²⁰⁶.

Concentration of the fluorophore has an important effect on the amplitude of the auto-correlation. Fluctuations in fluorescence at low concentrations have a large signal, thus, the amplitude of the auto-correlation curve is high. Likewise, the contrary is true, and such can be said that the fluorophore concentration is inversely proportional to the amplitude of the auto-correlation curve. By fitting the auto-correlation data to an equation suitable to the experimental parameters, diffusion co-efficient and concentrations can be determined.²⁰⁶ For a molecule diffusing freely in solution, a three dimensional fit model is then applied to the auto-correlation data. However, in a planar two dimensional system such as a lipid bilayer a two dimensional fit is the better option due to the spatial restrictions. In some cases where more than one fluorophore is present or where an intermediate non-fluorescent triplet state is obtained, additional parameters can be added to the equation.¹⁷² The time a fluorophore spends within the effective detection volume (V_{eff}), depends on the speed the molecule is diffusing at. A quickly diffusing molecule spends less time in the V_{eff} and results in the auto-correlation curve decaying to zero at a faster rate than a slowly diffusing molecule. The V_{eff} is also affected

by the dimensions of the observed area. V_{eff} does not match the confocal volume. Approximating the volumes using a 3D gaussian shape function, the V_{eff} is larger than the confocal volume. To obtain V_{eff} values FCS setups must be regularly calibrated in case of slight misalignments and changes in the optical system.²⁰⁷ A few options are available for calibration, for example, a freely diffusing dye of a known concentration, a freely diffusing dye with a known diffusion co-efficient or with imaging fluorescent beads of a known diameter. For the work in this thesis, the FCS set up calibration is obtained by a freely diffusing dye with a well-known diffusion co efficient.

Assuming that the confocal volume is approximated using a 3D gaussian function, dimensions can be obtained from fitting using:

$$G(\tau) = \frac{1}{N} \left[1 + \frac{4D\tau}{\omega^2} \right]^{-1} \left[1 + \frac{4D\tau}{z^2} \right]^{-1/2}$$

Where $G(\tau)$ is the auto-correlation function of fluorescence fluctuations, N is the average number of fluorophores in the focal volume, τ is the lag time, ω is the lateral radius of the confocal volume, z is the axial radius of the confocal volume and D is the diffusion co-efficient. K represents the axial ratio and the ratio between the z and ω axes. By a slight variation of the z component and using average retention time of the fluorophore, or diffusion time ($\tau_D = \omega^2/4D$), the above equation becomes:

$$G(\tau) = \frac{1}{N} \left[1 + \frac{\tau}{\tau_D} \right]^{-1} \left[1 + \frac{\tau}{k^2 \tau_D} \right]^{-1/2}$$

The experimental spatial values can be calculated by combining the above and below equations.

$$V_{\text{eff}} = (\pi)^{3/2} \omega^2 z = (\pi)^{3/2} \omega^3 k$$

However, in a 2D system, a separate 2D V_{eff} must be applied. In this case the effective value is the area of intersection between the confocal volume and the planar object. Experimentally this occurs at the widest point of the focal volume. Experimentally determining the lateral radius of the beam, the 2D V_{eff} is calculated by finding the area of a circle.^{207–209}

FCS is most commonly applied in literature to determine the diffusion co-efficient in both 3D and 2D systems, but the technique has not yet been perfected and still suffers from a number

of problems that can lead to artefacts in measurement. Conventional FCS experiences a number of unwanted signal components, and background contributions, which can appear in the auto-correlation curve. These can be caused by Rayleigh and Raman scattered excitation light, detector thermal noise and/or detector after pulsing.²¹⁰ In 2002, Jörg Enderlein introduced Time Resolved Fluorescence Correlation Spectroscopy or Fluorescence Lifetime Correlation Spectroscopy (FLCS) to combat these issues.²⁰⁹ FLCS is a modified version of FCS which incorporates Time-Correlated Single Photon Counting (TCSPC) to separate the auto-correlation function of various signal components. TCSPC is the process where a sample is excited using a short pulse of light and the temporal delay from the excitation pulse to the arrival of a photon that was emitted by the sample at the detector is recorded. This is repeated a number of times to obtain a statistically significant distribution of time differences.²¹¹ A histogram is used to represent the data obtained with time difference of the pulse and photon on the x axis and the frequency with each time difference on the y axis. This histogram then describes the decay of the excited state population after the pulse of light hits the sample, and by fitting this to an exponential decay function, the luminescent lifetime of the fluorescent probe can be obtained. The unwanted signals in FCS, the scattered light, noise, after pulsing, etc, occur on different timescales compared to the fluorophore and as such can be omitted from the autocorrelation curve and in turn improves the accuracy of the technique. By obtaining the fluorescent lifetime of the fluorophores, FLCS enables multichannel analysis, in which the two different fluorophores have sufficiently different lifetimes.

FCS and FLCS, within literature, have been studied for a number of applications in a range of environments. For example, to study the influence of lipid composition on lipid diffusion coefficients in liposomes¹³², to examine diffusion of protein interactions with lipid bilayers^{212,213}, and to determine the efficiency of cytosolic transport in cells.²¹⁴ Scherfeld et al investigated the effect of lipid domains in GUVs and found that lipid disordered membranes consisting of DOPC or DOPC/DSPC gave a diffusion coefficient of $6.5 \pm 0.4 \mu\text{m}^2/\text{s}$. Upon increasing the amount of cholesterol in the GUV the lipid diffusion coefficient decreased (to $1.7 \pm 0.2 \mu\text{m}^2/\text{s}$ at 50 mol% cholesterol) indicating that lipid ordered domains were forming and increasing membrane viscosity.¹³² LaRochelle et al used FCS to quantify the intracellular transport efficiency of cell penetrating peptides. They shown that molecules containing penta-arg motifs efficiently reach the cytosol whilst remaining intact. The high efficiencies of cellular transport correlated directly with results the obtained for cell based assays.²¹⁴ Berselli et al

investigated the binding of influenza receptor protein HA₁ to lipid bilayers containing different glycosphingolipids using microcavity arrays and FLCS. They reported that the HA₁ lateral diffusion coefficients varied depending on the glycolipid present in the membrane. For a DOPC/GD1a lipid bilayer, HA₁ was found to diffuse at 13 $\mu\text{m}^2/\text{s}$ compared to when GM1 or GM3 were present and HA₁ gave a diffusion coefficient of 5 $\mu\text{m}^2/\text{s}$, whilst all remaining at Brownian diffusion.²⁰¹

In this work, FCS and FLCS were used to determine lipid and protein diffusion coefficients in membrane aggregating protein systems. FCS and FLCS were chosen over Fluorescence Recover After Photobleaching (FRAP) due to the fact they are non-destructive to the fluorescent probe in the sample and can be used for to acquire additional data such as probe concentration and kinetic data. FRAP uses much larger volumes, typically μm^3 in its measurements making it more suited for slower moving molecules. FRAP also requires higher probe concentrations which may hinder the dynamics in the bilayer and have an impact on the protein binding.^{163,172} Guo et al reported a direct comparison of diffusion coefficients of NBD-PE in a POPC supported lipid bilayer using FRAP and FCS. The diffusion coefficients obtained for the tagged-PE were 3.1 $\mu\text{m}^2/\text{s}$ for FCS and 1.0 $\mu\text{m}^2/\text{s}$ for FRAP. The difference is was attributed to the larger region of interest in FRAP which could likely measure artifacts in the membrane or other defects which can lead to reduction in diffusion.²¹⁵

1.3.3 Electrochemical Impedance Spectroscopy

EIS is a widely used method applied to investigate the interfacial electrical properties of an electrode system. This technique is non-destructive with low amplitude sinusoidal perturbation of the systems function of frequency.^{216,217} Resistance is a measure of an electrical circuits ability to resist the flow of electrical current, defined by Ohm's law, $R = E/I$, where E is potential, and I is current. However, Ohm's law describes an ideal resistor, that exhibits resistance independent of applied alternating current (AC) frequency such that current and voltage pass through the resistor in phase. In more complex systems, such as supported lipid bilayers, this is not the case and a phase separation occurs between the applied AC voltage and current reflecting the impedance of the system.²¹⁸ Impedance is therefore, the measurement of the phase shift change in AC potential within the electrochemical cell. A small AC potential is applied to the system to give a pseudo linear response. A sinusoidal wave function is produced from the AC potential and if pseudo linear, the current response will also be sinusoidal at the same frequency, but phase shifted Φ .^{219–221}

Information from the electrode surface is provided from the frequency response. EIS measures the current response, where the impedance Z is governed by the applied voltage causing a perturbation of the sinusoidal wave of a given amplitude. The following equation gives the impedance of the system: $Z(t) = \frac{V(t)}{I(t)} = \frac{V_0 \sin(2\pi ft)}{I_0 \sin(2\pi ft + \phi)}$

Where V_0 and I_0 represent the maximum voltage and current signals applied to the system, f is the frequency of the sinusoidal wave, t is the time and ϕ is the phase shift between the current - time functions and the voltage - time.²¹⁶ Z can be considered as a complex value due to the fact that current can alter regardless of phase shift and/or amplitude, as opposed to the time – voltage function.

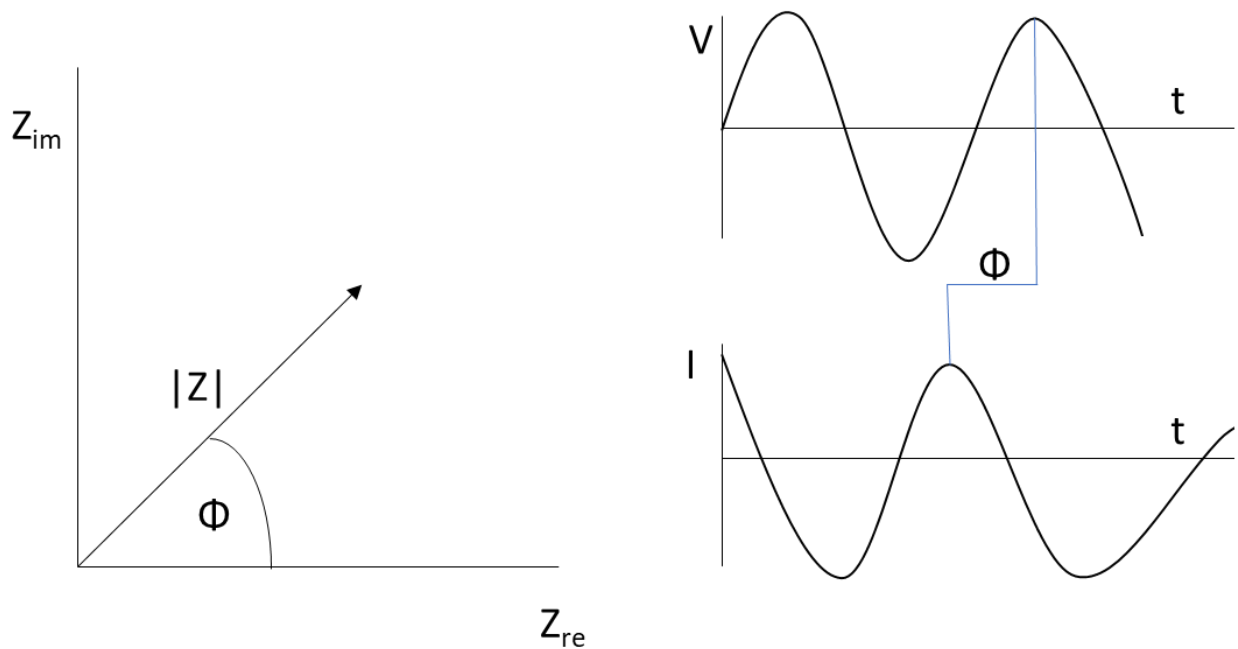


Figure 1.20: Figure representing the real and imaginary vectors of the impedance modulus where ϕ portrays the phase angle which is formed by the misalignment, or phase shift, of the V and I sinusoidal waves

Figure 1.20 shows the relationship between V , I , phase shift, impedance modulus which is the vector function of the real and the imaginary impedance. Data for impedance can be plotted in a number of formats but is most commonly analysed via Nyquist plots or Bode plots. Depending on the system of interest, each method has its advantages. Nyquist plots the real impedance (Z') against the imaginary data (Z''). Typically, a Nyquist plot is composed of a high frequency semi-circular segment relating to the electron transfer process and a low

frequency straight angled line representing the diffusion limited process shown in Figure 1.21 below.²²²

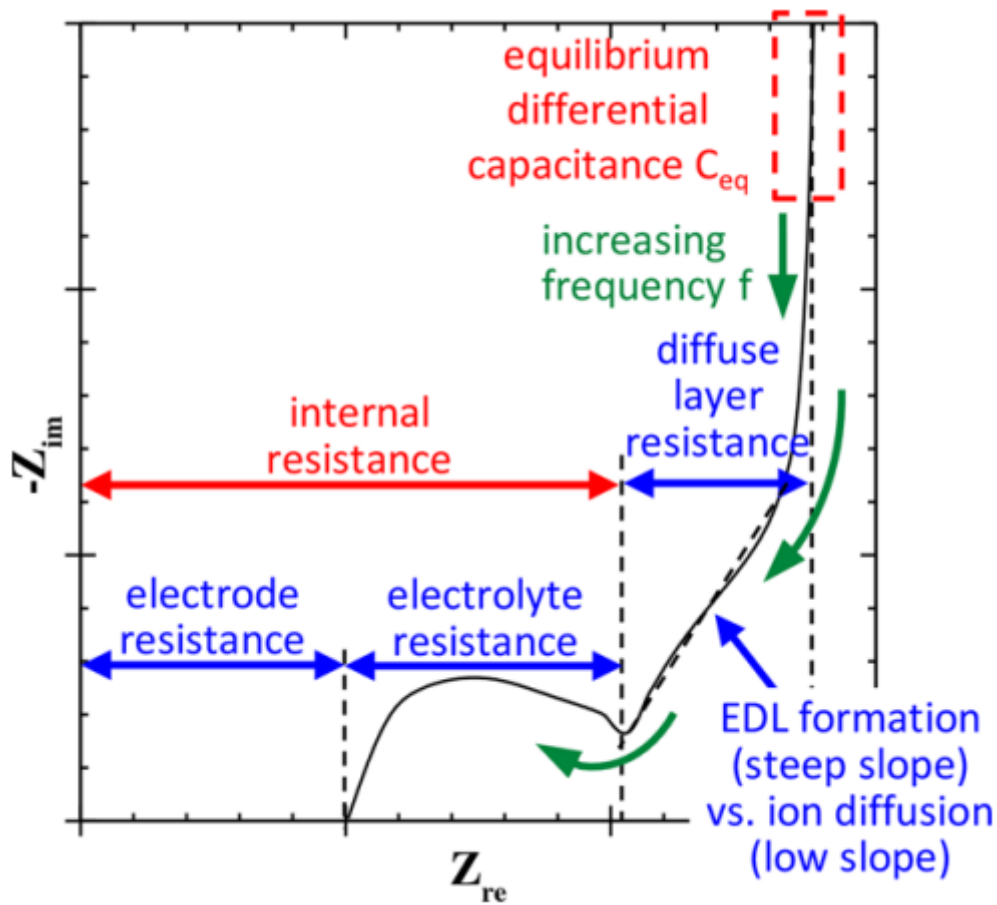


Figure 1.21: Typical Nyquist plot representation, showing the high frequency semi-circular region and the straight angled line region. Reproduced from reference²²³.

At a given frequency both real and imaginary components of impedance are represented within the point. The total impedance is given by the length of the vector from 0 to any given point, and the angle of the vector is the phase shift. Nyquist plots are ideal for studying lipid bilayers as the visual representation can give immediate insight to changes to the layer impedance, whilst resistance and capacitance information can be extracted to gain understanding of bilayer admittance and thickness. The lack of frequency data provided by Nyquist plots can be a disadvantage as EIS is the study of voltage response at different frequencies. Therefore, Bode plots can be used, by plotting the frequency in logarithmic scale against the phase shift.^{224,225}

Quantitative results can be obtained from EIS by fitting impedance data to an equivalent circuit model (ECM) by building circuits containing resistors and capacitors in series or in parallel.

ECMs are heuristic models of the impedance data that allow electrical and dielectric properties of the system to be extracted. Physical elements and electrical properties of the system of interest are important to understand in order to choose the correct ECM. The assignment of the components in the ECM is heuristic and makes assumptions however, in models like ours where the only parameter significantly changing in response to perturbation is the membrane impedance they are highly useful. A Faradaic process contains an electroactive compound such as ferricyanide. For EIS at a metal working electrode in a Faradaic system, the elements to be included in an ECM are known. A typical ECM for a faradaic process of lipid bilayers usually contain a double layer capacitance (C_{dl}), charge transfer resistance (R_{ct}), the ohmic resistance of the electrolyte (R_s) and the Warburg impedance (Z_w) and can be described further using a Randles circuit.^{217,219} In non-faradaic processes, where a redox probe is not present in the electrolyte solution, the impedimetric electrode surface and conductivity of the electrolyte are used. Since no redox probe is present in the electrolyte solution, a faradaic process is not occurring and the interfacial capacitance is usually observed.^{218,226} The capacitance value will change with the thickness of the double layer or when surface chemical reactions cause changes in the dielectric constant of the electrode. In some cases, particularly bioelectrochemical systems, an additional capacitor may be added in series or in parallel to the C_{dl} depending on the process of interest. The C_{dl} is seen to be the outer capacitance at the solid/liquid interface reliant on the ionic solution present. The added capacitor describes the electrode interface and any species adsorbed or attached on.²²⁷ Non-faradaic impedance has become a popular means of study of supported lipid bilayers due to the fact there is no probe required which may alter the membrane itself. A common ECM for supported lipid bilayers is shown below. It contains a solution resistor element (R_{sol}), a resistor and capacitor in parallel for the bilayer (R_{bl} , C_{bl}) and an electronic double layer capacitor for the electrode and solution interface (C_{dl}).^{202,228–}

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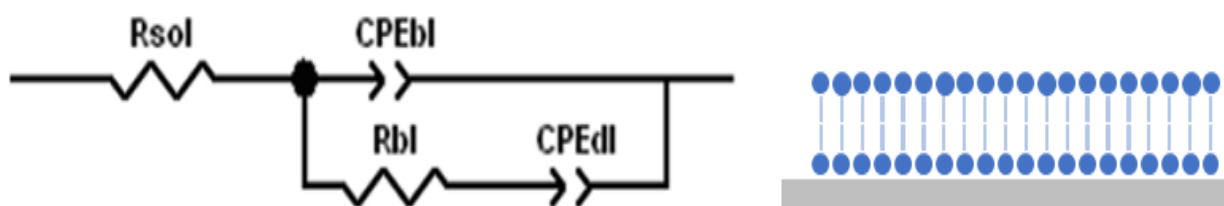


Figure 1.225: Typical equivalent circuit model (ECM) used for fitting of supported lipid bilayers in impedance data.

Steinem et al, have used impedance spectroscopy to analyse different preparation techniques for supported lipid bilayers and the properties associated with them. They reported that shorter phosphatidylcholine lipid chain lengths (C_{12}), micelles are likely to form leading to incomplete electrode coverage. They also reported that vesicle fusion onto 1,2-dimyristoyl-sn-glycero-3-phosphothioethanol (DMPTe) monolayers was in the range of hours however stability of the bilayer lasted for at least a day. When vesicle fusion was induced using calcium, the fusion process was faster however the resulting bilayer was only stable for 3 hours.²³¹ EIS has been used in particular for studying the effects of protein binding and interaction with a bilayer over various model membranes.^{68,232–235} Ren et al monitored polar lipid fraction extracted (PLFE) membranes suspended on thin PDMS films and found that the dielectric properties were distinctly different from diester and co-polymer planar membranes. Due to the tightly packed free standing PLFE membranes stability of the membrane was increased compared to POPC and POPC/POPS free standing membranes. The high resistance of PLFE membranes at a single electrical signal frequency obtain lead them to suggest that PLFE membranes are better for studying channel proteins and transmembrane events.²³⁴ Zhu et al reported the mechanical stability and lifetime for pore spanned nano DOPC BLM. Using EIS, they monitored pore diameters of 200, 400 and 700 nm. The membrane resistance for 700 nm pore size after 1 hour was found to be 51.3 G Ω on the BLM. Over time the resistance was observed to decrease to 0.8 G Ω after 36 hours. By decreasing the pore size to 200 nm the BLM's resistance was found to be 53.6 G Ω after 144 hours. They concluded that stability of the BLM was increased the smaller the pore sizes in the membrane.²³⁶

1.4 Conclusions and scope of this thesis

Cell membranes are organised, complex and dynamic assemblies that are challenging to study in the living system. This has led to the need for development of model lipid membranes that mirror the behaviour of membranes and more recently their integrated and associated proteins so that the key variables in the system are controlled. Research questions applied to model membrane models include lipid dynamics, investigation of drug membrane interactions, protein interactions, aggregation and diffusion and the symmetry effects of the membrane itself. The membrane model itself can have significant effect on the diffusivity of lipid membranes. SLB lipid diffusions were seen to be 2-fold slower than for free standing lipid

membrane models. Proteins are a challenge to study because in the most versatile model systems SLBs, the substrate can impeded diffusion and function. Many advances have been made in supported lipid bilayers in which membrane proteins can be incorporated, however, further developments are still needed to develop a stable, reproducible, and sophisticated mimic of the plasma membrane that can effectively model protein behaviour. This has led to the advancement of pore suspended lipid membranes, which incorporated the fluidity of free-standing models as well as the addressability of supported lipid membranes.

In this thesis, we demonstrate that lipid bilayers supported at water-water interfaces across micron dimensioned spherical cavity arrays offer a versatile, fluidic biophysical model that can be measured using different detection modalities to investigate both peripheral and integral protein aggregation and network formations. Specifically, by correlating electrochemical and fluorescence measurements we showed that membrane diffusion is comparably unimpeded by substrate interactions. This characteristic helps investigate processes that require lateral interactions at membrane interfaces such as protein aggregation and network formation.^{237–239} Furthermore, in preparation of the bilayer at the microcavity array a combination of Langmuir-Blodgett and vesicle disruption permits the assembly of asymmetric bilayers.^{104,240} This allows for targeted receptors for proteins to be investigated. The micron dimensions of the pores, stability and compositional versatility offer the opportunity of building biologically inspired interfaces that due to their inherent lateral mobility and stability of the transversal asymmetry can be switched. The main aims and objectives sought in this thesis were:

- To investigate the network formation abilities of AnV to zwitterionic and anionic lipids (Chapter 3).
- To investigate the flip flop of anionic DOPS from the interior leaflet to the exterior leaflet of the membrane to mimic the early stages of apoptosis (Chapter 3).
- To investigate the aggregation and oligomerisation capabilities of A β in the presence of anionic lipids (Chapter 4).
- To investigate the aggregation effect by the concentration of DOPS and concentration of A β (Chapter 4).
- To investigate the protein clustering effects of Galectin 3 in the absence and presence of integrin (Chapter 5).
- To investigate the protein clustering effect of Galectin 3 under different conformational states of integrin (Chapter 5).

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Chapter 2: Materials and Methods

2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), L- α -phosphatidylcholine [Egg, Chicken] (EggPC) and L- α -phosphatidic acid [Egg, Chicken] (EggPA) were purchased with maximum degree of purity (<99%) from Avanti Polar Lipids (Alabama, USA) and used without further purification. 1,2-dioleoyl-sn-glycero-3-phosphoethamine labelled Atto532 (DOPE-Atto532) & 1,2-dioleoyl-sn-glycero-3-phosphoethamine labelled Atto655 (DOPE-Atto655) was purchased from ATTO-TEC GmbH (Siegen, Germany). Phosphate saline buffer (PBS) tablets, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) salt, calcium chloride and sodium azide were purchased from Sigma-Aldrich (Wicklow, Ireland). Purified annexin V and labelled annexin V-Alexa647 were purchased from Biolegend UK Ltd (London, UK). Amyloid beta(1-42) was purchased from Biolegend UK Ltd (London, UK). Amyloid beta(1-42) HiLyte™ Fluor 555 was purchased from Eurogentec (Seraing, Belgium). Thioflavin T (ThT) was purchased from Sigma-Aldrich (Wicklow, Ireland). Gold wafers were purchased from Ams Biotechnology (Abingdon, UK). Gold wafers were purchased from Ams Biotechnology (Abingdon, UK). Polybead microspheres were purchased from Polysciences Europe GmbH (Baden-Württemberg, Germany). Aqueous solutions were prepared using MilliQ water (18m Ω cm) (Milipore Corp. Bedford, USA). Polydimethylsiloxane silicon elastomer (PDMS) was purchased from Dow Corning GmbH (Wiesbaden, Germany) and mixed following supplier's instructions. Electroplating gold solution Elevate gold 7990 RTU (8.2 g/L) was purchased from Technic Inc. USA.

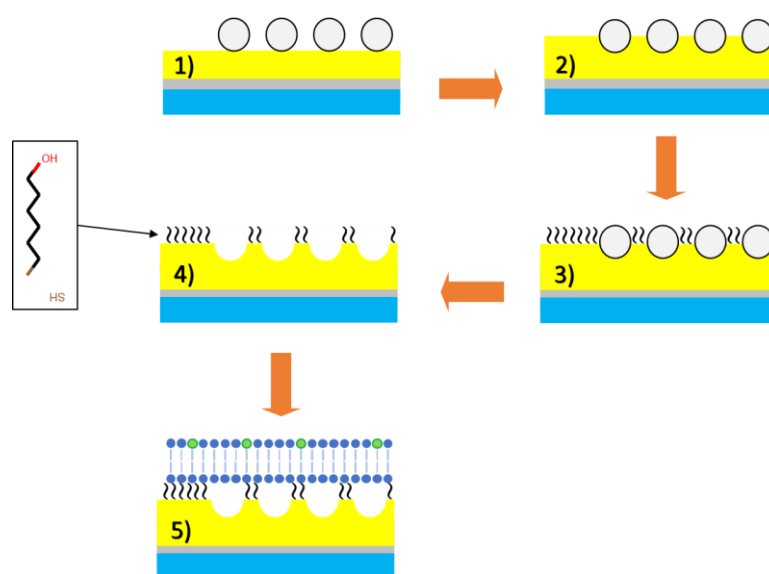
2.2 Instrumentation

A Hitachi 3400 Scanning Electron Microscope was used for imaging the MSLB array during the fabrication steps. Monolayer formation by Langmuir-Blodgett was performed using a NIMA 102D Langmuir-Blodgett trough. Electrochemical Impedance Spectroscopy was performed using a CH Instruments model 760E Electrochemical Workstation, using a three-electrode cell, Ag/AgCl reference electrode, platinum wire auxiliary electrode and the MSLB as the working

electrode. Equivalent circuit models were fit to raw data using Scribner Z-View software. A 405 nm laser, 3.12 mW, applied for 10s, was used to photodamage the bilayer. The diffusion coefficients of labelled DOPE (DOPE-Atto532/DOPE-Atto655) and labeled proteins were obtained using Fluorescence Lifetime Correlation Spectroscopy, FLCS, performed on a Microtime 200 system (Picoquant GmbH, Berlin, Germany) consisting of an inverted microscope model Olympus X1-71 with an Olympus UPlanSApo 60/1.2 water immersion objective. The fluorophores were excited with 532 nm and 640 nm PicoTA laser from Toptica (Picoquant).

2.3 Fabrication of Arrays

2.3.1 Gold Microcavity Arrays & Preparation



Scheme 2.1: Schematic representation of the stepwise fabrication of gold microcavity array and lipid bilayer formation over gold-MSLBs. 1) Dropcasting of polystyrene spheres. 2) Electrochemical deposition of gold to equator of sphere. 3) Functionalization of gold array with 6-mercapto-1-hexanol. 4) Removal of polystyrene spheres with THF. 5) Bilayer deposition using LB and Vesicle disruption on the MSLB.

Gold microcavity arrays were prepared following the method previously reported by Basit et al.¹ Gold wafer discs consisting of silicon wafer onto which a 100 nm thick layer of gold had been vapour deposited were cut into 1.5 cm × 0.8 cm chips and then air plasma treated for 5 minutes. The cleaned chips were placed onto a fabricated stage for this purpose. Polystyrene microspheres of 1 µm size (1 wt% v/v in milli-Q water) were drop cast onto the chips. A clean glass slide was placed over the sample causing a capillary effect. The polystyrene spheres were allowed to dry at 4°C in a refrigerator overnight, with a tilted front angle of 2° and a side angle of 1°. After polystyrene assembly and using the gold coated silicon as a working electrode, gold was then electrodeposited onto the wafer by holding the electrode at a potential of −0.6 V (vs Ag/AgCl) using a commercial gold electrodeposition solution which had been degassed for a minimum of 15 minutes. A platinum wire was used as the counter electrode. As slight variations might occur from batch to batch, best electrodeposition potential needs to be defined and was found here to be -0.6 V, until the metal had grown to the equator of the polystyrene spheres. The i-t curve was monitored and stopped when current reached the minimum corresponding to the equator level of the spheres monolayer, illustrated below. The resulting array was then rinsed with water to remove any remaining gold salt or electrolyte from the substrate surface.

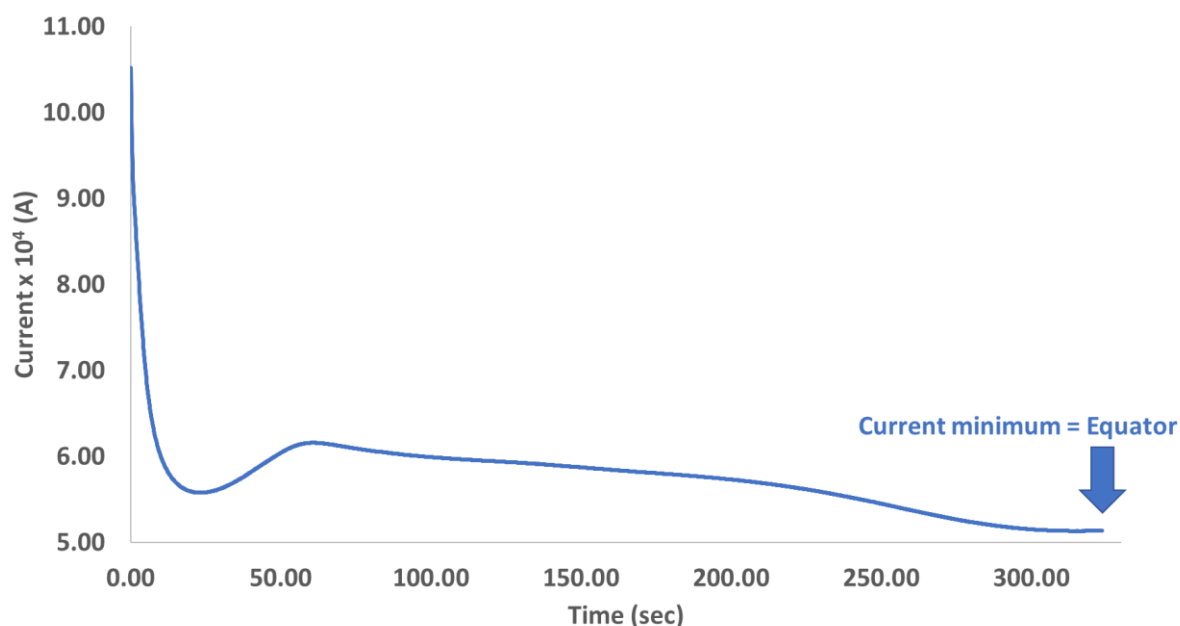
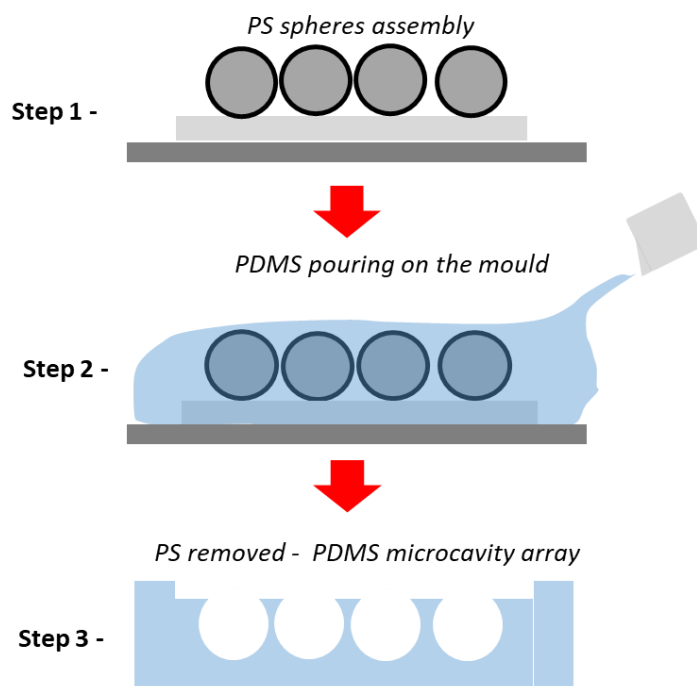


Figure 2.1: Representative I-t curve obtained during the electrodeposition of gold around the hexagonal close packed PS spheres monolayer assembled onto an Au/Si substrate, up to a depth of the equator of the spheres. ($E = -0.6$ V vs. Ag/AgCl) The electrochemical cell consists in the Au-Si wafer being the working electrode, an Ag/AgCl reference electrode and a platinum wire as counter electrode, linked to a potentiostat CH Instrument model 900a electrochemical station.

To selectively modify the top surface and leave the interior cavity surface bare, the arrays were placed in a solution of 10 mM 6-mercapto-1-hexanol in ethanol overnight while the templating spheres were left in the cavities. 6-mercapto-1-hexanol was chosen due to its ability to partially block the surface while promoting the formation of a lipid membrane due to its hydrophilic modification of the surface.^{2,3} To remove PS the substrates were sonicated in THF for 10 minutes, enough time to remove the PS spheres but not damage the self-assembled modified monolayer, and rinsed with ethanol. To pre-aqueous fill the cavities before lipid bilayer deposition, functionalized gold arrays were sonicated in HEPES buffer at pH 7.4⁴ for 30 min. Supported lipid bilayers were assembled across the microcavity array using a combination of the Langmuir-Blodgett (LB) and vesicle fusion methods as described below.⁵

2.3.2 Fabrication of PDMS Microcavity Arrays



Scheme 2.2: Stepwise representation of the procedure for the fabrication of microcavity array in PDMS substrate. Similarly, for gold electrodes preparation, in step-1 approximately 50 μL of 4.61 μm polystyrene spheres (0.1 wt% v/v in ethanol) was self-assembled on 1 cm x 1 cm fresh cleaved mica sheet. The PDMS mixture (10:1 ratio) is degassed in a vacuum chamber for 30 min and the bubble-free mixture is poured into a handmade aluminium mould to be cured at 90°C for 30 minutes. After curing, PS spheres were removed by sonicating the substrates in THF for 15 minutes and leftover for solvent evaporation in fume hood overnight.

FLCS measurements were carried out on polydimethylsiloxane (PDMS) arrays as previously reported, with slight protocol modifications.^{1,6,7} Briefly, 50 μL of polystyrene (PS) microspheres (0.1 wt% v/v in ethanol) of 4.61 μm diameter were drop cast onto mica (1cm²) the spheres were allowed to dry for 1 hour. PDMS was then poured over the spheres and mica, and then thermally cured at 90°C for 30 min to 1 hour. The cured PDMS was then removed from the mica template and soaked in THF to dissolve the polystyrene spheres for 30 minutes. The arrays were then left overnight to ensure all THF was evaporated off.⁸ Prior to lipid bilayer deposition, the array was air plasma cleaned and sonicated in 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) buffer (10 mM HEPES salt, 150 mM NaCl, 2 mM NaN₃) at pH 7.4⁴ for 30 min to pre-aqueous fill the cavities. The combination of the plasma treatment and the sonication was essential step to ensure that the majority of cavities buffer filled.

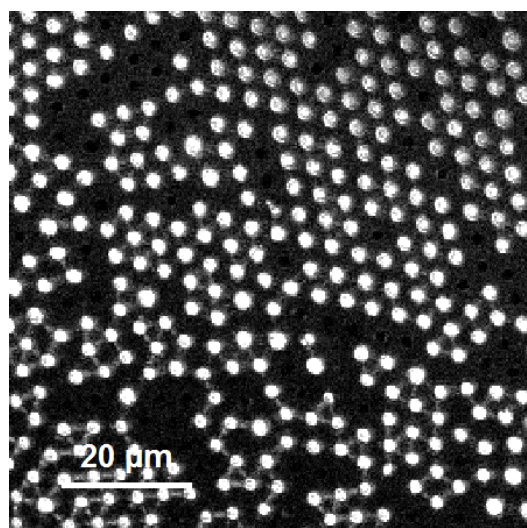


Figure 2.2: Reflectance image of PDMS cavity array following sonication with buffer. Due to the scattering of the laser light buffer filled cavities appear much brighter than the surrounding PDMS substrate.

Due to the refractive index difference of PDMS ($n=1.45$) and the buffer ($n=1.33$), and the way which incident laser light scatters at the site of cavities, reflectance images could be used to identify cavities. Filled vs unfilled cavities could be differentiated due to the degree of scattering in which buffer filled cavities were more intense than the unfilled cavities in reflectance images, as seen in Figure 2. Supported lipid bilayers were assembled across the microcavity array using a combination of the Langmuir-Blodgett (LB) and vesicle fusion methods as described below and previously done Keyes et al.⁵ Only buffer filled cavities were used when lipid membranes and proteins were studied.

2.4 Fabrication of Supported Lipid Bilayers on Microcavity Arrays

Microcavity Supported Lipid Bilayers (MSLBs) were then assembled across the microcavity array, both for gold and PDMS, using a combination of the Langmuir-Blodgett (LB) and vesicle fusion methods as described previously⁸. A 1 mg/mL lipid stock in chloroform solution was deposited onto the air-water interface of an NIMA 102D Langmuir-Blodgett trough. The chloroform was left to evaporate then the remaining lipids monolayer at the aqueous interface was compressed to a surface pressure of 32 mN/m. The MSLB was then immersed in the LB trough and withdrawn at a rate of 5 mm/s while a surface pressure of 32 mN/m was maintained

to form a monolayer of lipids. The array was removed from the trough, maintaining wet, and placed in a 0.5 mL solution of lipid small unilamellar vesicles prepared as reported before by the group⁹, for 1.5 hours to form the distal leaflet of symmetric or asymmetric bilayer. Vesicles were prepared in advance using stock solutions (50 mg/mL) of all vesicle components such as DOPC, DOPS prepared in chloroform and stored at -20 degrees. For fluorescence studies a mixture of unlabeled lipids and fluorescently labeled phospholipid DOPE-Atto532 or DOPE-Atto655 in a ratio of 50000:1 mol/mol were used. Electrochemical measurements were label-free and as such unlabeled lipids were used in preparation of vesicles. Aliquots of the appropriate amounts of stock solution were mixed in a clean glass vial and chloroform removed by a gentle stream of nitrogen forming a thin lipid layer on the glass vial. To ensure all chloroform was removed the lipid film was placed under vacuum for a minimum of 30 minutes. The lipids were rehydrated with 1 mL of buffer and vortexed for 60 seconds. A mini extruder (Avanti Polar Lipids) was used to extrude the multilamellar vesicle suspension through a polycarbonate membrane (100 nm pore size), to form monodisperse vesicles.

Where the proximal LB deposited leaflet had the same (symmetric) or different (asymmetric) composition to the vesicles, various lipid membranes could be formed for studying. Various bilayers are used in this work and we use the following short -hand to represent the compositions; Symmetric DOPC means both exterior and interior membrane leaflets are composed of DOPC, DOPC:DOPS(80:20)//DOPC meaning the exterior leaflet is composed of 80% DOPC and 20% DOPS while the proximal leaflet (closest to the substrate) is composed of just DOPC, and DOPC//DOPC:DOPS(80:20) meaning the exterior leaflet is composed of DOPC and the interior leaflet is 80% DOPC and 20% DOPS. The ratio of DOPC to DOPS changes in experiments and will be stated.

2.5 Scanning Electron Microscopy (SEM)

SEM images were collected on a Hitachi S3400 SEM. Samples were prepared by mounting upon a carbon backing on a metal stub. Gold microcavity arrays were imaged as made. PDMS samples were sputter coated with gold prior to imaging to make them conduct. All images were taken using an accelerating voltage in the range of 10 - 20 kV at a working distance of 10 mm with a probe current of 35 μ A. At least 3 areas of the array were examined to ensure a representative sample was observed.

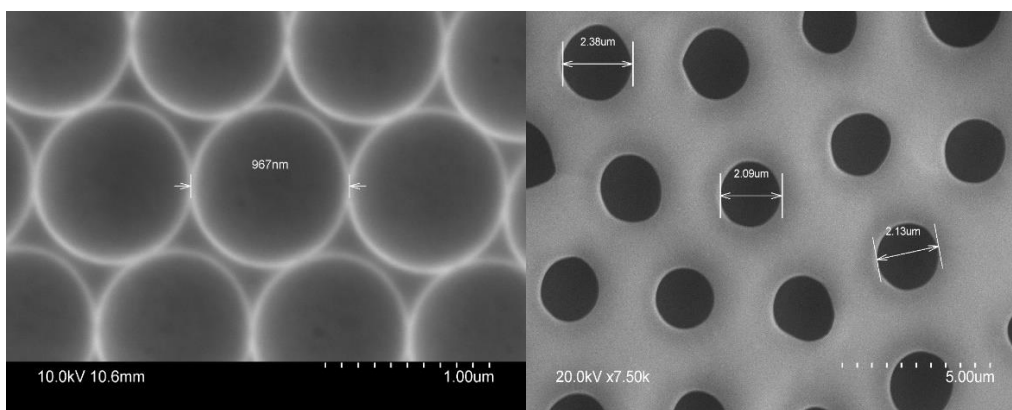


Figure 2.3: Right: SEM image of gold 1 micron microcavity array at 10 KV at a working distance of 10mm. The cavity arrays are seen to have a diameter close to 1 micron. Left: SEM image of PDMS 4.61 micron microcavity array after gold sputter coating at 20kV at a working distance of 10mm. The cavity is seen to be about 2 microns in diameter. Polystyrene spheres have been removed for both samples.

Microcavity arrays were characterised using SEM to ensure they were suitable for bilayer formation. The gold microcavity arrays form a uniform, closely packed hexagonal patterned upon formation. The electrodeposition to the current minimum leading to the equator means that the size of the PS spheres used is now the diameter of the cavity. Observed in the Figure 2.3 above, the gold cavity has a diameter of roughly 1 micron, the same sphere size that was used for drop casting. Growth was seen to be uniform and lead to a uniform formation with a consistent pore size, meaning the array batch was suitable for use. The PDMS arrays formed uniform and spherical cavities, however they are not as closely packed and form a more spontaneous pattern instead of hexagonal. Some of the differences in pore size, i.e. narrowing the pore aperture, on the PDMS array is attributed to the sputter coating which is required because the PDMS is insulating, prior to SEM analysis to ensure the sample was conductive and not holding charge from the electron beam of the system.

2.6 Fluorescence Lifetime Correlation Spectroscopy

Reflectance images were collected using an OD3 density filter for initial focus before FCLS measurements. The laser was directed onto a 532/640 rpc dichroic mirror and focused on the water immersion objective. Sample fluorescence was collected through an HQ550lp AHF/Chroma or HG670lp AHF/Chroma bandpass filter for the 532 or 640 nm laser, respectively. The sample fluorescence was then passed through a 50 μm pinhole, to eliminate

photons outside the confocal volume, onto a detection unit comprised of two single photon avalanche diodes (SPADs). The time dependent fluctuations of fluorescence intensity were recorded and analyzed by autocorrelation function (ACFs). The ACFs were fit using SymphoTime software (Picoquant GmbH, Berlin, Germany) to the following eqn (1) for a 2D model fit:

$$G(\tau) = \frac{1}{N(1-T)} \left[1 - T + T e^{\left(-\frac{\tau}{\tau_T}\right)} \right] \left[1 + \left(\frac{\tau}{\tau_D}\right)^\alpha \right]^{-1} \quad (1)$$

where $G(t)$ is the autocorrelation function of fluorescence fluctuations; N is the average number of diffusing fluorophores in the effective volume; T corresponds to the fraction of molecules in the triplet “dark” state; τ is the triplet state time; α is the anomalous parameter; τ_D is the diffusion time of the molecules and τ_T is the decay time for the triplet state. The anomalous parameter is related to the deviation of the diffusional regime from Brownian motion. i.e., the main square displacement does not relate linearly to time. The transit time (τ_D) is defined as the average time that a molecule requires to cross the confocal volume. The diffusion coefficient is related to the τ_D by the equation $D = \omega^2/4\tau_D$, where ω is the $1/e^2$ radius of the exciting laser beam. ω was determined by calibration using reference dyes. In the case of a second contributing diffusion component the 2D diffusion model could be adjusted to add a second component as shown in eqn 2:

$$G(\tau) = \frac{1}{N(1-T)} \left[1 - T + T e^{\left(-\frac{\tau}{\tau_T}\right)} \right] \left[1 + \left(\frac{\tau}{\tau_{D1}}\right)^{\alpha_1} \right]^{-1} \left[1 + \left(\frac{\tau}{\tau_{D2}}\right)^{\alpha_2} \right]^{-1} \quad (2)$$

Prior to performing FLCS measurements, fluorescent images of the MSLBs were obtained in order to locate and identify suitable cavities areas for measurement. Point FLCS measurements were then recorded for a duration of 30 seconds per cavity to generate an ACF trace and an average of 20 cavities were taking for every FCS data point. Each membrane and set of conditions were performed in a minimum triplicate, so that every recorded data point was collected from at least of three separately prepared MSLB. All measurements were recorded at room temperature 20 ± 0.4 °C.

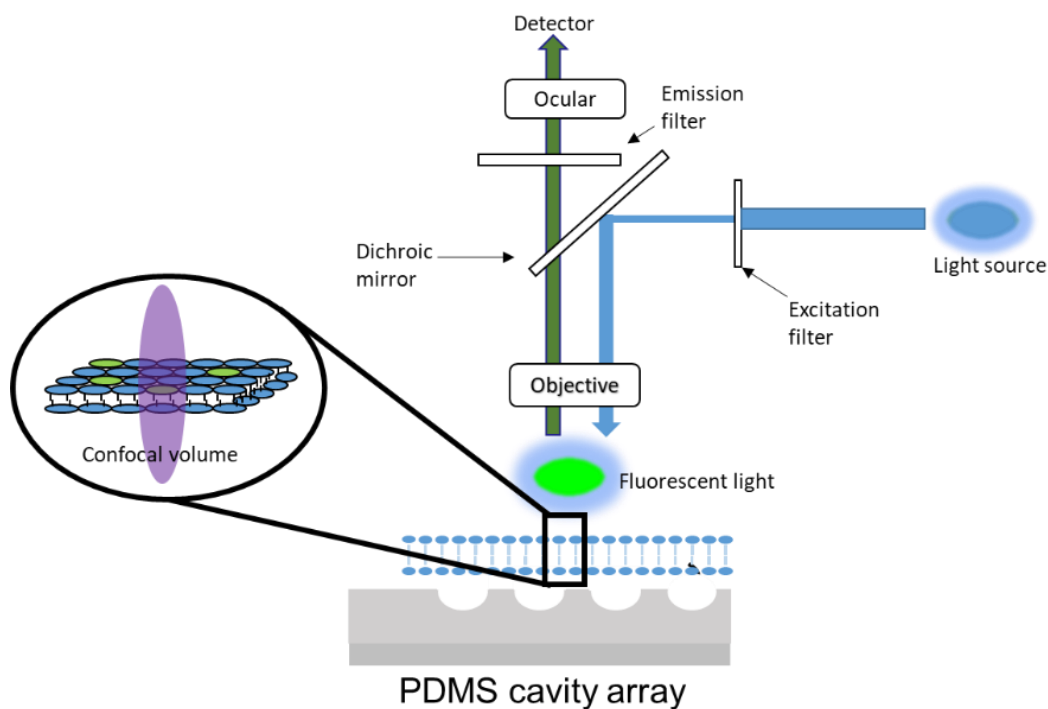


Figure 2.4: FLCS instrument set up showing the confocal volume seen on the lipid bilayer.

2.7 Electrochemical Impedance Spectroscopy

Electrochemical Impedance Spectroscopy was performed using a CH Instrument model 760E Electrochemical Workstation as potentiostat and a conventional three electrode cell (a platinum wire auxiliary electrode, an Ag/AgCl reference electrode and a gold MSLB as the working electrode). EIS was measured over a frequency range of 100,000 Hz to 0.01 Hz with an AC modulation amplitude of 0.01 V at a potential bias of 0 V (vs Ag/AgCl). All measurements were performed in a glass cell (approximate volume of 10 mL) in HEPES buffer at pH 7.4, unless stated otherwise. The gold MSLB with the desired bilayer was initially analyzed prior to the addition of protein or peptide to ensure signal stability. Proteins or peptides under investigation were then added into the glass cell with HEPES buffer at pH 7.4. The electrochemical impedance response of the bilayer and protein/peptide were measured every

30 minutes over 24 hours. Each measurement takes 10 minutes and was conducted at room temperature (20°C) unless stated otherwise.

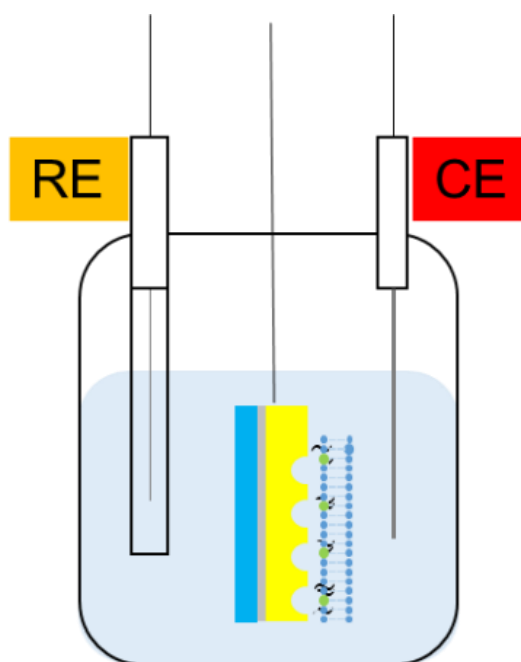
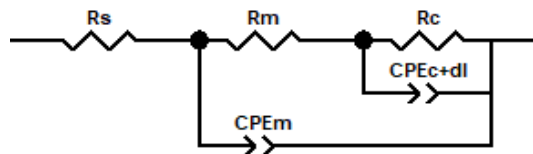


Figure 2.5: Electrochemical cell set up. Working Electrode is the MSLB, Reference electrode is Ag/AgCl, auxiliary is platinum wire. Within the cell is HEPES buffer solution at pH 7.4.

The EIS circuit model, shown in scheme 3 used to fit the measured AC impedance data of the microcavity supported lipid bilayers was performed on Z-View software to calculate the membrane resistivity and capacitance, as reported previously.^{9,10} In Scheme 2.3, R_s , R_m , R_c are resistance of the solution, membrane and cavity array respectively, CPE_{cdl} is the electrode double layer constant phase element and CPE_m is the constant phase element of the membrane. Constant phase elements were used over pure capacitors to account for defects in the MSLB and the bilayers. The circuit model provided an excellent fit of the measured EIS data over the full frequency range. The goodness of the fit from the ECM was accessed from visual inspection of the fits and also by χ^2 values which were around 0.8×10^{-3} . Upon fitting the impedance response, R_m and CPE_m were the only elements to undergo significant change upon addition with proteins or peptides indicating the model is valid. For all EIS measurements at

least 3 MSLBs were prepared per experimental conditions. The impedance values obtained were normalised and averaged between the replicates unless stated otherwise.



Scheme 2.3: The equivalent circuit model used to fit the AC impedance data of the MSLB. R_s , R_m and R_c are the resistance of the solution, membrane, and cavity array, respectively. CPE_m and CPE_{c+dl} are the constant phase element of the membrane and the electrode double layer constant phase element.

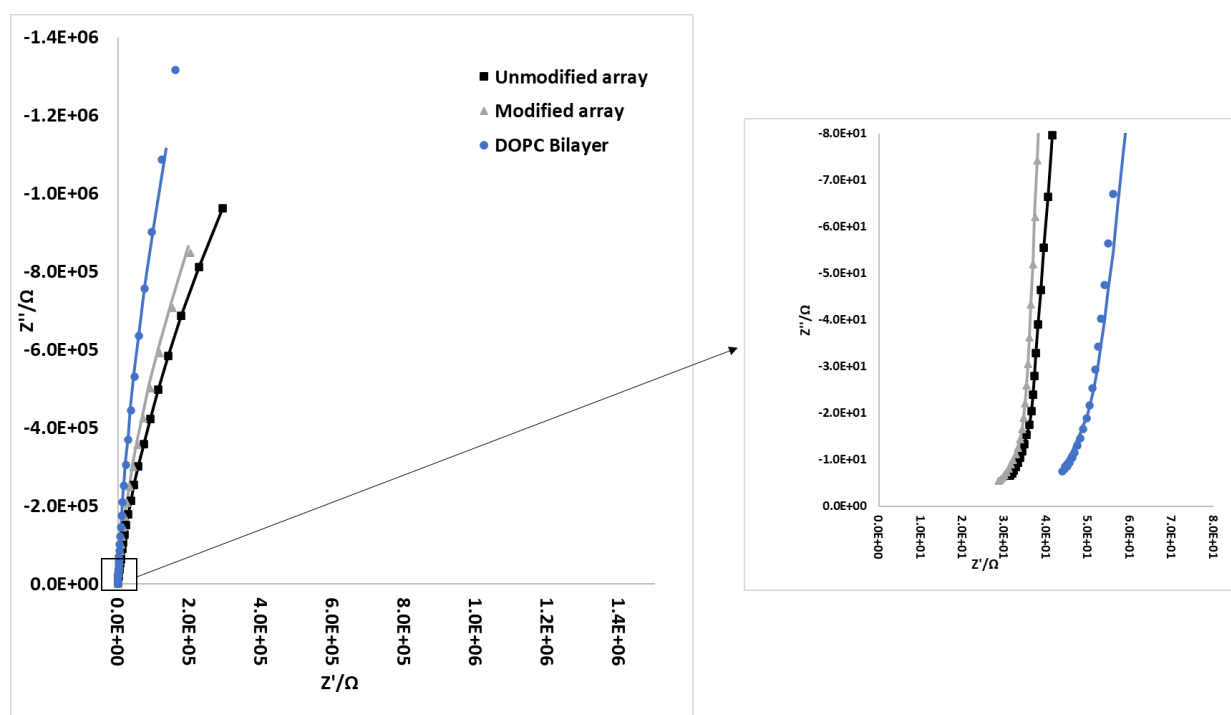


Figure 2.6: Representative Electrochemical Impedance Spectroscopy controls for the MSLB fabrication steps in HEPES buffer at pH 7.4 versus Ag/AgCl reference electrode at 0V. Coloured circles are the raw data obtained from impedance. Solid lines are the data fitted using the equivalent circuit model. Black data represents the bare microcavity array. Green data represents the microcavity array treated in 1-mercapto-6-hexanol. Blue data represents the microcavity array containing a DOPC symmetric bilayer. A zoom in of the high frequency region is included to enhance that the ECM fit is accurate for all frequency examined.

As a control, the EIS was obtained for bare cavities, following their SAM modification and then the final MSLB. Figure 6 shows a Nyquist plot in the conventional format representing the same dimensions on each axis, the microcavity array with the polystyrene spheres dissolved out had the smallest resistance, $4.7\text{ M}\Omega$ and the highest capacitance of $10.37\text{ }\mu\text{F}$. Upon soaking the array (still containing polystyrene spheres) in 6-mercaptohexanol overnight, a self-assembled monolayer formed on the topmost gold surface. The polystyrene spheres were then dissolved to leave the cavities bare and the flat surface areas covered in thiols. This causes the resistance of the array to increase, $5.3\text{ M}\Omega$, and causes the capacitance to decrease to $9.26\text{ }\mu\text{F}$. The lipid bilayer acts like parallel plates of capacitors, and the charge lines up on either side of the membrane. The decrease in capacitance can be attributed to the correlation between capacitance and thickness of a layer on the electrode, which follows the Helmholtz theory, that a membranes thickness is inversely proportional to the capacitance and directly proportional to the area.^{11–13} Since the area of the MSLB is constant throughout the experimental window, any change to the capacitance is due to membrane thickness. Assembly of the bilayer at the array via LB and vesicle disruption increases the resistance significantly, $27.2\text{ M}\Omega$, and this is accompanied by a large capacitance decrease to $6.73\text{ }\mu\text{F}$, which is to be expected.¹⁴

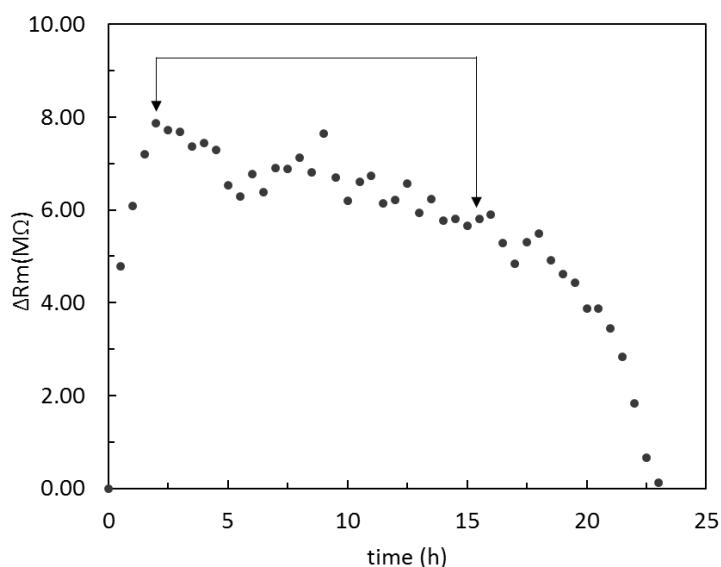


Figure 2.7: Relative resistance changes of DOPC symmetric lipid bilayer in HEPES buffer (pH 7.4). Black circles represent the relative resistivity change monitored by EIS for the full experimental run of 24 hours. The full time scaled graph represents the initial contact of the lipid bilayer with the electrochemical cell ($t = 0\text{ h}$), equilibrium ($t = 0.5$ to 2 h), stability of the

lipid membrane ($t = 2$ to 15 h, Indicated within arrows) and degradation of the bilayer ($t = 15.5$ - 24 h).

The lipid membrane stability was established using EIS over 24 hours. EIS is very sensitive to changes in permeability /admittance caused by alterations to the packing of the bilayer. As the arrays show some modest batch to batch variation due to cavity packing and thiol modification, the relative resistance change rather than the absolute impedance changes are reported throughout. The initial contact of the MSLB with the electrochemical cell was normalised to 0 and used to gather the relative resistance changes. Initially, during equilibrium, the resistance was seen to show an increase whilst stabilising until 1.5 – 2 hours. During this period, no experimental parameters were changed (i.e., no proteins/peptides would be added). Between 2 and 15 hours, the membrane is seen to be stable, and that resistance was only changing negligible, due to interactions with the buffer. After 15.5 to 18 hours, it was observed that the membrane was degrading. This was attributed to the natural degradation of the membrane on a gold platform. The DOPC membrane undergoes increased permeability due to looser lipid packing over time, leading to the greater decrease in resistance. As such, all bilayers for this thesis underwent stability experimentation to determine the experimental windows. Figures for EIS continuing will usually be presented containing mainly just the experimental window.

It is notable, however, for PDMS array, and consistent with previous reports (Gaul PhD and other papers) stability of a bilayer was found to persist for up to 5 days and was determined using FCS and other microscopy techniques.^{1,15,16} This may be attributed to the oxygen plasma treatment which renders the PDMS array superhydrophilic.¹⁶ It may also be the relatively greater % of top area thus hydrophilic. This level of hydrophilic is not easily achieved on gold and accounts for the difference in experimental windows. If evidence that the membrane was not stable or showed degradation, for either EIS or FCS techniques, the sample was discarded.

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Chapter 3: Annexin-V Drives the Stabilization of Damaged Asymmetric Phospholipid Bilayers

3.1 Introduction

Annexins are a family of soluble proteins that bind peripherally to plasma membranes through negatively charged phospholipids in a calcium regulated manner.¹⁻⁷ The crystal structure of annexin V⁸ shows a core domain, which is highly conserved in all annexins except annexin A6. The domain comprises four similar repeats, each being of 70 amino acid domains and containing a Ca^{2+} recognizing motif. In contrast, the N-terminal domain of the protein is highly variable across all annexins. This is thought to support a regulatory role annexins play in membrane organization, signaling and vesicle transport.⁸ This function is implicated in a number of normal and pathological processes including thrombosis and inflammation.^{6,9} In particular, annexin V appears to be critical in plasma membrane repair. While nano-dimensioned tears can be repaired by lipid refilling/diffusion into the damaged domain, plasma membrane repair for larger tears is a complex process.¹⁰ Typically triggered by calcium influx through the tear site, the process undergoes a number of routes for removing or reconstructing the damaged section of membrane,¹¹⁻¹³ in which annexin V is involved in stabilizing membrane tears.

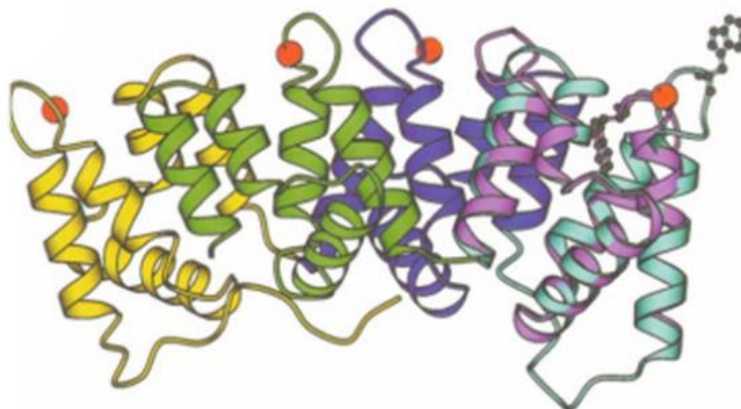


Figure 3.1: Structure of Annexin V composed of 4 repeating 70 amino acid unit domains. The orange circles are to represent the calcium binding²

Monomeric in solution, the protein self-assembles at the membrane interface to form a two-dimensional crystalline lattice network that extends over the lipid membrane. The assembly occurs in response to cell membrane damage that initiates the transversal movement of anionic

phospholipid phosphatidylserine (PS) to the extracellular leaflet of the membrane, i.e. migration of negatively charged phospholipids through the membranal tear to the extracellular leaflet of the membrane (Figure 3.2).^{14–17} Once bound, these monomeric AnV units then trimerize and the formed trimers cross-link into a crystalline layer running over the membrane surface.^{9,18,19} This relatively straightforward mechanism support repair in damaged membranes, which otherwise could lead to apoptosis.^{20,21}

Artificial biomembranes has been instrumental in understanding membrane -based processes without the complexity of live cells.^{22,23,24,25} Membrane assembly of annexin mediated by 1, 2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS)/Ca²⁺ was investigated in such model membranes, including liposomes and supported lipid bilayers (SLBs).^{26–31} For example, annexin V was shown to bind to target PS at the surfaces of unilamellar vesicles using techniques including quartz-crystal microbalance with dissipative monitoring or atomic force microscopy.^{5,32–35}

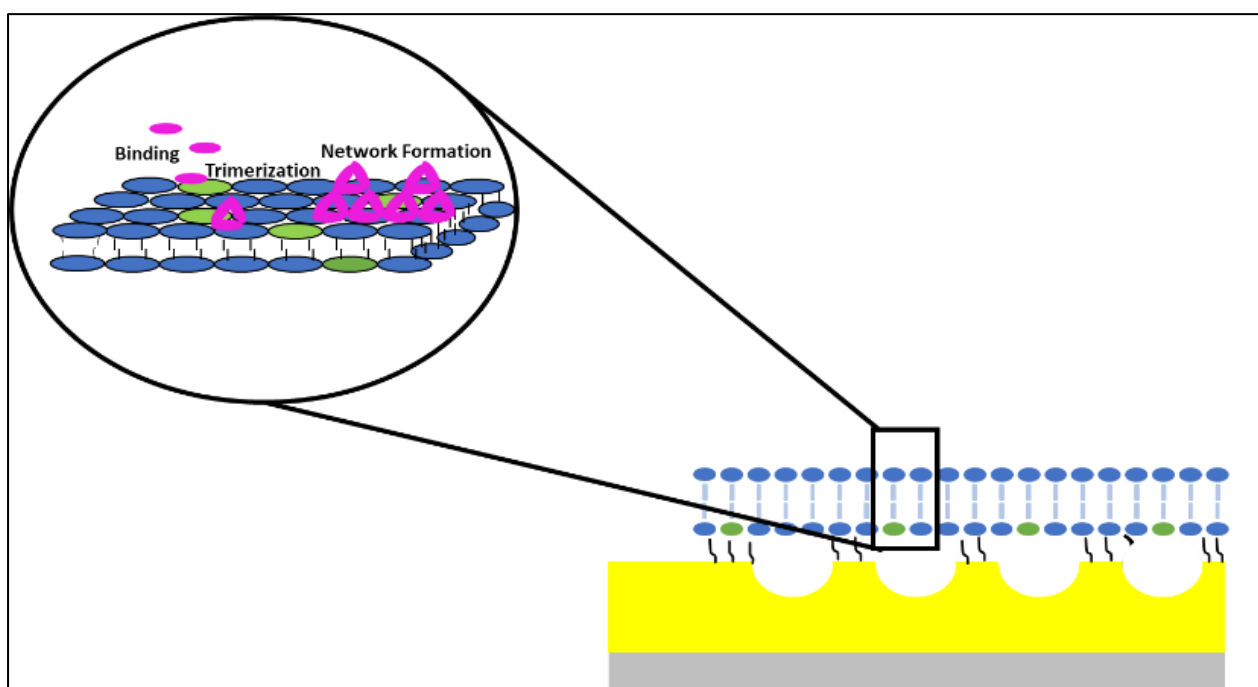


Figure 3.2: Schematic representation of annexin V forming a network over an asymmetric DOPC//DOPC/DOPS(80:20) phospholipid bilayer supported by a microcavity substrate, MSLB. Upon damage DOPS (green circles) migrates to the exterior of the membrane. Annexin

V monomers bind to DOPS in a calcium dependent manner, undergoes trimerization and then crystallizes to form a two-dimensional network.

However, the use of liposomes is associated with several challenges including poor stability and limited versatility in terms of asymmetric composition. While SLBs can address these issues, they can suffer from frictional substrate-membrane interactions that limit diffusivity of the bilayer or protein constituents. This limitation may prove to be important in studying processes that require lateral diffusivity of membrane components such as protein network formation.^{22,36} In part, this can be addressed by introducing a cushioning layer between the lower leaflet of the bilayer and the substrate. Double cushioned SLBs (protein and polymer layers) were shown to give a high degree of annexin mobility in bilayers³⁷. An alternative to ensure high fluidity in lipid bilayers is to use supported lipid membranes on pore or microcavity substrates with micron-sized pores³⁸ [Brisson et al]. This format allows to study annexin assembly on DOPC/DOPS bilayers at air/water interface using AFM and TEM.³⁹

In this chapter we focused on the aggregation of AnV on membranes and impact it has on the fluidity of microcavity-supported DOPC/DOPS bilayers. Electrochemical Impedance Spectroscopy (EIS) on gold microcavity arrays was used to study the effects of AnV network formation on the membrane admittance for symmetric bilayers without anionic phospholipids and asymmetric bilayers containing DOPS. Although electrochemical impedance spectroscopy has been used in biosensor applications using immobilized annexin V, this is one of the first reports of applying EIS to study the effects of AnV network formation at a lipid bilayer and the flip flop movement of DOPS. EIS is a particularly sensitive means of following changes to membrane permeability and thickness with both being affected by protein network formation at a bilayer.

The loss of asymmetry at the plasma membrane is a universal characteristic of early apoptosis. The migration of negatively charged lipids to the exterior leaflet of the membrane signals phagocytes for clearance of apoptotic cells and as such can lead to blood coagulation or facilitate tumour progression.⁴⁰ Damage or tearing of the plasma membrane can stimulate DOPS transversal migration leading to apoptosis whereas annexin V-DOPS recognition and annexin network formation, has been shown to provide a route to repair of membrane tears. MSLBs are an attractive route to mimic this process as they can be used to reliably form asymmetric lipid compositions and as described their high lateral mobility facilitates network

formation. Here we demonstrate that transversal lipid diffusion can be induced thermos-optically to mimic processes such as membrane damage and apoptosis and that this process stimulates annexin binding in the presence of Ca^{2+} at MSLBs.

Optically transparent PDMS microcavity arrays were exploited in the Fluorescence Lifetime Correlation Spectroscopy (FLCS) experiments to study the diffusion of fluorescently labelled AnV and evaluate the effect of AnV aggregation on the membrane fluidity in support of the electrochemical studies.^{41,42} By combining the two techniques, EIS and FLCS real time insights are gained of the dynamics of the annexin protein binding.⁴³ Moreover, we demonstrate the value of MSLBs in interrogating membrane protein network formation and demonstrate an approach to building a biologically inspired patch repair surface that may have broader device-based applications.

3.2 Materials and Methods

All samples were prepared according to the protocols described in Chapter 2, all experiments were performed at room temperature. All experiments were repeated a minimum of three times unless stated otherwise. For all experiments, after 1.5 hours vesicle fusion with the LB formed monolayer on the MSLB, the array was rinsed with buffer to remove excess vesicles. For EIS experiments the array was used as the working electrode in the electrochemical system. The MSLB was connected, and the membrane was allowed equilibrate and stabilise for 2 hours before the addition of protein to allow for the measurement to be made. All data shown in this chapter is a representative data set from the replicates of that experimental condition.

The following bilayer compositions were used;

An asymmetric DOPC:DOPS (80:20)//DOPC lipid bilayer where the distal leaflet is composed of 80 mol% DOPC and 20 mol% of DOPS while the proximal leaflet is composed of DOPC to represent an apoptotic cell, and DOPC//DOPC:DOPS(80:20) with distal leaflet comprised of DOPC and proximal leaflet of 80 mol% DOPC and 20 mol% of DOPS to represent a non-apoptotic cell. 1 mM calcium was used due to previous reports stating saturation of AnV/ Ca^{2+} occurs between 1 and 2 mM.⁴⁴ In this work annexin V was left to equilibrate with the lipid bilayers for 30 minutes prior to FLCS measurements. Other commercial apoptosis annexin assays require 10 minutes of incubation time to ensure binding and formation of a network therefore an incubation time of 30 minutes was used here and demonstrated to be adequate.⁴⁰

3.3 Results & Discussion

3.3.1 Fluorescence Correlation Spectroscopy on PDMS Array

Fluorescence correlation spectroscopy was used to characterize the binding and clustering of annexin V as well the MSLBs formation and lipid bilayer fluidity over PDMS microcavities arrays. Phosphate Buffered Saline (PBS) was used initially in preparation of the MSLBs, however, in the presence of Ca^{2+} the formed lipid bilayer was observed to be unstable and unreliable for the experimental window, both in FCS and EIS. This was attributed to the formation of calcium phosphate precipitation, which is normally observed in PBS buffer.^{45,46} As annexin-membrane binding is Ca^{2+} mediated, HEPES buffer was investigated, and found suitable for use in these experiments to avoid calcium phosphate precipitation. We previously reported that DOPC lipid bilayer supported across an aqueous filled PDMS cavity array exhibit a diffusion coefficient of approximately $10 \mu\text{m}^2/\text{s}$ in PBS buffer (pH 7.4).^{22,47–49} Interestingly, in contact with HEPES buffer we observed that the lateral diffusion of labelled DOPE-Atto532 in symmetric DOPC lipid bilayer was lower than in contact with PBS at $6.45 \pm 0.98 \mu\text{m}^2/\text{s}$. Similar reduced diffusivity has been noted for bilayer in contact with HEPES buffer at liposomes and was attributed to decreased van der Waals forces and increased electrostatic repulsion in the lipid-water interface as well as increased membrane rigidity caused by integration of the buffer between the lipids of the bilayer.⁵⁰ The anomalous coefficient (α) was unaffected by the buffer and remained at approximately 1.0 indicating Brownian diffusion. As noted previously for MSLBs, lipid diffusion at the microcavity array is comparable to values reported for GUVs of analogous compositions, but diffusion values are nearly twice those reported for bilayer diffusion at supported lipid bilayers (SLB) indicating good decoupling of the lipid from the underlying substrate.^{4,41,51,52}

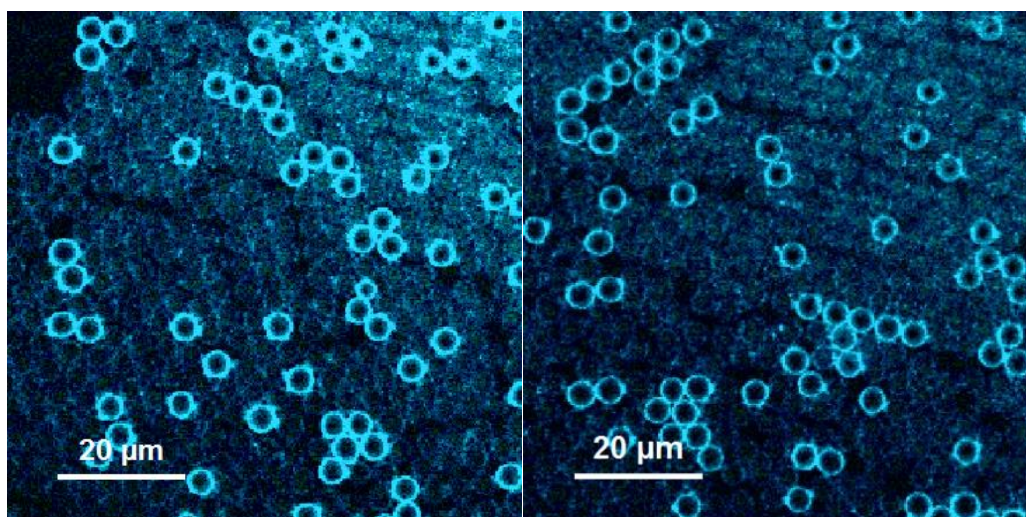


Figure 3.3: Left: DOPC symmetric bilayer with DOPE- Atto-532 labelled $\lambda_{\text{exc}} = 532 \text{ nm}$ in PBS buffer. Right: DOPC symmetric bilayer with DOPE- Atto-532 labelled $\lambda_{\text{exc}} = 532 \text{ nm}$ in HEPES

Initially, annexin V binding to negatively charged DOPS was studied in asymmetric lipid bilayers comprised of DOPC:DOPS(80:20) in the distal lipid leaflet. The proximal (substrate nearest) leaflet was comprised by DOPC only. The ratio of DOPC to DOPS of 4:1 mol/mol is consistent with previously reported studies on AnV lipid binding.^{20,40} Phosphoserine is predominately found on the cytoplasmic proximal leaflet of the plasma membrane as it forms a neutral to negative region of curvature and it's concentration within this leaflet can range from 8-20 mol%.^{53,54}

First, for a simple DOPC bilayer, a reflectance image of the array was taken to ensure that the cavities had been filled with buffer. As shown in Figure 3.4A, buffer filled cavities are easily distinguished from unfilled as the former appear as bright reflective circles and the latter appear as dark circles.

Figure 3.4B shows the fluorescent channel for the DOPE-Atto532 label in the DOPC bilayer, confirming the bilayers is in place (blue). Following 30 minutes incubation with either 1 nM, 10 nM or 100 nM Alexa647 labelled AnV in the presence of calcium (in apoptosis annexin assays 10 minute incubation is typically used so to ensure binding and formation of a network here an incubation time of 30 minutes was chosen.⁴⁰ and exciting at 640 nm, Figure 3.4C shows the second fluorescence channel, 647 nm, where no fluorescence is observed from the bilayer, indicating that under the conditions of Ca^{2+} at a DOPC bilayer there is no AnV membrane binding occurring.

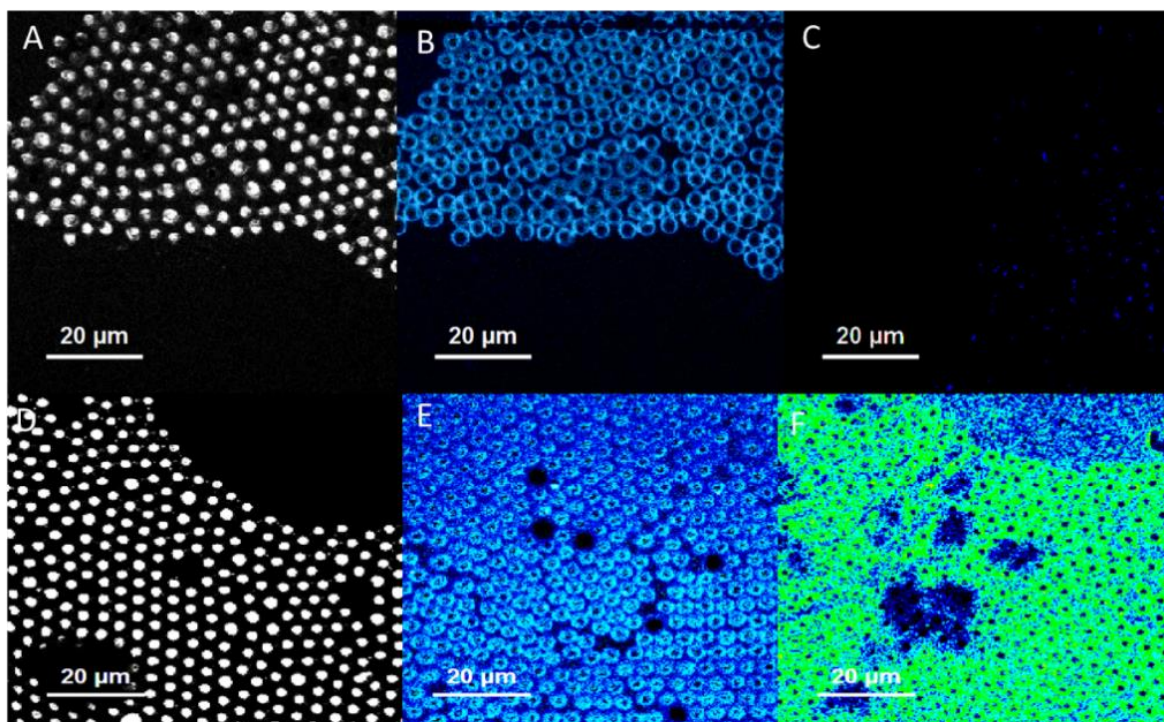


Figure 3.4: Confocal images of MSLBs spanned over PDMS microcavity arrays for symmetric DOPC lipid bilayer (A to C) and to asymmetric DOPC/DOPS (D to F). Figures A and D are reflectance images of 4.61 μm PDMS microcavity array obtained with an OD3 filter showing buffer filled microcavities are bright spots. B and E are fluorescence lifetime images of DOPE-Atto532 ($\lambda_{\text{exc}} = 532 \text{ nm}$). C and F are AnV-Alexa647 labelled ($\lambda_{\text{exc}} = 640 \text{ nm}$). In symmetric DOPC bilayers no fluorescence of labelled annexin was observed indicating no binding to the lipid membrane.

Figure 3.4D and 3.4E show the reflectance and fluorescence images respectively, for DOPE-Atto532 labelled DOPC/DOPS(80:20)//DOPC bilayer, confirming that the lipid bilayer was properly formed.⁵¹ In contrast to the symmetric DOPC bilayer, however, after incubation with AnV in the presence of calcium, intense and extensive fluorescence from the AnV-Alexa647 was observed from the bilayer at 640 nm excitation, indicating that AnV has associated with the bilayer. This confirmed that the protein binds to DOPS containing MSLBs in the presence of Ca^{2+} but not to DOPC alone and is consistent with data reported for other bilayer models and in cells. In the absence of Ca^{2+} no annexin was seen to bind.^{20,27}

FLCS was then used to obtain the diffusion coefficients for the asymmetric bilayer before and after exposure to annexin. Table shows that the asymmetric DOPC:DOPS//DOPC bilayer labelled with DOPE-Atto-532 exhibits a diffusion coefficient of $6.2 \pm 0.9 \mu\text{m}^2/\text{s}$ and α of 0.98

± 0.05 , consistent with Brownian diffusion and, within error, the same as the simple DOPC bilayer, $6.45 \pm 0.98 \mu\text{m}^2/\text{s}$.

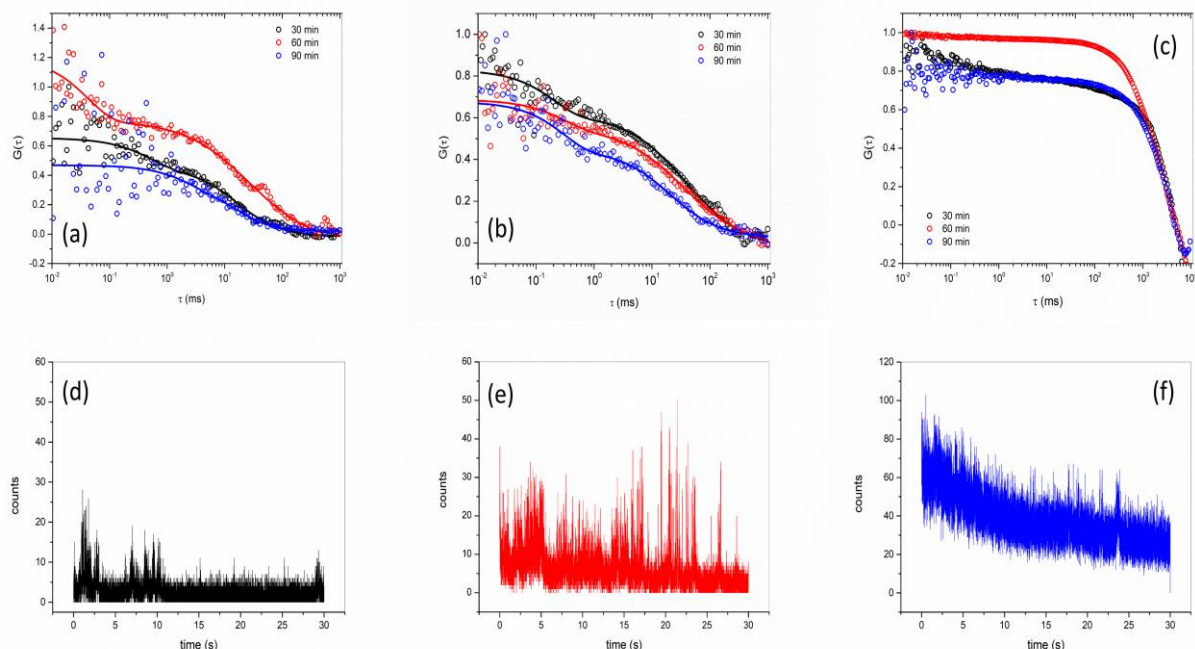


Figure 3.5: Representative FLCS results of bound AnV-Alexa647 to asymmetric DOPC:DOPS (80:20)//DOPC bilayer after 30, 60 and 90 minutes. (a) Fitted Autocorrelation Function (ACFs) of 1 nM Annexin-Alexa647, (b) 10 nM and (c) 100 nM. (d), (e) and (f) represents the fluorescence intensity of AnV-Alexa647 monitored over microcavities monitored for 30 seconds for 1 nM, 10 nM, and 100 nM, respectively.

Our studies herein, focus on 10 nM AnV, as this is a concentration, as reported previously, and shown below at which we expect aggregation without complete immobilization of AnV.^{4,55} We also evaluated AnV association at DOPC:DOPS (80:20) // DOPC at 1 nM and 100 nM of AnnexinV-Alexa647 to investigate concentration effects. The ACFs and intensity time traces obtained are shown in Figure 3.5. Following 30 mins incubation with 10nM, AnV in the presence of calcium, the diffusion coefficient of the DOPE-Atto-532 was reduced to $4.43 \pm 0.91 \mu\text{m}^2/\text{s}$. and α was recorded as 0.84 ± 0.12 . The reduction in diffusion coefficient is expected as similar effect has been reported (albeit much slower) for SLBs over similar AnV concentrations. The greater diffusion values observed here speak to the excellent fluidity of our system compared to SLB. That the anomalous factor, α , has decreased to below 1 is consistent with sub-diffusion indicating hindered labelled lipid diffusion typically it is attributed to

diffusion through a crowded environment and here is attributed to the formation of the AnV network. The diffusion coefficient of AnV, the monomer in free solution, was measured as $34 \mu\text{m}^2/\text{s}$, which corresponds with an earlier reported value.⁵¹ On incubation with the DOPC:DOPS//DOPC bilayer the diffusion of AnV-Alexa-647 was measured as $1.40 \pm 0.4 \mu\text{m}^2/\text{s}$, indicating that binding to the membrane had occurred, Figure 3.5B. Monomeric protein units that are bound peripherally through lipid usually exhibit a diffusion coefficient similar to the lipid diffusion coefficient.⁵⁶ In this case AnV's diffusion coefficient is 3-fold lower than the initial DOPE diffusion coefficient, which again we attribute to commencement of network formation rather than diffusion of single AnV-DOPS complexes. This is supported both by the anomalous diffusion behavior of the underlying lipid membrane, described *vide supra* and also by the α value for AnV which was recorded as 0.9 ± 0.1 , also indicating sub-diffusion.

These results are also consistent with the fluorescence images shown in Figure 3.4F that also indicated extensive AnV binding is occurring at the membrane attributed to a network of AnV forming across the bilayer at the AnV and Ca^{2+} concentration used.^{17,32,57,58} In Figure 3.5e, the intensity time trace of 10 nM AnV is indicative of aggregation of the protein. The time trace displays fluorescence fluctuations over time during the point measurements and therefore can be used to visualise the movement of the fluorescently labelled AnV molecules passing through the focal volume. At 10 nM AnV high frequency clusters spikes are observed in the trace and indicates that groups of labelled proteins are diffusing together through the confocal volume. At 10 nM AnV evidence of the start of photobleaching is observed in the fluorescence intensity trace by the slight decrease in intensity over time. This further confirms that a network is beginning to form. Notably, the AnV ACFs, Figure 3.5, fit well to single component models this suggests that extent of oligomerization was fairly uniform across the membrane at this AnV concentration. All ACF traces are representative time traces of a single cavity point measurement which is then fit with the software to gain diffusion coefficient and alpha values. The alpha value corresponds to the mean-squared displacements of the lipid scale as a power law of time with an exponent α of 1 indicating that the lipid is undergoing normal Brownian diffusion. 20 cavities ACF traces are all fit and values calculated and averaged to give an average diffusion coefficient. All ACFs are normalised with the Picoquant software and $G(\tau)$ is between 0 and 1. For Figure 3.5a at 60 minutes (red line), the ACF plotted was not normalised for comparison and as such the $G(\tau) > 1$. Concentration of the fluorophore has an important effect on the amplitude of the ACF. Fluctuations in fluorescence at low concentrations have a large signal, thus, the amplitude of the ACF curve is high. Likewise, the contrary is true, and

such can be said that the fluorophore concentration is inversely proportional to the amplitude of the ACF curve. The time a fluorophore spends within the effective detection volume depends on the speed the molecule is diffusing at. A quickly diffusing molecule spends less time in the detection volume and results in the ACF curve decaying to zero at a faster rate than a slowly diffusing molecule. For the earlier part of the AnV Alexa 647 ACFs, an artifact of the dye can be observed as a fast decaying portion. Visual examination of the fit, the residuals and the quantitative goodness of fit, the R^2 (approximately 0.99) value were all used to determine the quality of fit of data to the model for all ACFs.”

To confirm initial network formation is occurring at 10nM we evaluated AnV diffusion at DOPC:DOPS (80:20) // DOPC at an order of magnitude lower and higher concentration of the protein; 1 nM AnV and 100 nM respectively. At 1 nM AnV was also observed to bind to the membrane but diffused with a slightly higher D at $1.56 \pm 0.18 \mu\text{m}^2/\text{s}$, and binding was complete at 30 mins. However, notably at this concentration, the DOPE diffusion coefficient did not decrease significantly and the anomolous factor remained approximaely 1, Table 1.

Table 3.1: Table showing 1nM AnV with DOPC:DOPS(80:20)//DOPC asymmetric bilayer in HEPES buffer pH 7.4

Time of AnV incubation	Diffusion ($\mu\text{m}^2/\text{s}$); DOPE-532	alpha; DOPE-532	Diffusion ($\mu\text{m}^2/\text{s}$); AnV-647	alpha; AnV-647
Pre incubation	6.26 ± 0.46	0.98 ± 0.05	-	-
After 30 minutes	6.08 ± 0.35	0.97 ± 0.05	1.57 ± 0.20	1.03 ± 0.18
After 60 minutes	6.04 ± 0.41	0.96 ± 0.05	1.52 ± 0.21	0.97 ± 0.08
After 90 minutes	6.00 ± 0.54	0.97 ± 0.05	1.56 ± 0.18	0.97 ± 0.41

This suggested that at 1 nM, AnV binds to DOPS however although there is some aggregation there is no network formation, as reflected in the time trace (Figure 3.5d). The time trace at 1

nM AnV shows a less frequent and less intense cluster spikes when compared to the time trace at the 10 nM concentraion, indicating that less aggregation is occurring. At the higher concentration of Annexin V (100 nM) extensive binding of the protein was observed but it was found the time-scale of the FLCS experiment to be immobile, wherein significant photobleaching prevented reliable acquisition of FLCS data seen in the ACF in Figure 3.5C. This suggest an extensive aggregation and network formation of Annexin V at 100nM. The downward trend of fluorescence intensity over time observed in Figure 3.5f is indictive of photobleaching. The intensity of the fluorescene probe is diminishing and weakening due to the protein not diffusing out of the confocal volume. The photobleaching obtained from the ACF and the time trace are all consistent with an immobile network at 100nM. AnV-DOPS binding was observed but with limited aggregation was at 1 nM AnV thus the concentration of 10 nM was used for the study of flip flop of DOPS as it showed evidence of initial network formation but FLCS is still accessible.

Table 3.2: Diffusion values for a symmetric DOPC and an asymmetric DOPC:DOPS(80:20)//DOPC bilayer with 10 nM AnV and 1 mM CaCl₂ in HEPES buffer at pH 7.4

Lipid bilayer composition	Labelled marker	Diffusion Coefficient ($\mu\text{m}^2/\text{s}$)	Anomalous coefficient, α
Sym DOPC//DOPC	DOPE-Atto532	6.45 ± 0.98	0.99 ± 0.02
Asy DOPC:DOPS//DOPC	DOPE-Atto532	6.20 ± 0.90	0.98 ± 0.05
Asy DOPC:DOPS//DOPC	AnV-Alexa647	1.40 ± 0.40	0.90 ± 0.10
Asy DOPC:DOPS//DOPC + AnV	DOPE-Atto532	4.43 ± 0.91	0.84 ± 0.12

Fluorescence Correlation Spectroscopy on PDMS Array for an Asymmetric Bilayer with DOPS at the proximal leaflet only.

Since in the mammalian plasma membrane phosphatidylserine containing lipids are predominantly isolated to cytoplasmic leaflet, we assembled asymmetric lipid bilayers in which DOPS is similarly confined to the proximal leaflet at the array; DOPC//DOPC:DOPS(80:20).

We then incubated this MSLB (labelled with DOPE-Atto532) with 10 nM AnV in the presence of Ca^{2+} and monitored the diffusion coefficient of the DOPE-Atto532 periodically over 24 hours.

Table 3.3 shows the diffusion coefficient of DOPE-Atto532 (which is at the outer leaflet) and labelled AnV which was maintained in contact with solution at the exterior interface bilayer only, over time. Fluorescence imaging confirmed that over the first 19 hours, there is no evidence of AnV association with the DOPC//DOPC:DOPS(80:20) membrane confirming the isolation of DOPS to the lower leaflet. Typically, transversal diffusion of a lipid is challenging to measure as it is often accomplished by monitoring the flip-flop of a labelled lipid whose transversal diffusion will be influenced by the label. By using annexin binding to monitor DOPS arrival at the external leaflet we can monitor transversal diffusion of the unlabeled lipid directly. The absence of Annexin assembly at the bilayer when it is assembled with DOPS at the lower leaflet indicates that the lipid asymmetry is preserved over extended windows at the MSLB.

Table 3.3 shows, fluorescent signal at the bilayer for AnV could not be observed until after 21 hours incubation. We observed previously in uncharged lipid compositions that asymmetry is preserved for approximately 5 hours but here DOPS transversal diffusion from the interior leaflet to the exterior leaflet is likely slowed in the case of DOPS by the anionic charge on the lipid that inhibits movement through hydrophobic core of the membrane.

Indeed, degradation of film packing may contribute to the flip flop over this time scale. Notably, the diffusion coefficient of AnV at 21 hrs, at $1.50 \pm 0.85 \mu\text{m}^2/\text{s}$ is faster than that of AnV at the inverted DOPC:DOPS//DOPC bilayer composition. But, in particular, the anomalous coefficient is unity, suggesting, that there is not extensive network formation. Interestingly the diffusion coefficient of the DOPE-Atto 532 decreases concomitantly with appearance of AnV binding at 21 hours but unlike the anomalous factor at the DOPS proximal layer lipid continues to reflect Brownian diffusion. Overall, the behavior is rather similar to that reported above for 1nM AnV at the DOPC:DOPS//DOPC bilayer and can be attributed to very low concentrations of DOPS available at the outer leaflet on transversal diffusion which are expected to be significantly less than the 20% mol/mol in the lower leaflet of DOPC:DOPS//DOPC bilayer, as even after extensive equilibration, overall composition will be dispersed across both leaflets.

The very low levels of DOPS at the membrane exterior leaflet generated by flip flop will reduce the extent of AnV aggregation. The extent of decrease of diffusion of the DOPE-Atto 532 is notable and may also arise from contributions from other effects including aggregation of the Atto 532 label on the DOPE over time, some degradation of the film packing (which as stated may contribute to the flip flop over this time scale) in addition to the impact of DOPS-AnV association at the lipid membrane. However, the fact that temporally, both AnV binding and DOPE-ATTO diffusion decreases exactly coincides, strongly indicates that AnV aggregation is the main contributor to this effect. It is possible that there is some specific, interaction between the ATTO label DOPE which, as it is relatively hydrophilic and would be expected to orient at the membrane interface, and the membrane associated AnV may occur which exacerbates the apparent impact on the DOPE label diffusion.

Table 3.3: Diffusion coefficients for DOPC//DOPC:DOPS(80:20) bilayer labelled with DOPE-Atto 532 and annexin V labelled -Alexa647 in HEPES buffer at pH 7.4, monitored over time to determine when annexin V interacts with DOPS indicating a DOPS flip flop. (ACF Figure 3.6)

Time of AnV Incubation	Diffusion ($\mu\text{m}^2/\text{s}$); DOPE-532	alpha; DOPE-532	Diffusion ($\mu\text{m}^2/\text{s}$); AnV-647	alpha; AnV-647
Pre incubation	5.77 ± 0.90	1.00 ± 0.04	-	-
After 19 Hours	5.88 ± 0.92	0.97 ± 0.02	-	-
After 21 Hours	5.09 ± 0.58	0.99 ± 0.01	1.50 ± 0.85	1.00 ± 0.03
After 24 Hours	2.79 ± 0.67	1.01 ± 0.04	1.60 ± 0.62	1.01 ± 0.07
After 26 Hours	2.12 ± 0.85	0.97 ± 0.04	1.56 ± 0.53	0.99 ± 0.05
After 28 Hours	2.15 ± 0.86	1.01 ± 0.04	1.56 ± 0.59	0.99 ± 0.03

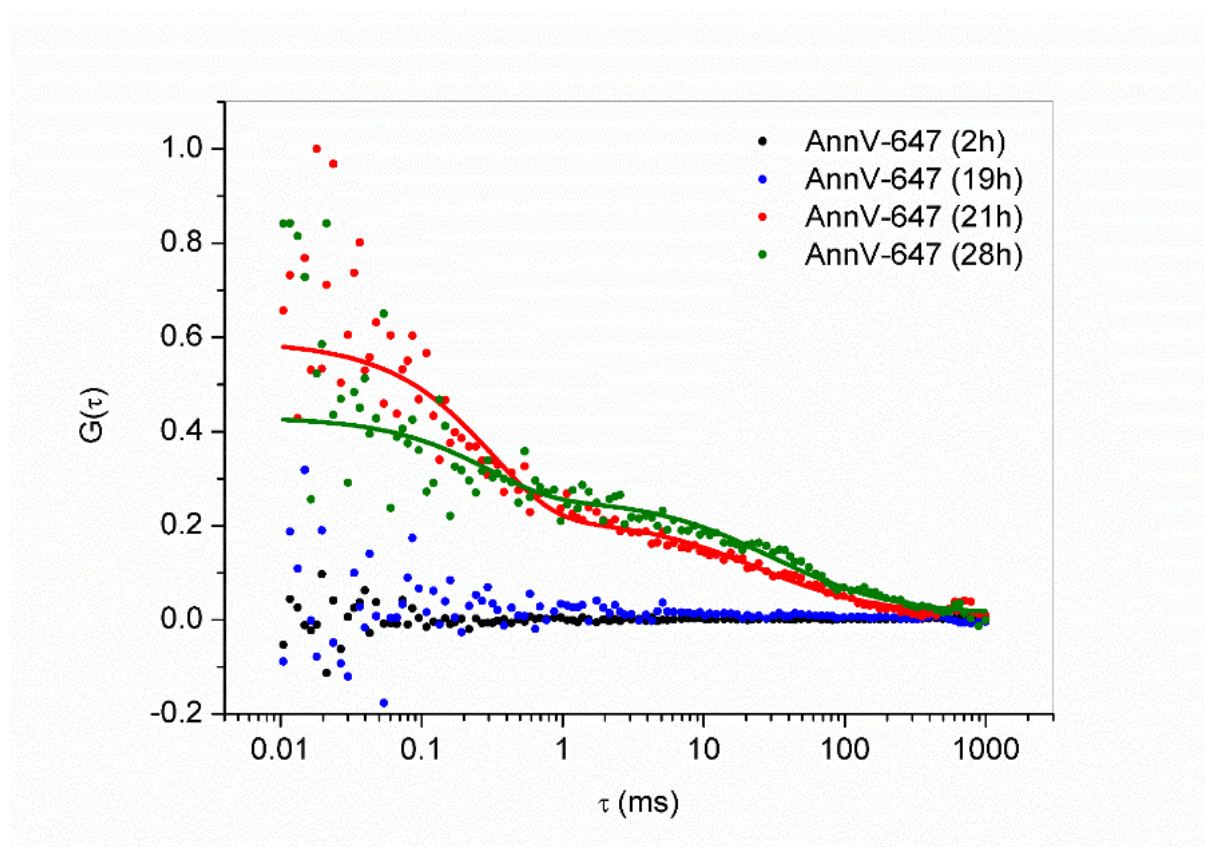


Figure 3.6: ACFs of AnV-647 with DOPS present in the distal lipid leaflet. Binding of AnV was observed after 21h in HEPES buffer pH 7.4.

To simulate membrane damage in an asymmetric bilayer which, in analogy to the mammalian plasma membrane, contains DOPS only at the cytoplasmic leaflet (here the proximal leaflet), the transversal diffusion of negatively charged DOPS from the interior leaflet of the bilayer to the exterior leaflet was induced by photo/thermal annealing of the bilayer using a laser source.

On applying localized heating to the DOPC//DOPC:DOPS bilayer using a white light source at the microscope at a power 2.73 mW, flip flop of DOPS from the inner leaflet to the exterior leaflet was confirmed from the ACFs and time traces (in Figure 3.7), alongside fluorescence imaging evident as labelled AnV can be observed binding to the bilayer (vide infra, Figure 3.8). Initial Brownian diffusion of $6.91 \pm 0.67 \mu\text{m}^2/\text{s}$ was measured for the asymmetric bilayer containing DOPS at proximal leaflet. Diffusion of the membrane post light damage was measured as $6.56 \pm 0.83 \mu\text{m}^2/\text{s}$ when left incubating with calcium and AnV for 1 hour, which

although decreased, is the same within the experimental error. The incubation period was to allow sufficient time for the flip flop of DOPS to occur.

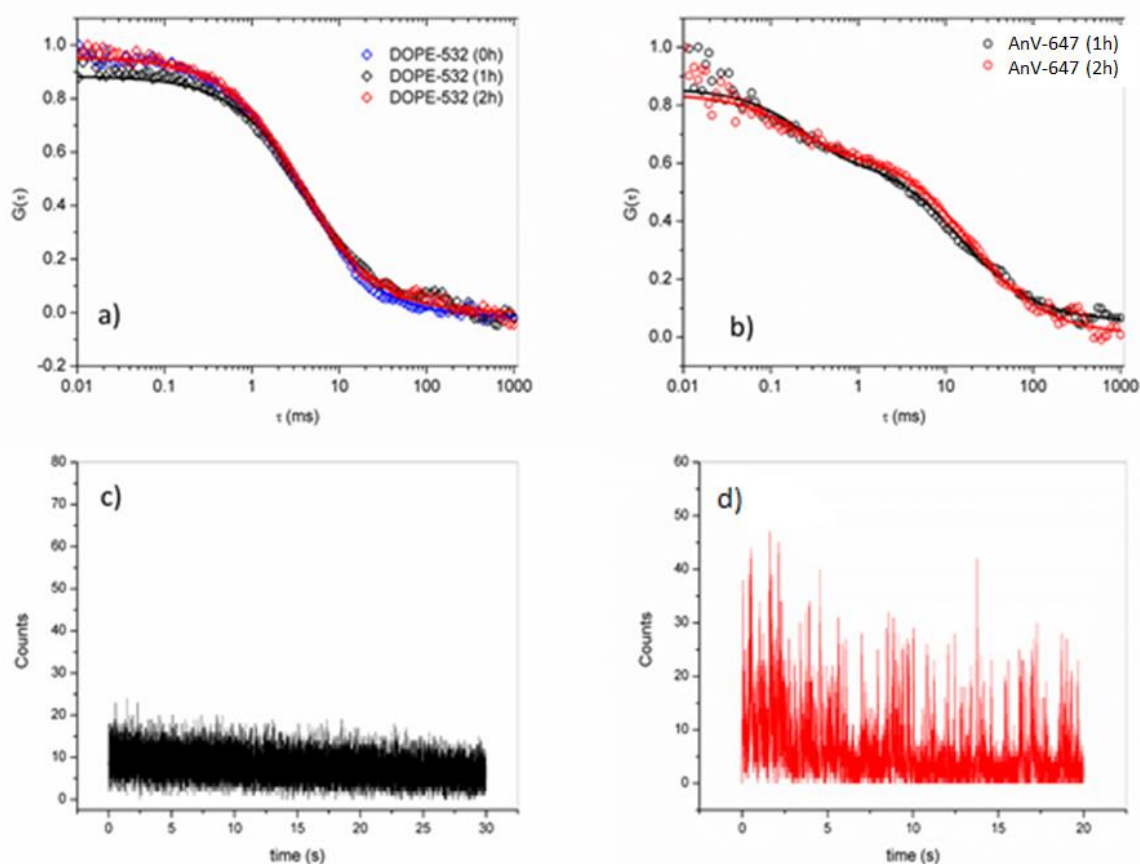


Figure 3.7: Representative auto-correlation function (ACF) curve for a DOPC//DOPC:DOPS Bilayer (a) before photo-damage labelled with DOPE-atto532 and (b) auto-correlation function curve for AnV-Alexa647 after photo-damage and incubation with the bilayer in HEPES buffer at pH 7.4. (c) and (d) representative fluorescence intensity time-trace of AnnexinV-647 before and after laser damage, respectively.

The slower diffusion of the membrane and AnV binding confirms flip flop of DOPS is induced but as, this is a highly localized effect the concentration of DOPS flipped to the distal leaflet would be much less than 20 mol% in the lower leaflet. The fluorescence intensity time trace of AnV-647 before laser damage, Figure 3.7c, shows that there is negligible change in fluctuations over the time scale. This confirms that the membrane was asymmetric and that the AnV was not interacting with the DOPC at the distal leaflet. After photothermal damage to the membrane and incubation with 10 nM AnV and Ca^{2+} the fluorescence intensity time trace of

AnV-647, Figure 3.5d, shows evidence for aggregation. The appearance of bursts in fluorescence intensity clusters and a decreasing trend of intensity, that was similarly observed when DOPS was at the distal leaflet, strongly confirm that a flip flop of DOPS occurred and that AnV has begun aggregation and network formation. Further confirmation of AnV binding is derived from FCS from which AnV diffusion coefficient was measured as $1.60 \pm 0.64 \mu\text{m}^2/\text{s}$ which is similar to the results reported above for DOPS at the proximal leaflet after natural flip flop after 21 hours, and at low AnV concentration at the DOPC:DOPS//DOPC membrane. However, the anomalous factor has decreased below 1, indicating sub-diffusion indicating some aggregation may be occurring. The behavior is also consistent with the impedance data outlined below.

Overall, this result indicates that localized optothermal treatment of an asymmetric bilayer containing DOPS at the cavity proximal leaflet, simulates damage, stimulating DOPS transversal diffusion to the exterior leaflet and AnV binding.

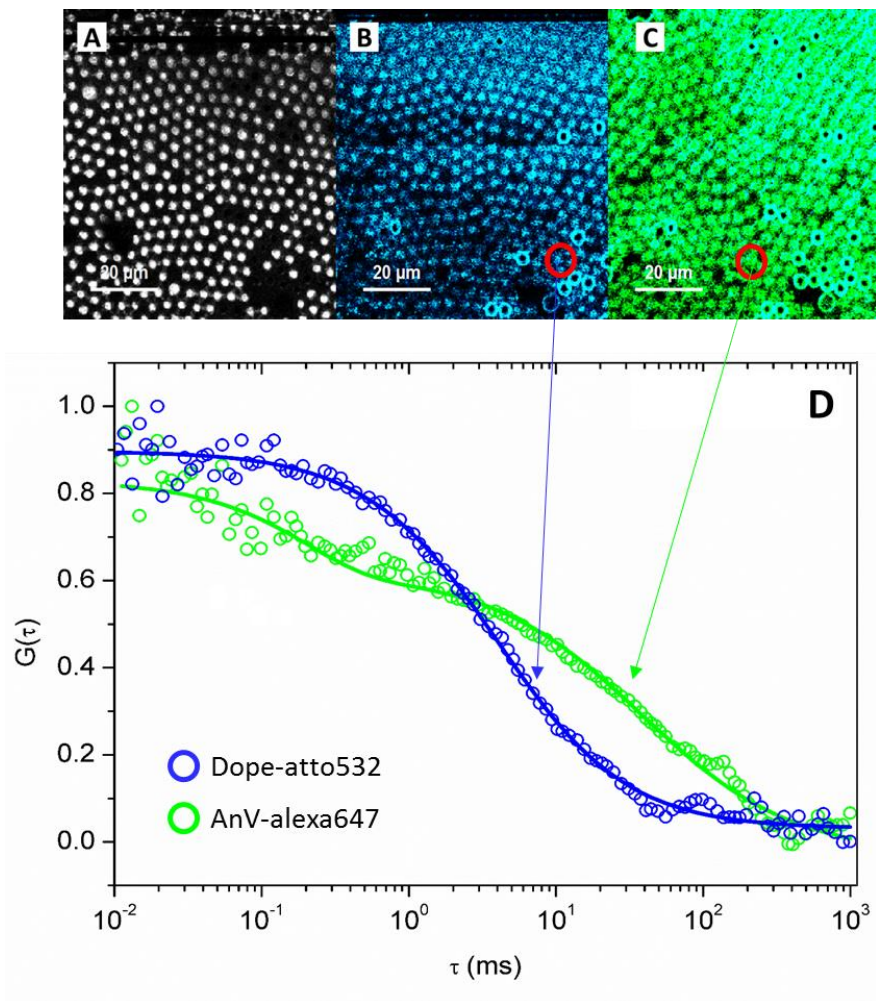


Figure 3.8: Fluorescence Spectroscopy of annexin V after laser damage on asymmetric MSLBs containing DOPS in the proximal lipid leaflet. A) Represents the reflectance images of 2.66 μm PDMS cavity array obtained with an OD3 filter. B) Fluorescence image obtained for DOPE-Atto 532 labelled ($\lambda_{\text{exc}} = 532 \text{ nm}$). C) Fluorescence image of AnV-647 labelled ($\lambda_{\text{exc}} = 640 \text{ nm}$) after localized DOPS flip flop induced by photothermal damage. D) Represents the autocorrelation functions (ACFs) for labelled DOPE-atto532 and annexin-alexa647 obtained above buffer filled microcavities.

Figure 3.8 shows the fluorescence imaging on photodamage of the DOPC//DOPC:DOPS bilayer. As described, in the absence of DOPS in the bilayer, no annexin V binding is observed and similarly at the asymmetric bilayer DOPC//DOPC:DOPS, in the absence of stimulation, over the first 21 hours after bilayer formation and in contact with a solution of AnV/ Ca^{2+} no annexin binding is observed. This experiment confirms, (a) that the bilayers are asymmetric with DOPS localized to the lower leaflet (b) that transversal diffusion of DOPS takes at least 21 hours, likely corresponding to the natural degradation (i.e., the loss of tight packing of lipids) of the lipid bilayer contained in the MSLB. Upon localized heating of the asymmetric DOPC//DOPC:DOPS(80:20) bilayer, translocation of DOPS from interior to exterior leaflets, leads to loss to rapid loss of asymmetry of the bilayer mimicking the early stages of apoptosis.⁵⁹ Consistent with the diffusion values flip flop was observed to have occurred wherein annexin V bound to the membrane after 1 hour incubation with the photothermally damaged bilayer, as seen in Table 3.4.

Table 3.4: Diffusion coefficients for DOPC//DOPC:DOPS(80:20) bilayer labelled with DOPE-Atto 532 and annexin V labelled Annexin V-Alexa647 in HEPES buffer at pH 7.4 Following Localized heating of bilayer to induce the flip flop of DOPS. Time indicates incubation time of the bilayer with AnV.

3.3.2 Electrochemical Impedance Spectroscopy of MSLBs on Gold Array

To understand the impact of annexin binding/network formation on the electrochemical

Time of AnV Incubation	Diffusion ($\mu\text{m}^2/\text{s}$); DOPE-532	alpha; DOPE-532	Diffusion ($\mu\text{m}^2/\text{s}$); AnV-647	alpha; AnV-647
Pre incubation	6.91 ± 0.67	0.97 ± 0.09	-	-
After 1 Hours	6.94 ± 0.98	0.89 ± 0.09	1.60 ± 0.64	0.90 ± 0.09
After 2 Hours	6.96 ± 0.63	1.01 ± 0.10	1.49 ± 0.48	0.90 ± 0.10
After 3 Hours	6.71 ± 0.80	0.97 ± 0.12	1.56 ± 0.57	0.88 ± 0.15
After 4 Hours	6.56 ± 0.83	0.97 ± 0.90	1.50 ± 0.67	0.95 ± 0.08

properties of the MSLB, Electrochemical Impedance Spectroscopy (EIS) was carried out. EIS is highly sensitive to changes in permeability and packing of the bilayer but takes a global perspective of the whole bilayer unlike FCS which takes a molecular perspective, so the methods are quite complimentary. As the arrays show some modest batch to batch variation due to cavity packing and thiol modification, the relative resistance change rather than the absolute impedance changes are reported here.

3.3.3 Electrochemical Impedance Spectroscopy of Symmetric DOPC Bilayer

As a control, we evaluated the impact of AnV/calcium incubation on the impedance of a simple DOPC bilayer in the absence of DOPS.⁶⁰ As shown in Figure 3.9, the resistance of the DOPC symmetric bilayer was found to be stable for 15 hours. And, the impedance profile is analogous to that observed in the absence of AnV and can be attributed to the natural degradation of the film integrity over extended windows, consistent with other reports this degradation is faster for DOPC on gold MSLBs than the PDMS arrays.^{19,21,26} The control, as expected on the basis of fluorescence measurements above, indicates that the DOPC bilayer is unaffected by incubation with AnV. Resistance of the bilayer was measured at AnV concentrations of 1 nM, 9 nM and 100 nM in triplicate and the same trend was seen across all measurements at DOPC alone, indicating that AnV has no interaction with the zwitterionic

lipid bilayer. The capacitance of the DOPC bilayer as expected, also exhibited negligible change over the course of the run over these concentrations indicating bilayer thickness remained constant.

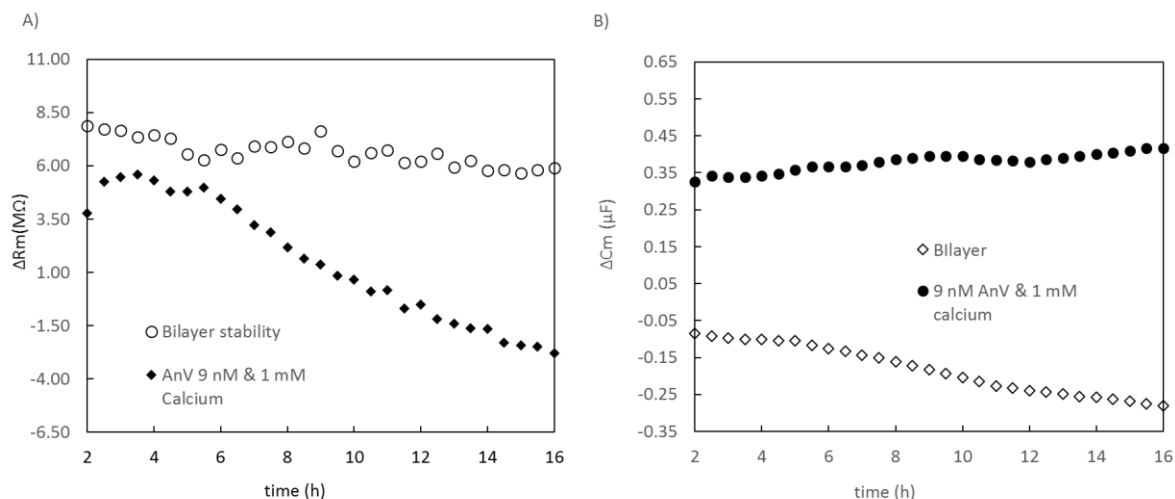


Figure 3.9: The relative resistance changes and capacitance on a DOPC symmetric bilayer with and without the addition of annexin V in HEPES buffer at pH 7.4 within the experimental window 2-16 hours (full 24 hours shown in chapter 2). A) Stability of DOPC lipid bilayer over the experimental window of 16 hours (White circles) and after the addition of 9 nM AnV (black diamonds). B) The relative capacitance changes on the effect of a DOPC symmetric bilayer over 16 hours. annexin V was added after 1.5 hours equilibration in presence of 1 mM $CaCl_2$. N=3

3.3.4 Electrochemical Impedance Spectroscopy of asymmetric DOPC:DOPS(80:20)//DOPC Bilayer

An asymmetric bilayer containing DOPS at the exterior leaflet was then studied to examine the impedance response when AnV binds to negatively charged DOPS in the presence of calcium.⁶¹ As shown in **Error! Reference source not found.3.10**, the resistance of a DOPC:DOPS//DOPC bilayer was confirmed to be stable for at least 10 hours, well beyond our experimental window. Following initial 1.5 hours equilibration, resistance change was negligible across the following 12 hours, defining our experimental window. Upon the addition of calcium and annexin V, to the equilibrated film, the resistance was observed to increase rapidly by over 2 $M\Omega$ compared to the initial bilayer, before equilibrating at this higher resistance and remaining stable beyond 12 hours. The resistance increases on AnV exposure

are attributed to the reduced the permeability of the bilayer on network formation.⁵ AnV concentrations of 1 nM, 9 nM and 100 nM were all added in triplicate and the same trend in resistance observed. The resistance change of the bilayer with 1 nM AnV was less than 1 M Ω . When 100 nM of AnV was incubated with the bilayer containing DOPS on the exterior the magnitude of resistance change was the same as when 9 nM was added. However, similar magnitude of resistance change was observed for 9 nM and 100 nM studies suggesting that DOPS binding, and network formation is at equilibrium concentration under the higher conditions used here and increasing AnV concentration further does not impact the film resistance. Therefore, 10 nM of AnV was chosen for the fluorescence studies and gave optimum signal to determine diffusion co-efficient values.

In parallel, the maximum capacitance change, (for the 9 and 100 nM AnV) for the asymmetric bilayer increased roughly 3-fold over the course of the run. Interestingly though, the dynamic response was slower for capacitance compared to resistance change. The reason for this difference was unclear, however we speculate that as capacitance reflects membrane thickness, it may suggest that resistance changes follow AnV binding to the membrane and the consequential influence of AnV on membrane packing and the membrane capacitance reflects slower lateral organization of the protein network or even that there may be some multilayer formation, though this was not evident from diffusion studies. Figure 3.10C shows the representative Nyquist plot for the DOPC:DOPS(80:20) membrane before the addition of AnV, blue, and after AnV aggregation and network formation at 10 and 15 hours, grey and orange respectively.

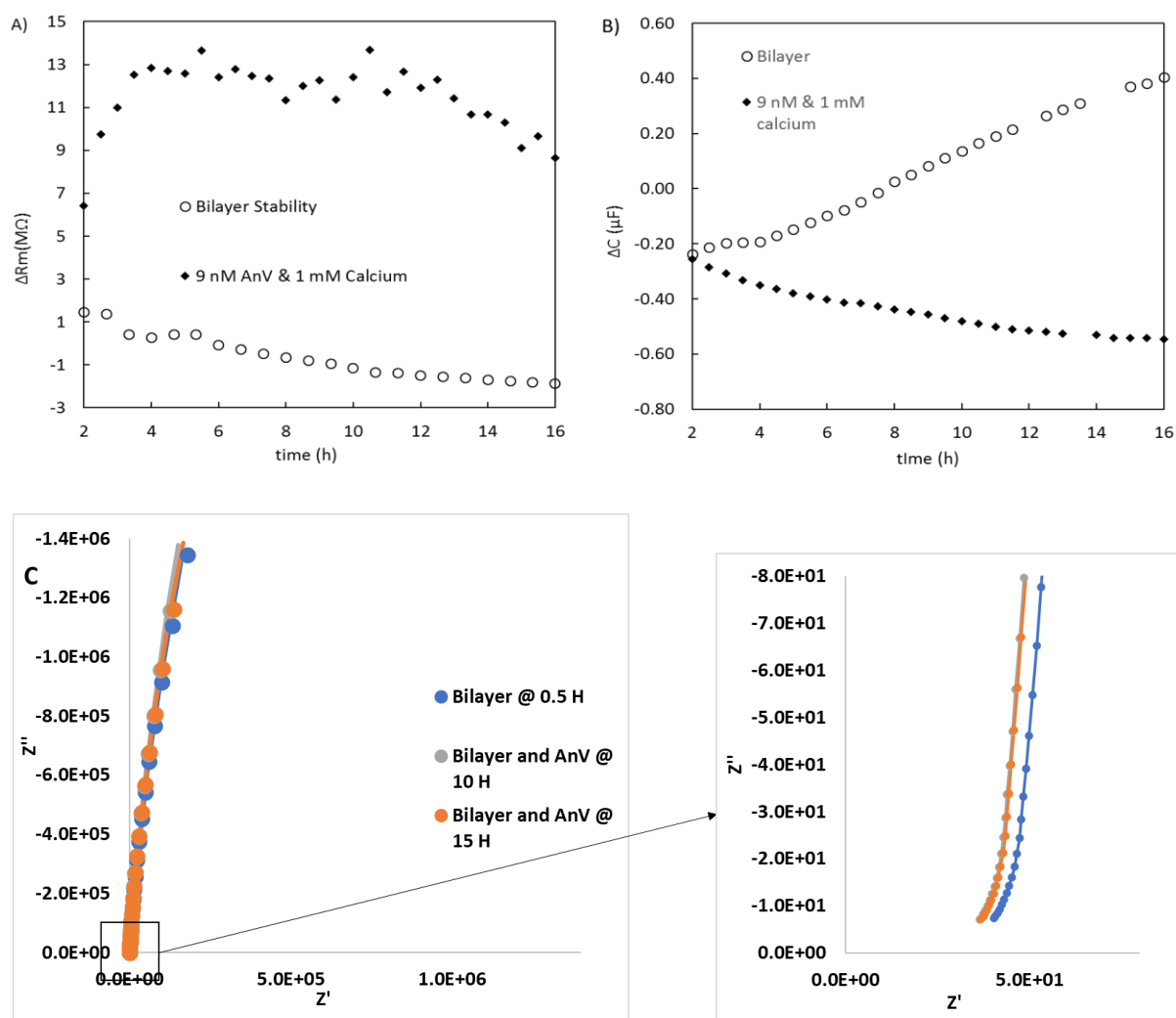


Figure 3.10: A) The relative resistance changes on the effect of a DOPC:DOPS(80:20)//DOPC asymmetric bilayer with and without the addition of annexin V in HEPES buffer at pH 7.4 within the experimental window 2-15 hours. White circles: DOPC:DOPS(80:20)//DOPC bilayer stability over 24 hours. Black Diamonds: DOPC:DOPS(80:20)//DOPC bilayer after the addition of 9 nM AnV and 1 mM $CaCl_2$. B) The relative capacitance changes on the effect of a DOPC:DOPS//DOPC asymmetric bilayer over 24 hours. $N=3$, annexin V was added after 1.5 hours of equilibration of the lipid bilayer in the electrochemical cell. C) Nyquist plot of DOPC:DOPS(80:90) before addition of 9 nM AnV (blue) and after addition of AnV at 10 and 15 hours (grey and orange, respectively). Zoom in of the high frequency range on the left. Coloured circles are the raw data obtained from impedance. Solid lines are the data fitted using the equivalent circuit model.

3.3.5 Electrochemical Impedance Spectroscopy of asymmetric DOPC//DOPC:DOPS(80:20) Bilayer

To mimic apoptosis/membrane damage, optothermally induced damage was applied as described above, whilst monitoring the impedance response at asymmetric lipid bilayers comprising of DOPC:DOPS (80:20) at the proximal lipid leaflet and DOPC at the distal leaflet. The asymmetric bilayer was found from EIS measurements to be stable for at least 10 hours. In the absence of photodamage, the bilayer initially acted like a symmetric DOPC bilayer, in that on incubation with AnV and calcium there was no evidence of AnV binding, i.e., changes to film impedance, and the bilayer resistance remained stable for approximately 12 hours after which it decreased, attributed to some natural degradation of the film.

For optothermal stimulation of the transversal diffusion of DOPS to the exterior leaflet of the bilayer, after initial equilibration the MSLB was irradiated with a 405 nm laser, 3.12 mW power. The resistance was observed to decrease by 5 M Ω immediately on irradiation consistent with increased permeability associated with bilayer annealing and associated increased local fluidity. The bilayer resistance decreased about 30 % of its initial resistance once the light was extinguished in the absence of AnV. On treatment with AnV and calcium shown in Figure 3.11, the resistance was observed to rise rapidly and increased by approximately 40% of the original resistance of the bilayer before optothermal exposure. Therefore, AnV binding was observed to partially seal and stabilize the damaged film, which is attributed to network formation, similar to the bilayer with DOPS on the exterior. The result also confirmed that AnV was binding to the bilayer as DOPS had migrated to the exterior layer, similar to what occurs naturally during apoptosis.⁴⁰ AnV concentrations of 1 nM, 9 nM and 100 nM were all added in triplicate and the same trend observed across all concentrations. As per when DOPS was located on the exterior of the bilayer, a smaller resistance change of less than 1 M Ω was seen when 1 nM AnV was added to the electrochemical cell. The magnitude of change between the additions of 9 nM and 100 nM were again similar which indicates that at 9 nM the AnV concentration is at saturation for the available DOPS within the bilayer.

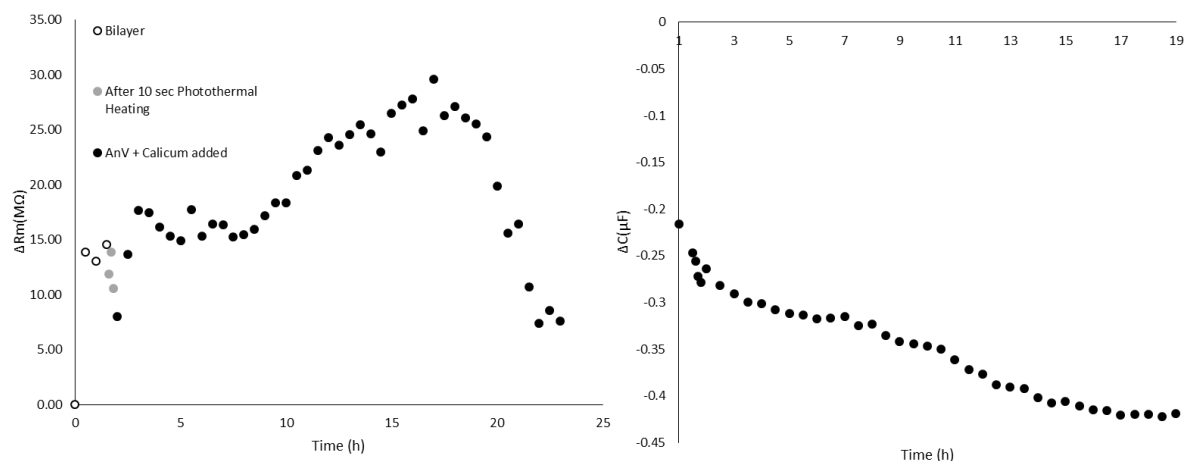


Figure 3.11: Right: The relative resistance changes on the effect of a DOPC//DOPC:DOPS(80:20) asymmetric bilayer with the addition of annexin V after photodamage in HEPES buffer at pH 7.4 within the experimental window 2-19 hours. White circles: Bilayer stabilization. grey Circles: Relative resistance of the bilayer after 10 seconds of 405 nm photodamage. Black diamonds: Network formation of AnV with 1 mM $CaCl_2$ on the bilayer suggesting DOPS flip-flop. $n = 3$. annexin V was added after 2.5 hours equilibration. Left: Relative capacitance change on the effect of a DOPC//DOPC:DOPS(80:20) asymmetric bilayer with the addition of annexin V after photodamage in HEPES buffer at pH 7.4 within the experimental window 2-19 hours.

3.4 Conclusions

This chapter explored a microcavity array supported asymmetric lipid bilayer system applied as a biomimetic model of cell membrane damage induced annexin V-phosphoserine binding.

Microcavity supported lipid bilayers comprising DOPC//DOPC:DOPS(80:20), with DOPC only in the outer leaflet and a DOPC/DOPS composition in the lower (proximal) leaflet or the reverse; DOPC:DOPS(80:20)//DOPC where DOPS is at the distal leaflet were evaluated for annexin V binding in the presence of Calcium(II). Electrochemical impedance studies, in agreement with fluorescence imaging and FCS data, confirmed that AnV associates with the membrane only when DOPS was present at the external bilayer leaflet and in the presence of Ca^{2+} ion. Association of three concentrations of AnV, 1, 10 and 100 nM were examined at DOPC:DOPS(80:20)//DOPC bilayer were studied by FLCS and whereas binding was observed for all three concentrations, the diffusion values and anomalous factors varied systematically

with concentration. At 1 nM AnV the diffusion of AnV was measured as $1.57 \pm 0.20 \mu\text{ms}$ and the anomalous coefficient, α was obtained as 1, consistent with Brownian diffusion. The diffusion of the underlying lipid probe, DOPE ATTO532 decreased modestly on AnV binding but diffusion remained Brownian. At 10 nM AnV the diffusion of AnV was measured as $1.4 \pm 0.40 \mu\text{ms}^{-1}$ and the anomalous component decreased to 0.9 indicating sub-diffusion. In parallel, the diffusion of lipid probe also decreased from 6.2 to $4.43 \pm 0.91 \mu\text{ms}^{-1}$ and interestingly, α was obtained as 0.84 ± 0.12 , indicating sub-diffusion of the lipid probe. On incubation of DOPC:DOPS(80:20)//DOPC bilayer with 100 nM AnV, the AnV was immobile, reflected in photobleaching of both the AnV fluorescent label and that of the lipid probe. The data indicates that at 1nM AnV the annexin binds without significant aggregation to the DOPS containing membrane but progressively aggregation occurs at 10 and 100 nM AnV which exhibits extensive immobile network formation at the membrane interface.

Conversely for DOPC//DOPC:DOPS(80:20), where DOPS resides only at the lower, proximal leaflet, both electrochemical impedance and fluorescence measurements concur that AnV does not associate with the membrane. Thus, confirming that membrane asymmetry is maintained, mimicking a healthy plasma membrane. Incubating the DOPC//DOPC:DOPS(80:20) with 10 μM AnV over a 30 hour window, it was observed that the membrane asymmetry was preserved for at least 19 hours. Annexin assembly at the membrane interface was only observed after 21 hours where the diffusion coefficient of the AnV was measured by FLCS as 1.50 ± 0.85 with an anomalous factor of 1 consistent with Brownian diffusion.

Upon localized optothermal damage of the asymmetric DOPC//DOPC:DOPS (80:20) bilayer, translocation of DOPS from interior to exterior leaflets, led to loss to the asymmetry of the bilayer which mimics the early stages of apoptosis. The damage was reflected with a dramatic decrease in resistance, while. capacitance of the membrane remained constant after photodamage indicating that the light treatment increased permeability of the bilayer, but the thickness of the bilayer is unaffected, confirming that the bilayer was not destroyed by the laser. In the absence of AnV some recovery of the bilayer resistance is observed to occur after photodamage. In the presence of AnV, DOPS flip flop was confirmed to have occurred upon phototreatment of the bilayer a resistance and capacitance as shown above by fluorescence and in EIS this was evident as gradual recovery of resistance which then stabilized for over 15 hours indicating AnV bound to the negatively charged lipids appearing on the exterior of the

membrane after 1 hour incubation.^{60–62} The slow recovery of the bilayer after photodamage is attributed to the low concentration of DOPS that translocates to the distal leaflet and mimicking early stages of apoptosis. The stabilization of the bilayer for an extended period of time with when AnV is present indicated suggests that AnV seals and stabilizes the membrane. Overall, this works demonstrates the utility of MSLBs in studying network aggregation at membrane interfaces and beyond direct biophysical insights the work offers interesting prospects for building bio-inspired repair of surface chemistries.

Thus, annexin V can both act as a probe of transversal stability of asymmetric bilayer compositions permitting monitoring of this process label free, and it can be used to introduce a means of spontaneous repair into cavity supported bilayers and related films.

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Chapter 4: Role of Phosphatidyl Serine in Beta Amyloid Oligomerization at Asymmetric Phospholipid Bilayers

4.1 Introduction

Alzheimer's disease (AD) is the most common form of dementia. Mainly associated with older adults, it is an irreversible brain disorder that causes progressive and irreversible loss of cognitive function.¹ Amyloid beta (A β), a naturally secreted peptide, in its oligomeric form has been detected in the cerebrospinal fluid and brain homogenates of patients in the femto to picomolar range.^{2,3} There is substantial evidence indicating that the self-aggregation of monomeric A β to fibrils but in particular oligomers play an important role in the progression of AD disease.⁴⁻⁷ Although there is some controversy regarding the amyloid model and the relative role for example of tau and amyloid in disease progression, the recent FDA approval of Aducanumab suggests causation that these peptides are involved, although the drug is not currently approved by the European Medicines Agency.⁸ In Alzheimer's disease, A β peptide is released from cells by the action of β - and γ -secretases on amyloid precursor protein (APP) where it undergoes amyloidogenesis; aggregation to oligomers, multimers, and fibrils in the extracellular space, eventually forming the plaques that are characteristic of the disease.⁹ Furthermore, pathogenic amyloid aggregates are not confined to Alzheimer's but play a major role in Parkinson's, and Huntington's and in Diabetes.¹⁰ A β peptides are composed of 36 to 43 amino acids that spontaneously self-aggregate through hydrophobic- hydrophobic interactions especially at higher concentrations (micro to submillimolar range).¹¹⁻¹³ All amyloid aggregates can serve as AD cellular hallmarks, however,, soluble small oligomers formed from A β 1-42 have recently been shown to be more toxic than larger fibrils.^{14,15} Currently, AD has no cure and cannot be prevented so understanding the mechanisms of A β aggregation, can provide insights into early detection strategies and is of great importance for understanding possible prevention approaches and development of therapy.^{15,16} Furthermore, given the prevalence of across multiple disease states beyond AD tools to investigate

A β aggregation is known to occur on the surface of neuronal cell membranes, leading to plaques of amyloid in brain tissue of AD patients. The current understanding of amyloid action at the cellular interface has been gained through study by either targeting specific receptors or by examination of non-specific interactions with lipids within membranes.^{17,18} Numerous in vitro studies have revealed that lipidic membrane in general and negatively charged

phosphatidylserine, in particular, can promote A β oligomer formation at the bilayer interface.^{15,19,20 21–23} At high concentration (μ M to mM), A β oligomers can be formed in the solution due to self-assembly but at low concentration (pM to nM) oligomers can form upon interaction with lipidic environment. A β oligomers have the ability to disrupt the integrity and impermeability of cell membrane.^{24,25}

To date, a number of models have been hypothesized to explain A β oligomer induced membrane toxicity, e.g., (a) the poration hypothesis suggests amyloid form stable transmembrane pores at the neuronal membrane leading to disruption in intracellular Ca²⁺ homeostasis (b) the carpet model, hypothesizes that peripheral adsorption of amyloid at the membrane interface destabilizes the membrane structure and c) membrane dissolution model, where the amyloid precipitates strip off lipid from the bilayer acting like a detergent.^{16,26,27} In all such models, membrane disruption can lead to oxidative stress, a key effector in AD progression that further leads to cellular apoptosis.^{28–31} Under conditions of oxidative stress, phosphatidylserine, normally confined in the naturally asymmetric neuronal membrane to the cytosolic leaflet of lipid bilayer migrates to the extracellular leaflet, a primary step in apoptosis initiation. A number of studies have indicated that anionic lipids including phosphatidylserine (PS) influence A β oligomerization, fibrilization and fibril growth.^{32–38} PS is particularly important as it is prevalent in the plasma membrane of neuronal cells where A β peptide interaction.³⁹ This is mainly driven by electrostatic association between positively charged A β and negatively charged PS.^{40–42} And, while evidence, particularly from in-vitro models is strong that A β and PS interaction plays a role in inducing the formation of oligomer scaffold^{43–47}, other membrane factors also play a role including cholesterol content, the packing density of the membrane, and lipid rafts.^{14,20,48–52} However, the impact of variable concentrations of PS at physiological concentration and its asymmetric distribution within bilayer towards the recognition and assembly of A β as well as their associated kinetics of oligomer formation under these conditions not yet been explored extensively. Very recently Kim et al reported on the impact of anionic phospholipid Phosphatidyl Serine (as DOPS) or Phosphatidyl glycerol (DOPG) concentration on aggregation of A β 1–42 (A β 42) at lipid vesicles comprising PC/PS or pure DOPS. They observed, using thioflavin T and transmission electron microscopy, that the liposome packing density (controlled by varying fatty acid on the PS in terms of length and saturation) and DOPS/DOPG concentration controlled the rate of fibrogenesis and the length of the resulting fibrils.⁵³ Although the concentration of PS was much greater than anticipated

in natural membranes the study reflected the impact of membrane packing/fluidity and charge on amyloidogenesis. Rocha et al reported similar experiments involving Amyloid fibrinogenesis of the synaptic brain protein α -synuclein (α S) at vesicles comprised of anionic lipids for the protein and observed using circular dichroism spectroscopy, that the anionic lipids formed nucleation sites for oligomer formation at vesicles.⁵⁴

Such biophysical models of the lipid membrane have provided deep insights into A β cell-membrane interactions since they permit a reductionist approach to separate the variables that affect oligomer growth at membranes and address these questions. Diverse models, including Liposomes, Black lipid membranes and supported lipid membranes (SLBs) have been applied to study amyloid-membrane interactions.^{2,21,36,55–59} For example recently the first 3-D visualization of amyloid- β oligomer interactions with lipid membranes by cryo-electron was enabled at liposomes. However, there are limitations in addressing some aspects of interaction. Liposomes for example, suffer relatively poor stability, lack of control over compositional asymmetry, and are generally not addressable with surface-sensitive analytical tools. Substrate Supported lipid bilayers (SLBs) and their variants address these issues but they suffer different limitations, particularly in study of dynamics of membrane/protein interactions that require lateral or transversal diffusion of lipid or components due to frictional lipid-substrate or protein/peptide-substrate interaction that in turn leads to hindered diffusion and/or even protein/peptide denaturation.^{60–64}

Ideally, for greater bioanalogy the model should incorporate PS asymmetrically within the bilayer and requires a sensitive detection method to follow the interaction, binding, and oligomer formation event in real time at A β concentrations at close to physiological conditions. In this context, microcavity supported lipid bilayers (MSLBs) in combination with electrochemical impedance spectroscopy (EIS) offer a useful approach. The microcavity pore provides aqueous reservoirs at each lipid leaflet interface avoid any loss of activity or assembly of peptide and the layer by layer approach to fabrication enables facile building of asymmetry into the bilayers structure.^{32,33} EIS is a sensitive, label-free technique that can reveal details of molecular mechanism of A β activity in real time by monitoring the admittance of bilayer membrane.

Exploiting these methods, herein we study the role of DOPS content within DOPC membrane on membrane association and assembly of A β ₁₋₄₂. At a fixed concentrations of A β ₁₋₄₂ using

EIS, we established that 10 mol% DOPS is sufficient to facilitate A β oligomerization without damaging the membrane. At 5 mol% DOPS, no large-scale perturbation of membrane or oligomers are detected in contact A β (1 μ M,) whereas at membranes comprising 20 mol% of DOPS, the activity of A β triggered the assembly processes leading to membrane damage. Large scale oligomer formation and membrane damaging effect caused by A β was further visualized using atomic force microscopy and confocal microscopy.

In reality, in the homeostatic cell, PS is localized to the cytoplasmic leaflet of the neuronal membrane. But under conditions of oxidative stress, it is exposed at the outer leaflet and so to imitate this process we induced optothermal damage of asymmetric DOPS /DOPS membrane, to induce DOPS transversal diffusion and observed that the assembly of A β is triggered under these conditions.

4.2 Materials and Methods

All samples were prepared according to the protocols described in Chapter 2, all experiments were performed at room temperature. All experiments were repeated a minimum of three times unless stated otherwise. For all experiments, after 1.5 hours vesicle fusion with the LB formed monolayer on the MSLB, the array was rinsed with buffer to remove excess vesicles. For EIS experiments the array was used as the working electrode in the electrochemical system. The MSLB was connected, and the membrane was allowed equilibrate and stabilise for 2 hours before the addition of protein to allow for the measurement to be made. All EIS data shown is the average between the replicates (N=3), with error bars representing the mean squared error.

The lipid bilayer compositions were used in this chapter: symmetric DOPC lipid bilayer where both lipid leaflets are comprised of DOPC, Asymmetric DOPC:DOPS (80:20)//DOPC, DOPC:DOPS (90:10)//DOPC or DOPC:DOPS (95:5)//DOPC, lipid bilayers where the distal leaflet is composed of 80 mol% DOPC and 20 mol% of DOPS (or 90 mol% DOPC and 10 mol% DOPS or 95 mol% DOPC and 5 mol% DOPS) while the proximal leaflet is composed of DOPC to model an apoptotic cell membrane and DOPC//DOPC:DOPS(80:20), DOPC//DOPC:DOPS (90:10) or DOPC//DOPC:DOPS (95:5) with distal leaflet comprised of DOPC and proximal leaflet of 80 mol% DOPC and 20 mol% of DOPS (or proximal leaflet of 90 mol% DOPC and 10 mol% of DOPS or 95mol% DOPC and 5mol% DOPS) as an analogue of a non-apoptotic cell membrane.

The following deviations were also completed;

4.2.1 Amyloid Beta 1-42 Pretreatment:

Pretreatment of A β ₁₋₄₂ is required for controlled aggregation studies, to destroy possible preformed aggregates.^{36,65–69} 1 mg of Amyloid was dissolved in 1mL of HFIP, to disassemble any preformed aggregates or oligomers. The 1 mg/ml amyloid solution was then aliquoted out and the HFIP was left to evaporate completely, under vacuum. HFIP is a volatile and corrosive alcohol that is not compatible with in vitro, or cell-based studies and therefore must be removed by evaporation. The peptides are stored as thin films at -20 °C. Just before experimentation, the thin film was resuspended in HEPES buffer.^{49,57,68,70,71} A similar pretreatment procedure for A β ₅₅₅ was adopted in order to discard any preformed aggregates.

4.2.2 Atomic Force Microscopy:

Atomic force microscopy measurements of lipid bilayers were carried out using the Veeco Bioscope II system (Nanotec House, Cambridge) coupled with Zeiss Axiovert inverted optical microscope IX70. Topographic AFM images were obtained in HEPES buffer in tapping mode, using silicon nitride cantilevers PNP-TR-20 (NANO WORLD) with a nominal force constant of 0.32 N/m and a tip radius of 20 nm. During image acquisition, the resonance frequency in buffer was kept as low as possible, and the scan rate was kept at 0.5 Hz. Lipid bilayers were prepared on freshly cleaved mica by depositing DOPC monolayer by Langmuir-Blodgett method followed by vesicle fusion of liposomes of varied compositions such as DOPC, DOPC:DOPS(90:10) and DOPC:DOPC:DOPS(95:5). All AFM images were analyzed using Nanoscope 7.30 software, and the whole images were plane fitted with a 1st order polynomial.

4.2.3 Confocal Fluorescence Imaging:

Confocal measurements were carried out on MSLBs formed at optically transparent PDMS arrays as previously reported and in chapter 2.^{61,63,72,73} Confocal imaging was carried out using a Leica TSP DMi8 confocal microscope with a 63X oil immersion objective lens. Membrane impermeable probe pyranine was introduced into the pores of the array before assembly of the bilayer by pre-sonicating the microcavity array in 1 μ M pyranine solution of HEPES. The pyranine was excited at 405 nm and emission detected between 440 and 470 nm.⁶² A β ₅₅₅ at the designated concentration was allowed to interact to the PDMS spanning membrane and was excited at 555 nm with a detection window of 565 – 630 nm. 20 μ M Thioflavin T (tht) was dissolved in the contact solution from EIS studies (HEPES buffer used for the electrochemical cell) and excited at 405 nm with a detection window 430-580 nm.

4.3 Results & Discussion

Based on observations of A β beta (A β) oligomerization in contact with a DOPC/DOPS(65/35%) membrane by Supercritical Angle Fluorescence Seeger et al hypothesized the following steps: 1) A β monomer initially adsorbs at the membrane, facilitated by long range electrostatic interactions with anionic lipid 2) A β monomers diffuse along the membrane and self-aggregate, whilst concomitantly causing lipid clustering that leaves nanopores within the membrane causing additional membrane strain; 3) further aggregation of A β removes some of the lipids that are tightly bound to the oligomers scaffold; 4) the oligomer of A β is ejected from the membrane as a plaque leaving a porated/damaged membrane as shown schematically in Figure 4.1.^{16,25–27,55}

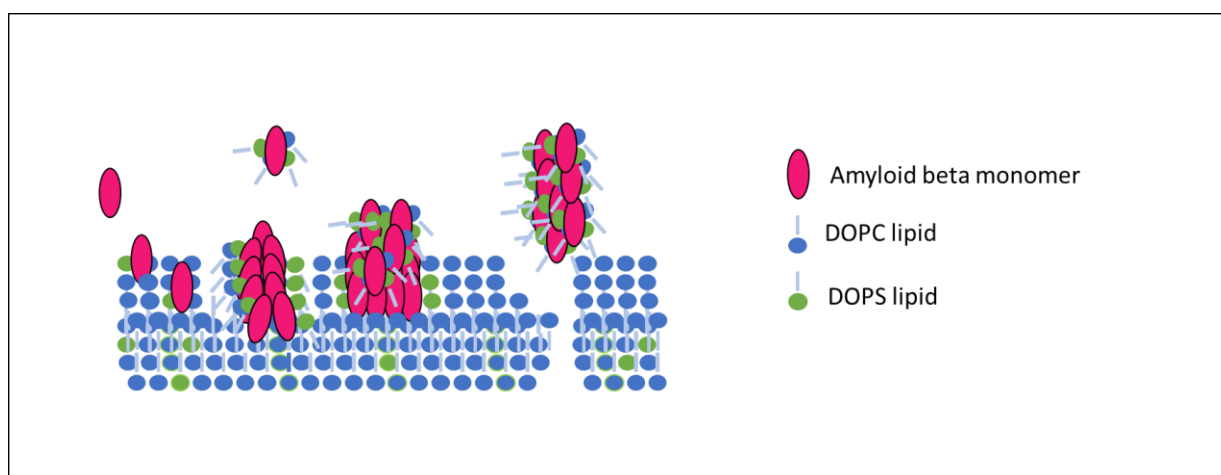


Figure 4.1: Hypothesized mechanism of Amyloid-beta interaction within a lipid bilayer.⁵⁵ Zwitterionic lipids (Blue) make up the majority of the membrane and the amyloid beta peptide monomer (pink oval) may adsorb to the membrane. Negatively charged lipids (green) attract amyloid beta peptides to bind and cause the monomers in the membrane to aggregate within the membrane, however some monomers might pull lipids out of the membrane. The monomers continue to aggregate to form oligomers within the membrane. Fibrils of amyloid beta fall out of the membrane as a plaque and leave the membrane damaged.

4.3.1 Effects of A $\beta_{(1-42)}$ on a Symmetric DOPC Membrane

A central tenet of this and other hypothesis is that the membrane becomes porous on amyloid aggregation. Electrochemical impedance spectroscopy (EIS) is a particularly powerful and highly sensitive method to explore such effects as it directly addresses the admittance of the bilayer and was used here distinguish each step starting from initial adsorption, role of negatively charged phospholipid, oligomerization, and membrane dissolution pathways.

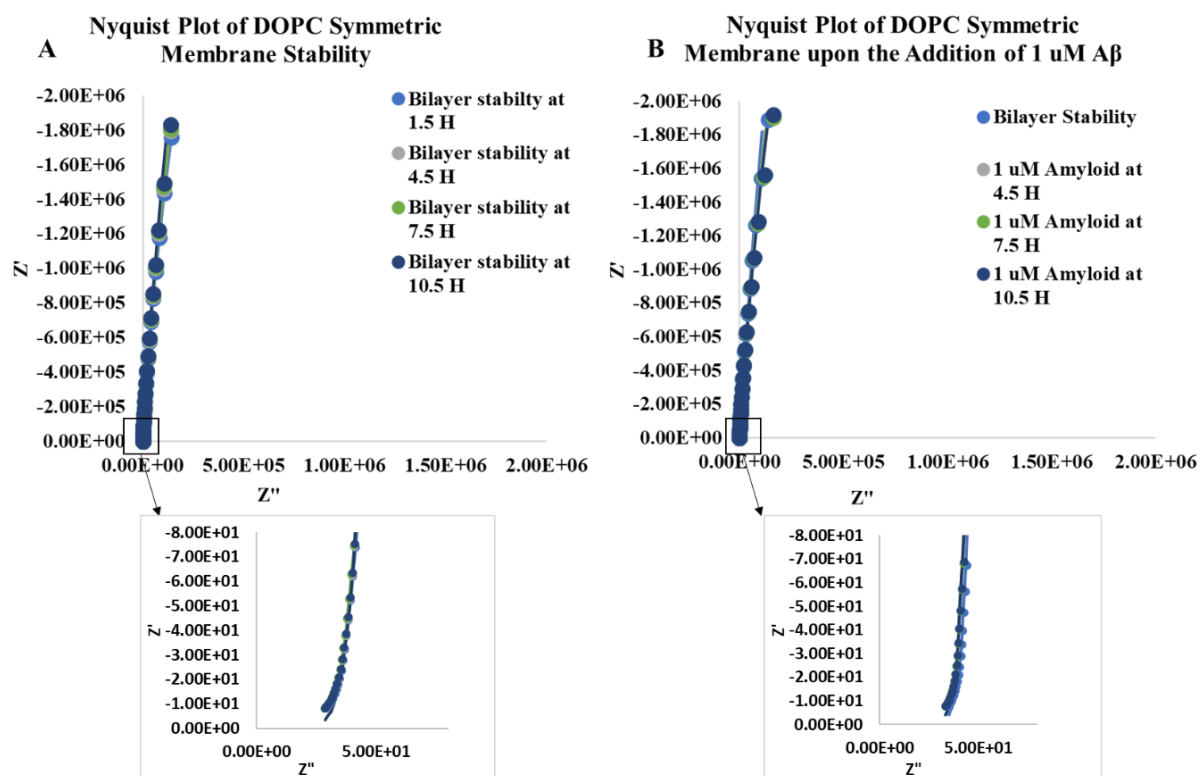


Figure 4.2: Representative non Faradaic Nyquist Plots of DOPC symmetric MSLB in the A) absence and B) presence of 1 μ M A β in the contact buffer solution, HEPES pH 7.4 versus Ag/AgCl reference electrode at 0V. Coloured circles are the raw data obtained from impedance. Solid lines are the data fitted using the equivalent circuit model. Zoomed in regions of the high frequency range below main plot.

First to establish the electrochemical characteristics of the membrane prior to exposure to A β ₁₋₄₂, non-Faradaic EIS measurements were carried on symmetric DOPC bilayers. The DOPC membrane was prepared by LB-VF method and spanned across the gold microcavity array and used as a working electrode. Representative non Faradaic Nyquist plots of DOPC MSLB in absence and presence of 1 μ M A β in the contact buffer are shown in Figure 4.2. Each membrane was prepared in triplicate and each experiment was performed at least N=3. The corresponding data was modelled using the ECM (Scheme 2.3) to extract the membrane resistance and capacitance values and fits of the data to the ECM model were confirmed visually and quantitatively using the χ^2 value to ensure a good fit. As the arrays naturally show some batch to batch variation in electrode area due to variations in cavity packing and thiol modification, the relative resistance/capacitance change rather than the absolute impedance changes are

reported in Figure 4.3. The normalised resistance and capacitance values have been averaged and the standard error of the mean has been incorporated as error bars for all plots.

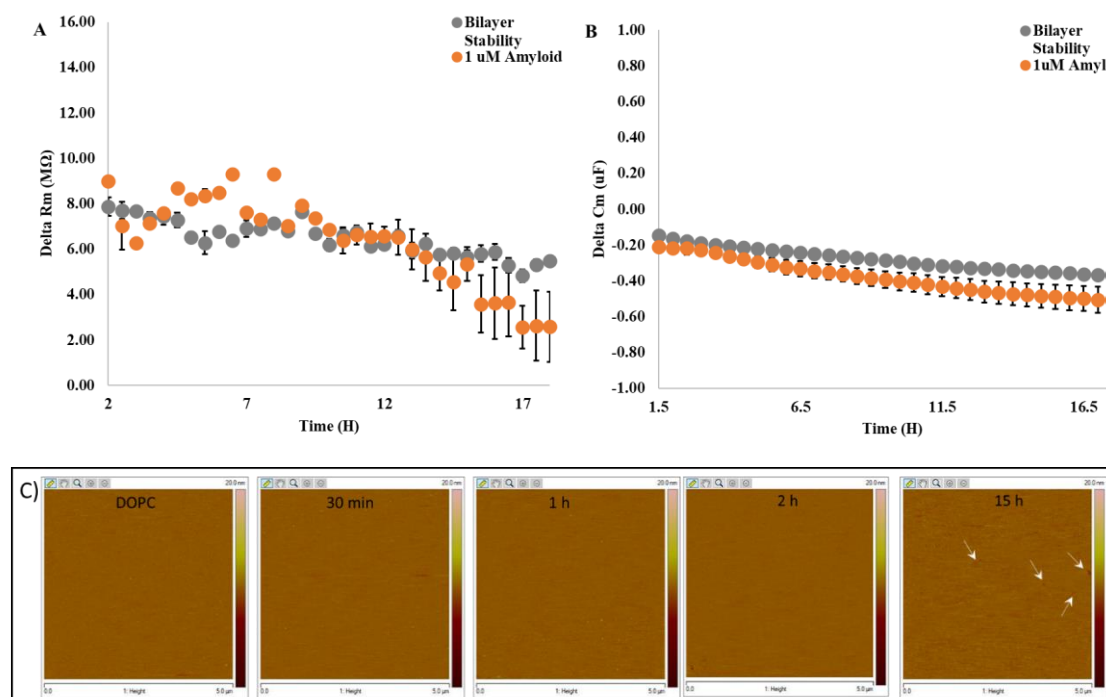


Figure 4.3: Top representative temporal electrochemical data from DOPC bilayers showing relative change in membrane A) resistance and B) capacitance of DOPC symmetric bilayer versus time before (grey) and after (orange) the addition of Amyloid Beta (1uM) in HEPES buffer at pH 7.4 over 24 hours. Equilibration of DOPC lipid bilayer was achieved within 1.5 hours prior to Aβ addition (for clarity the data from 0-1.5 h are omitted) and the impedance change remains constant for over 24 hrs. The data recorded are the mean of $n = 3$ and error bars as standard error. C) Topographic tapping mode AFM images ($5 \times 5 \mu\text{m}^2$) of DOPC bilayer without (0 min) and in the presence of $1 \mu\text{M}$ Aβ at different time intervals. The white arrow marked in the 18h panel shows the pore-like features caused by Aβ. For AFM images the bilayer is prepared on freshly cleaved mica by the LB-VF method and imaged under the liquid. AFM courtesy of Dr. Nirod Kumar Sarangi.

Impedance spectra were collected every 30 minutes and showed a systematic increase in resistance and decrease in capacitance of DOPC membrane which equilibrates within 2 h, beyond which the signal remain constant. Figure 4.3 A and B (grey) shows the temporal evolution of relative changes in membrane resistance and capacitance respectively for pristine DOPC, after electrochemical equilibration, which indicate that the membrane is stable. After initial MSLB equilibration, $1 \mu\text{M}$ Aβ in its monomeric form was added to the contact buffer

and impedance measurements were sampled for at least 18 h. The corresponding relative change in resistance and capacitance with respect to equilibrated membrane are shown in Figure 4.3A and B (orange) respectively. Although a modest fluctuation of membrane resistance was observed within 1-2 h of A β introduction, within experimental error, the overall change in resistance remain undistinguishable from that of pristine membrane for at least 10 h suggesting limited interaction/binding of A β to the DOPC membrane. Beyond 10 h of A β incubation, the resistance starts to decrease. The capacitance on the other hand does decreases systematically over the experimental window after exposure to A β . This suggests some weak association of A β at the membrane occurs over time. Our results are consistent with a previous report, where the A β monomers were observed to weakly bind to zwitterionic DOPC bilayer.¹⁹ It is worth mentioning that a systematic but modest decrease in capacitance of the pristine DOPC membrane (grey, Fig. 2B) also occurs over the course of 24 h in absence of A β , which may be attributed to impact of HEPES buffer that has been reported to cause the membrane to rigidify.^{61,74} Nonetheless, the effect is greater in the presence of A β but it is difficult to decouple the contribution of both effects. Therefore, to gain more insight, we carried out complementary measurements with atomic force microscopy (AFM) to visualize any changes caused by A β to the DOPC membrane within the time scale of EIS measurements. EIS gives a global outlook on to what is happening across the array, whereas AFM allows visualization of the topological changes across on the array. AFM was performed on flat mica as due to the tapping mode used to obtain the AFM images, the bilayers on the MSLBs were found to be damaging and the smoother surface was easier to gather images and small changes by AFM. By using a flat mica array for imaging, the time scales to EIS cannot be directly compared as the fluidity of the membrane is hindered and thus the timescale of A β aggregation will likely differ, possibly slowed down. In line with EIS, the membrane remains fairly homogeneous in presence of A β peptide up to 10 h with some modest heterogeneity after 15 h, evident in the membrane topography, with what appear to be a small number of nanoscale inclusions (cf. white arrow mark, Figure 4.3C). This modest extent of the perturbation and time-scale correlates well with observations from EIS resistance drop.

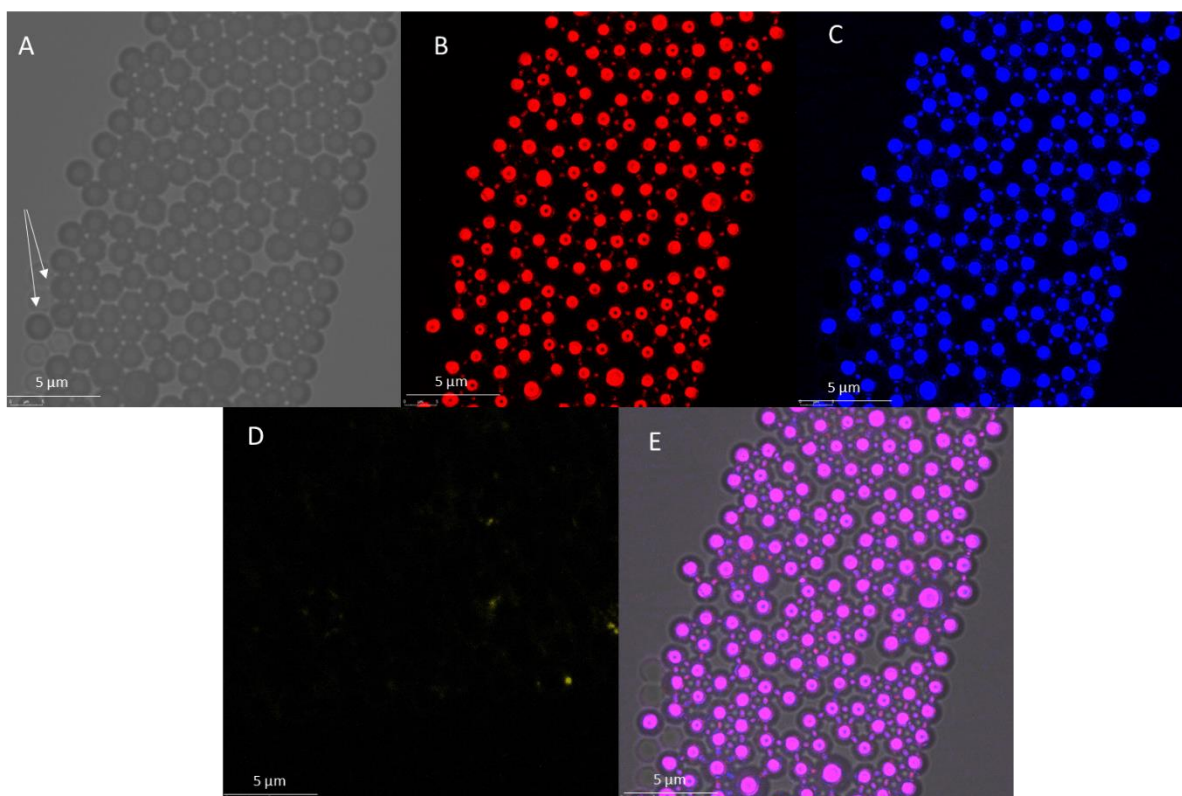


Figure 4.4: Confocal images for symmetric DOPC bilayer containing DOPE-Atto 655 with 1 μM $\text{A}\beta$ incubation after 3 hours. A) Reflectance image of the PDMS microcavities. White arrows mark cavities that did not fill with buffer B) Confocal image of DOPC membrane labelled with DOPE-Atto655 excited at 655 nm with detection window 665-780 nm. C) Confocal image of 1 μM $\text{A}\beta$ 555 incubated with the DOPC membrane, excited at 555 nm with detection window of 565 to 630 nm. D) Pyranine image focused on the membrane above cavities, excited at 405 nm, detection window of 440 - 470 nm. E) Overlay of 4 confocal images together indicating that the $\text{A}\beta$ is interacting with the membrane. Scale bar on bottom left of each image is 5 μm .

Confocal fluorescence microscopy was carried out on DOPC MSLBs supported at PDMS arrays following 3 hours incubation with fluorescently labelled $\text{A}\beta$ ($\text{A}\beta$ -hy555) to confirm that the amyloid does associate with the membrane. In this experiment, prior to the bilayer formation, the cavities were filled with 1 μM pyranine dye which is impermeable to the bilayer and thus expected to remain trapped in the cavity if the membrane is intact.⁶² The reflectance image Figure 4.4A allow clear distinction between cavities that are filled (dark circular regions) with buffer and the small number of cavities that are not buffer filled, (marked with white arrows). Figure 4.4B shows the corresponding confocal fluorescence image for labelled lipid:

DOPE-Atto655 doped at the distal leaflet of DOPC membrane spanned over the buffer/pyranine filled cavities. Figure 4.4C shows the fluorescence image collected from the fluorescently labelled, A β -hy555 and confirms emission from the membrane from this probe, that is further confirmed when the DOPE-Atto655 and A β -hy555 channels are overlaid, Figure 4.4E indicating adsorption of A β at the bilayer interface. Within the 3 hours incubation with A β the distribution of A β -hy555 at the membrane looks uniform and there is no impact on the membrane integrity since the pyranine signal remains intact, originating from the pore with no leakage evident in the contacting solution, i.e. it remains sealed by the bilayer inside the array pores (Fig. 4.4D). The above results agree with EIS and AFM data indicating physical adsorption of the A β at the DOPC membrane without extensive aggregation and the A β did not cause pore-formation or significant membrane damage.

4.3.2 Effects of A $\beta_{(1-42)}$ on an Asymmetric DOPC:DOPS(90:10)//DOPC Membrane

Next, we explored the impact of A β on the electrochemical properties of an asymmetric membrane comprised of DOPC at proximal leaflet and DOPC:DOPS (90:10) at distal leaflet. Phosphatidylserine (PS) is an important physiological lipid localized to the cytosolic proximal leaflet in healthy cells and it is particularly enriched in plasma membranes of neuronal cells. Plasma membrane concentrations of PS range between 5 - 10% (mol/mol) and is present at up to 15% in neuronal cell membranes.⁷⁵ Upon damage to the membrane e.g. following oxidative stress, the PS translocates to the exterior leaflet where it serves as an “eat me” signal for cellular apoptosis.^{76–80} As oxidative stress is a hallmark biomarker of Alzheimer’s this may mean that translocation of anionic lipids serves as a seed reaction for amyloid aggregation in AD.²⁹

A β is present in low femto to picomolar levels in healthy brains but is elevated in Alzheimer disease.^{81,82} Therefore, a binding curve for the A β_{1-42} monomer fragment was determined at asymmetric DOPC:DOPS MSLB array by varying the concentration of A β_{1-42} within picomolar range corresponding to the range expected to lead to oligomer formation, and emulate conditions for the interstitial fluid in the Alzheimer brain.^{2,81,83,84} This is particularly enabled by the high sensitivity of electrochemical impedance spectroscopy. In many studies high micromolar concentrations have been necessary to instigate oligomer/fibril formation, especially in the case of zwitterionic membranes or particularly in solution.^{55,57,71,85–87} More recently, at low but more physiologically relevant concentrations have been used to mimic oligomer formation at complex membranes consisting of cholesterol and/or charged lipids.^{49,70}

Besides the closer physiological analogy, by working within the picomolar range we are well below the critical micelle concentration for the A β and so avoid complications due to adsorption of aggregates at the membrane.⁸⁸

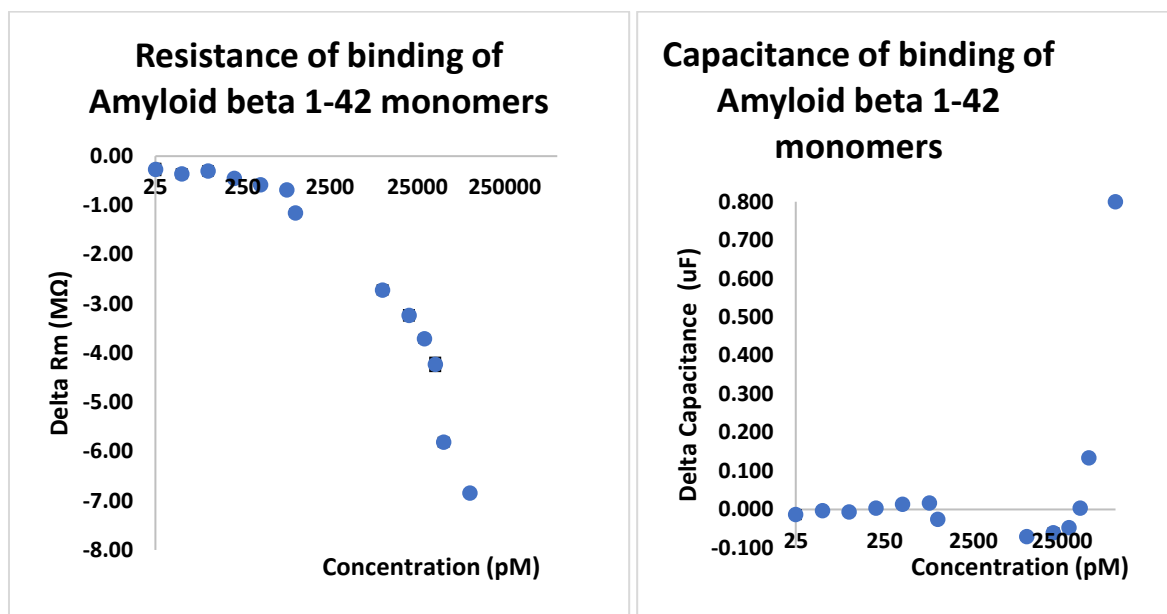


Figure 4.5: Binding Curve of A β picomolar range on a DOPC:DOPS(90:10) membrane. The resistance change is on the left whilst the capacitance change is on the right hand side. Stability of membrane was run for 1.5 hours before addition of the first dose of amyloid concentration. At each concentration of A β , a 30 min incubation was set and after which the next concentration was added. Any change to the membrane impedance caused by A β is normalized with respect to the equilibrated pristine membrane impedance (before any amyloid addition). N=3 and standard deviation included as error bars.

Figure 4.5A shows the relative resistance change of an asymmetric DOPC:DOPS(90:10)//DOPC membrane (in which the distal leaflet contains 90 mol% DOPC and 10 mol% DOPS and the proximal leaflet contains 100 mol% DOPC) upon titration of A β . Figure 4.5B shows the corresponding relative capacitance plot. For each concentration of A β introduced to the contact buffer, 30 mins incubation time was applied, and the induced impedance signal was extracted and normalized w.r.t the pristine impedance value. Incubation of A β at DOPC:DOPS(90:10) membrane over the concentration range 25-200 pM showed a negligible change to either membrane resistance or capacitance. However, upon application of

400 pM A β and above, the membrane resistance proceeded to decrease, this suggests that at 400 pM oligomerization commences or perhaps the limit of detection in our experimental set-up for oligomerization process to occur. Since oligomerization and fibril formation at bilayer interface is a slow process even at elevated (micromolar) concentration, the decrease in resistance after 30 min incubation could be due to partial membrane insertion, which maybe a first step in the oligomerization. Further addition of 800 pM and 1 nM A β resulted in dramatic decreases to membrane resistance, indicating significant interaction of A β with the anionic membrane. The bilayer capacitance on the other hand remains unchanged across the concentration range 400 pM to 1nM. Together the decrease in resistance without change to capacitance (which reflects changes to bilayer thickness) suggests some insertion of monomeric A β into the membrane, leading to increased membrane admittance. The monomeric A β 1-42 is known to have amphiphilic properties, and this is consistent with surfactant behaviour at the membrane. After addition of 10 nM A β , a notable drop in resistance to 2.7 M Ω is accompanied by a modest decrease in capacitance indicating both increased permeation of the membrane and modest thickening of the bilayer. As the concentration of amyloid increased in the nanomolar range above 10 nM, the resistance continued to decrease sharply, whilst the capacitance now increases. This indicates that the membrane is being damaged at higher concentrations of amyloid. Since our titration reveals a significant change in bilayer impedance data at higher concentrations, wherein the capacitance increased to $\sim 0.8 \mu\text{F}$ and the resistance decreased to -6.84 M Ω , this is suggestive of pore formation, likely indicating that oligomerization of amyloid is underway from 400 pM. The titration revealed that a binding curve was undeterminable due to the nature of the A β beginning to form oligomers at higher concentrations and as such the electrochemical cell was not in full equilibrium upon the addition of the next concentration after 30 minutes. We thus extended the incubation period of A β with membrane at different fixed concentrations of 100 nM, 1 μM and 2 μM to dynamically follow the oligomerization process at the DOPC:DOPS asymmetric membrane.

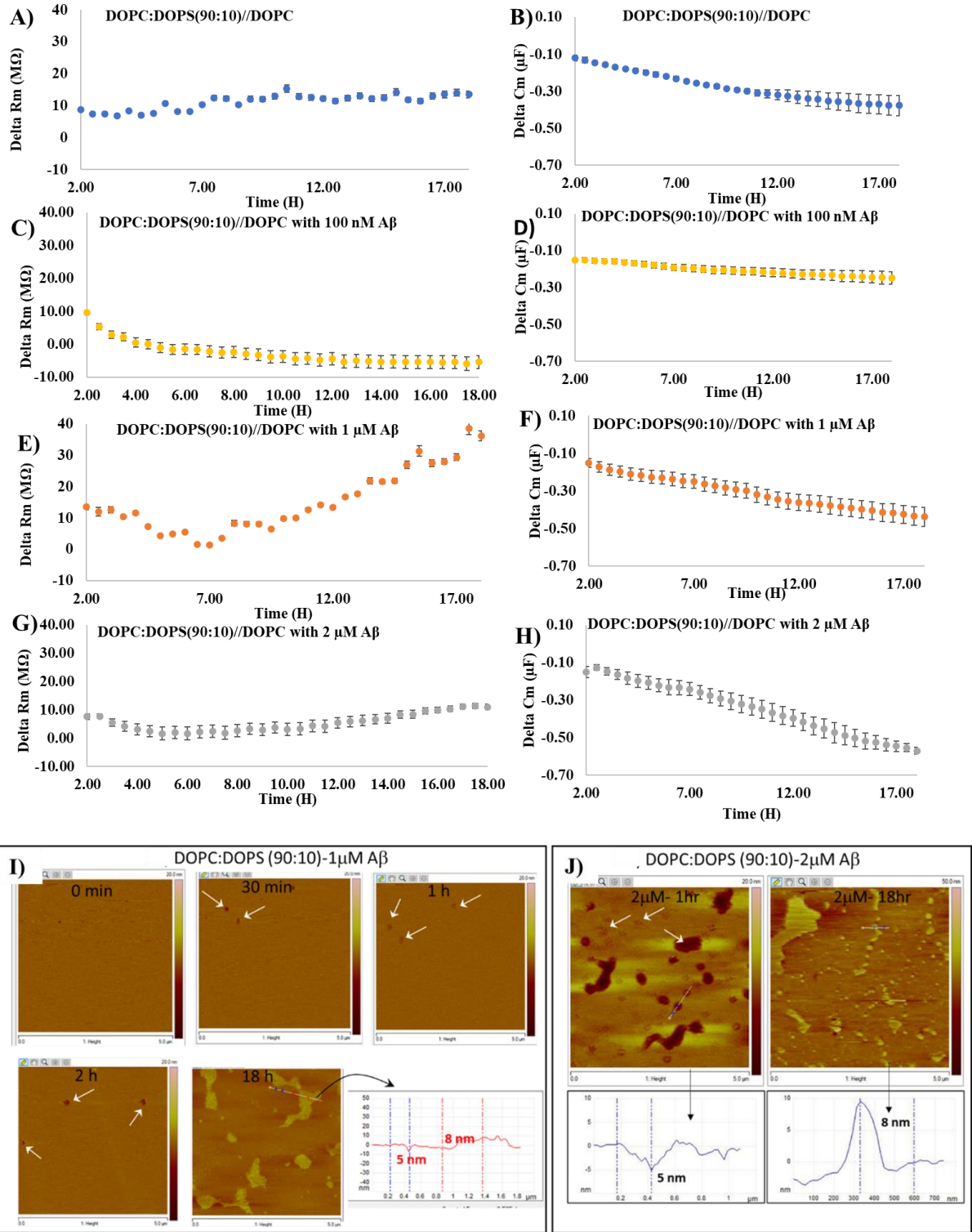


Figure 4.6: Relative resistance and capacitance changes DOPC:DOPS(90:10)//DOPC asymmetric bilayer. A) The resistance stability of asymmetric DOPC:DOPS(90:10) lipid bilayer in the absence of Aβ. B) The capacitance stability of asymmetric DOPC:DOPS(90:10) lipid bilayer in the absence of Aβ. [stability=blue] C) Illustrates the resistance effects of the

addition of 100 nM A β . D) Illustrates the capacitance effects of the addition of 100 nM A β . [100 nM A β = yellow] E) Illustrates the resistance effects of the addition of 1 μ M A β . F) Illustrates the capacitance effects of the addition of 1 μ M A β . [1 μ M A β = orange] G) Illustrates the resistance effects of the addition of 2 μ M A β . H) Illustrates the capacitance effects of the addition of 2 μ M A β . [2 μ M = grey] All samples were in HEPES buffer at pH 7.4 over 24 hours. Experimental range of 1.5 to 18 hours shown. Initial 1.5 hours are bilayer stabilization before addition of desired amyloid concentration addition, $n = 3$ for all concentration ranges, error bars are the standard error across the samples. Temporal evolution of topographic AFM images along with height profile analyses of asymmetric DOPC:DOPS (90:10) bilayer deposited on mica before and after incubation with I) 1 μ M A β and J) 2 μ M A β . The image size in panel I and J were 5 $\times$ 5 μ m². The white arrow in panels I and J indicates A β induced lipid disruption. The line profile analyses are indicated by black arrow sign corresponding to the line cross-section in the respective image. All images are acquired under liquid and the membrane was prepared via LB-VF method.

Figure 4.6A shows a representative stability profile of an asymmetric bilayer comprised of DOPC:DOPS(90:10) in the outer leaflet and DOPC at the inner leaflet. Electrochemical equilibrium was achieved within two hours of preparing the bilayer and we observed a small rise in resistance and a drop in capacitance (these data points are not included in Fig. 4.6). For our A β ₁₋₄₂ binding and assembly studies, we injected the peptide after the membrane had been equilibrated for 2 hours. The data show that the membrane was stable for 22 h, similar to the pristine DOPC bilayer. And, in prior work it was demonstrated that asymmetric DOPS MSLBs, containing DOPS on the inner leaflet only, are stable toward transversal DOPS diffusion for at least 19 hours.⁶¹ At each concentration of A β (100 nM, 1 μ M and 2 μ M), the membrane resistance dropped as seen in Figure 4.6C, E and G for at least up to 6-7 h of incubation. The large drop in resistance can be linked to membrane damage consistent with detergent-like behaviour of A β leading to damaging of the membrane, loss of lipid or may alternatively suggest pore formation, but is inconsistent with the carpet model.²¹⁻²³ Such a large resistance drop was not observed at the pristine DOPC membrane (without DOPS), and implies that the anionic charge of the DOPS on the exterior facilitates the observed effect. This may further suggest, consistent with previous observations, that PS in the bilayer triggers a catalytic effect leading to A β aggregation on the membrane.^{36,40} In the case of 100 nM A β , the membrane resistance, as shown in panel Figure 4.6C, beyond 6-7 h, exhibited a further

exponential decrease in resistance but to a lesser extent which levels off, indicating that the binding of the amyloid with the DOPS on the exterior can cause membrane damage or pore formation persisted without further concentration dependent aggregation at the bilayer interface. Damage is also inferred in the capacitance data since A β negligibly impacts the membrane thickness (Figure 4.6D). Over the same experimental window in the absence of A β , the membrane capacitance decreased by roughly 0.5 μ F. In the presence of 100 nM A β , a change of 0.15 μ F was observed. The overall trend, a modest decrease in capacitance, for both the pristine DOPC and the exterior containing DOPS membranes in presence of 100 nM A β is speculated to be due to membrane ordering effect caused by HEPES buffer as observed in our earlier study.⁶¹ Increasing the A β , in the electrochemical cell to 1 μ M the resistance dropped over the first 6 to 7 hours. After which a recovery of resistance that exceeded the initial membrane was observed. (Figure 4.6E). We speculate, supported by the AFM data below, that this is due to formation of large-scale A β oligomers at the bilayer interface. A large decrease in membrane capacitance (Figure 4.6F) accompanies the resistance recovery and this is indicative of membrane thickening, again attributed to oligomer deposition at the membrane interface. The result suggests that an A β concentration of 1 μ M A β is sufficient for A β oligomerization at DOPC:DOPS (90:10) membrane, in which DOPS is on the exterior. When 2 μ M A β is present in the contact buffer, the recovery in resistance (oligomer formation) commences after a shorter window of resistance decrease 4-5 hours (vs. 6-7 hours for 1 μ M A β) suggesting the kinetics of oligomer formation is faster. Notably, the final resistance recovery did not exceed the initial membrane resistance, this may be due to the concomitant damage that the resulting oligomers are inflicting on the membrane at the higher A β concentration. Although, the trend toward decreasing capacitance was observed for all A β concentrations over long timescales (Figure 4.6), on initial addition of 2 μ M A β the capacitance was observed to initially increase over the first 1-2 hours before the capacitance proceeded to systematically decrease up to 18 h (cf. Figure 4.6H). This initial increase may be due to the removal of bilayer patches due to high concentration of A β (2 μ M). Comparing the resistance trend at lower A β concentrations (0.1 and 1 μ M) to the magnitude of decrease in relative resistance for 2 μ M A β , the latter was lower, but the kinetics of the process was faster which is reflected by the resistance plateau at 4 hours.

The EIS data suggests membrane damage occurs at high A β concentrations, potentially through lipid erosion or pore formation. AFM imaging was therefore compared at exterior DOPS

containing membranes on incubation with A β concentration of 1 and 2 μ M. Figure 4.6I shows the topographic images of DOPC:DOPS exterior membrane on cleaved mica, before and after incubation of 1 μ M A β at different time intervals. Mica was used over the microcavity arrays for AFM experiments to ensure stability of the membrane for the duration of the experiment. Optimisation of AFM with the use of MSLBs to ensure damage to the membrane does not occur from the technique itself is in progress within the group. Before the addition of A β , the DOPC:DOPS exterior membrane is quite homogeneous (Figure 4.6I, 0 min). Within 30 mins incubation with 1 μ M A β , nanoscale depressions (see arrow in Figure 4.6I) appear on the bilayer surface. The density and dimensions of the features increase over time until 4-6 h (for reference the impact of A β at different time intervals such as 0.5, 1 and 2 hr data are shown in Figure 4.6I). This observation is strongly consistent with EIS data where membrane resistance was observed to decrease, Figure 4.6E. After 18 hours, the bilayer was imaged again and, showed A β oligomer is stacked in patches distributed heterogeneously across the membrane's surface. The patches which are attributed to network-like structures of A β oligomers, protrude with a height of $\sim$ 8-10 nm from the bilayer surface as shown in height profile analyses in the bottom right panel of Figure 4.6I which is consistent with a report by D'Ursi et al. for AFM studies on A β ₂₅₋₃₅ interaction with DOPC and DOPC/DHA bilayers.⁸⁷ The AFM data shows that formation of the A β oligomer network commences after 6 hrs of incubation (data not shown) and continues to grow over 18 hrs which is further consistent with our electrochemical data, in which membrane resistance increased while capacitance decreased.

In contrast, addition of 2 μ M A β to the membrane contacting solution caused dramatic changes to the AFM images of the DOPC:DOPS exterior membrane with extensive pore formation and membrane damage within just 1 hour of incubation (cf. Figure 4.6J). At this concentration, A β seems to exhibit detergent-like behaviour removing patches of membrane from the bilayer surface and creating nanoscale pore like features with dimensions of (10-30 nm). The regions where bilayer patches are completely removed are shown in dark (Figure 4.6J, left). The line profile analyses (bottom left, in panel J) distinguish two effects: where areas of lipid removal with a height difference between mica and the top surface membrane of 5 nm. This was consistent with the known thickness of DOPC membrane. Whereas circular pore like regimes with a depression of 1.6 nm from the bilayer surface is also observed. At the centre of these circular features, globular A β aggregates with lateral dimensions of $\sim$ 4-6 nm, can be distinguished, likely the oligomeric seed. These observations correlate well with the EIS where

within 0-2.5 h of incubation, the capacitance increases due to membrane thinning. Interestingly after 18 h of incubation, the minimal oligomer size of an aggregate amyloid seed is grown laterally and covered up to $\sim 20\%$ of the bilayer surface. The representative image of the layered aggregates covering the bilayer surface protruding a height of $\sim 8\text{nm}$ is shown in Figure 4.6J (right panel). These AFM results which were completed over multiple replicates, further correlates with the EIS data, where the resistance recovery as a result of oligomeric growth and drop in capacitance causing the membrane to thicken. Again, the AFM images are fully consistent with the EIS data where upon increasing the concentration of A β from 1 to 2 μM , the aggregation and oligomerization capacity is increased in DOPS exterior contained membrane. Combined, the data agree well with the hypothesized theory illustrated in Figure 4.1 that the interaction of A β at the negatively charged membrane follows initial binding, pore-formation, lipid dissolution and growth in oligomer formation and also provides additional insights towards the concentration dependency and the time scale of A β oligomer formation.

To investigate further pore formation by 1 μM A β to the DOPS exterior containing membrane, confocal imaging was carried out. Figure 4.7 shows the impact of 1 μM A β after 3 hours on an asymmetric DOPC:DOPS(90:10) membrane. Figure 4.7A is the reflectance image of the microcavities filled with buffer/pyranine. Pyranine is a membrane impermeable probe and cannot diffuse across the membrane. Figure 4.7B is the corresponding confocal image obtained from DOPE-Atto655 lipid label doped to the distal leaflet indicating that the membrane has properly formed over the buffer filled cavities. The association of A β -hy555 to the membrane is shown in Figure 4.7C. As expected, pore-like features could not be distinguished due to diffraction limit of optical microscopy. However, evidence of pore-formation can be gleaned by monitoring for leakage of pore encapsulated pyranine by focusing on the membrane plane (Figure 4.7D). From both panel Figure D and E, it is evident that upon 1 μM A β -hy555 after 3 hours incubation, pyranine dye which was sealed by the bilayer, extensively leaks from the cavities. This is clear comparing these images with those of the pristine DOPC membrane, shown in Figure 4.4D, where now pyranine emission is seen from areas across the top surface.

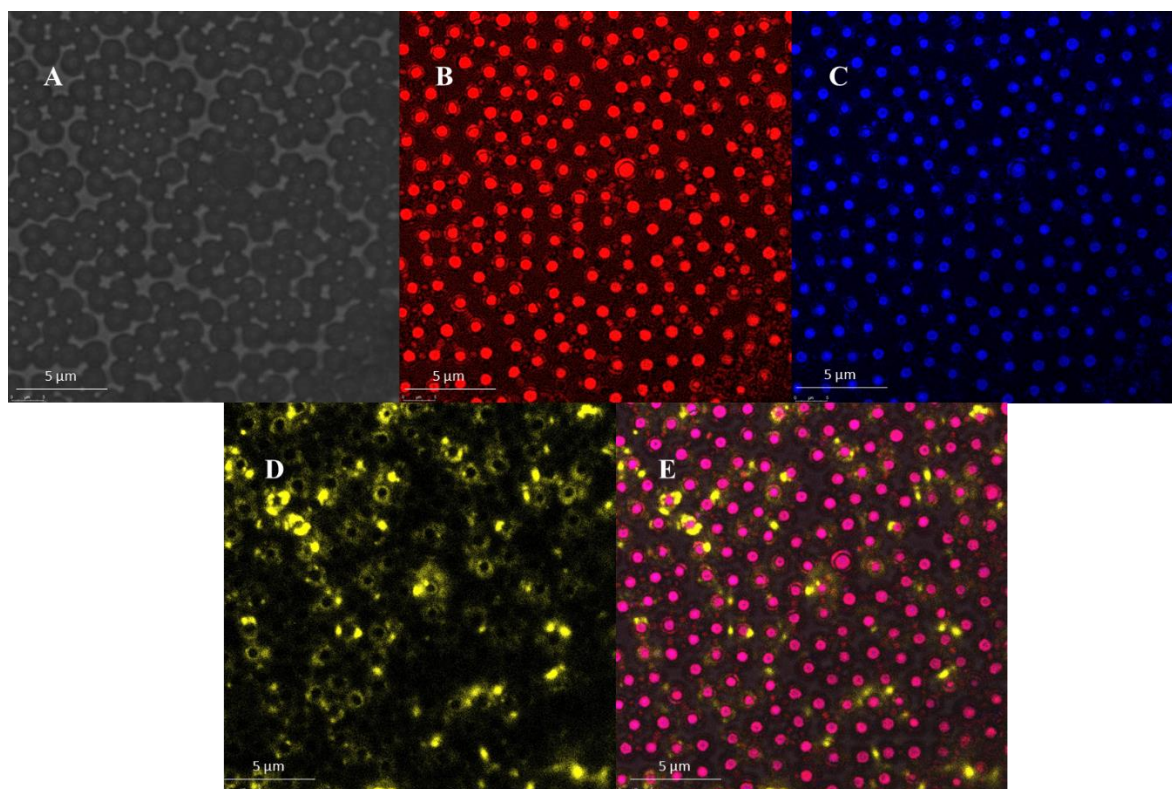


Figure 4.7: Confocal microscopy images of asymmetric DOPC:DOPS(90:10) bilayer spanned across PDMS microcavity array after 3 hr incubation with 1 μ M A β -Hy555. A) Reflectance image of the PDMS microcavities. Confocal image of asymmetric DOPC:DOPS membrane showing the fluorescence signal of labelled B) DOPE-Atto655 excited at 655 nm with detection window 665-780 nm and C) A β -Hy555 excited at 555 nm with detection window of 565 to 630 nm. D) Pyranine fluorescence image obtained at the membrane plane, excited at 405 nm with detection window of 440 -470 nm. E) Overlay images showing the association of A β -Hy555 to the bilayer surface along with the adsorbed pyranine leaked from the cavity. In each panel the scale bar was 5 μ m.

According to the hypothesized mechanism described in Figure 4.1, when A β aggregates lead to large scale oligomers/fibrils, they can be released from the membrane and into the contact solution.^{55,89,90} To explore this, the contact solution from the electrochemical experiments after 24 h of A β incubation with the membrane were collected and studied via UV/Vis and Fluorescence and treated with thioflavin T (ThT). ThT is a fluorescent probe is known to bind specifically to amyloid fibrils/aggregates, but not to the amyloid monomers. In the absence of amyloid, the absorbance of ThT alone in HEPES buffer was 411.5 nm and the emission was $\sim$ 480 nm. However, when ThT is added to the contact buffer from EIS studies, along with the

emission peak at 480 nm, another red shift peak at 510 nm appears (Figure 4.8). The later band corresponds to the ThT tightly bound to the A β fibrils/aggregates^{91,92} indicating that fibrils/oligomers are released from the membrane into the contact solution after 24 hours incubation of 1 μ M A β with the asymmetric DOPC:DOPS(90:10)//DOPC membrane.

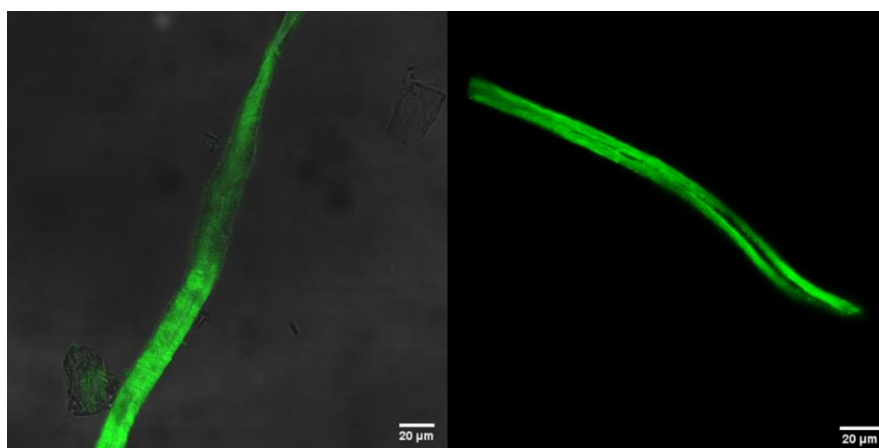
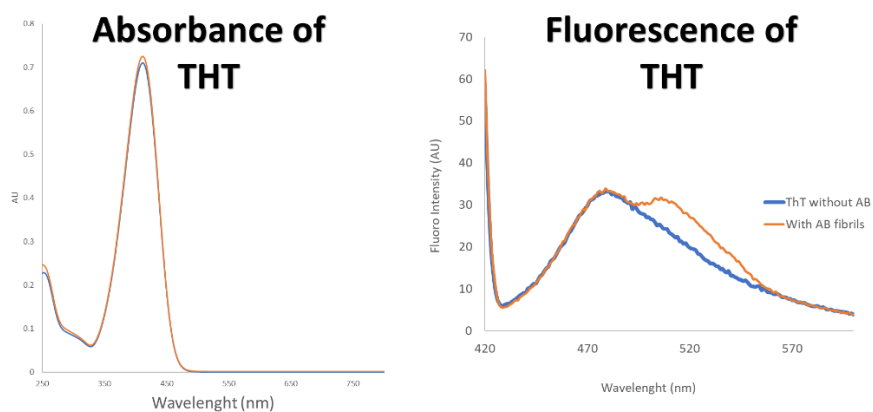


Figure 4.8: Absorbance (left) and emission (right) of ThT with (orange) and without (blue) the contact solution from electrochemistry containing A β . (HEPES buffer solution containing A β after EIS run at pH 7.4) For emission results, solution was excited at absorbance maximum. $N = 3$. The contact solution was then analysed with Confocal imaging. Excited at 405 nm, detection window 430 – 580 nm.

4.3.3 Effects of DOPS Concentration within Asymmetric DOPC:DOPS//DOPC Membrane

Given the observed A β concentration dependence on oligomerization pathways at pristine DOPC and the asymmetric DOPC:DOPS(90:10) membrane, we extended our study and evaluated the impact of DOPS content on oligomerization. Initially reducing DOPS was to mimic a damaged or aging cell content of DOPS at the outer leaflet which reflects translocation from interior to the exterior leaflet of cellular membrane. For membrane's containing 5 mol%

in the distal (exterior) leaflet the stability was again confirmed (Figure 4.9A, B) post 2 h equilibration to 18 h.

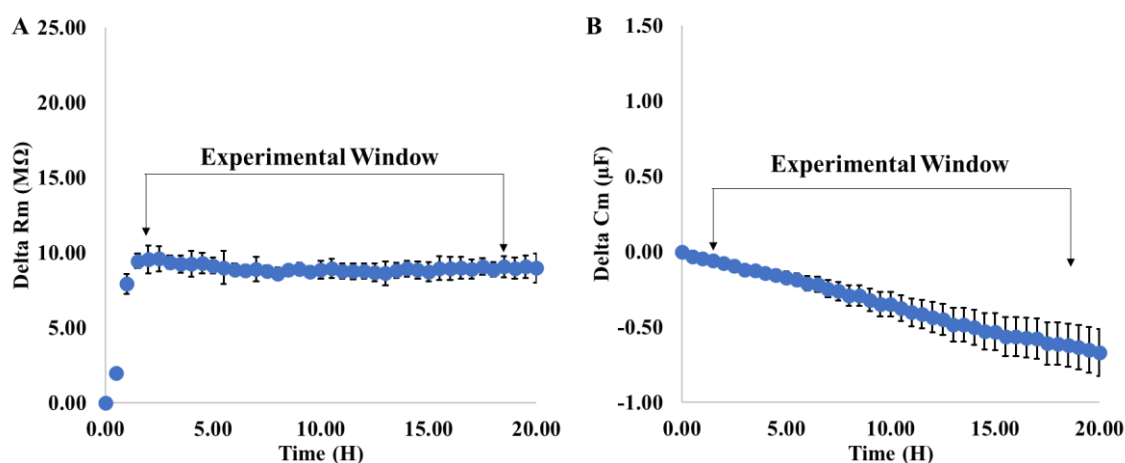


Figure 4.9: A) Relative resistance changes and B) capacitance on a DOPC:DOPS(95:5)//DOPC asymmetric bilayer stability in HEPES buffer at pH 7.4 over 24 hours. Equilibration of lipid bilayer over the initial 1.5 hours and then experimental window is shown between 2 and 18 hours. $N=3$ and standard error is shown using error bars.

5 mol% DOPS on the exterior of the membrane did not elicit oligomer formation when incubated with 1 μM A β . The resistance decreased over time with no evidence of recovery. (Figure 4.10A, orange). This is in contrast to what was observed at 10% DOPS on the exterior of the membrane (cf. Figure 4.6E). Simultaneously, the membrane capacitance increased (Figure 4.10B, orange) suggesting thinning of the membrane. This is further confirmed by AFM images described in Figure 4.10E which reveal pores but no evidence of aggregates or layers after prolonged incubation times. Combined EIS and AFM data indicate that the membrane is damaged by pore formation at 1 μM A β for the DOPC:DOPS (95:5) membrane without concomitant fibril/oligomer formation.

Incubation of 2 μM A β with 5 mol% DOPS on the exterior of the membrane, on the other hand shows resistance decreases over 3-4 h and then recovers (grey, Figure 4.10A). Simultaneously membrane capacitance decreases modestly after 4 h (grey, Figure 4.10B) suggesting thickening of membrane or layer formation. The resistance and capacitance profiles are reminiscent to those for 1 μM A β at 10 mol% DOPS on the exterior of the membrane (*vide infra*). Suggesting that more A β is required to form layered aggregates when DOPS content is reduced.

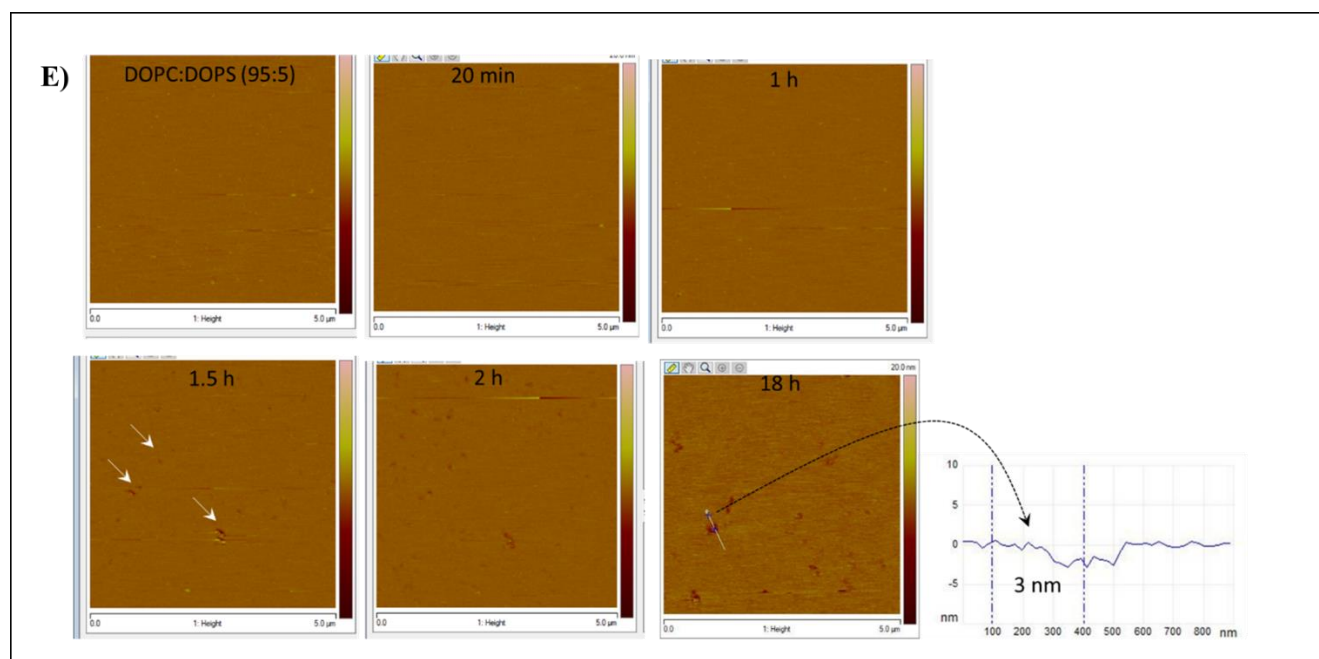
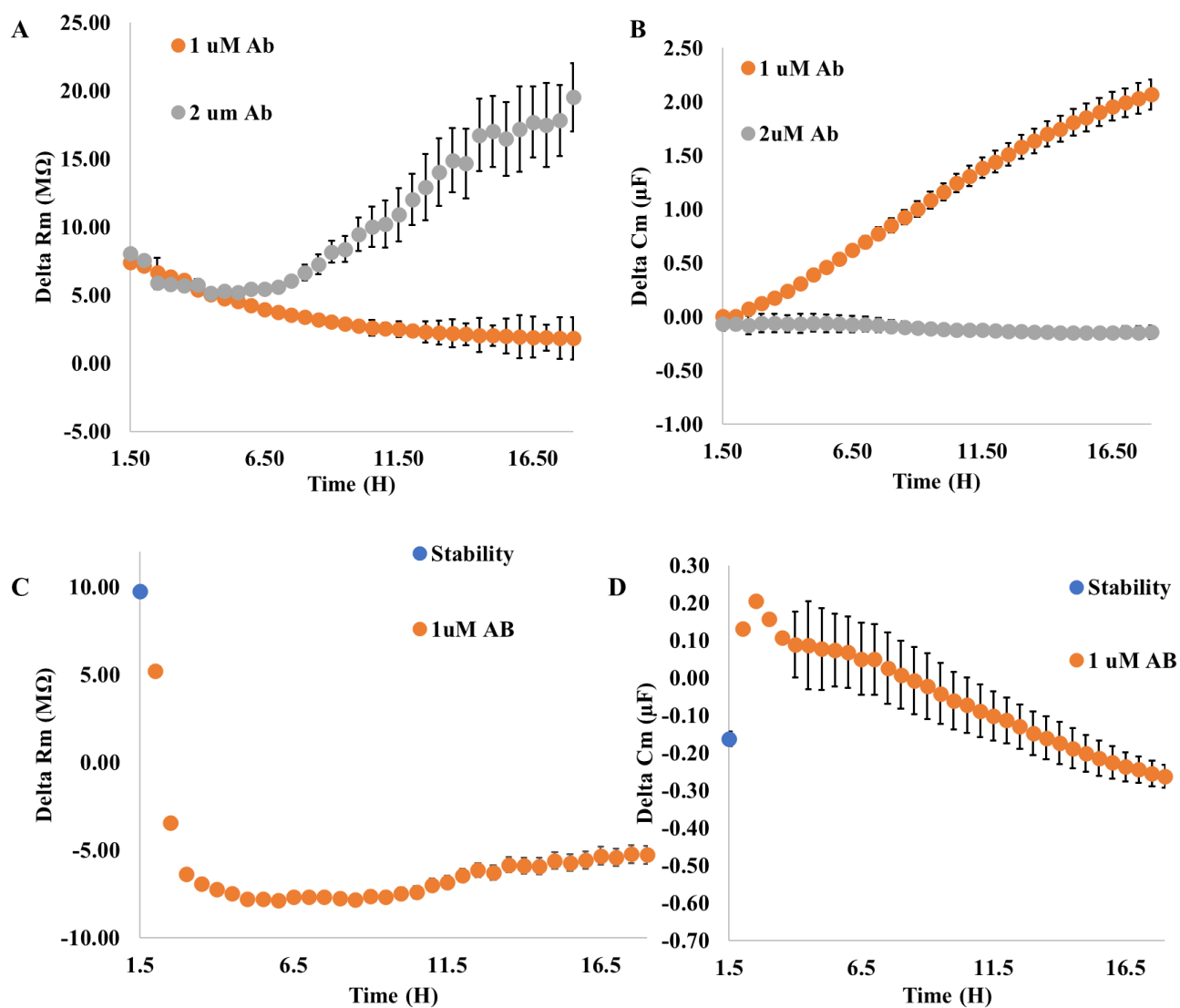


Figure 4.10: A) Relative resistance changes and B) capacitance on a DOPC:DOPS(95:5)//DOPC asymmetric bilayer with the addition of Amyloid Beta (1 or 2 μM) in HEPES buffer at pH 7.4 over 24 hours. Equilibration of lipid bilayer over the initial 1.5 hours and then Amyloid added. Figure shows time points after the addition of 1 μM Amyloid Beta (orange circles) & after the addition of 2 μM Amyloid Beta (grey circles). (C-D) Relative changes of DOPC:DOPS(80:20)//DOPC asymmetric bilayer membrane (C) resistance and (D) capacitance upon addition of Amyloid Beta (1 μM) in HEPES buffer at pH 7.4 over 24 hours. Equilibration of lipid bilayer is reached after initial measurements of bilayer for 1.5 hours (blue) and following which $A\beta$ was added to the contact buffer and the impact of $A\beta$ to the EIS response are shown in orange symbol. after 1.5 h. $N = 3$ and error bars are standard error. E) Temporal evolution of change in DOPC:DOPS (95:5) membrane topography upon incubation of 1 μM $A\beta$. The pore like features can be visualized at or above 1.5 hr window. All images are taken under HEPES at $22 \pm 1^\circ\text{C}$. The image size was $5 \times 5 \mu\text{m}^2$. AFM courtesy of Dr. Nirod Kumar Sarangi.

At membranes containing 20 mol% DOPS on the exterior of the membrane, 1 μM $A\beta$ elicited a dramatic drop in resistance (Figure 4.10C) within 1 h of incubation remained steady over ~ 9-10 h. Resistance began to recover after this time point but remained low. Simultaneously capacitance of the membrane increased modestly within 1 h then systematically decreased (Figure 4.10D) suggesting the formation of layered aggregates. Overall, the data suggest that the rate and extent of oligomer formation are dependent on concentration of both DOPS and $A\beta$. When the membrane concentration of DOPS is decreased on the exterior, which is anticipated in for the aged brain, a high content of $A\beta$ is required for the formation of oligomers, whereas when the concentration of DOPS is increased, oligomers formation commences at a relatively low $A\beta$ concentration.

4.3.4 Effects of $A\beta_{(1-42)}$ on an Asymmetric DOPC//DOPC:DOPS(90:10) Membrane Utilizing optothermal damage.

Varying the concentration of DOPS (20,10 and 5 mol%) in the proximal (inner) leaflet instead of distal (exterior) leaflet (as discussed in previous section), we attempted to mimic the PS translocation that occurs in oxidative stress, apoptosis and aging of the brain. This was accomplished by optothermally damaging the membrane with a laser to initiate the flip flop effect. In the absence of photodamage, DOPC//DOPC:DOPS(90:10) bilayers were found to be stable for at least 10 hours, as shown previously by us while assessing the transversal

diffusion of DOPS (from the interior to the exterior) by annexin.⁶¹ After initial equilibration, the MSLB was irradiated with a 405 nm laser, 3.12 mW power for 10 seconds.⁶¹ As reported previously, for all DOPS concentrations used in this study, irradiation of the membrane led to a decrease in resistance. This confirms an increase in membrane admittance associated with bilayer annealing and increased local fluidity. Correspondingly, the capacitance does not change dramatically with this process. When 20 mol% DOPS was present at the proximal side of the membrane, the resistance decreased dramatically upon the addition of 1 μ M A β after photothermal damage (Figure 4.11A). This observed effect was similar to the case when 20 mol% DOPS was present on the distal site of asymmetric membrane (Figure 4.10C). Within 1-2 hr the corresponding capacitance, Figure 4.11D, increases but then systematically decays up to 18 h. As expected, the magnitude of decrease in capacitance is not the same irrespective of whether 20% DOPS is isolated to the proximal or distal leaflet. Since DOPS transversal migration from DOPC//DOPC:DOPS membranes will be much less concentrated than that of DOPC:DOPS//DOPC membranes, it is not surprising to see the observed capacitance differences. However, the former membrane's DOPS concentration is likely to be very high at the point of laser focus and thus can rapidly seeds the amyloid binding and aggregation causing immediate damage to the membrane.

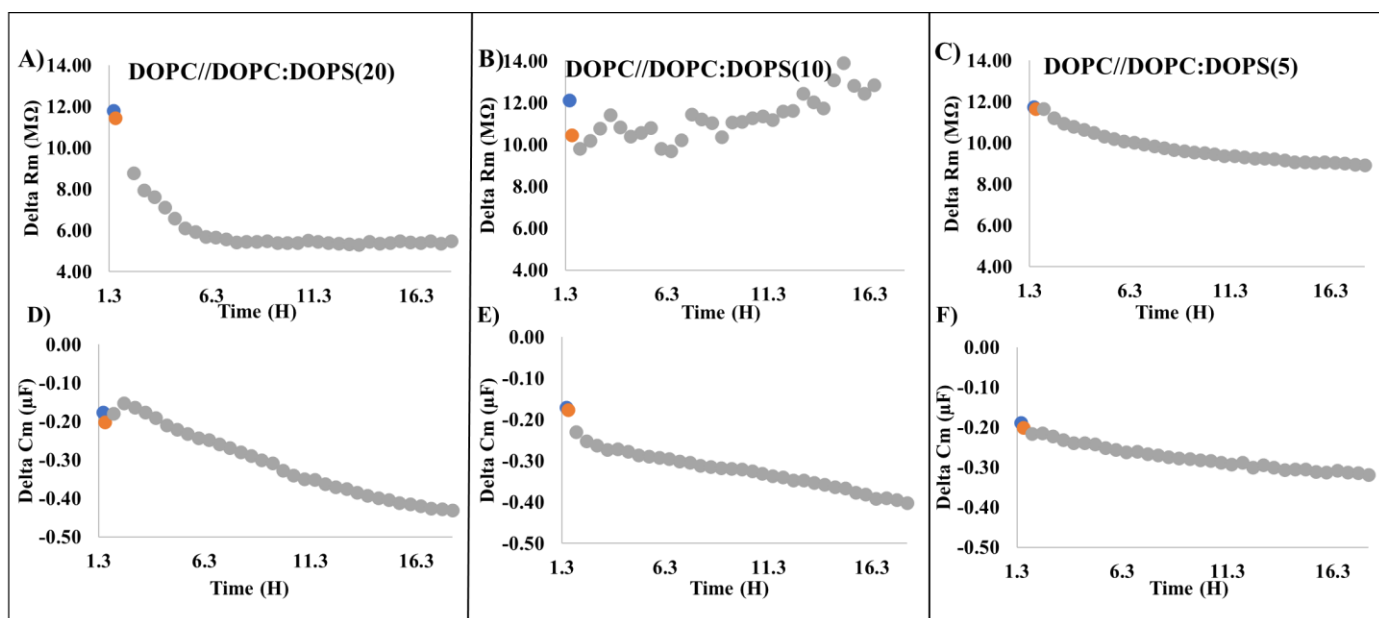


Figure 4.11: Representative relative resistance (A-C) and relative capacitance (D-F) changes on the effect of DOPC//DOPC:DOPS asymmetric bilayers, A & D) DOPC//DOPC:DOPS(80:20), B & E) DOPC//DOPC:DOPS(90:10) & C & F)

DOPC//DOPC:DOPS(95:5), upon addition of 1 μ M A β after photodamage in HEPES buffer at pH 7.4. Blue circles: Bilayer left to reach equilibrium [for clarity only the last time point of stabilization is show]. Orange circles: Representative relative resistance of the bilayer after 10 s of 405 nm photodamage. Grey circles: Amyloid beta 1 μ M addition and incubation with bilayer after DOPS flip-flop. $n = 3$ for all DOPS concentrations.

When 10 mol% DOPS is present at the proximal leaflet, laser damage induced a modest increase in membrane resistance, although the resistance change fluctuated over this incubation window (Figure 4.11B). This is surprising as in all other type of membranes or at different concentrations of A β , we did not observe this behaviour. The trend of decreasing capacitance persisted (Figure 4.11E) over multiple replicates. We speculate that transient A β interactions at the exposed DOPS and fast flip-flop rate may explain this dynamic interaction and tentatively attribute the observed electrochemical changes to competing effects of membrane damage/pore formation and rate of A β aggregation. In contrast to 20 and 10 mol% of DOPS, when 5 mol% DOPS (Figure 4.11C) was present at the proximal leaflet of the membrane, after photothermally initiation, a slow decrease of membrane resistance was observed, similar to the case when 5 mol% DOPS was present at distal leaflet (Figure 4.10A). The capacitance change however showed the opposite trend suggesting the membrane is thickening. This suggests the increase in local DOPS content likely facilitates a faster A β binding and integration within the membrane (Figure 4.11F). Note that none of the membranes containing DOPS isolated to the lower leaflet showed evidence of any large scale oligomerization, as no recovery in resistance is evident, with the exception of a modest overall increase in resistance for 10 mol% DOPS (Figure 4.11B).

4.4 Conclusions

Microcavity supported lipid bilayers comprised of symmetric DOPC, asymmetric bilayers composed of DOPC:DOPS//DOPC (with varying DOPS concentrations on the exterior leaflet) and DOPC//DOPC:DOPS (with varying DOPS concentrations on the interior leaflet) were evaluated for Amyloid beta 1-42 binding and aggregation using electrochemical impedance spectroscopy, AFM, and confocal fluorescence microscopy.

At the pristine DOPC membrane, A β appears to adsorb to the zwitterionic membrane but aggregates were not observed to form even at 1 μ M of peptide. Using a membrane integrity

test with pyranine, we observed that the DOPC membranes remained intact and impermeable to the probe in the presence of A β . This is highly suggestive of the carpet model, in which the A β absorbs with the phospholipids of the membrane.

The presence of anionic DOPS in the membrane dramatically alters the way A β interacts with the membrane. Oligomerization of A β monomers was observed through EIS, AFM, and confocal microscopy when DOPS was exposed at the outer membrane leaflet. There was evidence of both pore formation (at short time frames) and oligomerization with extensive membrane-oligomer multilayer binding and A β fibril formation (3 to 18 hours). The extent of oligomerization and membrane damage was depended on the concentration of both DOPS in the membrane and A β in the contacting solution. The aggregation of the peptide was fastest at highest amyloid and DOPS concentrations and the extent of membrane damage increased with A β concentration. The data suggested that the pore formation model and the dissolution model of membrane-A β interaction occur but what model prevails is dependent on the concentration of both A β and DOPS.^{16–18}

In a simple model of PS translocation that occurs in damaged neuronal membranes associated oxidative stress and apoptosis, asymmetric membranes with DOPS localized to the lower leaflet were prepared and, DOPS lipid transversal migration was instigated optothermally to create local regions of high concentration of DOPS. Our data indicated that at higher concentrations of DOPS (>10 mol%) within the lower leaflet, migration of anionic lipids can seed A β aggregation. DOPS appears to have a catalytic effect on A β oligomerization.

4.5 References

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Chapter 5: Galectin-3 binding to conformationally different, resting, and activated, $\alpha_5\beta_1$ integrin in pore suspended biomembranes.

5.1: Introduction

Galectins are present in many forms of life and in humans they have a wide range of functions which are linked to immunity and disease, cell migration, cell signalling and early developmental processes. Galectins are a family of 15 soluble proteins, namely lectins, that contain a highly conserved carbohydrate-recognition domain (CRD) that recognise carbohydrate binding sites such as β -galactosides.¹ Galectins therefore can recognise, a range of glycosylated proteins and lipids at the surface of cells or in the extracellular matrix, including integrins. Unconventionally secreted at the extracellular milieu, galectins can be classified according to three subtypes, i) the prototype group, ii) the tandem repeat group and iii) the chimera group.²⁻⁸

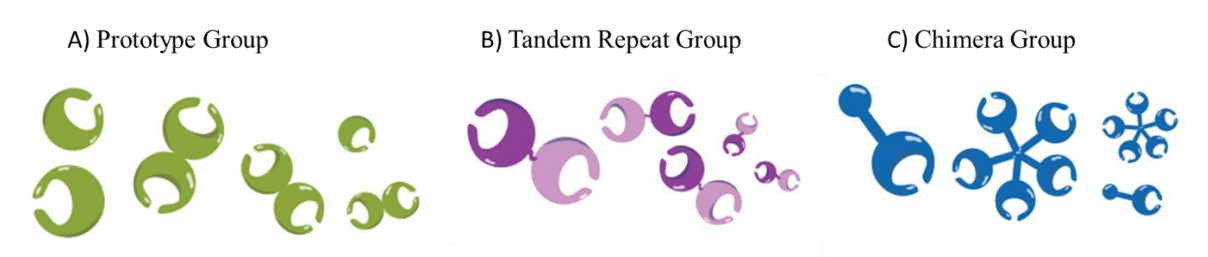


Figure 5.1: Galectin family groups. A) Prototype galectins, containing one carbohydrate-recognition domain (CRD) Galectins 1,2,5,7,10, 11, 13, 14 & 15. B) Tandem-repeat-type galectins, which contain two distinct CRDs in tandem, connected by a linker, Galectins 4, 6, 8, 9 & 12 and C) Chimaera-type galectin consisting of unusual tandem repeats of proline- and glycine-rich short stretches fused onto the CRD, Galectin 3.⁹

The prototype group, Figure 5.1A, has one carbohydrate-recognition domain, whereas the tandem repeat group, Figure 5.1B, consists of two carbohydrate-recognition domains separated by a linker sequence.¹⁰ Each galectin has individual carbohydrate binding preferences and can be either bivalent or multivalent. Prototype Galectins can exist as dimers, whereas two-CRD galectins have two carbohydrate binding sites meaning they are at least bivalent.^{9,11,12} One of the most studied galectin family members is Galectin-3, (wild type Galectin 3, WTGal3), has an important role, believed in part to be mediated through integrin recognition, in physiological

and pathological phenomena.¹¹ WTGal3, the focus of this chapter, is the sole chimera galectin in vertebrates, Figure 5.1C, and is widely distributed in many tissues.¹³ WTGal3 carries two specific domains, one carbohydrate-recognition-domain, as for all galectin family members, but also an intriguing nonlectin N-terminal domain comprising of proline/glycine/tyrosine-rich repeats. WTGal3 can form pentamers upon binding to multivalent carbohydrates that have reportedly linked with its oligomerization capacity upon binding to surface glyco-receptors, possibly involving liquid-liquid phase separation.^{9,14–18}

WTGal3 is reported to stimulate neutrophil adhesion and migration, spreading of cancer cell lines, and furthermore, promotes focal adhesions turnovers and fibronectin (Fn) fibrillogenesis in tumour cells.^{19–22} WTGal3 has been linked to all sorts of diseases such as various types of cancers, type 1 diabetes, depression and Alzheimer's disease.^{4,13,23–25} Galectin and notably WTGal3 have been directly implicated in the regulation of the cell surface homeostasis of glycoproteins.²⁶ They have been shown to favour the endocytic uptake of these glycoproteins (e.g., $\alpha_5\beta_1$ integrin) via a mechanism that has been termed the glycolipid-lectin or GL-Lect hypothesis.^{22,27,28}

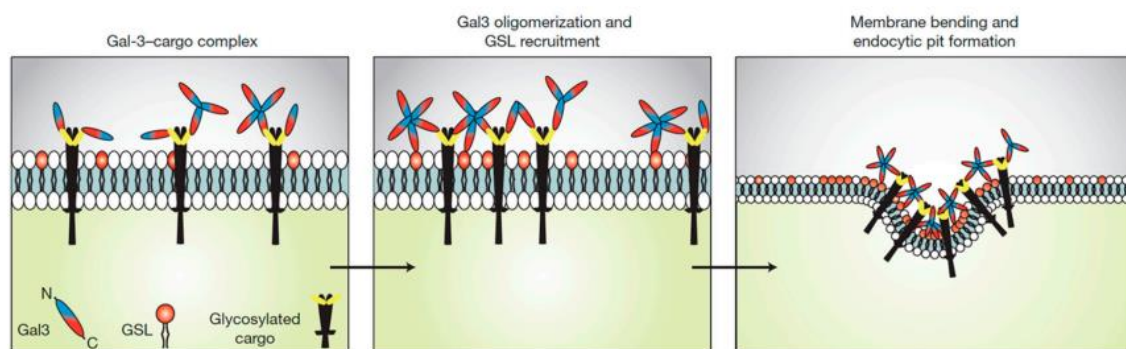


Figure 5.2: Glycolipid-lectin (GL-Lect) hypothesis showing the formation of endocytic pits. The WTGal3 is monomeric in solution and binds to complex carbohydrates of the plasma membrane glycoproteins (glycosylated cargo e.g., integrins). The N terminus of WTGal3 undergoes oligomerization to enable WTGal3's ability to interact with glycosphingolipids (GSLs) in a way to bend the membrane resulting in endocytic pits recruiting the glycosylated cargo.^{27,29}

According to this model, Figure 5.2, monomeric WTGal3 in solution oligomerizes upon binding to cell surface glycoproteins such as integrins. Oligomeric WTGal3 then acquires the capacity to interact with glycosylated lipids of the glycosphingolipid family in a way such as

to drive the formation of tubular endocytic pits from which so-called clathrin-independent endocytic carriers form for the cellular uptake of the cargo glycoproteins.^{11,27,28} Initially described for WTGal3 and the cellular uptake of CD44 and $\alpha 5\beta 1$ integrin, the GL-Lect hypothesis has more recently also been documented for galectin-8 and a specific cargo protein, CD166.³⁰ Although these findings are in apparent contradiction with earlier ones on galectins including lateral lattices that negatively affect endocytosis, we now envision a model in which galectin lattices and the GL-Lect mechanism cooperate to control the homeostasis of glycoproteins at the plasma membrane.^{18,31–35}

Integrins are, heterodimeric transmembrane glycosylated proteins comprised of alpha and beta subunits that integrate and mediated signals between the extracellular and intracellular spaces in the cell and bind to extracellular matrix ligands. They also have numerous binding partners present at the cytosolic side of the plasma membrane.³⁶ 24 integrins are known in vertebrates and all are of significant biomedical importance as they act as a bidirectional link between the cell and its environment.

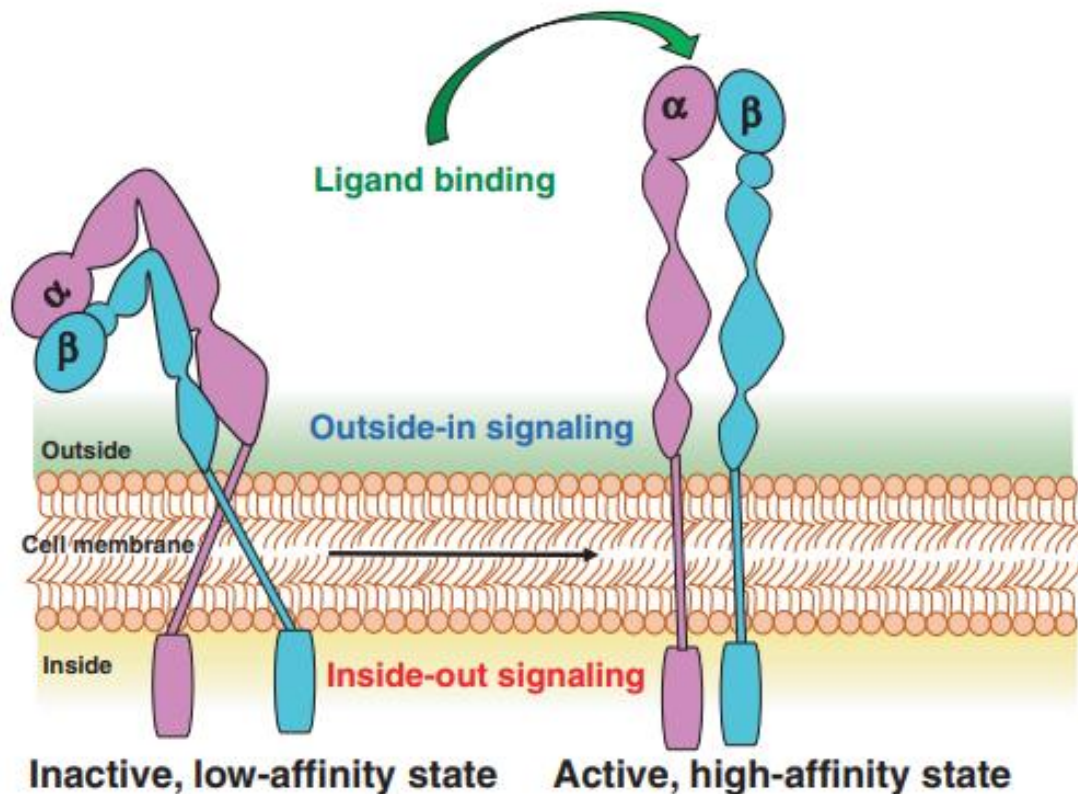


Figure 5.3: Cartoon representation of transmembrane Integrin structure at different activation states, bent inactive state and extended active state. Structurally each integrin consists of $\alpha\beta$ subunits, in which each contain a large multi domain extracellular segment, a transmembrane helix and a small tail with the cytoplasm.³⁷

All integrins have common structural motifs including in the α domain a 7-bladed β propeller connected to a thigh and two calf domains. A metal ion-dependent adherent site (MIDAS) is also conserved and is crucial for ligand binding.³⁸ The beta subunit is composed of a hybrid domain with a beta ion domain. Each subunit consist of one large multidomain extracellular segment, one transmembrane helix and a short cytoplasmic tail, shown in Figure 5.3.³⁷ Several β 1-induced phenomena are associated with interactions with a large family of matricellular proteins, β -galacto-side-binding lectins, galectins.² As mentioned earlier, galectins have been shown to cluster or form oligomers upon the interaction of glycosylated proteins, integrins, as shown in Figure 5.2.^{27,29,31,32,39}

Integrins are believed to exist in three conformational states that regulate their affinity to their ligands, proteins or the extracellular matrix.^{31,40,41} Integrins are normally resident in the cell

membrane in their resting state, characterized across many studies, including cryo electron microscopy by a bent conformation.^{42–44} In their bent resting state integrins have been shown to have low affinity to ligands, due to the head groups blocking access for the ligands as the extracellular domain is facing the membrane.^{45–47} Activation can be stimulated by the integrin binding to its ligand or by binding to an activator protein or indeed through chemical activation by reduction of the disulphides, removal of the metals from the MIDAS region or through treatment with Mn^{2+} , the latter leads to partial activation by enhancing the adhesive properties of many integrins.^{23,42,48–51} Manganese which mimics intracellular signals in vitro has been shown to “prime” integrins into having a higher affinity for its ligands.^{45,46} Priming of integrins, both by inside-out and outside-in signalling or in the presence of Mn^{2+} in vitro studies, results in a gross conformation change from bent to an extended arrangement and as such has an effect on the affinity of the integrin.^{48,52} The conformational change allows for the integrin to capture the ligand more efficiently, and upon capture of a ligand, full activation occurs in to which the β hybrid domain undergoes a swing out motion and the cytoplasmic tails spread apart termed “open of the legs”.^{47,53–55} Integrins regulate various biological functions including cell adhesion, migration, proliferation, differentiation and spreading, and the remodelling of the extracellular matrix.² This ability to regulate crosstalk between the cell and the surrounding environment positions integrins as key players in the process of tumour progression and different specific integrins are indeed expressed in different tumors.⁵⁶ Overexpression of integrins has led to resistance in therapies, tumour reoccurrence and survival issues. $\alpha_5\beta_1$, integrin, also known as the fibronectin receptor, has been shown to have therapeutic effects on certain solid tumors.²³ Breast cancer cell lines that have been ligated with β_1 integrins, such as $\alpha_5\beta_1$ and $\alpha_2\beta_1$, have been reported to significantly reduce drug-induced apoptosis from chemotherapeutic agents.^{57,58} Integrins have been shown to regulate cellular life and death, highlighting their vital role in apoptosis. In cell culture experiments a form of apoptosis termed anoikis has been induced by lack of attachment of endothelial and epithelial cell to proper matrix proteins such as integrins.^{59,60} $\alpha_5\beta_1$ and its adhesion to fibronectin has also been reported to promote cell survival in conditions such as hypoxia, serum starvation and chemotherapy.⁶¹ The alpha domain is responsible for $\alpha_5\beta_1$ ’s high specificity towards fibronectin. An initial interaction of the Arg-Gly-Asp (RGD) binding site of fibronectin with integrin has been shown to be a key attachment site, which is then followed by secondary interactions of the fibronectin synergy site and the primed MIDAS site to ensure full adhesive activity for $\alpha_5\beta_1$ integrin and fibronectin.⁶²

Understanding and regulating the response of Galectin3-integrin interactions is a critical biomedical challenge because Glycolectin-Integrin signalling is integral to many disease states.^{41,63} However, cells are a challenging environment to understand WTGal3-integrin interactions due to their highly complex and heterogenous membrane and as such a more reductionist approach is required to investigate elementary interaction. In vitro studies at artificial membrane platforms such as liposomes and supported lipid bilayers have been successfully applied to interrogate the interaction of membrane lipids as well as membrane embedded receptors with external biomolecules such as proteins and peptides.^{64–66} Goennenwein et al reported that for proteoliposomes above a planar substrate lead to complete immobilisation of $\alpha_{IIb}\beta_3$.⁶⁷ Purucker et al have reported that transmembrane platelet integrin in polymer tethered membranes exhibits stronger adhesion functions of integrin receptors whilst integrins incorporated onto substrates directly are 30 times less effective, due to the substrate friction effect.⁶⁸

In this chapter we study the interaction of the Wild Type Galectin 3 (WTGal3) or the truncated CRD of galectin 3 where the N terminus is deleted (Gal3 Δ Nter) with $\alpha_5\beta_1$ integrin, in both its resting and activated conformations, reconstituted into natural membranes, and evaluate the impact WTGal3 has on the fluidity of microcavity-supported EggPC:EggPA bilayers. Optically transparent PDMS microcavity arrays were exploited in the Fluorescence Lifetime Correlation Spectroscopy experiments to study the diffusion of fluorescently labelled $\alpha_5\beta_1$ -Atto488 and N-terminus WTGal3 or truncated Gal3 Δ Nter-Alexa647 to evaluate the effect of WTGal3 aggregation on the membrane fluidity in support with the electrochemical studies.^{69,70} EIS at gold microcavity arrays supporting EggPC:EggPA bilayers was used to study the effects of WTGal3 or Gal3 Δ Nter network formation on the membrane admittance for bilayers containing resting or activated integrin. To our knowledge, this is the first report applying FCS and EIS to study the effects of WTGal3 aggregation at a lipid bilayer with reconstituted integrin. The study is critically enabled by the MSLB as it provides the appropriate environment to support integral protein reconstitution and fluidity. EIS is a particularly sensitive means of following changes to membrane permeability and thickness, and both are affected by protein network formation at the bilayer. By combining the two techniques, FLCS and EIS real time insights are gained of the dynamics of the WTGal3- $\alpha_5\beta_1$ interaction.⁷¹

5.2 Materials and Methods

All samples were prepared according to the protocols described in Chapter 2, all experiments were performed at room temperature. All experiments were repeated a minimum of 3 times unless stated otherwise. For all experiments, after 1.5 hours vesicle fusion with the LB formed monolayer on the MSLB, the array was rinsed with buffer to remove excess vesicles. For EIS experiments the array was used as the working electrode in the electrochemical system. The MSLB was connected, and the membrane was allowed equilibrate and stabilise for 2 hours before the addition of protein to allow for the measurement to be made. All data shown is the average between the replicates (N=3), with error bars representing the mean squared error.

The following experimental procedures were used in this chapter in addition to those stated in Chapter 2;

Integrin ($\alpha_5\beta_1$, labelled and unlabeled), Wild Type Galectin 3 (labelled and unlabeled), Gal3 Δ nter (labelled and unlabeled), RGD (labelled and unlabeled) and Fibronectin (labelled and unlabeled) were obtained from Ludger et al, in the Marie Curie Institut, Paris.

5.2.1 Reconstitution of $\alpha_5\beta_1$ integrin in SUVs

The reconstitution of integrin into the bilayers was performed at a lipid/protein ratio (LPR) of analogous 10/1 (w/w). 200 μ g of EggPC:EggPA (9/1, w/w) solubilized in chloroform were mixed and excess of organic solvent evaporated under nitrogen atmosphere. Lipidic film was dried under vacuum at room temperature for 2 h, and then resuspended in 150 μ l of 20 mM HEPES, 150 mM NaCl pH 7.4. Lipids are then solubilized by adding 0.25 % (v/v) Triton X-100 and incubated for 10 min at 21 °C under gentle stirring. 20 μ g of purified $\alpha_5\beta_1$ integrin was added to the solubilized lipids, the Triton X-100 concentration was adjusted to 0.5 % (v/v) in a 200 μ l final reconstitution volume and incubated for 10 min at 21 °C under gentle stirring. The detergent was eliminated by three consecutive additions of Bio-Beads SM2 (prepared according to manufacturer's instruction), 2 times with 10 mg, and one time with 20 mg, after 2h, 1h and 1h time intervals, respectively. SUVs were transferred to a new tube and stored at 4 °C until use as vesicles for bilayer preparation of MSLB. Final lipid and $\alpha_5\beta_1$ integrin concentration in SUVs were 1 mg/ml and 0.1 mg/ml respectively. The integrin reconstituted SUVs were characterized using floatation in a sucrose gradient followed by immunodetection of the protein in the sucrose fragments and cryo-electron microscopy, all carried out by Ludger

et al. The size of the integrin reconstituted SUVs was estimated from Dynamic Light Scattering (DLS) using a Malvern, Zetasizer.

Pore suspended membranes were prepared as described in Chapter 2. Briefly the proximal leaflet of EggPC was transferred using LB technique. Approximately 50 μ L of EggPC (1 mg/mL in chloroform) were deposited onto the air-water interface of LB trough and the solvent allowed to evaporate for 15 min. The resulting lipid monolayer at the air/water interface was compressed four times to a surface pressure of 33 mN/m at 25 mm/min. Then, the micro-cavity arrays were immersed into the LB trough until all of the cavities were submerged completely into the subphase. The micro-cavity array was then withdrawn from the trough at a rate of 5 mm/min whilst the surface pressure of the lipids was retained at 32 mN/m to ensure an adequate transfer of the EggPC monolayer. The distal leaflet was assembled using liposome fusion using the Integrin containing SUVs described above, comprising of EggPC:EggPA:integrin. For experiments in the absences of integrin then SUVs were prepared as described in Chapter 2.

5.3 Results & Discussion

5.3.1 Fluorescence Correlation Spectroscopy on EggPC;EggPA membrane in the absence integrin.

Fluorescence lifetime correlation spectroscopy was carried out using the PDMS-MSLB platform to understand the interaction between Atto488- $\alpha_5\beta_1$ integrin and galectin, labelled with Alexa647 for both WTGal3 and Gal3 Δ nter. However, firstly the effect of galectin on the membrane alone was required to determine if any underlying nonspecific interactions occur in the absence of integrin. To interrogate lipid diffusivity in the absence of protein, the EggPC:EggPA(90:10) membrane was labelled with a fluorescent lipid, DOPE-Atto532 (0.01 mol%). Labelled WTGal3-Alexa647 and Gal3 Δ nter were used to examine their influence on an EggPC:EggPA membrane in the absence of integrin. Microcavity array suspended EggPC lipid monolayers were first prepared by LB transfer and followed by vesicle fusion with EggPC:EggPA(90:10) liposomes, without reconstituted $\alpha_5\beta_1$ integrin.

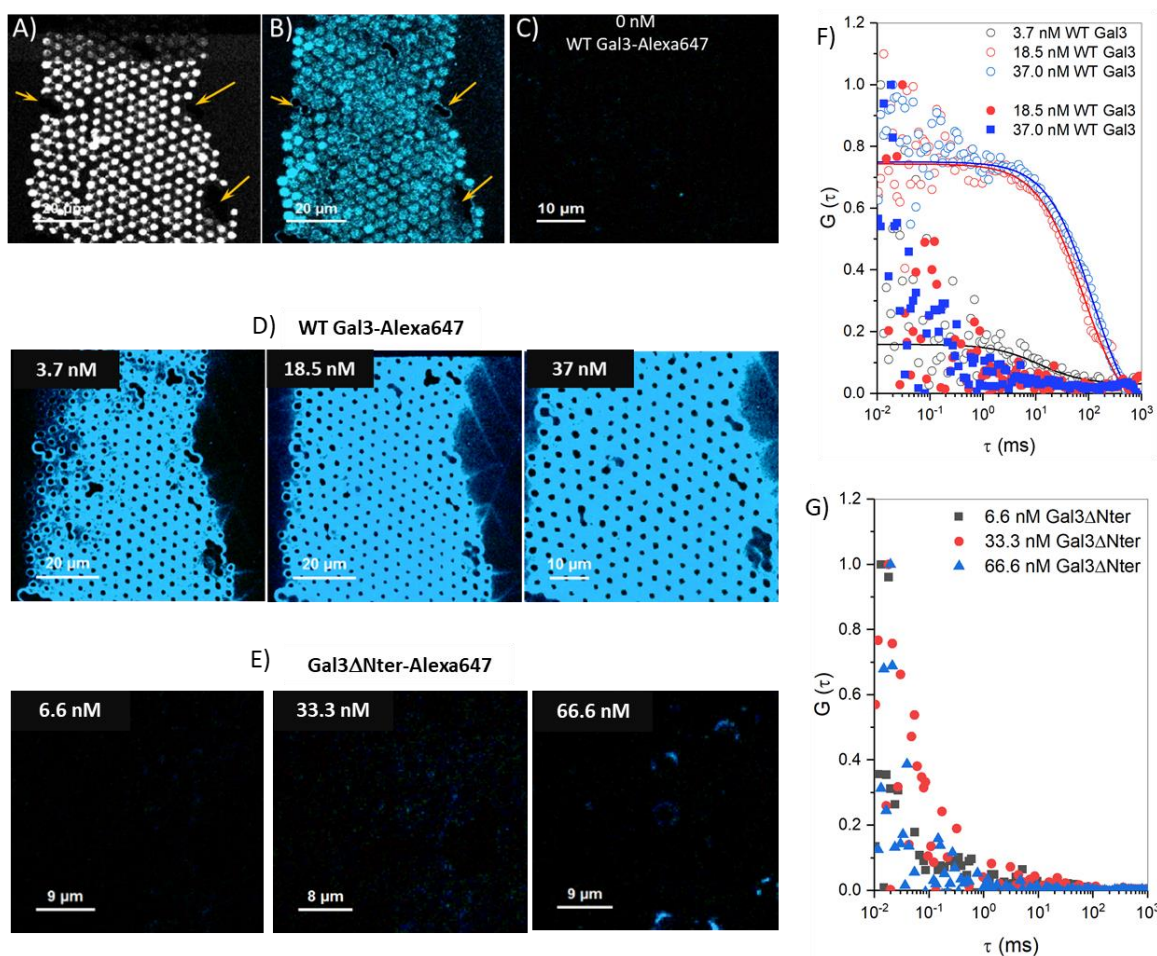


Figure 5.4: A) Reflectance and B) FLIM image of pristine EggPC:EggPA membrane labelled with ATTO532-DOPE (0.01 mol %). C) FLIM image from the emission channel corresponding to the galectin label i.e., at 647 nm, when no WTGal3-Alexa647 (0 nM) is added to EggPC:EggPA membrane. Arrow mark in panel A and B shows the buffer unfilled cavities where no bilayers are formed. D) and E) represent FLIM image of Alexa647 labelled WTGal3 and Gal3 Δ Nter at varying concentrations upon binding to EggPC:EggPA membrane respectively. F) ACFs showing the diffusion of WTGal3-Alexa647 upon binding to EggPC:EggPA membrane at different concentrations. Two regions could be distinguished in the array; open symbols (black, red, and blue) show the experimental ACF for 3.7, 18.5 and 37 nM of WTGal3-Alexa647, where extensive aggregation causes slow diffusion, and filled circles (red:18.5 nM and blue: 37 nM) are not diffusing due to weak binding to membrane. Solid lines are the 2D fit G) ACFs of Gal3 Δ Nter-Alexa647 at varying concentrations (square: 6.6nM, circle: 33.3 nM and triangle: 66.6 nM) upon binding to EggPC:EggPA membrane. For each concentration of WTGal3-Alexa647 and Gal3 Δ Nter-Alexa647, FLIM imaging and FLCS

are acquired after lectin incubation for 30 min and washes of the EggPC:EggPA membrane with fresh 0.01 M PBS at 22±1 °C. Courtesy of Dr. Guilherme Berselli.

The diffusion coefficient of the membrane was estimated from fitting the resulting ACF using 2D diffusing model equation and found to be $6.66 \pm 0.38 \mu\text{m}^2/\text{s}$ with an anomalous factor of 0.94 ± 0.17 indicating Brownian motion in PBS buffer pH 7.4. The assessment of fit was determined by visual examination of the fitted line, and residuals and from the R^2 regression coefficient (which was typically approx. 0.99). The diffusion coefficient for the EggPC:EggPA/EggPC MSLBs was considerably lower than recorded for simple DOPC MSLBs in PBS, which were previously reported as approximately $10.5 \mu\text{m}^2/\text{s}$.^{72,73} The value matches very well to diffusion values reported for DNA tethered lipid bilayer membrane patches of EggPC.⁷⁴ This reflects the greater viscosity of the natural EggPC:EggPA lipids that were used here.^{73,75,76} This mixture of natural lipids are expected to contribute both liquid and gel phases at room temperature.⁷⁴ In control experiments, the EggPC:EggPA membrane was confirmed stable by FCS for at least 48 hours, which well exceeds the experimental window for subsequent lectin binding studies (Data not shown).

Figure 5.4A shows a representative reflectance image obtained from the PDMS array, showing buffer filled cavities. The successful assembly of bilayer formation over the cavities was ascertained from FLIM images of 0.01 mol% DOPE-Atto532 contained in the upper leaflet of EggPC:EggPA, Figure 5.4B. As expected in the absence of WTGal3, no emission was observed from the membrane when excited with the 647 nm channel, Figure 5.4C. In EggPC:EggPA-only membranes, WTGal3 was observed using FLIM imaging, Figure 5.4D, to adsorb at the membrane, even at the lowest concentration of WTGal3 showing fluorescence from the WTGal3 across the array. Upon further addition of WTGal3, the emission remained largely unchanged after 18.5 nM indicating that adsorption was saturated at this concentration, consistent with the EIS data presented later. To confirm the adsorption of WTGal3 at the membrane, the diffusion coefficient of labelled WTGal3 was monitored. The WTGal3-Alexa467 bulk/solution diffusivity was measured at various concentrations from 3.7 to 37 nM and was found to be $83 \pm 3 \mu\text{m}^2/\text{s}$ in 0.01 M PBS (Table 1, Figure 5.5F grey). From the intensity-time trace from 20 independent recordings over 10s, no aggregate intensity spikes or any bleaching was observed, suggesting that even at the highest concentration of WTGal3, the lectin remains in monomeric state in solution. The data showed two populations of WTGal3 emerge on the basis of diffusivity of WTGal3, a lower prevalence population that was mobile

(Figure 5.4F, filled symbol) observed at lower concentration of WTGal3 and a dominant population that showed very low mobility (Figure 5.4F, open symbol) observed at high concentrations of WTGal3 and was also observed to bleach during the FLCS acquisition. The diffusion coefficient of WTGal3 reduced significantly upon incubation with the EggPC:EggPA membrane to a mobile fraction 6.5 ± 0.15 (Table 5.1), which matches closely to the diffusivity of the lipid probe, and an immobile/aggregated fraction $0.1 \pm 0.08 \mu\text{m}^2/\text{s}$ (Table 1), further confirming that the WTGal3 adsorbs at the membrane. This immobile population may due be to WTGal3 self-aggregation or preferential association of this population with the membrane. Interestingly over the described range of concentrations, a modest increase of lipid marker DOPE-Atto532 diffusion was recorded at the addition of 37 nM WTGal3, from 6.6 to 6.9 $\mu\text{m}^2/\text{s}$, Table 1. This could suggest that the membrane packing was being disrupted by the WTGal3.

Table 5.1: Estimated diffusion coefficient values of lipid and protein along with anomalous factor, α values. The SD are from triplicate measurements in PBS pH 7.4. Courtesy of Dr. Guilherme Berselli.

Diffusing fluorophores	D ($\mu\text{m}^2/\text{s}$)	α
ATTO532-DOPE in EggPC:EggPA	6.6 ± 0.38	0.94 ± 0.17
ATTO532-DOPE in EggPC:EggPA after 37nM WTGal3	6.99 ± 0.12	0.86 ± 0.05
ATTO532-DOPE in EggPC:EggPA after 66.6 nM Gal3 Δ Nter	6.7 ± 0.11	0.97 ± 0.15
WTGal3-Alexa647 in solution	83 ± 3	1 ± 0.1
WTGal3-Alexa647 in EggPC:EggPA (mobile fraction)	6.5 ± 0.15	0.94 ± 0.17
WTGal3-Alexa647 in EggPC:EggPA (immobile/aggregates)	0.1 ± 0.08	0.86 ± 0.11
Gal3 Δ Nter-Alexa647 in EggPC:EggPA	27 ± 5	0.98 ± 0.10

Conversely, under identical conditions, much lower association of the Gal3 Δ ter with the EggPC:EggPA membrane, was observed Figure 5.4E, 5.4G. From imaging, little signal from the labelled Gal3 Δ ter was observed over the membrane and the very weak ACFs from the bilayer interface, exhibited a diffusion coefficient of $27 (\pm 5) \text{ um}^2/\text{s}$. This diffusion coefficient was too high to indicate membrane insertion but also too low for a freely diffusing Gal3 Δ ter. This diffusion coefficient value is likely attributed to a weak physisorption of the protein at the bilayer interface possibly due to electrostatic interactions between the Gal3's C-terminus domain and the EggPA in the membrane. It has been previously reported that WTGal3 has the ability to self-associate through intermolecular interactions in the absence of ligands.⁷⁷ In WTGal3, the N-terminus is known to be hydrophobic, and indeed N-terminal hydrophobic-hydrophobic interactions, are thought to drive oligomerization. Overall, the data indicate that membrane adsorption/association is driven by the WTGal3 N-terminus.

5.3.2 Fluorescence Correlation Spectroscopy on EggPC;EggPA membrane in the presence of resting integrin

PDMS arrays were used for the investigation of Microcavity array suspended EggPC lipid monolayers were first prepared by LB transfer and followed by vesicle fusion with

EggPC:EggPA(90:10) liposomes, with reconstituted $\alpha_5\beta_1$ integrin. Dynamic Light Scattering was used to follow the steps of proteoliposome preparation.

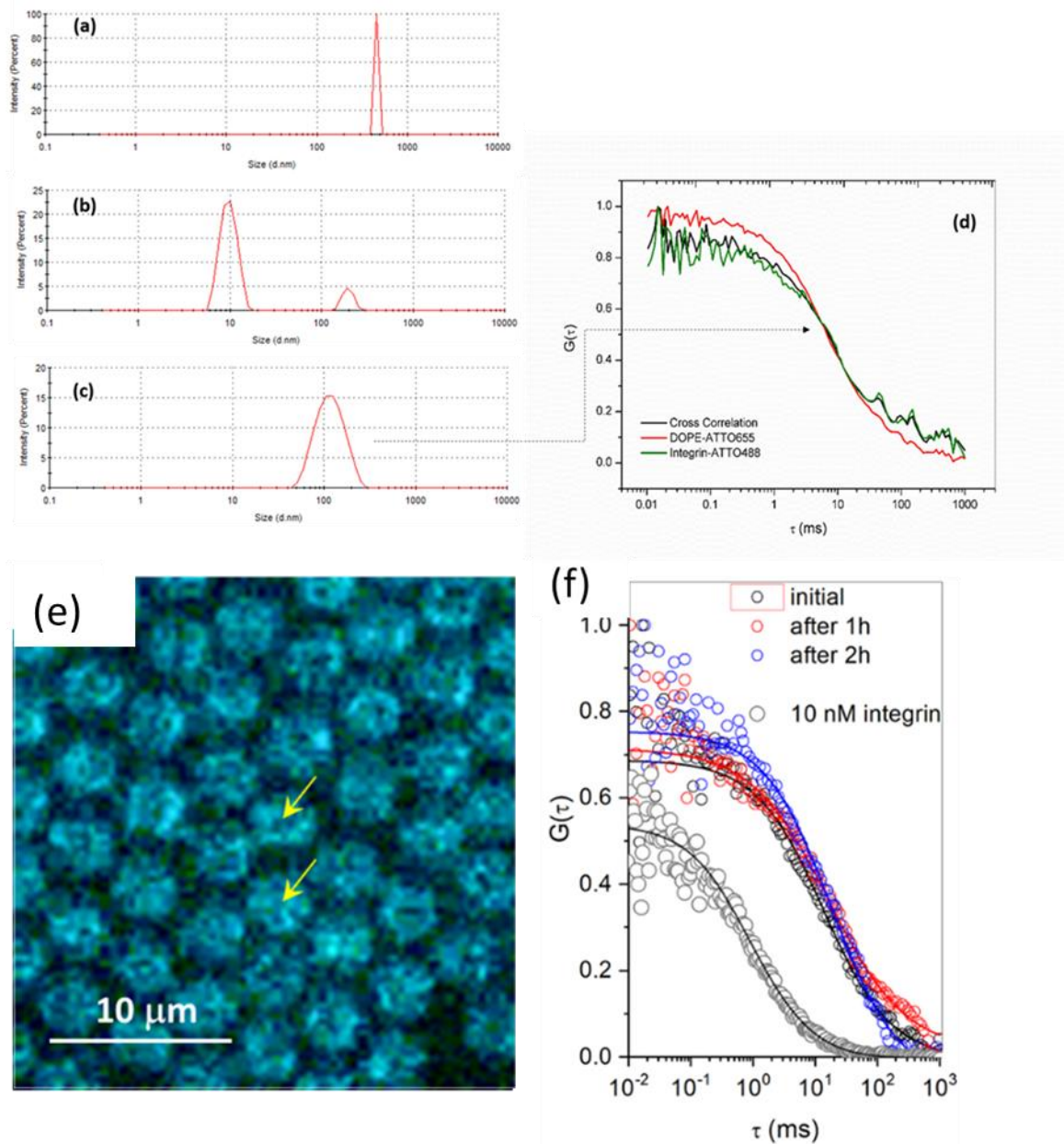


Figure 5.5: Dynamic Light Scattering (DLS) and Auto correlation functions (ACFs) obtained for Fluorescence Lifetime Cross-Correlation Spectroscopy (FLCCS) of proteoliposomes after reconstitution integrin using two independent fluorophores. DLS represents size distribution of (a) liposomes comprised of EggPC:EggPA (9:1) with integrin at a lipid to protein 10 after resuspension into HEPES buffer, (b) after destabilization with Triton X-100 and (c) after detergent removal using bio-beads. (d) Shows the ACFs obtained from FLCS and FLCCS of

proteoliposomes. The red and the green line represent the ACFs of fluorescent labelled ATTO488- $\alpha_5\beta_1$ and DOPE-ATTO655 (0.01 mol%) respectively and the black line indicates the cross-correlation between the two dyes. E) FLIM image of ATTO488-labelled $\alpha_5\beta_1$ integrin reconstituted into a EggPC:EggPA MSLB. Brighter circles indicate zones in which the membrane was suspended over pores. Arrows indicate typical regions at the centre of the pores where FCS was acquired from. F) ACFs for ATTO488-labelled $\alpha_5\beta_1$ integrin after different time points (black, red, and blue) post-reconstruction in MSLBs, along with 10 nM ATTO488- $\alpha_5\beta_1$ integrin in micellar form diluted in PBS solution (grey). Solid lines are the fitted data for integrin diffusion across MSLB and solution using 2D diffusion model, and pure diffusion model respectively. The FCS data are collected and averaged from approximately 80-100 points from pores across the substrate. ACFs traces show no changes over 6 hours windows. Both FLCS and FLIM image were taken over PDMS cavity array substrate sealed within a microfluidic chamber filled with 0.01 M PBS buffer (pH = 7.4) at $22 \pm 1^\circ\text{C}$. (Figure E) FLCCS was Courtesy of Dr. Guilherme Berselli.

The initial EggPC:EggPA liposomes resuspended in HEPES buffer formed vesicles of narrow size distribution with a mean diameter of 500 nm (Figure 5.5A). Detergent disruption was confirmed from the change in the size distribution of liposomes (Figure 5.5B) and following detergent removal, the reformed proteoliposomes containing integrin showed single liposome distribution with fairly homogeneous diameter of 120 nm (Figure 5.5C). Additionally, using fluorescence life-time cross-correlation spectroscopy (FLCCS), the diffusion of labelled integrin-ATTO488 and DOPE-ATTO655 in proteoliposomes bilayer was evaluated following simultaneous excitation of both fluorophores (Figure 5.5D). the ACFs indicate that integrin and DOPE co-diffuse, indicating that both are reconstituted into the same proteo-liposomes, confirming that integrin was well inserted in the liposomes formed after detergent removal. The integrin was reconstituted from proteoliposomes prepared with lipid to protein ratio of 10 (L/P=10 wt/wt). As FLCS requires only low labelling level, the integrin, $\alpha_5\beta_1$, was labelled with ATTO488 (Integrin-ATTO488) and mixed with unlabelled integrin while maintaining L/P of 10 and reconstituted at EggPC:EggPA membrane. The proteoliposome was then allowed to fuse over EggPC monolayer spanned across PDMS array to form the MSLB. This proteoliposome fusion protocol has been demonstrated successfully to reconstitute other membrane proteins.^{78,79} Successful reconstitution and formation of MSLB with embedded integrin was evaluated using FLIM imaging (Figure 5.5E) and FLCS (Figure 5.5F).

MSLB/Integrin diffusion was assessed over a minimum window of 6 hours, which exceeds the experimental window and found to be stable with ATTO 488.

Successful reconstitution of integrin into the MSLBs was confirmed by monitoring both FLCS and FLIM of the ATTO488-labelled integrin at PDMS platform. Compared to the pristine bilayer ($6.66 \pm 0.38 \mu\text{m}^2/\text{s}$), the decreased diffusion coefficient of the lipid marker to $5.40 \pm 0.40 \mu\text{m}^2/\text{s}$ indicates the integrin influences membrane viscosity, but as α was measured as 0.98 ± 0.10 , indicating that lipid diffusion remains Brownian in the presence of integrin, Table 2. Representative FCS autocorrelation data obtained from integrin reconstituted in MSLB spanned over PDMS along with FLIM images are shown in Figure 5.5E and 5.5F. The average integrin diffusivity before the addition of WTGal3 or Gal3 Δ nter was found to be $1.99 \pm 0.56 \mu\text{m}^2/\text{s}$ and the anomalous exponent, α was determined as 0.86 ± 0.05 indicating sub-diffusion, in PBS. This value is roughly 22 times slower than the solution diffusivity of $\alpha_5\beta_1$, $46 \pm 5 \mu\text{m}^2/\text{s}$ taken from the micellar form in PBS at 10 nM (Figure 5.5F, Table 5.2). The diffusion of integrin, its molecular brightness and α value were observed to remain unchanged over a measurement period of 6-8 h which well exceeded the time window for the lectin binding studies. The data indicate strongly that the protein was well reconstituted into the membrane, the values are comparable to previously reported data for reconstituted integrins in GUVs.⁸⁰ Our values also compare quite well with platelet integrin $\alpha_{\text{Ib}}\beta_3$ reconstituted into DOPC MSLB exhibited a diffusion coefficient of $2.80 \pm 0.59 \mu\text{m}^2/\text{s}$, and a complex membrane composition which exhibited diffusion coefficients of $3.20 \pm 0.59 \mu\text{m}^2/\text{s}$, both with an alpha value of approximately 1.^{78,80,81} The similar diffusivity of $\alpha_5\beta_1$ here, strongly supports proper reconstitution of the integrin in MSLBs; the slightly lower value may be attributed to the greater viscosity of the EggPC:EggPA mixture and in particular to the greater protein loading in the current protocol where the in the originating liposomes a LPR of 10 is expected to be faithfully translated to the MSLB. Moreover, the α is less than one, indicating sub-diffusion of $\alpha_5\beta_1$ under our conditions, which is attributed to the somewhat crowded protein environment compared to reports where integrin was much less concentrated and the LPR was 100:1 (w/w).

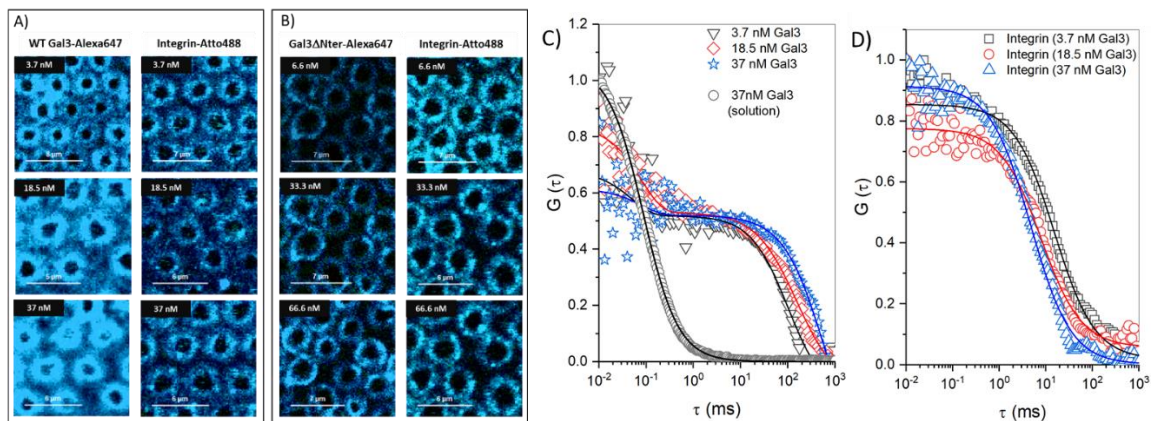
^{78,80,82}

Table 5.2: Estimated diffusion coefficients and corresponding anomalous (α) parameter of ATTO488- $\alpha_5\beta_1$ integrin in solution and in MSLBs along with lipid diffusion in EggPC:EggPA MSLBs in the presence or absence of integrin. EggPC:EggPA:Int indicates membranes

reconstituted with integrin, and EggPC:EggPA membranes without integrin. Data from FLCS studies in PBS at pH 7.4. SDs are from triplicate measurements.

Diffusing fluorophores	D ($\mu\text{m}^2/\text{s}$)	α
ATTO488- $\alpha_5\beta_1$ in solution	46 ± 5	1.01 ± 0.03
ATTO488- $\alpha_5\beta_1$ in EggPC:EggPA:Int MSLBs	1.99 ± 0.56	0.86 ± 0.05
ATTO655-DOPE in EggPC:EggPA MSLBs	6.66 ± 0.38	0.94 ± 0.17
ATTO655-DOPE in EggPC:EggPA:Int MSLBs	5.40 ± 0.40	0.98 ± 0.10

Once the integrin was established to have been successfully incorporated into the membrane, investigation of the effects of WTGal3 and Gal3 Δ nter addition were performed. In contrast to the pristine membrane, when the integrin was reconstituted into the membrane, FLIM imaging of fluorescently labelled galectin (WTGal3 and Gal3 Δ nter), confirmed that treatment with either lectin resulted in the association of both to the membrane. The extent of association is greater for WTGal3, and it occurs with amplifying FLIM intensity in a concentration dependent manner, as shown in the FLIM image of WTGal3-Alexa647 channel (Figure 5.6A, left).



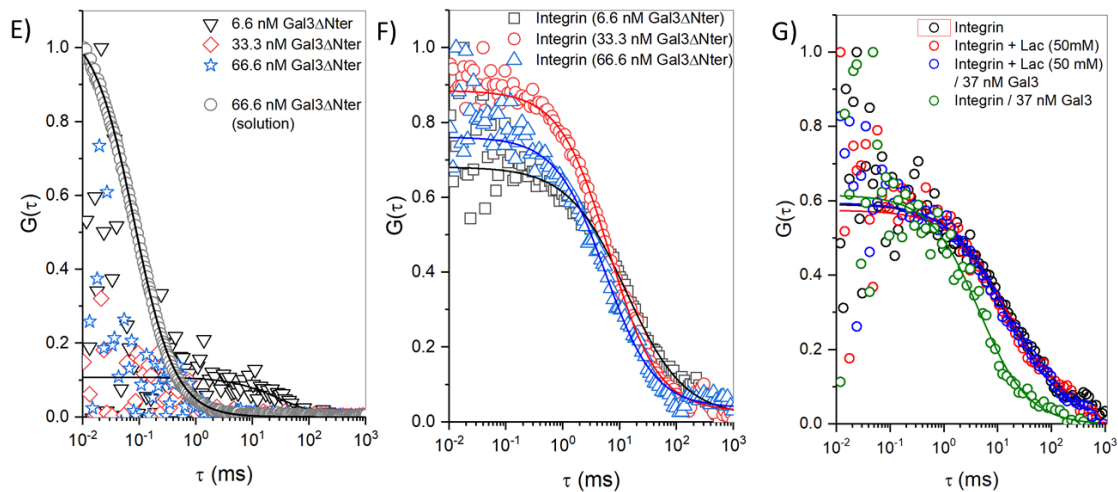


Figure 5.6: FLIM images of A) WTGal3-Alexa647 (left) and B) Gal3 Δ Nter-Alexa647 (left) at varying concentrations upon binding with EggPC:EggPA/Int membrane. The corresponding integrin-Atto488 FLIM image are shown in both panel A,B (right). C) and E) illustrates ACFs of WTGal3-Alexa647 and Gal3 Δ Nter-Alexa647 at varying concentrations when bound to integrin containing EggPC:EggPA membrane. ACFs in D) and F) are the evolution of integrin-Atto488 diffusion upon binding of different concentrations of WTGal3-Alexa647 and Gal3 Δ Nter -Alexa647 respectively. G) ACFs of Integrin-Atto488 reconstituted into EggPC:EggPA membrane (black circle); in the presence of 50 mM β -lactose (red circle); in the presence of 37 nM WTGal3 preincubated with 50 mM β -lactose (blue circle); after exchanging the contact solution of WTGal3+Lac with fresh 37 nM WTGal3 (olive circle). In all panels (C-G) open symbol represents the experimental data and solid lines are the fit using eq. (2) except a pure diffusion model equation (Eq.S1, SI) was utilized to extract the diffusion coefficient values for Alexa labelled WTGal3 and Gal3 Δ Nter solution diffusivity measurement. All measurements were carried out in 0.01M PBS buffer of pH 7.4 and at 22 ± 1 °C.

The WTGal3-Alexa467 bulk/solution diffusivity was measured at various concentrations from 3.7 to 37 nM and was found to be 83 ± 3 $\mu\text{m}^2/\text{s}$ in 0.01 M PBS (Figure 5.6C, grey circle). From the intensity-time trace throughout 20 independent recordings over 10s, it was not observed that any aggregate intensity spikes or any bleaching, suggesting that even at the highest concentration of WTGal3, the lectin remains in monomeric state in solution. Upon exposure to the integrin containing membrane, its diffusivity reduced drastically to below 4 $\mu\text{m}^2/\text{s}$, Table 3.

Table 5.3: Estimated diffusion coefficients of ATTO647-WTGal3 and ATTO488-integrin alongside with the anomalous values; integrin is reconstituted at EggPC:EggPA (90:10) at a L/P of 10. The SD are from triplicate.

Diffusing fluorophore	D ($\mu\text{m}^2/\text{s}$)	α
Integrin-Atto488 before WTGal3	1.99 ± 0.56	0.85 ± 0.26
Integrin-Atto488 after 3.7 nM WTGal3 addition	4.31 ± 0.24	1.02 ± 0.26
Integrin-Atto488 after 18.5 nM WTGal3 addition	5.01 ± 0.09	0.93 ± 0.20
Integrin-Atto488 after 37 nM WTGal3 addition	5.26 ± 0.23	0.92 ± 0.02
3.7 nM WTGal3-Alexa647	2.51 ± 0.28	0.96 ± 0.12
18.5 nM WTGal3-Alexa647	3.54 ± 0.11	0.96 ± 0.08
	1.07 ± 0.15	0.84 ± 0.10
37 nM WTGal3-Alexa647	3.59 ± 0.16	0.92 ± 0.12
	0.43 ± 0.15	0.75 ± 0.18

On analysis, this comprised of two diffusing components from WTGal3: a slow diffusing component, especially at higher concentrations, (1.07 and $0.43 \mu\text{m}^2/\text{s}$ for 18 nM and 37 nM WTGal3 respectively), (Figure 5.6C, Table 5.3) likely indicating clustering or oligomerization and a mobile fraction with a diffusion coefficient of WTGal3 that reaches a plateau at highest protein concentration (3.54 ± 0.11 and $3.59 \pm 0.16 \mu\text{m}^2/\text{s}$ for 18 nM and 37 nM WTGal3-Alexa647 respectively) (Figure 6D, Table 3). The alteration of diffusivity cannot merely be attributed to membrane association as there are very clear distinctions between diffusive behaviour of the WTGal3 when the integrin is present in the membrane and when it is not. While two mobile populations emerge in both situations, the more mobile population of WTGal3-Alexa647 matches closely to the membrane diffusivity at $6.5 \pm 0.15 \mu\text{m}^2/\text{s}$ (Table 1) in the pristine membrane. But at the integrin containing membrane the more mobile WTGal3-Alexa647 component exhibits a dramatically lower diffusivity of 2.51 and $3.54 \pm 0.20 \mu\text{m}^2/\text{s}$ at 3.7 and 18 nM respectively. In both cases, the immobile population diffusivity is below 1 , however D is equal to $0.1 \pm 0.08 \mu\text{m}^2/\text{s}$ at the pristine membrane and to $0.43 \pm 0.15 \mu\text{m}^2/\text{s}$ when the integrin is present. This observation clearly distinguishes the impact of integrin, the closer match of integrin and galectin diffusivity and that they vary in tandem suggests co-association

in both slow and fast moving components. Galectin lattices have been reported to be resistant to lateral movement of the cell surface once the oligomers have formed and therefore regulate the lateral movement and recycling of membrane receptors.^{83,84}

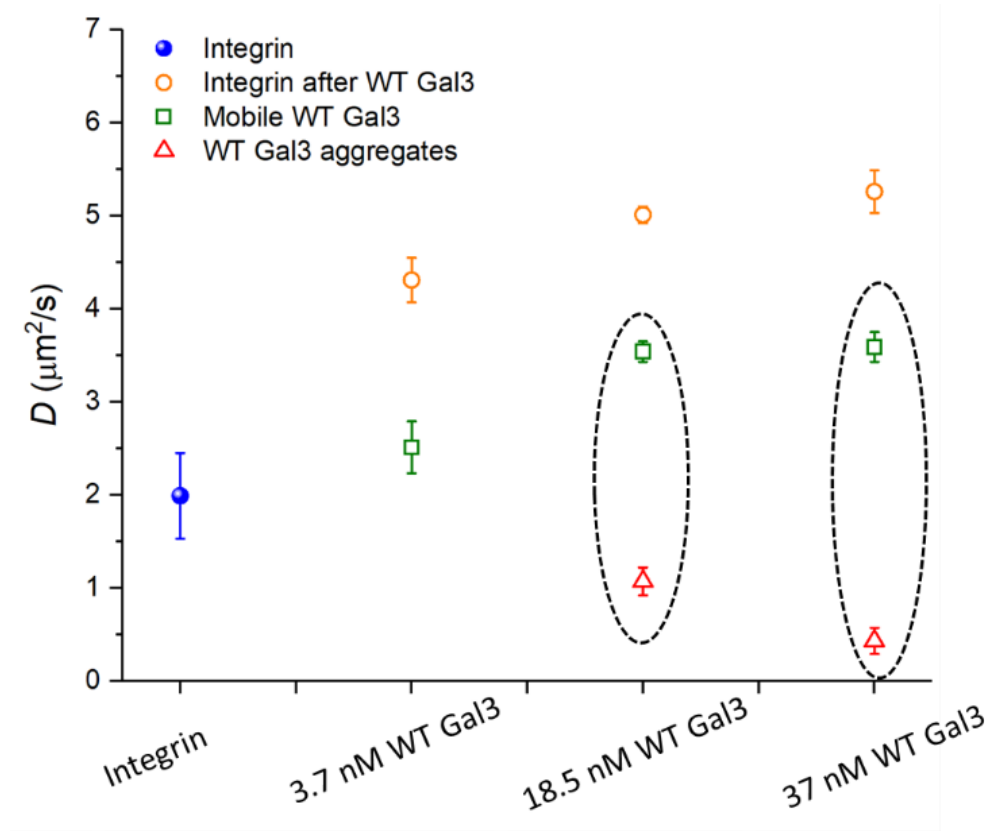


Figure 5.7: Graphical representation of diffusivity trends of ATTO488-integrin before and after WTGal3 addition, along with the diffusivity trends of WTGal3-Alexa647. The blue filled circle shows the diffusion coefficient values for Integrin-Atto488 at-MSLB before WTGal3 addition. Orange circles are the diffusion coefficient values of integrin following incremental addition of WTGal3-Alexa647 (3.7 nM, 18.5 nM, and 37 nM); each diffusion value was obtained following 30 minutes incubation of WTGal3-Alexa647 at the membrane. Green squares show the concentrations dependent diffusion coefficient values for WTGal3-Alexa647 after binding to integrin containing EggPC:EggPA membrane; diffusion values are measured after 30 min incubation at the MSLB. Red triangles show the diffusion coefficient values of WTGal3 aggregates originated at higher concentration after binding to integrin containing membranes. The dotted oval mark highlights the bimodal diffusivity values of WTGal3-Alexa647.

From the perspective of integrin diffusivity, incubation of WTGal3 with integrin reconstituted in membrane was observed to cause integrin diffusivity to increase, by at least 2-fold compared to the original diffusion value (Figures 5.6D, figure 5.7 and Table 5.3). This is accompanied by an increase in alpha to nearly 1, indicating a switch from sub-diffusion to normal diffusion regime. As described, the interaction of the WTGal3 with the underlying membrane is observed to decrease its viscosity and this is expected to contribute, in part, to this effect. However, both the greater magnitude of the change and alteration in diffusion regime suggests the situation may be more complex than changes to membrane induced by the WTGal3 and that other parameters might also be at play. These may include membrane phase changes or modulation of nanopore formation by WTGal3 when the integrin is present. However, it is important to note that it is also likely that only a subpopulation of integrin remained mobile after galectin addition, as observed by FCS. Indeed, integrin tied up in immobile lattices would not contribute to the FCS signal, and immobilisation of the integrin at the lattice would deplete the concentration of mobile integrin in the bilayer. It has been shown previously that increased concentrations of membrane proteins increase membrane viscosity, thereby slowing the diffusion of lipids and of the proteins themselves⁸⁵⁻⁸⁷. This may well explain why we observe an increased diffusion of integrin and an increase in alpha, as the remaining mobile integrin in the bilayer would be in a less crowded environment after galectin sequesters the integrin into immobile networks.

Figure 5.7 traces the trends in integrin and galectin diffusivities upon incubation with increasing concentrations of WTGal3. It shows that integrin and the fast moving component of WTGal3 diffusivities follow the same increasing trend when the concentration of WTGal3 was increased. However, the diffusivity values are not identical between integrin and WTGal3. It may be because of some WTGal3 sub-populations that will exist, are not involved in binding to integrin but merely associated to the membrane, thus contributing to lower the mean the diffusion values of WTGal3 fast component. In addition, the integrin was randomly orientated in reconstitution membranes, with 50% of the molecules not exposed to WTGal3. The contribution of integrin sub-population not exposed to WTGal3 will introduce some bias in the mean diffusivity values. At 18.5 nM and above concentrations of WTGal3, where the immobile fraction of WTGal3 is only observed, the integrin diffusivity tends to reach a plateau. This behaviour could be due to the emergence of large clusters of integrin consecutive to the recruitment of more WTGal3 and oligomers formation. Our results are in line with a recent

study where upon addition of WTGal3 to integrin expressing HeLa cells, the lateral mobility as well as the cluster size of integrin is increased.¹⁸

Compared to WTGal3, FLIM imaging of integrin containing membrane incubated with Gal3 Δ Nter-Alexa647 shows weaker fluorescence intensity (Figure 5.6B, left panel), indicating a lower degree of association. The solution diffusivity of Gal3 Δ Nter in the absence of membrane was found to be $90 \pm 5 \mu\text{m}^2/\text{s}$ (Figure 5.6E, grey circle) and the Gal3 Δ Nter ACF signal was too low (Figure 5.6E, black, red, and blue items) to reliably measure diffusivity from the membrane, indicating that binding to integrin is too low to be detectable by FCS. Furthermore, in contrast to WTGal3, the diffusion coefficient of the integrin was unaffected by incubation with Gal3 Δ Nter and remained constant at approximately $1.9 \pm 0.2 \mu\text{m}^2/\text{s}$ over all concentrations of the Gal3 Δ Nter explored (Figure 5.6F). These measurements are consistent with a weak binding of Gal3 Δ Nter to integrin, which in the absence of the N-terminus oligomerization domain did not translate into the type of robust interaction and clustering capacity that was seen with WTGal3.

Finally, to confirm that these changes in WTGal3 and integrin diffusivities are specifically driven to the interaction between both proteins, we carried out β -lactose inhibition studies (saturation of Gal3 CRD domain) by FCS. The disaccharide β -lactose binds into CRD of the glycan recognition site on galectins.¹¹ It can therefore be used as a competitive inhibitor of interactions that depend on this binding pocket. In a control experiment that the diffusivity of integrin in the absence of WTGal3 was not influenced by 50 mM β -lactose (Lac) incubation and remains stable at $1.9 \pm 0.7 \mu\text{m}^2/\text{s}$ (Figure 5.6G). In the presence of WTGal3 pre-incubated with 50 mM β -lactose, the increase of integrin diffusivity previously described upon WTGal3 binding in the absence of β -lactose was no longer observed (Figure 5.6G). Hence, the initial diffusivity of reconstituted ATTO488-integrin ($1.99 \pm 0.56 \mu\text{m}^2/\text{s}$) remains stable at $2.2 \pm 0.4 \mu\text{m}^2/\text{s}$ after the addition of 37 nM WTGal3 pre-incubated with 50 mM β -lactose (Figure 5.6G).

After incremental additions of WTGal3 in a background of β -lactose, blank PBS buffer was then flushed across the bilayer to remove unbound Gal3- β -lactose complex and the integrin containing MSLB exposed to WTGal3 (37 nM) in fresh buffer without β -lactose. The diffusivity of integrin was observed to increase to $5.5 \pm 0.8 \mu\text{m}^2/\text{s}$ ($\alpha=0.98$) (Figure 5.6G). This

data confirm that enhanced integrin diffusivity is caused by WTGal3 association to $\alpha 5\beta 1$ integrin through a carbohydrate recognition dependent process.

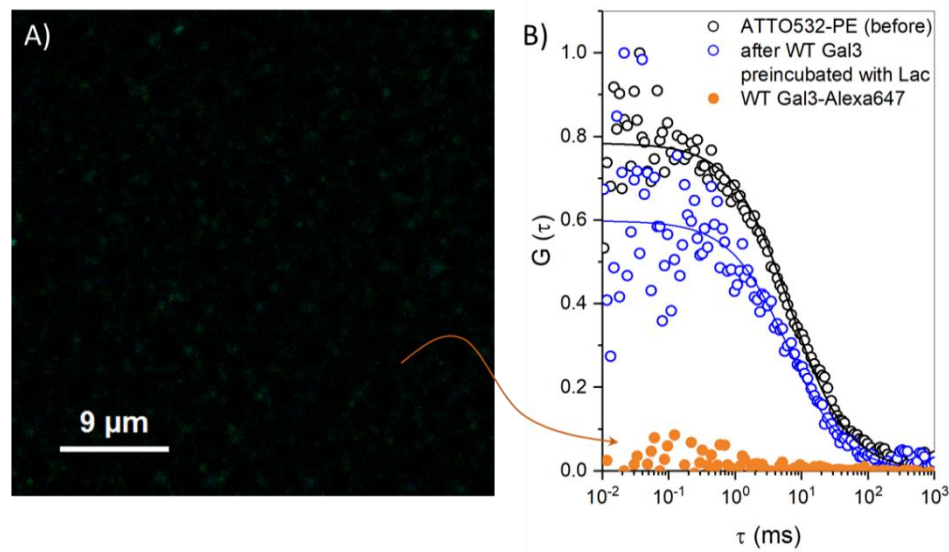


Figure 5.8: FLIM image of 37 nM WTGal3-Alexa647 preincubated with 50 mM Lac after binding to EggPC:EggPA membrane spanned over microcavity array showing no association to the membrane. B) ACFs of showing the lipid (ATTO532-PE) diffusivity before (open black), and after 50 mM Lac preincubated WTGal3-Alexa647 binding (open blue). Filled orange symbol in panel B illustrates the protein ACF (WTGal3-Alexa647 preincubated with Lac) acquired from the MSLB pores, shown by curved arrow. Solid lines in panel B are the 2D fit. Courtesy of Dr. Guilherme Berselli.

Unexpectedly though, when 37 nM WTGal3-Alexa647 was preincubated with 50 mM lactose and allowed to interact with a pristine EggPC:EggPA membrane very little signal was observed from FLIM imaging, a low ACF signal in FLCS and a much more modest increase in lipid diffusivity was seen (Figure 5.8). In other words, lactose seems to inhibit the non-specific membrane interaction by preventing its lactose occupied CRD domain to interact with the membrane. Overall, we speculate that native WTGal3 may behave rather like an amphiphile, with its hydrophobic N-terminus and its positively charged C-terminus may both interact with the membrane bilayer and alter the viscosity.¹⁵ Why this occurs is not clear but Lactose, which is water soluble may alter the lipophilicity of protein or may alter the conformation of the N terminus and its availability to penetrate the membrane. Our observations are strongly consistent with findings of Lukyanov et al who reported, on the basis of fluorescence studies, that galectin-3 binds to phospholipids and can penetrate liposomal and cell membranes.⁸⁸ As

described, the effect is inhibited by lactose and suggests that the sugar induces structural changes in the galectin beyond the CRD. Indeed it has been reported that self-association between Gal3 molecules (which are not glycosylated) is impeded by lactose.^{89,90}

5.3.3 Electrochemical Impedance Spectroscopy on EggPC;EggPA membrane in the absence or presence of resting integrin

EIS was then used as a highly sensitive, label-free measurement modality to study how WTGal3 influences membrane phase or packing in the absence or presence of resting $\alpha_5\beta_1$ integrin, as a way to confirm the effects observed with FCS. Unlike FCS which is a single molecule technique, EIS exploits the electrical properties of the whole membrane on the electrode, and as such the gold MSLBS were exploited as for the previous chapters and reports from Keyes et al.^{75,91–94} For the same membrane compositions a greater equilibration time was allowed for EIS studies in comparison to FLCS studies to ensure a global equilibrium was reached due to the larger volume in the electrochemical cell. First, in control experiments, the impact of WTGal3 on the membrane resistance of the EggPC;EggPA MSLBs were compared to determine if interactions between the lectin and membrane that were observed from FCS occur in the absence of integrin. After an equilibration time of 2 h, the WTGal3 concentration was increased in a stepwise manner in the contacting solution at the EggPC;EggPA membrane. After each WTGal3 addition, an incubation time of roughly 30 minutes was applied before EIS was recorded. The changes in membrane resistance and capacitance values were extracted by fitting the EIS data to an equivalent circuit model. These values were reported as ΔR and ΔQ rather than absolute values to avoid the influence of batch-to-batch variation in substrate dimensions. However, in each instance the absolute resistance of the protein-reconstituted membrane was evaluated, to ensure it was conform to the expected R and Q values of stable lipid bilayers, as described above and previously.^{75,76,95}

As shown in Figure 5.9A (black), incubation of WTGal3 with the EggPC;EggPA membrane elicited a modest but systematic decrease in membrane resistance with increasing protein concentration. The response saturated at a WTGal3 concentration of ~37 nM. The decreased membrane resistance was consistent with the increased diffusivity of the membrane reported in FCS studies *vide infra* and may be due to phase changes in the membrane induced by WTGal3. In contrast, the Gal3 mutant in which the unstructured N-terminal oligomerization domain was deleted (Gal3 Δ anter) did not elicit any measurable change in membrane resistance (Figure 5.9A, red). Correspondingly, capacitance of the membrane was not influenced

significantly up to 60 nM of Gal3ΔNter (Figure 5.9B, red), whereas WTGal3 caused a small initial increase to capacitance that stabilised at 10 nM (Figure 5.9B, black). An increase in capacitance typically suggests membrane thinning. Although the capacitance change was very small at only $\sim 0.1 \mu\text{F/s}$, taken together with the decreased membrane resistance indicates that WTGal3 interacts with the membrane and increases its permittivity. This may be explained by an electrostatic interaction of negatively charged PA head groups with a density on the carbohydrate recognition domain of Gal3 that is positively charged at physiological pH of 7.4⁹⁶, or by penetration of the partially lipophilic proline-rich N-terminal domain⁹⁷ into the lipid bilayer possibly inducing some surfactant-like nanoporation⁸⁸. The fact that Gal3ΔNter did not affect the electrical membrane properties argues in favour of the latter explanation.

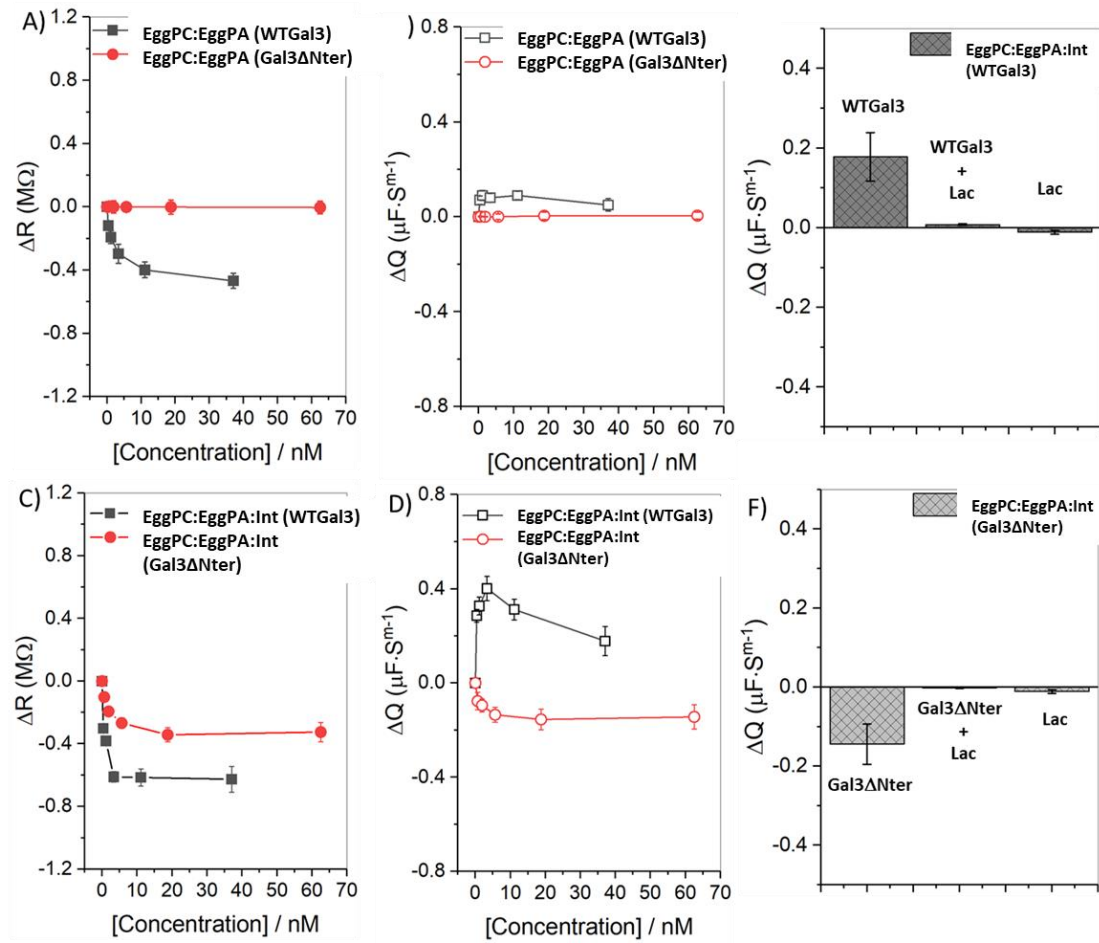


Figure 5.9: A,B) Relative change in (A) resistance, ΔR (filled symbol), and (B) capacitance, ΔQ (open symbols) values obtained upon binding of different concentrations of WTGal3 (black) or Gal3ΔNter (red) to EggPC:EggPA membranes. (C,D) Relative changes in resistance (ΔR) and capacitance (ΔQ) values respectively obtained upon binding of different

concentrations of WTGal3 (black) and Gal3 Δ Nter (red) to EggPC:EggPA membranes into which $\alpha_5\beta_1$ integrin was reconstituted. The solid lines in each panel A-D are shown to guide the eye. Data are means $\pm$ SD from triplicate experiments. (E,F) Bar charts showing the change in capacitance values with respect to the pristine EggPC:EggPA/Int membrane capacitance values when WTGal3 or Gal3 Δ Nter were incubated with $\alpha_5\beta_1$ integrin-containing membranes in the presence (+Lac) or absence of β -lactose. Lactose fully abolished the WTGal3 or Gal3 Δ Nter effects on ΔQ . In the absence of Gal3, lactose did not affect ΔQ . The concentration of WTGal3, Gal3 Δ Nter and β -lactose were 37 nM, 62.5 nM, and 50 mM, respectively. EIS measurements were performed in 0.01 M PBS buffer, frequency ranges were from 0.05 Hz to 105 Hz at 0 V DC bias potential vs Ag/AgCl (1 M KCl) with an AC amplitude of 10 mV at 22 ± 1 °C. Cell was a three-electrode set-up where gold cavity/MSLB, Ag/AgCl (1 M KCl) and Pt coiled served as working, reference, and counter electrode respectively. Courtesy of Dr. Nirod K Sarangi.

EIS experiments were then performed on EggPC:EggPA membranes containing $\alpha_5\beta_1$ integrin (L/P=10, w/w). Substantially greater changes to membrane resistance and capacitance were observed upon incubation of WTGal3 with integrin reconstituted MSLB compared to pristine membrane (Figure 5.9C, D). In addition, the slope of the curves of the EIS response in presence of integrin are steeper. Furthermore, both WTGal3 and Gal3 Δ Nter cause a decrease in resistance of the integrin-containing bilayer (Figure 5.9C). The magnitude of resistance changes was found to be consistently roughly twice as great for WTGal3 compared to Gal3 Δ Nter. Strikingly, opposing results were observed for WTGal3 and Gal3 Δ Nter when capacitance data were recorded. While for WTGal3, the membrane capacitance increased at concentrations up to 10 nM, and then decreased at higher concentration (Figure 5.9D, black), we observe a continuous decrease in capacitance for Gal3 Δ Nter that saturated at 10 nM (Figure 5.9D, red). That we saw evidence for weak physisorption of Gal3 Δ Nter in EIS measurement but not by FCS can be attributed to the fact that in the EIS experiments, the galectin was retained in the contacting solution during measurements whereas in the microfluidics device used for FCS, blank buffer was exchanged to reduce any background contribution from unbound fluorophore.

For WTGal3, the initial increase in capacitance indicated, along with the decreased resistance, that membrane permittivity increased, suggesting, along with EIS data in absence of integrin, that WTGal3 reorganizes the membrane and influences its packing. Although the initial

capacitance increase was also observed in the absence of $\alpha_5\beta_1$ integrin, its amplitude was much higher on integrin-containing membranes (Figure 5.9B, black), which indicates that the glycoprotein strongly favoured the recruitment of the lectin. Since no capacitance increase was observed for Gal3 Δ Nter, it was concluded to be due to the N terminal domain of WTGal3. It is speculated that the formation of galectin 3 oligomers with integrin organise and/or positions the N terminal domain of WTGal3 to perturb the membranes organisation. WTGal3 has been shown to directly interact with lipids.⁸⁸

At WTGal3 concentrations above 10 nM, the membrane resistance stabilised but capacitance decreased suggesting thickening of the membrane (Figure 5.9B, black). This might come from the formation of WTGal3- $\alpha_5\beta_1$ integrin clusters that would grow laterally as membrane invagination cannot occur in the MSLB system. This view is supported by imaging and FCS data. The WTGal3 effect on permittivity persisted under these conditions as resistance remained at a low plateau level (Figure 5.9C, black) and capacitance globally also remained above the level that was observed in the absence of the lectin (Figure 5.9D, black). This indicated that the permittivity and thickening effects occur simultaneously at WTGal3 concentrations above 10 nM.

Since WTGal3 and other galectins drive endocytosis at the threshold concentration of 10 nM in an oligomerization-dependent manner, the effect of the oligomerization deficient Gal3 Δ Nter in our system were tested.^{27,30} This mutant had a weak impact on the membrane electrical properties (Figure 5.9C,D), similar to the response at the pristine membrane (Figure 5.9A,B). This striking finding clearly indicates that its oligomerization capacity is required for efficient WTGal3 recruitment at concentrations up to 10 nM and for lateral clustering at concentrations above 10 nM.

The effects of WTGal3 and of Gal3 Δ Nter on the capacitance of $\alpha_5\beta_1$ integrin-containing membranes were fully cancelled out in the presence of β -lactose (Figure 5.9E,F). It could thereby be concluded that these effects were indeed dependent on glycan-lectin interactions. In agreement with this conclusion, the corresponding FCS lactose data also showed that lactose inhibits the effects of WTGal3. In contrast, lactose had no effect in the absence of WTGal3 (Figure 5.9E,F), which confirmed the specificity of our findings, both with EIS and FCS.

5.4 Activation of $\alpha_5\beta_1$ integrin, from the resting state to the extended conformational state.

5.4.1 Introduction of $\alpha_5\beta_1$ integrin activation with Fibronectin.

Integrins are believed to exist in three conformational states that regulate their affinity to their ligands, proteins or the extracellular matrix.^{31,40,41} Integrins are normally resident in the cell membrane in their resting state, characterized across many studies, including cryo electron microscopy by a bent conformation.^{42–44} Activation can be stimulated by the integrin binding to its ligand or by binding to an activator protein or indeed through chemical activation by reduction of the disulphides, removal of the metals from the MIDAS region or through treatment with Mn^{2+} .^{23,42,48–51} Despite being a major class of integrin, $\alpha_5\beta_1$, has been proposed to not follow the traditional bent-closed-open activation, stated above in section 5.1.⁴⁶ Reports have indicated that $\alpha_5\beta_1$ is only half bent whilst in its resting state.^{46,98,99} In contrast to other integrins, upon priming of the integrin with intracellular signals, ligand-mimetic peptides or specific ions or by using Mn^{2+} in vitro to simulate the MIDAS binding site, $\alpha_5\beta_1$ appeared to not undergo any conformational change and remained in this half bent state, shown in Figure 5.10. However this priming of the MIDAS is suspected to play a critical role in the increasing affinity of $\alpha_5\beta_1$ to its ligands.⁴⁶

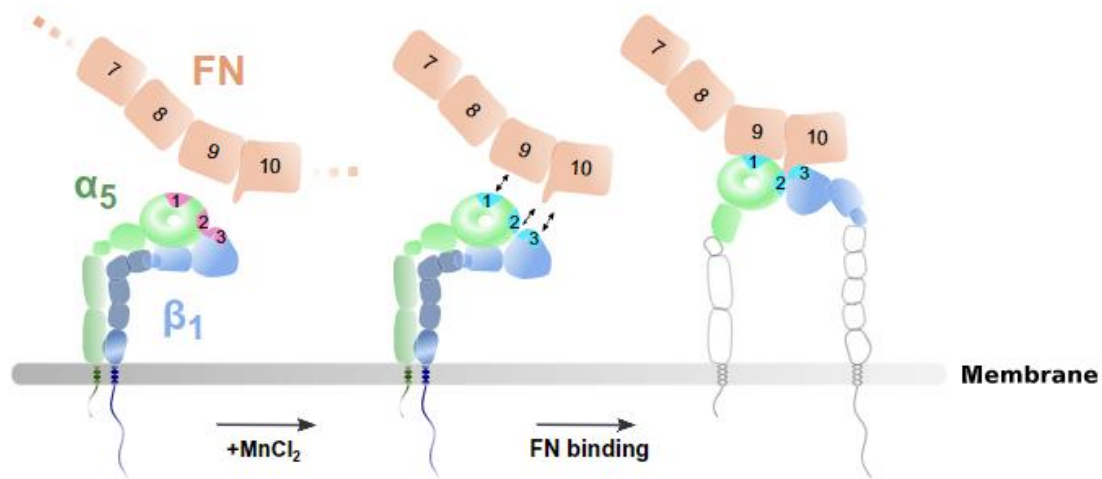


Figure 5.10: Integrin $\alpha_5\beta_1$ exists in different conformational states, fluctuating between a resting bending state and an activated extension state. Fibronectin (Fn) binding secures the extended and open conformation of integrin $\alpha_5\beta_1$ through three binding sites (marked as 1, 2, and 3) at the integrin heads. Mn^{2+} primes binding sites, and brings integrin to a ready state in which it is ready to bind to Fn. Full engagement of Fn takes place, resulting in the integrin $\alpha_5\beta_1$ opening.⁴⁶

Integrin $\alpha_5\beta_1$ is the primary fibronectin (Fn) receptor which is responsible for cell migration and adhesion.^{23,40,45,100–102} Both fibronectin and $\alpha_5\beta_1$ are commonly expressed in various cells and tissues to ensure communication between cells and the extracellular matrix. $\alpha_5\beta_1$ and its adhesion to Fn has been reported to promote cell survival in conditions such as hypoxia, serum starvation and chemotherapy.⁶¹ An initial interaction of the Arg-Gly-Asp (RGD) binding site of Fn with integrin has been shown to be a key attachment site, which is then followed by secondary interactions of the fibronectin synergy site and the primed MIDAS site to ensure full adhesive activity for $\alpha_5\beta_1$ integrin and Fn.⁶² Fn can bind to multiple integrins through its RGD motif on Fn(III)₁₀ fragment. The RGD motif of Fn(III)₁₀ is recognised by the extracellular alpha domain of $\alpha_5\beta_1$, however, then further binding occurs using a synergy site in Fn(III)₉ domain which is specific to $\alpha_5\beta_1$, once the MIDAS domain has been primed, e.g. with Mn^{2+} .^{62,104–106}

$\alpha_5\beta_1$ connects to the Fn ligand through its extracellular domain. Fn is a fibular dimer that acts as a core component of the extracellular matrix.¹⁰⁰ Fn is a large protein comprising type I, type II and type III domains that repeat into a “beads on a string morphology”, show in Figure 5.10.¹⁰³ Fn is linked to cell adhesion, migration, growth, and differentiation.¹⁰⁴ Upon the binding of Fn to $\alpha_5\beta_1$, a conformational shift from half bent to an extended opened state occurs. The dual binding of Fn(III)₉ and Fn(III)₁₀ stabilises this open confirmation of $\alpha_5\beta_1$. When priming of the MIDAS did not occur, then recognition of the RGD site alone did not cause a conformational shift in $\alpha_5\beta_1$ state. Both priming of the MIDAS site and interaction with Fn was needed to ensure and stabilise the extended confirmation of $\alpha_5\beta_1$.^{107,108} Fn matrix assembly has been linked to contribute to tissue cohesion and this specific activation of $\alpha_5\beta_1$ using Fn to form matrixes in the extracellular matrix could have an effect on the clustering and aggregation of lectins.¹⁰⁹ Galectins and Fn both depend on integrins, namely $\alpha_5\beta_1$, to aggregate or cluster, and thus could potentially lead to a competitive inhibition of galectin binding upon activation of the integrin.¹¹⁰ Gal1 aggregation has been seen to be reduced and inhibited when $\alpha_5\beta_1$ undergoes activation with Fn.¹¹¹ Similarly Fn has been used to block Gal3 interaction with $\alpha_5\beta_1$ whilst investigating cell matrix adhesions in live cells with an immunofluorescence assay.³⁹ Herein, we investigate the effect of activation of $\alpha_5\beta_1$ with Fn and with cyclic RGD. This was to determine if the activation state of $\alpha_5\beta_1$ effected the clustering effects observed for WTGal3 when integrin was in the resting state.

5.4.2 Fluorescence Correlation Spectroscopy on EggPC:EggPA:Integrin membrane in the presence of different buffers.

In order to determine if activation of integrin, $\alpha_5\beta_1$, had an effect on WTGal3 clustering observed in the resting state of integrin, the underlying effects of the activators were firstly examined on the pristine EggPC:EggPA membrane. In order to prime the MIDAS site of the integrin to enhance the affinity of $\alpha_5\beta_1$ for its ligand, Mn^{2+} was applied. Initial studies employed PBS as the buffer system, but this was found to be unsuitable for studies employing $MnCl_2$ as it caused precipitates to form. HEPES buffer was then used as buffer in all subsequent studies. Interestingly, in HEPES a modest increase in the average diffusion coefficient of integrin in the EggPC:EggPA membrane was observed, from an average of 1.99 ± 0.56 in contact with PBS and an average of $2.76 \pm 0.36 \mu m^2/s$ in contact with HEPES, Table 5.4.

In both cases alpha was approximately 0.8, indicating sub-diffusion. The diffusion coefficients obtained for EggPC:EggPA labelled with DOPE-Atto532 in PBS were seen to be similar to the diffusion coefficients obtained for DOPE-Atto532 in HEPES buffer, around $6.66 \mu m^2/s$.⁷⁵ This is interesting because whereas alterations to membrane fluidity is expected to have a large impact on integrin diffusivity this was not the sole cause of the change indicated by the large integrin-atto488 diffusivity change and small DOPE-Atto532 diffusivity change. The HEPES buffer may affect the integrin directly, perhaps reducing aggregation within the membrane.

Table 5.4: $\alpha_5\beta_1$ Integrin reconstituted in EggPC:EggPA (90:10 mol%) using PBS Buffer (pH 7.4) in the MSLB versus $\alpha_5\beta_1$ Integrin reconstituted in EggPC:EggPA (90:10 mol%) using HEPES buffer (pH 7.4) in MSLB.

Diffusing fluorophore in EggPC:EggPA:Int membrane	Diffusion ($\mu m^2/s$)	Alpha
Integrin Atto488 in PBS buffer	1.99 ± 0.56	0.85 ± 0.26
Integrin Atto488 in HEPES buffer	2.76 ± 0.36	0.81 ± 0.10

The buffer interaction with the membrane does not affect the stability or integrity of the membrane and as such was suitable to continue activation studies in HEPES buffer.^{75,112} All subsequent experiments were prepared with HEPES buffer.

5.4.3 Fluorescence Correlation Spectroscopy on EggPC;EggPA membrane in the presence of the different activating materials

As mentioned, the integrin of interest, $\alpha_5\beta_1$ is known as the fibronectin receptor. An initial priming of the MIDAS site occurs allowing for the interaction of the RGD sequence of the Fn(III)₁₀ fragment with $\alpha_5\beta_1$. This is reportedly followed by a secondary reaction of the fibronectin synergy site on the Fn(III)₉ fragment, shown in Figure 5.10.^{62,104–106} As such a fragment of fibronectin (FnIII₉₋₁₀) and cyclic-RGD were used to investigate activation effects of $\alpha_5\beta_1$ integrin on the aggregation of WTGal3.

Table 5.5: Pristine EggPC:EggPA:DOPE-Atto532 (90:10 mol%) investigation of the effects on the membrane of treatment protocols for integrin activation. The diffusion coefficient of the lipid marker within the membrane was used for investigation. Incubation of the membrane with 5 mM MnCl₂ for 30 minutes, incubation of 10 μ M FnIII₍₉₋₁₀₎ for 2 hours and incubation with 1 mM of cRGD for 2 hours completed in HEPES buffer, pH 7.4. N=3

DOPE-Atto532 diffusion In EggPC:EggPA	Diffusion ($\mu\text{m}^2/\text{s}$)	Alpha
Untreated membrane	6.66 ± 0.38	0.96 ± 0.06
5 mM MnCl ₂ treatment	5.10 ± 0.27	1.00 ± 0.10
1 mM RGD treatment (no Mn ²⁺ present)	4.80 ± 0.14	0.88 ± 0.02
10 μ M FnIII ₉₋₁₀ treatment (no Mn ²⁺ present)	4.36 ± 0.25	0.98 ± 0.05
RGD & Mn ²⁺ treatment	4.32 ± 0.13	0.92 ± 0.07
Mn ²⁺ & FnIII ₉₋₁₀ treatment	2.53 ± 0.34	1.00 ± 0.01

Initially before evaluating the activation of the integrin, controls were completed to understand any effects of the activating species on the underlying membrane viscosity. Table 5.5 shows the diffusion coefficient of membrane label DOPE-Atto532, following membrane treatment with the chemical activators on the pristine membrane. Following Mn²⁺ incubation for 30 minutes there was an initial decrease in lipid diffusion from 6.66 $\mu\text{m}^2/\text{s}$ to 5.10 $\mu\text{m}^2/\text{s}$. This is

tentatively attributed to interaction of the negatively charged EggPA with Mn^{2+} which, from a previous report, imposes inhomogeneity in phase from liquid disordered to a gel phase.¹¹³

Upon incubation of the pristine membrane with 10 μM FnIII₉₋₁₀ or 1 mM RGD, for 2 hours in the absence of manganese, a decrease of membrane diffusion was observed to 4.36 ± 0.25 and 4.80 ± 0.14 $\mu m^2/s$ respectively. This data shows that both RGD and Fn bind with the EggPC:EggPA membrane slowing the diffusion of the lipid marker (from 6.66 ± 0.38 $\mu m^2/s$). This is attributed to RGD or FnIII₉₋₁₀ adsorption at the membrane, even in the absence of Mn^{2+} . In the presence of both Mn^{2+} and RGD, the membrane diffusion was seen to further decrease slightly from 4.80 ± 0.14 with RGD alone to 4.32 ± 0.13 $\mu m^2/s$ when Mn^{2+} was also present. This decrease in the presence of Mn^{2+} is attributed to the viscosity change within the membrane to gel phase when the cation is present. Interestingly, both with and without Mn^{2+} , RGD caused the anomalous parameter of DOPE-Atto532 to decrease from 1, showing sub-diffusion away from Brownian motion which also suggests that a viscosity change to gel phase is caused. This indicated that the adsorption onto the membrane is mainly driven by the incorporation of RGD in the system.

Whilst the membrane was incubated with Mn^{2+} and FnIII₉₋₁₀, a significant decrease of DOPE-Atto532 diffusion was observed to 2.53 ± 0.34 $\mu m^2/s$ compared to in the absence of Mn^{2+} , 4.36 ± 0.25 $\mu m^2/s$ (Table 5.5). The alpha value remained at 1 indicating Brownian motion, when FnIII₉₋₁₀ with and without Mn^{2+} . This significant decrease of lipid marker diffusion can be attributed to the bulk of Fn interacting with the membrane alongside the increased viscosity of the membrane from the Mn^{2+} . The further lipid marker diffusion decreases upon the addition of Fn after Mn^{2+} could suggest that the pristine membrane's viscosity is further increased from the gel phase shift that Mn^{2+} caused to the membrane. Perhaps instead of a viscosity increase, the presence of Mn^{2+} promotes Fn binding to the pristine membrane and with the enhanced binding the diffusion of the lipid marker is slowed.

5.4.4 Fluorescence Correlation Spectroscopy on EggPC:EggPA:Integrin membrane in the presence of Mn^{2+} , Fibronectin and RGD

Table 5.6 shows the diffusion values of $\alpha_5\beta_1$ Integrin-Atto488 and FnIII₉₋₁₀ Alexa647 in the presence and absence of Mn^{2+} in an EggPC:EggPA(90:10) bilayer reconstituted with $\alpha_5\beta_1$ Integrin (LPR 10). Initially the membrane was incubated with manganese for 30 minutes. Mn^{2+} treatment has been shown across a wide range of integrins to promote ligand binding, attributed to partial activation appears to cause epitope exposure without full conformational activation

of the integrin.^{101,102,114} Correspondingly, here, the diffusion coefficient of the integrin was not observed to change, within experimental error, when incubated with manganese, from 2.76 ± 0.36 to $2.69 \pm 0.08 \mu\text{m}^2/\text{s}$. Whilst remaining in a sub-diffusion regime similarly reported for the resting integrin above.

Table 5.6: The effect of 10 μM FnIII₉₋₁₀, with and without 5 mM MnCl₂, on the diffusion coefficient of EggPC:EggPA(90:10) membrane reconstituted with $\alpha_5\beta_1$ Integrin LPR 10 in HEPES, pH 7.4.

Diffusing fluorophore in EggPC:EggPA	Diffusion ($\mu\text{m}^2/\text{s}$)	Alpha
Integrin-Atto488	2.76 ± 0.36	0.83 ± 0.03
Integrin-Atto488 after Mn ²⁺	2.69 ± 0.08	0.78 ± 0.18
Integrin-Atto488 after FnIII ₉₋₁₀	2.24 ± 0.30	0.86 ± 0.09
Integrin-Atto488 after Mn ²⁺ & FnIII ₉₋₁₀	2.03 ± 0.09	1.01 ± 0.07
FnIII ₉₋₁₀ Alexa 647 in absence of Mn ²⁺	4.36 ± 0.18	0.99 ± 0.03
FnIII ₉₋₁₀ Alexa 647 with Mn ²⁺ present	2.00 ± 0.20	1.01 ± 0.04

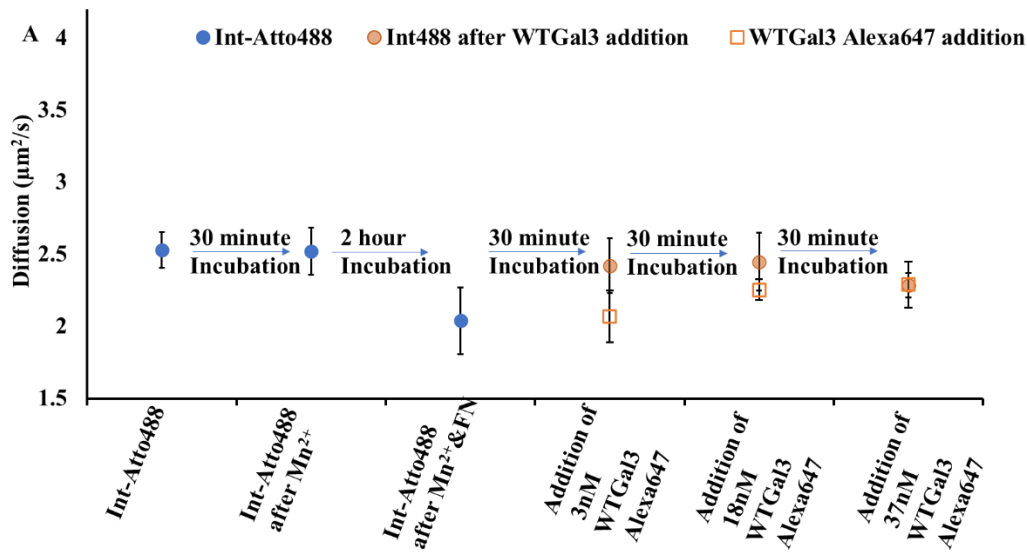
Following Mn²⁺ treatment, FnIII₉₋₁₀ was incubated at the membrane for 2 hours in the presence of manganese. Thereafter, the diffusion coefficient of Integrin was seen to decrease to $2.03 \pm 0.09 \mu\text{m}^2/\text{s}$ from $2.69 \pm 0.08 \mu\text{m}^2/\text{s}$. The FnIII₉₋₁₀-Alexa647 was observed to diffuse at $2.00 \pm 0.20 \mu\text{m}^2/\text{s}$ the alpha value for both species was 1. This indicates unhindered, Brownian motion for both. Interestingly when treated with FnIII₉₋₁₀ (10 μM , 2 hours) in absence of Mn²⁺, FnIII₉₋₁₀ elicits a different response. Integrin diffusion decreases slightly to $2.24 \pm 0.30 \mu\text{m}^2/\text{s}$ (within experimental error) whilst the alpha value remains unchanged, at 0.85. The Fn Alexa647 was observed to diffuse at the integrin reconstituted membrane with a diffusion of $4.36 \pm 0.18 \mu\text{m}^2/\text{s}$. This is the same as reported for the DOPE-Atto532 lipid marker of the pristine membrane upon treatment with just Fn (4.36 ± 0.25 , Table 5.5 above). This suggests that when no manganese is present, the FnIII₉₋₁₀ does not bind to or activate the integrin but does appear

to adsorb at the membrane. This indicates that Mn^{2+} promotes Fn binding to both the integrin reconstituted and pristine membranes. By comparison FnIII₉₋₁₀-Alexa647 was observed to have a diffusion coefficient of 124 $\mu m^2/s$ whilst in bulk solution.

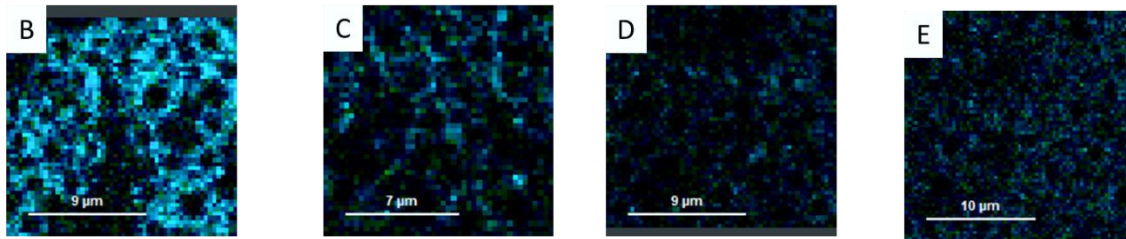
Therefore, the modest decrease in integrin diffusion to $2.24 \pm 0.30 \mu m^2/s$ upon treatment with FnIII₉₋₁₀ is attributed to viscosity changes of the membrane induced by Fn adsorption. The Fn(III) diffusion matches the integrin diffusion when Mn^{2+} is present indicates that the MIDAS has been primed and the integrin interacts directly with the ligand. Manganese pre-treatment was therefore used for all proceeding experiments.

5.4.5 Fluorescence Correlation Spectroscopy on Activated $\alpha_5\beta_1$ with Mn^{2+} and Fibronectin in an EggPC;EggPA membrane in the presence of WTGal3

Figure 5.11A and Table 5.7 shows the impact of the Fn activation on $\alpha_5\beta_1$ integrin diffusion in contact with varying concentrations of WTGal3.



FLIM - Integrin-Atto488 (0, 3.7, 18, 37 nM WTGal3 additions)



FLIM – WTGal3-Alexa647 (3.7, 18, 37 nM WTGal3 additions)

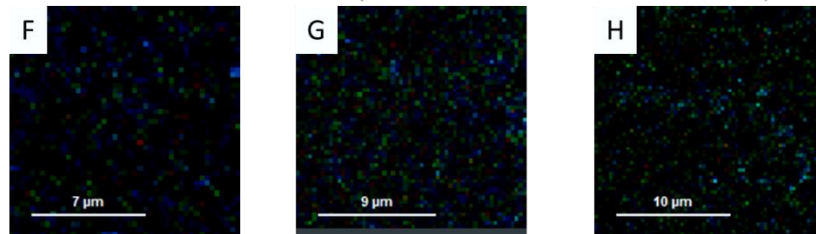


Figure 5.116: (A) Graphical representation of diffusion co-efficient trends of EggPC:EggPA(90:10) with reconstituted Integrin-Atto488 (LPR 10) in HEPES buffer pH 7.4 following the activation of $\alpha_5\beta_1$ with 5 mM $MnCl_2$ for 30 minutes & 10 μM FnIII₉₋₁₀ for 2 hours (blue circles). Diffusivity values of both activated integrin-atto488 (orange circles) and WTGal3 Alexa647 (orange squares) after the additions of 3 nM, 18 nM & 37 nM WTGal3 respectively, each after 30 minutes incubation with membrane. Each plotted point is obtained from 3 arrays ($N=3$), and 15-20 cavities measured per sample point per array. (B) FLIM image of activated Integrin-Atto488 after treatment with 5 mM $MnCl_2$ & 10 μM FnIII₉₋₁₀. C-E) FLIM

images of activated integrin-Atto488 after the addition of WTGal3 Alexa647 at 3 (C), 18 (D) & 37 (E) nM, respectively in HEPES pH 7.4 containing 5 mM MnCl₂ & 10 μ M FnIII₉₋₁₀. (F-H) FLIM images of WTGal3 Alexa647 upon incubation with the EggPC:EggPA:activated-integrin membrane at 3 (F), 18 (G), & 37 (H) nM, respectively in HEPES pH 7.4 containing 5 mM MnCl₂ & 10 μ M FnIII₉₋₁₀. .

Addition of 3.70 nM WTGal3 caused an increase in integrin diffusion to $2.42 \pm 0.19 \mu\text{m}^2/\text{s}$ and WTGal3 diffusion of $2.07 \pm 0.18 \mu\text{m}^2/\text{s}$. Interestingly, the intensity of the integrin-Atto488 emission diminishes slightly as 3.70 nM WTGal3 was incubated with the membrane, Figure 5.11C. The image of WTGal3 Alexa647 (Figure 5.11F) exhibits a weak signal from the 647 nm channel which was strikingly different when compared to WTGal3 interacting with $\alpha_5\beta_1$ in the resting state (Figure 5.6A).

As the concentration was increased to 18 nM WTGal3 the diffusion value of WTGal3 increased to $2.25 \pm 0.08 \mu\text{m}^2/\text{s}$. The integrin-Atto488 label diffusion remained the same at $2.45 \pm 0.20 \mu\text{m}^2/\text{s}$. Upon incubation with 37 nM WTGal3, the diffusion coefficients of both integrin and WTGal3 normalised to the same value $\sim 2.29 \mu\text{m}^2/\text{s}$ (2.28 ± 0.09 and $2.29 \pm 0.16 \mu\text{m}^2/\text{s}$ respectively) and the anomalous value was 1, indicating Brownian motion for both. The intensity of integrin-Atto488 obtained in the FLIM images as WTGal3 concentration increased, Figure 5.11D & E, remained significantly lower than before WTGal3 incubation. The WTGal3 Alexa647 signal, Figure 11G & H, also remained significantly weaker in the activated conformational state.

In contrast to the resting state of $\alpha_5\beta_1$, only one population of WTGal3 was observed from FCS and FLIM after the activation. Notably, integrin and WTGal3 diffuse at the same rate, graphically represented in Figure 11A. The absence of clustering in the FLIM and the presence of just one diffusional population seem to suggest upon $\alpha_5\beta_1$ activation with Mn^{2+} and FnIII₉₋₁₀, that WTGal3 oligomerization and clustering is inhibited.¹¹⁰ Galectins have also been shown to bind to Fn in the extracellular matrix and cause a reorganisation of Fn. Fn could be blocking the WTGal3 from interacting with the activated integrin.^{3,4,110,115,116} This reorganisation of Fn from the WTGal3 could also account for the increase of diffusion of integrin within the MSLB while alpha remained around 1 for all WTGal3 concentration additions.

Table 5.7: The effects of varying WTGal3 concentrations on an EggPC:EggPA (90:10 mol%) membrane with reconstituted $\alpha_5\beta_1$ Integrin (LPR 10) after activation using 5 mM MnCl₂ (30

minutes) & 10 μM FnIII₍₉₋₁₀₎ (2 hours) in HEPES buffer, pH 7.4 containing 5mM Mn & 10 μM FnIII₍₉₋₁₀₎ throughout experiment.

Diffusing Fluorophore	Diffusion ($\mu\text{m}^2/\text{s}$)	Alpha
Integrin-Atto488	2.76 ± 0.36	0.83 ± 0.03
Integrin-Atto488 after Mn^{2+}	2.69 ± 0.08	0.78 ± 0.18
Integrin-Atto488, after activation, 0 WTGal 3	2.03 ± 0.09	1.01 ± 0.07
Integrin-Atto488, after 3.70 nM WTGal3	2.42 ± 0.19	0.99 ± 0.19
Integrin-Atto488, after 18.52 nM WTGal3	2.45 ± 0.20	0.98 ± 0.11
Integrin-Atto488, after 37.04 nM WTGal3	2.28 ± 0.09	0.94 ± 0.03
WTGal3 Alexa 647, 3.70 nM	2.07 ± 0.18	0.94 ± 0.08
WTGal3 Alexa 647, 18.52 nM	2.25 ± 0.08	1.05 ± 0.11
WTGal3 Alexa 647, 37.04 nM	2.29 ± 0.16	1.14 ± 0.31

5.4.6 Fluorescence Correlation Spectroscopy on Activated $\alpha_5\beta_1$ with Mn^{2+} and Fibronectin in an EggPC;EggPA membrane in the presence of Gal3 ΔNter

Similar to the resting state of $\alpha_5\beta_1$, investigation into Gal3 ΔNter after the activation of $\alpha_5\beta_1$ with Mn^{2+} and FnIII₉₋₁₀ was completed. This was to determine if truncating the N terminus of the galectin 3 inhibited its capacity to aggregate and impact its influence on the activated integrin.

Gal3 ΔNter exhibited a very weak signal at the reconstituted integrin membrane, to the extent that there was insufficient signal to determine diffusion coefficients or images for the truncated protein.

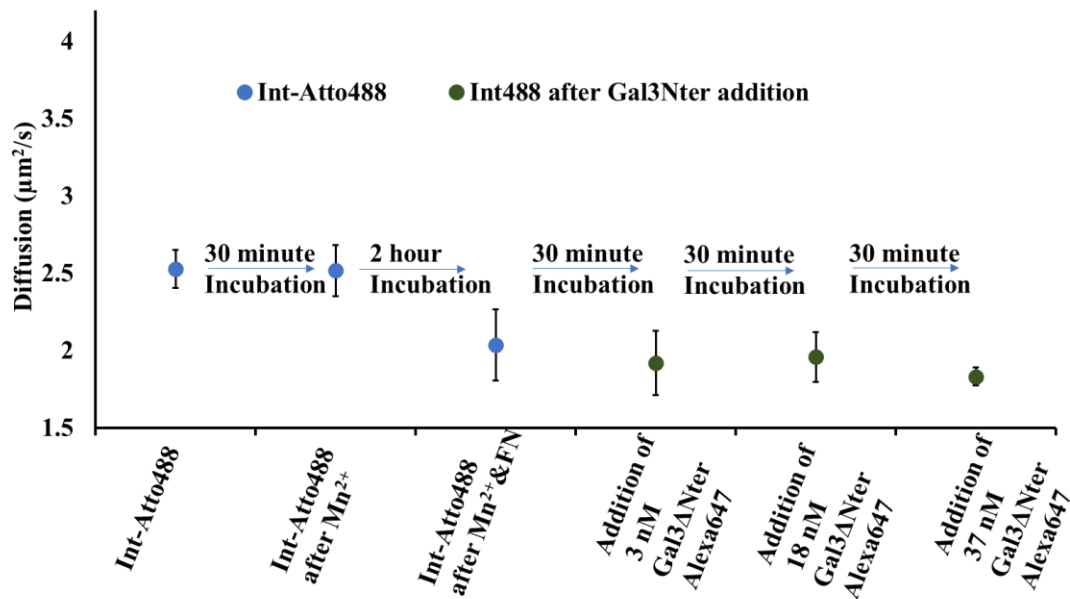


Figure 5.12: Graphical representation of diffusion co-efficient trends of EggPC:EggPA(90:10) with reconstituted Integrin-Atto488 (LPR 10) in HEPES buffer pH 7.4 following the activation of $\alpha_5\beta_1$ with 5 mM $MnCl_2$ for 30 minutes & 10 μM $FnIII_{9-10}$ for 2 hours (blue circles). Diffusivity values of activated integrin-atto488 (green circles) after the additions of 3 nM, 18 nM & 37 nM $Gal3\Delta Nter$ Alexa647 respectively, each after 30 minutes incubation with membrane in HEPES pH 7.4 containing 5 mM $MnCl_2$ & 10 μM $FnIII_{9-10}$. Each plotted point is obtained from 3 arrays ($N=3$), and 15-20 cavities measured per sample point per array in HEPES pH 7.4 with $MnCl_2$ & $FnIII_{9-10}$. $Gal3\Delta Nter$ signal was observed very weakly at the membrane, to the extent that there was insufficient signal to determine diffusion co-efficient for the truncated protein.

Furthermore, integrin-Atto488 diffusion with the addition of increasing concentrations of $Gal3\Delta Nter$, 3.7 and 18.5 nM remained similar to the integrin diffusion after activation with Mn^{2+} and $FnIII_{9-10}$ (1.92 ± 0.20 , 1.96 ± 0.16 and $2.03 \pm 0.09 \mu m^2/s$, respectively). At the highest concentration, 37 nM of $Gal3\Delta Nter$, the integrin diffusion coefficient decreased slightly to $1.83 \pm 0.06 \mu m^2/s$, Figure 5.12. This could suggest a weak binding of the $Gal3\Delta Nter$ with the activated integrin, which is consistent with the lower affinity observed at the resting state for $Gal3\Delta Nter$ versus the WTGal3 (section 5.3.2 above). This lack of interaction of the $Gal3\Delta Nter$ with integrin (in either resting or activated conformational states) further suggests that the N terminus tail is required for measurable integrin interaction between Galectin 3 and $\alpha_5\beta_1$. This implies that the N terminus tail is required for the clustering and aggregation of WTGal3 with $\alpha_5\beta_1$ in the inactive form.

5.4.7 Fluorescence Correlation Spectroscopy on Activated $\alpha_5\beta_1$ with Mn^{2+} and RGD in an EggPC;EggPA membrane in the presence of Gal3 Δ Nter

Integrin activation using cyclic RGD (cRGD) was investigated. RGD has been discovered to be the minimal integrin binding motif for multiple integrins including, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$.¹¹⁷ Herein, WTGal3 was added to an integrin reconstituted membrane that was first treated with 5 mM $MnCl_2$ for 30 minutes and then 1 mM cRGD for 2 hours.

Fn has a higher affinity for $\alpha_5\beta_1$ due to the RGD motif and the synergistic site used in activation, Figure 5.10, and as such a much higher concentration of RGD was used compared to the FnIII₉₋₁₀, to enhance the probability of the RGD recognition by $\alpha_5\beta_1$.^{118,119}

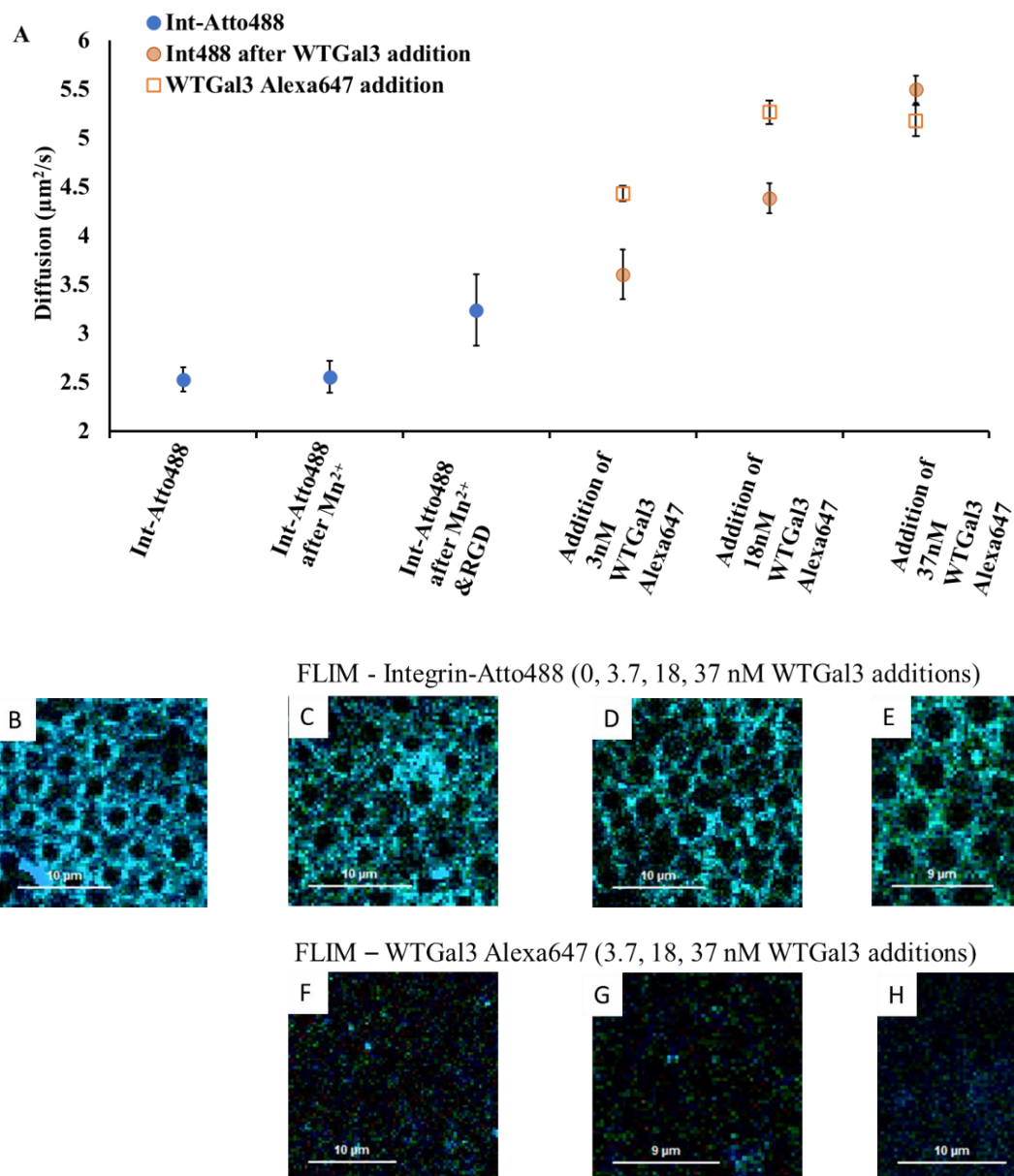


Figure 5.137: (A) Graphical representation of diffusion co-efficient trends of EggPC:EggPA(90:10) with reconstituted Integrin-Atto488 (LPR 10) in HEPES buffer pH 7.4 following the activation of $\alpha_5\beta_1$ with 5 mM $MnCl_2$ for 30 minutes & 1 mM cRGD for 2 hours (blue circles). Diffusivity values of both activated integrin-atto488 (orange circles) and WTGal3 Alexa647 (orange squares) after the additions of 3 nM, 18 nM & 37 nM WTGal3 respectively, each after 30 minutes incubation with membrane. Each plotted point is obtained from 3 arrays ($N=3$), and 15-20 cavities measured per sample point per array. (B) FLIM image of Integrin-Atto488 after treatment with 5 mM $MnCl_2$ & 1 mM cRGD. C-E) FLIM images of integrin-Atto488 after the addition of WTGal3 Alexa647 at 3 (C), 18 (D) & 37 (E) nM,

respectively in HEPES pH 7.4 containing 5 mM MnCl₂ & 1 mM cRGD. (F-H) FLIM images of WTGal3 Alexa647 upon incubation with the EggPC:EggPA:activated-integrin membrane at 3 (F), 18 (G), & 37 (H) nM, respectively in HEPES pH 7.4 containing 5 mM MnCl₂ & 1 mM cRGD .

Table 5.8 and Figure 5.13 show data associated with the reconstituted $\alpha_5\beta_1$ membrane following treatment with 5mM MnCl₂ and 1 mM cRGD, to confirm if the $\alpha_5\beta_1$ integrin recognises and activates with just the cRGD sequence itself. After incubation of the integrin reconstituted membrane with 1 mM cRGD, a moderate increase in integrin diffusion was observed from 2.69 ± 0.08 to $3.24 \pm 0.37 \mu\text{m}^2/\text{s}$. The anomalous parameter increased to 0.99 ± 0.08 signifying the diffusion regime changed to Brownian motion. Mn²⁺ and cRGD had less of an impact on the pristine membrane (DOPE-Atto532; $4.32 \pm 0.13 \mu\text{m}^2/\text{s}$) compared to Mn²⁺ and FnIII₉₋₁₀ (DOPE-Atto532; $2.53 \pm 0.34 \mu\text{m}^2/\text{s}$), (Table 5.5). The pristine membrane's decreased fluidity, alongside the increased integrin diffusivity (when reconstituted) with Mn²⁺ and cRGD, indicated that cRGD was interacting with both the membrane and the $\alpha_5\beta_1$. Figure 5.13B ascertained the integrin reconstituted membrane remains intact and stable after activation using Mn²⁺ and cRGD.

Upon the addition of 3.7 nM WTGal3, integrin diffusivity was seen to slightly increase to $3.61 \pm 0.25 \mu\text{m}^2/\text{s}$, whilst WTGal3 Alexa647 was observed to diffuse at $4.44 \pm 0.08 \mu\text{m}^2/\text{s}$. Figure 13C shows the FLIM of $\alpha_5\beta_1$ -Atto488 after the addition of 3.7 nM WTGal3. The intensity of integrin-Atto488 appeared to be less concentrated around the circumference of the pores in the presence of WTGal3. This along with the increase of diffusivity, which was recorded from the centre of the pore, suggests that the integrin was reorganising within the membrane. Contrastingly to when FnIII₉₋₁₀ was in the MSLB, the integrin-Atto488's fluorescence intensity maintained a strong signal when WTGal3 was added to the array with cRGD. However, compared to the resting state of $\alpha_5\beta_1$, the intensity of $\alpha_5\beta_1$ -Atto remained uniform across the membrane (Figure 5.6A). This suggests that the integrin is not undergoing any clustering from WTGal3 addition. Figure 5.13F of 3.7 nM WTGal3 Alexa647, exhibited a weak intensity, similar to that obtained when integrin was activated with FnIII₉₋₁₀ (Figure 5.11).

As the concentration of WTGal3 was increased to 18 and 37 nM, a further increase of integrin diffusion was exhibited, 4.39 ± 0.15 and $5.50 \pm 0.14 \mu\text{m}^2/\text{s}$ respectively. The FLIM images obtained for integrin at the increased WTGal3 concentrations remained at a similar intensity to when 3.7 nM was added (Figure 13D & E). The WTGal3 Alexa647 also increased in diffusion

values as the concentration was increased, 5.27 ± 0.12 and $5.18 \pm 0.16 \mu\text{m}^2/\text{s}$. All anomalous factors remained ≈ 1 to suggest that upon binding of WTGal3, integrin $\alpha_5\beta_1$ and WTGal3 remained in Brownian motion. Figures 13G and H show the intensity of WTGal3 at the higher concentrations, which similar to the FnIII₉₋₁₀ show a significantly weaker signal compared to the resting state of integrin.

For both cRGD and FnIII₉₋₁₀ the slower moving aggregated population of WTGal3 observed in the resting state of integrin did not exist. For both ligands, at the higher concentrations of WTGal3, diffusion for integrin and WTGal3 appear to diffuse at a similar rate. The lack of emission intensity of WTGal3 observed by FLIM in both FnIII₉₋₁₀ (Figure 5.11 F-H) and cRGD (Figure 5.13 F-H), in association with only one population of WTGal3 obtained from FCS are indicative that no WTGal3 clustering was occurring. The reconstitution of $\alpha_5\beta_1$ into the membrane is expected to give a mixture of correctly and incorrectly orientated $\alpha_5\beta_1$ in the membrane meaning that not all WTGal3, cRGD, or FnIII₉₋₁₀ can bind as expected. Due to roughly 50% of the integrin being orientated in the wrong direction, a diffusion value close to the membrane value is impossible to distinguish. However, it is certain that a change was occurring to the effects of WTGal3 binding upon the suggested activation of $\alpha_5\beta_1$. The change in WTGal3 behaviour indicated that cRGD and FnIII₉₋₁₀ cause a conformational change to the $\alpha_5\beta_1$.

FnIII₉₋₁₀ is a large bulky protein which has a molecular weight of roughly 22000 Da, compared to the cRGD sequence which has a molecular weight of 603.7 Da. Due to this size discrepancy between the two activators used, it is hard to distinguish if both cause a conformational shift to the extended open state of $\alpha_5\beta_1$ from FCS. The effects of FnIII₉₋₁₀ and cRGD on the membrane itself are significant (more significant for FnIII₉₋₁₀) and could be masking the effects of WTGal3 with $\alpha_5\beta_1$. However, it is evident that the resting state of integrin in the presence of WTGal3 responds strikingly differently in both FLIM and diffusion coefficient values. Clustering of WTGal3 occurred only for resting integrin.

Table 5.8: The effects of varying WTGal3 concentrations on an EggPC:EggPA (90:10 mol%) membrane with reconstituted $\alpha_5\beta_1$ Integrin (LPR 10) after activation using 5 mM MnCl_2 (30

minutes) & 1 mM cRGD (2 hours) in HEPES buffer, pH 7.4 containing 5mM Mn & 10uM FnIII₍₉₋₁₀₎ throughout experiment.

Diffusing Fluorophore	Diffusion ($\mu\text{m}^2/\text{s}$)	alpha
Integrin 488	2.76 ± 0.36	0.83 ± 0.03
Integrin 488 after Mn^{2+}	2.69 ± 0.08	0.78 ± 0.18
Integrin 488 after Mn^{2+} & RGD, 0 WTGal3	3.24 ± 0.37	0.99 ± 0.08
Integrin 488, after 3.70 nM WTGal3	3.61 ± 0.25	1.00 ± 0.05
Integrin 488, after 18.52 nM WTGal3	4.39 ± 0.15	1.00 ± 0.03
Integrin 488, after 37.04 nM WTGal3	5.50 ± 0.14	0.93 ± 0.02
WTGal3 Alexa647, 3.70 nM	4.44 ± 0.08	1.02 ± 0.07
WTGal3 Alexa647, 18.52 nM	5.27 ± 0.12	1.00 ± 0.08
WTGal3 Alexa647, 37.04 nM	5.18 ± 0.16	0.98 ± 0.01

5.4.8 Electrochemical Impedance Spectroscopy on EggPC;EggPA membrane in the absence or presence of integrin and the effects of the activating materials

EIS was employed to gain a global insight to the impacts of activated $\alpha_5\beta_1$ -WTGal3 interactions on the whole membrane's electrical properties. Insight into the membrane phase or packing in

the presence of activated $\alpha_5\beta_1$ integrin was obtained as a way to better understand the effects observed from the fluorescence studies.

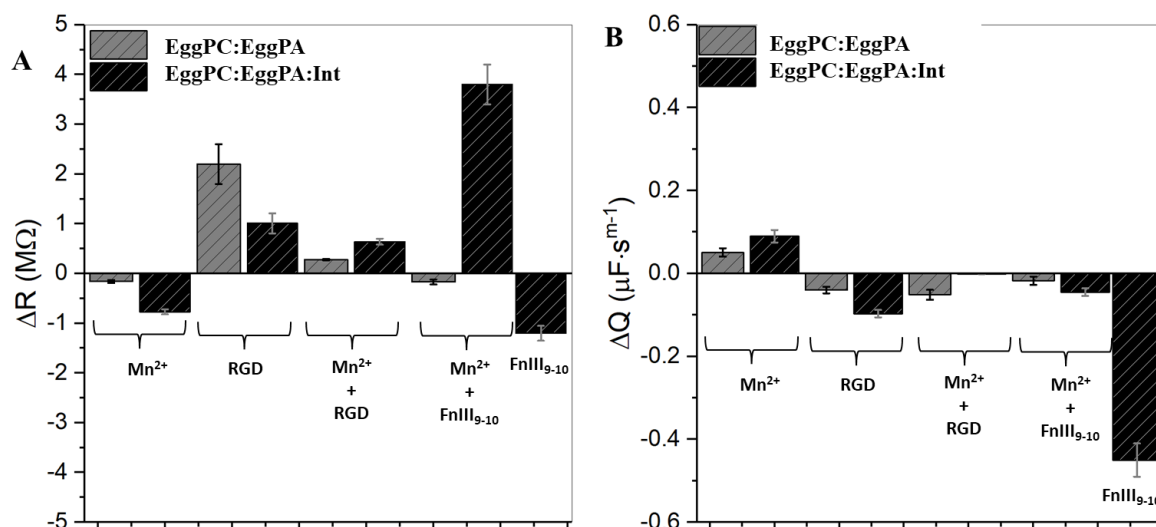


Figure 5.14: A) The relative resistance change and B) the relative capacitance change of the impact of Mn^{2+} and RGD and $FnIII_{9-10}$ on EggPC:EggPA pristine membrane (grey) and integrin reconstituted EggPC:EggPA:Int (black) in HEPES buffer pH 7.4. The error is SD for triplicates. EIS measurements were performed within the frequency ranges between 0.05 Hz to 10^5 Hz at 0 V DC bias potential vs Ag/AgCl (1 M KCl) with an AC amplitude of 10 mV at $22 \pm 1^\circ C$. A three-electrode set-up where gold cavity spanned MSLB, Ag/AgCl (1 M KCl) and Pt coiled served as working, reference, and counter electrode respectively. Courtesy of Dr Nirod Kumar Sarangi.

Alongside the FCS experiments, the impact of 5 mM Mn^{2+} , 1 mM cRGD and 10 μM $FnIII_{9-10}$ were investigated on their effects to the membrane, pristine (grey) and integrin reconstituted (black), Figure 5.14. As determined from FCS, Mn^{2+} was found to have a significant impact on effects of the activators by priming the MIDAS site of the integrin and as such was used for all activation experiments.

The impact of the activators in the presence and absence of Mn^{2+} (as well as Mn^{2+} alone) were investigated electrochemically. From Figure 14A, it is evident that the relative membrane resistance is decreased. The relative capacitance is increased for both pristine and integrin containing membranes upon the incubation with Mn^{2+} . However, the extent of change is greater seen when the membrane contains $\alpha_5\beta_1$. This decrease in resistance and increase in capacitance was due to electrostatic interactions between the anionic EggPA and cationic Mn^{2+} which is

known to cause a shift from lipid disordered phase to gel phase leading to an overall increase in admittance.¹¹³

When the membrane was incubated with RGD the membrane resistance increased for both the pristine and integrin reconstituted membranes. The capacitance decreased upon incubation with RGD for both pristine and integrin reconstituted membranes. The increase of resistance and decrease of capacitance is a strong indicator, in agreement with the diffusional decrease of the pristine membrane, that cRGD is binding to the membrane, both with and without $\alpha_5\beta_1$. The binding of RGD could be from nonspecific electrostatic interaction with the anionic EggPA or specific interaction between RGD and the integrin. The membrane thickening indicated by capacitance suggests a RGD layer was forming. In the absence of integrin, the membrane increases in resistance and decrease in capacitance when both Mn^{2+} and cRGD were in the electrochemical cell, indicated a layer was forming. However, when Mn^{2+} was absent, the layer of RGD formed appeared to be greater, Figure 5.14.

Interestingly when Mn^{2+} and cRGD were incubated with integrin reconstituted membranes, a modest increase in resistance was observed, whilst almost no change in capacitance was observed. This suggests specific RGD interaction with $\alpha_5\beta_1$. However due to the large excess of RGD present, non specific interactions between the membrane and RGD cannot be ruled out.

In the case of FnIII₉₋₁₀ in the absence of Mn^{2+} a decrease in resistance was observed, whilst capacitance also decreased significantly by 0.45 μF for the integrin reconstituted membrane, Figure 5.14. This large decrease in capacitance, along with the lipid marker diffusion coefficient decrease indicates that Fn is interacting with the membrane itself. When both Mn^{2+} and Fn were present in the integrin reconstituted membrane, resistance increased by 4 $M\Omega$ and capacitance decreased slightly. This suggested that the Fn, even at a low concentration was forming a layer on the membrane and strongly implicates interaction with the integrin. This was also observed with the FCS data that upon incubation of the pristine membrane with FnIII₉₋₁₀ and Mn^{2+} , the lipid markers diffusion decreased (Table 5.5). The reconstituted integrin membrane also exhibited an integrin-atto488 diffusion decrease (Table 5.6). The diffusion decrease of both the lipid marker and the integrin indicated binding of Fn to both the membrane and integrin.

Both cRGD and FnIII₉₋₁₀, in the presence of Mn²⁺ were observed to interact greatly with the membrane as well as with $\alpha_5\beta_1$, the EIS data was normalised to 0 before the addition of WTGal3 or Gal3 Δ Nter to better resolve the lectin effects on integrin.

5.4.7 Electrochemical Impedance Spectroscopy on activated $\alpha_5\beta_1$ in an EggPC;EggPA membrane and the effects of the Galectin 3

Figure 5.15A and B illustrate respectively, the relative change in membrane resistance (filled symbols) and capacitance (open symbols) obtained upon binding of varying concentrations of WTGal3 or Gal3 Δ Nter to the activated integrin post treatment with 5 mM Mn²⁺ and 10 μ M FnIII₉₋₁₀.

Figure 5.15C and D illustrate respectively the relative changes in membrane resistance and capacitance for activated integrin, but the treatment was carried out using 5 mM Mn²⁺ and 1 mM RGD. At each concentration of WTGal3 or Gal3 Δ Nter, upon incubation with the integrin containing membrane, an equilibrium EIS spectrum was obtained, and the resistance/capacitance values were normalized with respect to the resistance of the activated integrin containing membrane. The relative change in membrane resistance (ΔR , filled symbol, Figure 5.15A) and capacitance (ΔQ , open symbol, Figure 5.15B) were then plotted against the administered WTGal3 concentration (0 – 37.04 nM).

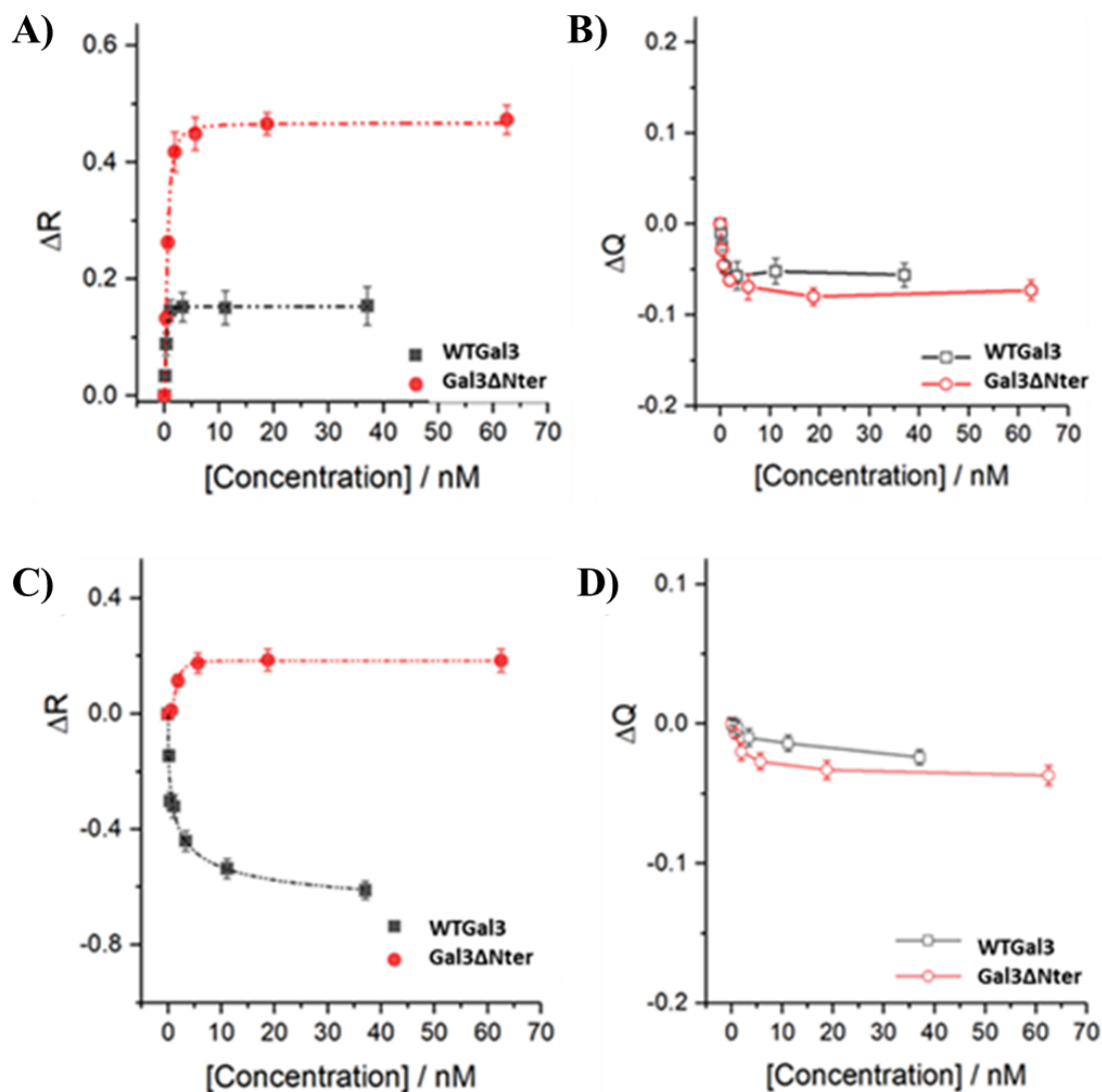


Figure 5.15: Graphical representation of relative change in A) resistance and B) capacitance of EggPC:EggPA(90:10) with $\alpha_5\beta_1$ Integrin reconstituted upon varied concentration of WTGal3 (black) or Gal3 Δ Nter (red) binding when the Integrin is in treated with 5 mM Mn^{2+} & 10 μ M FnIII $_{9-10}$. Incubation times of Mn^{2+} were 30 minutes prior to incubation time of FnIII $_{9-10}$ for 2 hours, before lectin concentration additions. Graphical representation of relative change in C) resistance and D) capacitance of EggPC:EggPA(90:10) with $\alpha_5\beta_1$ Integrin reconstituted upon varied concentration of WTGal3 (black) or Gal3 Δ Nter (red) binding when the Integrin is in treated with 5 mM Mn^{2+} & 1 mM RGD. Incubation times of Mn^{2+} were 30 minutes prior to incubation time of RGD for 2 hours, before lectin concentration additions. Each panel, the membrane is comprised of integrin reconstituted EggPC:EggPA(90:10)

membrane at a L/P=10, in HEPES buffer pH 7.4. The lines in the panels are lines guide to eye. Courtesy of Dr. Nirod K Sarangi.

A systematic, saturable increase in resistance was observed with galectin concentration when the integrin is activated using 5 mM Mn^{2+} and 10 μM FnIII₉₋₁₀ for both WTGal3 and Gal3 Δ Nter. The resistance increase was greater for the truncated lectin compared to the WTGal3, Figure 15A. Relative capacitance decreased on addition of either WTGal3 or Gal3 Δ Nter, the change was greater for Gal3 Δ Nter, Figure 5.15B. This suggests layer formation leading to thickening of the membrane. As stated, the capacitance of the membrane is inversely proportional to membrane thickness and so changes to this parameter can reflect protein assembly at the membrane interface.

As discussed earlier for the resting integrin, the capacitance increases initially up to 10 nM WTGal3 and decreases at higher concentration. This observation was linked to oligomerization of WTGal3 when integrin is in its resting state. It was attributed to thinning of the membrane from lectin insertion at low concentrations and then aggregation at concentrations above 10 nM that decreased membrane capacitance. Post FnIII₉₋₁₀ activation the initial increase of capacitance was not observed. The different trends across the resting state and FnIII₉₋₁₀ activated integrin suggest a layer of WTGal3 is indeed forming on the membrane in the latter case, but that oligomerization of WTGal3 is unlikely. This is in agreement with the observations obtained from the fluorescence studies, that upon activation of $\alpha_5\beta_1$ then WTGal3 does not seem to exhibit clustering behavior.

Integrin activated with Mn^{2+} and cRGD (Figure 5.15C & D) exhibited a resistance decrease and a slight capacitance decrease upon WTGal3 binding. Overall, the extent of electrochemical change is evidently influenced by the conformational status of the integrin and the nature of its ligand. This decrease trend of capacitance is more indicative of a complete layer formation of WTGal3 with the RGD in contrast to the thinning and rethickening when integrin was resting, that suggested oligomer formation. The fluorescence studies indicated that only one population of WTGal3 is present in the activated integrin case and the EIS data support this conclusion. The WTGal3 appears to be binding and diffusing tandem with the RGD and the membrane.

When Gal3 Δ Nter was titrated to $\alpha_5\beta_1$ that was activated with RGD, the relative resistance change was observed to increase. The capacitance upon incubation with Gal3 Δ Nter was

observed to decrease. This suggests that the Gal3 Δ Nter appears to be associating minimally to the activated integrin containing membrane.

Whilst WTGal3 on the resting state of integrin was observed to cause an initial thinning of the membrane at low concentrations of WTGal3, followed by a capacitance decrease indicative of clusters and oligomer formation on the membrane. Integrin activated through either approach, FnIII₉₋₁₀ and cRGD, do not exhibit this effect upon WTGal3 addition. Upon incubation of WTGal3 increasing in concentration a layer begins to form with the ligand or the membrane, in both activation approaches. Both ligands used for the activation appear to hinder the oligomerisation effects of WTGal3 in the presence of $\alpha_5\beta_1$ for both our fluorescence and electrochemical studies.

5.5 Conclusions

In this chapter, we successfully reconstituted the purified transmembrane glycoprotein $\alpha_5\beta_1$ integrin, into a microcavity suspended lipid bilayer (MSLB) platform of complex lipid composition and applied fluorescence microscopy, and backed up with electrochemical tools, to study $\alpha_5\beta_1$ -integrin/Galectin-3 complex formation and lateral membrane diffusivity at different conformational states. Notably, this original and sensitive approach allowed us to provide a novel investigative angle to emphasize the key and specific functions of the C-terminal glycan binding and the N-terminal oligomerization domains of Gal3, in the presence of a resting and activated glycosylated cargo protein, $\alpha_5\beta_1$ integrin.

Our FCS, and EIS, studies demonstrate that the WTGal3 has some propensity to unspecifically associate with the pristine membrane, but specifically binds to the reconstituted $\alpha_5\beta_1$ integrin via the CRD domain. Indeed, the carbohydrate-mediated nature of the binding between integrin and galectin-3 was clearly confirmed by β -lactose inhibition study.

Intriguingly, FCS measurements revealed the existence of two WTGal3 populations with distinct diffusivity behaviours formed in both the pristine and the resting integrin containing membrane: a dynamic mobile and a slow diffusive one. However, in the absence of integrin, the fast component of WTGal3 has a diffusivity value in the range of the one observed for the DOPE-Atto532 (6.5 ± 0.15 vs 6.99 ± 0.12 , respectively), suggesting a passive binding of the lectin on the pristine membrane. In contrast, this diffusivity is significantly decreased in integrin containing membrane compared to the corresponding DOPE-Atto532 lipid one (3.54 ± 0.11 vs 5.40 ± 0.40 , respectively), which is suggestive of integrin binding. Moreover, in the

latter condition, the diffusivity of both integrin and the fast component of WTGal3 follow the same trend upon increasing concentration of WTGal3, suggesting a partial cooperative diffusion rate. However, it was evident from FLIM imaging that a portion of the resting integrin was also immobilised upon galectin treatment. The observed increase in diffusion of the mobile $\alpha_5\beta_1$ integrin at the membrane was attributed to the sequestration of a fraction of the total integrin pool into galectin lattices. This would result in a reduced concentration of mobile integrin and thereby to faster protein diffusion by normal Brownian motion in regions of the membrane that now should have been less crowded. The potential capacity of Gal3 to induce pore formation may also have contributed to increased general membrane fluidity.

Regarding the immobile pool of WTGal3 appearing at higher concentration, it was unexpected to not see a similar slow population at the integrin level. One possible explanation could be that there is formation of integrin clusters through multivalent interaction with Gal3-mediated lattices, which in turn dramatically decrease its diffusivity down to immobilization, thus not measurable by FCS anymore. Upon the addition of WTGal3, a systematic and significant increase, followed by a gradual decrease of capacitance were observed for the reconstituted integrin membrane, suggesting an initial thinning and then re-thickening of the membrane. The initial thinning at low nanomolar concentrations might come from the oligomerization of WTGal3 on resting $\alpha_5\beta_1$. It is likely that at the cell surface, this would lead to the construction of endocytic sites.^{27,30} At concentrations above 10 nM, WTGal3 appears to drive the lateral condensation of $\alpha_5\beta_1$ integrins into lattices, which would result in the global thickening of the bilayer and thereby explain the observed decrease in membrane capacitance. This proposal is supported by FLIM imaging, where integrin clusters seem to reorganize between 3.7 nM and 18.5 nM of WTGal3, to then increase in size between 18.5 and 37 nM.

Conformation state of the integrin from a bent inactive state to an extended open state was investigated using two activator ligands, FnIII₉₋₁₀ and cRGD. Both activator ligands were firstly examined on their effects on the pristine membrane. Both FnIII₉₋₁₀ & RGD were found to have an effect on the pristine membrane and decreased the lipid labels diffusion to 4.36 ± 0.25 and $4.80 \pm 0.14 \mu\text{m}^2/\text{s}$, respectively. Manganese was reported and observed to be key in the interaction of integrin with its ligand, most likely attributed to the partial activation effect and priming of the integrins MIDAS site.^{88,89} Manganese was observed cause a phase change in the membrane by interacting with the anionic EggPA.¹¹³ In the presence of Mn^{2+} , the lipid marker diffusion was observed to decrease further for both ligands, $2.53 \pm 0.34 \mu\text{m}^2/\text{s}$ (Fn) & $4.32 \pm$

0.13 $\mu\text{m}^2/\text{s}$ (RGD). For the reconstituted integrin, upon treatment with Mn^{2+} , the diffusion remained within error. FnIII₉₋₁₀ caused integrin diffusion to decrease to $2.03 \pm 0.09 \mu\text{m}^2/\text{s}$ when manganese was involved however alpha increased to 1 indicating that Brownian diffusion was occurring. RGD caused the integrin diffusion to increase to $3.24 \pm 0.37 \mu\text{m}^2/\text{s}$ when manganese was involved, and alpha also increased to 1. Both ligands used for activation were observed to have an impact on the membrane and on the integrin itself.

Interestingly, with the use of manganese and RGD/FnIII₉₋₁₀ to force a conformational change to $\alpha_5\beta_1$ integrin, the clustering and oligomerisation of WTGal3 was ceased. The activation state of integrin effecting the clustering capabilities of WTGal3 were evident in the existence of only one WTGal3 population within the FCS and FLIM data. By changing the integrin from the bent conformation to the extended conformation state, the ability of WTGal3 to cluster was not observed and a more layer like binding affinity was observed from the lectin/integrin complex. This was evident when both WTGal3 and integrin were seen to diffuse at a similar rate, 2.29 $\mu\text{m}^2/\text{s}$ with Fn activation and 5.50 $\mu\text{m}^2/\text{s}$ with cRGD activation (37 nM WTGal3). The capacitance was also seen to decrease compared to the resting state of integrin. This confirms that the membrane, whilst thickening, was not thickening to the same extent when the integrin was resting, further suggesting that upon activation of integrin a non-clustered WTGal3 association occurred. This effect was observed whether integrin was activated with RGD or the fibronectin fragment.

The overall the results reported in this chapter demonstrate that WTGal3 binds to $\alpha_5\beta_1$ integrin reconstituted in MSLB in a carbohydrate-dependent manner. Lactose inhibited binding of WTGal3 to the integrin by saturating the CRD of the galectin3, and the clustering effect of WTGal3 was not observed. FCS studies indicate that this binding occurs through a cooperative process, likely involving the oligomerization of WTGal3 upon binding to resting integrin, thanks to its N-terminus domain. This observation is in agreement with the recent literature where the Gal3 oligomerization/condensation process initially triggered by binding to glycosylated proteins, could be assimilated to liquid-liquid phase separation, which is especially controlled through its apolar and partially hydrophobic proline-rich N-terminal domain.⁹⁷ The effect of activating the $\alpha_5\beta_1$ integrin seems to prevent this clustering effect of lectin. By understanding and regulating the conformational state of integrins and the response of Galectin3 interactions it can lead to a critical biomedical understanding because Glycolectin-Integrin signalling is integral to many disease states.^{41,63}

Importantly, there have been very few biophysical models of integrin-galectin interaction reported to date, but with the emerging importance of this interaction across a diverse range of disease states models, microcavity spanned bilayers represents a versatile platform to study such interactions and may provide important biomedically relevant insights at the molecular level extricated from the complexity of the cell.

5.6 References

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Chapter 6: Conclusions and Future Work

Interactions of protein-protein including network formation and aggregation are key factors across diverse signalling and in disease. Due to the high complexity of the cellular environment, these processes are very difficult to follow in cells. Biophysical models can therefore provide valuable insight into protein aggregation, clustering, and network formation. In order for biophysical models to be effective they must be capable of robust protein reconstitution to allow for correct membrane insertion and protein lateral diffusion is enabled.

This thesis sets out to evaluate aggregation and network formation of proteins in a biophysical model that could address these issues; by exploiting Microcavity supported lipid bilayers at gold microcavity arrays and PDMS microcavity arrays. Herein we have shown aggregation and network formation abilities of Annexin V, Amyloid beta (1-42) and Galectin 3 in the presence of different activation states of integrin $\alpha_5\beta_1$ all through biophysical methods such as impedance and fluorescence spectroscopy. This thesis has sought enabled by these models, to advance upon what is currently known within the literature to better understand mechanisms of aggregation of proteins and peptides within the cell membrane.

Chapter 1 detailed the cellular complexity and the need for robust biomimetic models. The current models used for study of membrane models include vesicles, droplet interface bilayers, SLBs and modified SLBs. Vesicles are effective for use as free standing membranes, however they are not easily electrochemically addressable, and the movement of GUVs can affect the focus in microscopy techniques. Asymmetry is difficult to achieve in vesicles which causes great difficulty in studies that need true biomimicry, especially with proteins. DIBs are versatile platforms for studying the cellular environment including proteins, however the surrounding high viscosity environment can impact the diffusivity of membranes and proteins directly. SLBs are hugely beneficial in regard to the formation of asymmetric bilayers. However due to the substrate interaction difficulty in protein incorporation and diffusivity measurements is commonplace. Diffusion coefficients are usually 2 folds lower in SLBs when compared to GUVs. The modification of SLB has led to a great increase of possibility for the investigation into protein aggregation, however even with cushioned and polymer SLBs diffusivity was still reported to be lower than free standing membranes and interaction with the cushion/tether was affecting the incorporation of proteins. Pore suspended lipid membranes are of huge benefit to protein aggregation studies as both sides of the bilayer are assessable and lateral movement of

proteins is successful due to the pore. Utilising MSLBs ability to access free standing membranes across multiple substrates and the retained fluidity of the membrane, protein aggregation could be investigated in this thesis.

In chapter 2 the fabrication methods and the experimental procedures used for the investigation of the aggregation of different proteins was explained. The microcavity array fabrication has been optimised and widely published by the Keyes group and these methods were applied herein.¹⁻⁵ The bilayers were formed using a combination of Langmuir Blodgett and vesicle disruption. This method was faithfully reproducible and the resulting bilayers were found, from EIS and microscopy to be stable for many hours spanned across our cavity arrays.⁶ Preparation of symmetric and asymmetric membranes were demonstrated and stability was maintained at both gold and PDMS substrates, consistent with previous membrane compositions reported by Keyes research group.⁷⁻¹⁰ The stability windows defined in this chapter defined the experimental windows for our subsequent investigations into different protein aggregation and network formations.

Chapter 3 explored network formation of Annexin V on a biomimetic apoptotic cell membrane. AnV has been reported to be critical to the plasma membrane repair of small tears.¹¹ Firstly, confirmation of AnV in the presence of calcium binding to anionic DOPS was investigated. In the absence of DOPS, no association of AnV was observed either via FCS or EIS at a symmetric DOPC membrane. When 20 mol% of DOPS was assembled at the distal leaflet of the membrane, 3 concentrations of AnV were observed to bind. At the lowest concentration of AnV (1nM), Brownian diffusion was maintained for the lipid marker. At the middle concentration of AnV (10 nM), a decrease in the anomalous coefficient of both lipid probe and protein indicated that aggregation was occurring. The highest concentration of AnV (100nM) exhibited a photobleaching upon incubation with the negatively charged membrane. These effects indicate that binding is occurring of the 1 nM AnV and progressively aggregating as the concentration of AnV was increased, to the point of extensive immobile network formation at the anionic membranes interface. Conversely, whereupon the DOPS is isolated at the asymmetric membrane to the proximal, lower leaflet, no AnV association at the membrane was observed. This was important confirmation of the lipid asymmetry of our membranes at MSLBs. AnV assembly was not observed for at least 19 hours, on FCS, showing the natural “flip flop” of the negatively charged lipid to the exterior of the membrane which a precursor to apoptosis.¹²⁻¹⁵ Utilising localized optothermal damage to this asymmetric membrane,

translocation of the DOPS to the outer leaflet of the membrane could be induced, mimicking the early stages of apoptosis. In the absence of AnV, some recovery of the membrane's resistance was observed. However, when AnV was present, the flip flop effect of DOPS was confirmed by the bilayer's resistance increase and capacitance decrease indicating that AnV was binding to the damaged membrane and forming a network. This chapter demonstrated the MSLBs capacity to support asymmetric bilayers and to mimic apoptosis whilst also showing the seal and stabilisation capabilities of AnV through network formation. This observation correlates well with previously reported hypothesis that AnV repairs small tears in the cell membrane. For future work, and had time permitted, more complex membranes containing cholesterol or various other lipids, or proteins could be formed. More complex membranes leading to different domains could also determine if the effects of apoptosis could be investigated further using AnV as a probe in a more biomimetic environment, having proved the anionic lipid is needed.

Chapter 4 investigated the association and oligomerization of amyloid beta at a zwitterionic, anionic and damaged membranes, to mimic an aging or damaged brain cell. Upon investigation of A β it was found that interaction of A β monomers with a zwitterionic membrane were possible, however aggregates were not seen to form at 1 μ M, which is beyond physiological concentration. When negative DOPS was introduced to the membrane, oligomerization of these A β monomers was observed both through a change in impedance and through imaging techniques, AFM, and confocal imaging. Initially upon incubation of A β with anionic DOPS containing membranes, damage to the membrane occurred and when left over the course of 24 hours oligomerisation of A β was observed indicated by the systematic increase of resistance and decrease of capacitance. From our results, it is suggested that all models of membrane interaction occur (pore formation, dissolution and carpet effect)^{16–18} but what prevails is dependent on the concentration of both A β and DOPS. This is important insight into A β at the neuronal membrane. As our brains age, or under the influence of inflammation, more anionic lipids will flip flop from the interior, or upon damage to the cell membrane and act as an “eat me” as a precursor of apoptosis.^{13–15,19,20} This may be the instigator that led to more plaques forming as our cells damage. From our results it was evident that when DOPS was at the interior leaflet, upon optothermal DOPS induced lipid transversal migration by the method established in chapter 3, A β binds to the anionic lipids this process seeds A β aggregation to form oligomers.

From fluorescence experiments, it was observed that these fibrils leave the membrane surface and can signify that aggregation of A β occurs within the membrane leading to the plaques that cause symptoms of AD. Formation of oligomers within the membrane appear to stay and cause further damage and upon fibrilization, the fibrils leave the membrane as plaques which block the synaptic regions in the brain. A β aggregation has been seen in membranes with high cholesterol content or on the addition of GSL to the membrane. Upon more investigation with MSLBs to include these more complex elements, study of A β oligomerisation could be broadened to influence of other membrane constituents. Firstly by monitoring cholesterol's effect on A β aggregation, followed by introducing the anionic DOPS and eventually the addition of GSLs.^{21,22} The MSLBs are highly useful for the investigation into A β aggregations as full control of the membrane components and asymmetry is possible and if time permitted further investigation into more complex membranes would be completed. The MSLBs could then be further used for analytical interrogation of spinal fluid for the detection of A β in patients suspected to be in the early stages of AD. Lee et al have reported that AnV could be a competitive inhibitor to A β aggregation as the common interaction site is the anionic PS.²³ Investigation into competitive protein interactions could be an interesting advancement for the use of MSLBs.

Chapter 5 examined the role of $\alpha_5\beta_1$ integrins activation state on the aggregation of galectin 3. The successful reconstitution of purified transmembrane $\alpha_5\beta_1$ into a MSLB was completed and investigated using fluorescent and electrochemical tools. WTGal3 was observed to have unspecific association to a pristine membrane of EggPC:EggPA but specifically binds to $\alpha_5\beta_1$ when reconstituted into the membrane. When integrin was in its resting state two populations of WTGal3 with distinctive diffusional behaviour were observed, one fast diffusing and one slow diffusing component. The fast diffusing component of WTGal3 in the pristine membrane, showed a diffusion coefficient of $6.5 \pm 0.15 \mu\text{m}^2/\text{s}$, which remained in the range of the lipid marker diffusion, however in the presence of integrin the fast diffusing component of WTGal3 was significantly reduced, $3.54 \pm 0.11 \mu\text{m}^2/\text{s}$, suggesting binding to integrin. From FLIM it was evident that a portion of integrin when resting was immobilised upon WTGal3 treatment. This may suggest that a fraction of the integrin is tied up in the formation of WTGal3 clusters, leading to its immobilization so it is not observed with FCS. The capacitance data suggested that upon addition of WTGal3 an initial thinning, indicated by an increase in capacitance, followed by a thickening, indicated by a capacitance decrease, occurred as the concentration

increased. This effect in a cell could lead to the formation of endocytic sites upon galectin binding. The thickening of the membrane hereafter then confirms, alongside the two populations in the diffusional results, that clusters and oligomers of WTGal3 were forming as concentrations increased. Interestingly, when the galectin was truncated so that its N terminus was deleted from the carbohydrate region domain, the binding and clustering effects were not observed, showing that the N-terminus of Galectin 3 is required for the protein association and aggregation.

Switching the conformational state of the integrin to its active state where the integrin is extended state revealed that WTGal3 does not appear to cluster with the integrin when it is activated. Manganese was both reported and found to be necessary for the binding of ligands to integrins by priming the MIDAS site.²⁴⁻²⁷ A fibronectin fragment and cyclic RGD were found to both impact the pristine membrane and slow the diffusion on the lipid marker in the absence of Mn^{2+} , 4.80 ± 0.14 & $4.36 \pm 0.25 \mu m^2/s$ respectively. In the presence of Mn^{2+} , the fibronectin fragment had a greater effect on the diffusion of the lipid marker, 2.53 ± 0.34 , than RGD, $4.32 \pm 0.14 \mu m^2/s$. For both activator ligands upon addition of WTGal3, no evidence of oligomerization or clustering was observed by FLIM or from the diffusion of integrin. Only one population of WTGal3 was evident after activation. As the concentration increased for WTGal3, both integrin and galectin appeared to diffuse at similar rates, in the presence of the activator ligands and Mn^{2+} . Capacitance compared to the resting state of integrin, did not observe the initial thinning effect, and indicated that while the membrane was indeed thickening due to WTGal3 binding, the effect was weaker signifying no clustering of WTGal3 was occurring. In a similar response to the resting state of $\alpha_5\beta_1$, the truncated galectin, Gal3 Δ Nter, had no effect on the activated integrin and negligible binding of the truncated protein was observed. From our investigations, while $\alpha_5\beta_1$ is in the resting state, aggregation of WTGal3 can occur, however once a conformational shift occurs through activation with fibronectin, and also RGD, after priming of the MIDAS site the clustering effect of WTGal3 is ceased. This suggests that the activation of integrin, $\alpha_5\beta_1$, can regulate Glycolectin signaling which has been linked to many disease states. To further this investigation a next step in the process would be to introduce GSLs to the bilayer. As per the Glycolipid-lectin hypothesis to form endocytic pits, firstly oligomerization of WTGal3 with integrin occurs which is then followed by interaction of WTGal3 with GSL which causes the membrane to bend leaving pits

in the membrane.^{28,29} It would be interesting to see the effects of GSLs and to see if clustering of WTGal3 would still be prevented upon activation.

Overall, this thesis demonstrated through bilayer spanned cavity arrays with EIS, FLCS and some other techniques that valuable insight into aggregation mechanisms of proteins can be achieved with these biophysical models. The MSLBs represent a highly versatile platform to study protein aggregation and network capabilities that can provide important biomedically relevant insights at the molecular level extricated from the complexity of the cell.

The further development of a more reliable fabrication method for the MSLBs to obtain more reproducible substrates is currently ongoing. For both gold and PDMS microcavity array a drawback of dropcasting the polystyrene spheres lead to variation in the arrays structure. The dropcasting is also prone to lead to the spontaneous formation of multilayer of sphere, leading to instability of the membrane if used. Gold MSLBs have been found to be lack temporal stability when compared to the PDMS array. Similar to liposomes, stability of the bilayer can last hours before degradation which limits the experimental window. Stability of the membrane may be increased if the fabrication method was improved, and if larger cavity sizes were possible, they could act as a cellular endosome to store and deliver lipids to the suspended membrane leading to a seal healing array. A major drawback is also the length of the experimental runs, especially for EIS. If the testing of MSLBs could be increased then the MSLB could be used for rapid testing of protein/ membrane interactions, which could lead to advancements in a commercial device, or indeed could potentially identify protein aggregation process quicker which could advance the knowledge of protein effects and aggregation knowledge further.

6.1 References

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