ENGINEERING ARTIFICIAL PROTEIN-PROTEIN INTERACTIONS THROUGH MEMBRANES WITH CONTROLLABLE ARCHITECTURES

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I, Stuart Haylock, declare that the following thesis and the material presented in it is my own, except where specific reference is made to the work of others. This work has not been previously submitted in any form to satisfy any degree requirement at this or any other university.

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Abstract

Cell membranes are a complex mixture of lipids and proteins, all of which vary in size, shape and composition. The ability for a cell to control its membrane, and regulate its contents, is in large part due to the regulation of local composition, curvature and tension. These changes to membrane properties can in turn alter the behaviour of membrane bound proteins. Understanding how these membranes, and their fundamental properties, can influence proteins in vitro is essential for building true synthetic cells, and building artificial cellular networks.

In this thesis, work is presented which investigates the effect of membrane composition on the activity of the mechanosensitive channel of large conductance (MscL) from E.Coli. Using different model membrane systems, and many activation mechanisms of the protein, a greater understanding of MscL function has been gained. The first reconstitution, and activity measurement, of MscL into droplet interface bilayers (DIBs) is presented. Being able to measure MscL activity in the DIB model membrane has then lead to whole new avenues of research.

The DIB system allowed for electrophysiological recording of single MscL channels, exploring the ability to see in-depth changes to MscL activity as DIB composition changes. Then, by linking multiple DIBs together, a network of bilayers connected by MscL channels has been presented. The first time a synthetic bilayer network linked by a gated ion channel has been demonstrated. Finally, a new device has been built allowing the generation of a measurable amount of shear force inside a DIB, and MscL activity was measured.

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List of Abbreviations

MscL – Mechanosensitive channel of Large conductance

MscS – Mechanosensitive channel of Small conductance

sPLA₂ / PLA₂ – secretory Phospholipase A₂

DOPC / PC - 1,2-dioleoyl-sn-glycero-3-phosphocholine

DOPE / PE - 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

DOPG / PG - 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol)

DMPC - dimyristoyl glycero-sn-3-phosphocholine

DTPM - 1,2-ditetradecyl-glycero-sn-3-phosphomethanol

DPPC - 1,2-dipalmityl-sn-glycero-3-phosphocholine

E.Coli - Escherichia Coli (bacterium)

DIB – Droplet Interface Bilayer

E-Phys – Electrophysiology / Electrophysiological
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Conclusions and Outlook

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Thesis Overview

There are several large research questions that must be answered within this thesis, with a range of topics and disciplines, from optimising new model membrane studies to assessing membrane protein kinetics and rheology. Thus, the thesis is structured to allow for each chapter to resolve its own research question. Each chapter has its own small overview which should act like a sub-abstract. Following the overview there is a detailed introduction to the specific topics that are addressed within the chapter; a materials and a methods section (if chapters share materials or methods, a brief outline followed by modifications to the procedure is provided); a detailed results and discussion; finally a chapter conclusion aims to complete the local research question, and provides a link to the overarching project.

Chapter 1 is a broad introduction to several themes covered within this thesis, giving a grand overview of the field of membrane biophysics and synthetic biology, as well as a review of a number of literature studies within the field.

Chapter 2 covers the transformation of competent cells, as well as the expression and purification of the membrane protein, the Mechanosensitive Channel of Large Conductance (MscL), which became the focus of all following experiments. This chapter also covers changes made to known protocols to improve efficiency, in addition to calculations of MscL concentration.

Chapter 3 looks at the reconstitution of the membrane protein, the MscL, into a liposome based fluorescent dye release assay. First, the MscL was examined to ensure activity was reproducible. Following, lipid composition studies were completed to explore the effect of changing membrane properties on MscL activity.

Chapter 4 takes the vesicle based assays into a different model membrane system, the Droplet Interface Bilayer (DIB). Within this chapter, a significant proof of concept experiment was carried out, the first successful reconstitution of the MscL into a DIB. This work was published in the Journal of the Royal Society, Interface in 2014.

Chapter 5 explores the movement from fluorescence based activity measurements, into electrophysiological measurements. These electrophysiological measurements allow for closer control over, and more accurate measurement of, the kinetics of MscL gating events. Within this chapter, a substantial piece of work was carried out in which we measured signal propagation across multiple DIBs mediated by gating of the MscL. This work is currently in review in Nature Communication Chemistry as of July 2018.
Chapter 6 follows the application of the work in the previous chapters, optimising MscL reconstitution into a DIB based assay, into a new device capable of generating shear force inside a DIB. The assay was intended to show that MscL could open in response to a purely mechanical stimulus. Parts of this chapter formed a publication on the design and building of the rheological device, published in Nature Scientific Reports in 2017³.

Chapter 7 concerned hopeful future work that could be carried out in field following on from the work completed in this thesis. Preliminary data of future work is included, with assays looking at activating MscL using a range of amphipathic proteins, and a circular dichroism experiment examining MscL reconstitution structure.

A final conclusion brings each chapter’s research question together, in order to answer the overarching research question, and summarises the project as a whole.

Published work is highlighted within each chapter, along with a description of the personal author contribution to the article.
1. Overview

The complexity of life, even in its simplest form, makes any effort to synthesise truly synthetic cells an extremely difficult task. In recent years, progress has been made in all aspects of the creation of synthetic cells, ranging from studies looking at artificial complex cell membranes, to work on cell-free transcription and translation of proteins. While significant advances have been made in many areas, there is still a very large ‘knowledge gap’ that needs to be overcome in the area of cell membrane mechanics and how these affects membrane proteins. The cell membrane itself cannot be thought of as just a ‘box’ to keep cell machinery in, the material of the ‘box’ is constantly shifting and changing, and is just as important as the contents of it.

The aim of this chapter is to provide a broad overview of the field of membrane biophysics, and to "set the scene" for the current scientific landscape within membrane protein research and how this relates to synthetic biology. A detailed summary of lipid behaviours and interactions is given, especially with respect to membrane proteins. Additionally, there is a review of several membrane protein studies and methods for studying them, along with a short review of the area of cell-cell signalling. Included is also a summary of the current knowledge of the Mechanosensitive Channel of Large Conductance (MscL) from *E. Coli*, upon which the rest of this thesis is based.
1.1. Lipids

Many similarities can be found in the estimated 100 million distinct species of organism on Earth today, both on the molecular and cellular level. The biggest similarity is the ability for the organism to maintain a separate inner environment from the outer environment using a membrane, or in certain cases several membranes. In almost all cases this membrane is composed of a series of amphipathic molecules called lipids.

Lipids are organic molecules generally composed of a hydrophilic head group and a hydrophobic tail which are linked by an ether or ester group. Lipids form two opposing leaflets, these leaflets together are then called a bilayer (Figure 1.1). These Lipids can be either absorbed from the digestive system or produced through lipid biosynthesis. Lipid biosynthesis involves the enzyme-mediated creation of fatty acyls, such as palmitate, from isoprene or thioester molecules. These fatty acyls can then be modified further to create the diverse range of lipids found in membranes.

![Figure 1.1 – Schematic of the hydrophilic and hydrophobic components of a lipid. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) is used an example.](image)

Lipids are grouped into 8 separate categories; these categories can then be split into multiple subcategories to further refine their function. The primary categories are: fatty acyls, glycerolipids, glycerophospholipids, sterol lipids, sphingolipids, prenol lipids, saccharolipids and polyketides. Fatty acyls, or fatty acids, consist of a simple, long chain hydrophobic hydrocarbon chain attached to a hydrophilic group such as an organic acid. Fatty acyls are the most biologically important of these lipid structures as they are the predominant building block of almost all complex lipids; they also form part of complex lipid formations such as organelle membranes.

The other lipid types are less common in biological membranes but each have important roles. Glycerolipids include triglycerides, and are used as energy storage in the body. Sterol lipids include cholesterol and any derivatives; they are constituent parts of the cell membrane providing structure and are also used in cell signalling. Sphingolipids are a very large family of lipids synthesised from the amino acid serine, are found in large proportions in neural tissue and play roles in signal transduction and cell recognition. Prenol lipids are the simplest of all the lipid groups and are
synthesised from 5-carbon organic alcohols. They play a role in cellular metabolism: for example, carotenoids are very simple prenoids and while they have a function as antioxidants they are also used to synthesise vitamin A. Saccharolipids or glycolipids are a series of lipids which have sugar groups covalently attached to them; they play a role in cell recognition and signalling. Polyketides are very structurally complex often comprising of a functionalised macrocycle. Polyketides have many different functions but their main function is as a secondary metabolite. Each of these then has further subclasses which divide their chemistry and function.

The final type of lipid are glycerophospholipids, or phospholipids, and are the most abundant lipid in model membrane research. These phospholipids are the main component of biological membranes, as well as being involved in cell-cell signalling and energy storage in multicellular organisms. Phospholipids consist of a mono- or di- acyl chain substituted glycerol with the third hydroxy substituted with a phosphate group which is then coupled with various small, functional headgroups. The head groups, chain lengths, chain saturation and charge vary to create the many different membrane properties found in biological membranes, and therefore replicate them in artificial membranes.

1.1.1. Phospholipid Bilayer

The biological cell membrane consists of two flat leaflets of phospholipids arranged into a bilayer with the hydrophobic chains creating a hydrophobic space in the middle of the bilayer. While most of the bilayer consists of phospholipids, there are many other molecules present including proteins and complex oligosaccharides. These molecules provide functions for the membrane such as channels to allow specific molecules in or out, and receptors to allow cell communication.

While the membrane is well studied, there are many models for how the membrane is structured. The most accepted model is the fluid mosaic model. The fluid mosaic model states that the entire membrane can be thought of as just one continuous and homogenous phase; lipids, proteins and sugars are free to diffuse within the bilayer and can be found at any position (Figure 1.2).

The fluid mosaic model is a very simplistic view of membrane organisation. The model doesn’t allow for specific protein-membrane interactions, and has no explanation for why proteins have preferences for specific membrane conditions such as charge or curvature. Techniques such as atomic force microscopy (AFM), as well as electron spin resonance spectroscopy (ESR), have shown that the membrane displays lateral organisation of lipids and components into different phases. These lipids phases are described as liquid ordered when certain types of lipids and other molecules organise into
small domains, whereas the liquid disordered phase occurs when there is no obvious organisation to any of the components. There is a definite and measurable difference in the mobility of the lipids and the constituent membrane components between these phases\textsuperscript{20}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{The three models for the structure of cell membranes. A) Fluid Mosaic Model, B) ‘Lipid Raft’ hypothesis C) Modern Interpretation. Image taken from Owen, 2012\textsuperscript{21}}
\end{figure}

While lipid domains are often rich in particular types of lipids and membrane molecules, studies have shown that they are frequently short-lived and therefore hard to show spectroscopically. These transient nano-domains of organised lipids are controversially known as ‘Lipid Rafts’ (Figure 1.2). These ‘rafts’ are often organised by the build-up of cholesterol and glycolipids which provide interactions with lipids and other molecules to create the organisation\textsuperscript{12,22,23}. Even this level of organisation is explained as too simplistic for cellular behaviour, as Lipid Rafts are too transient and uncontrolled\textsuperscript{24}.

Super-resolution microscopy of the cell membrane has revealed a more nuanced interpretation of organisation of the membrane. These microscopy studies have shown many complex interactions between small, dynamic lipid domains of between 20 and 200nm, and the underlying cellular cortical actin network (Figure 1.2). Evidence suggests that these lipid domains are even shorter-lived then ‘lipid rafts’ but their lifetime and activity are controlled directly by the cell\textsuperscript{21}.

The ability of the cell membrane to organise specific locations for proteins and other molecules is integral for many cellular functions including signalling and motion\textsuperscript{25,26}. The study of how the cell organises the cell membrane to create these domains has been applied in physiological applications
(such as the toxicology of poisons; endocytosis and exocytosis; analysis of membrane bound organelles)\textsuperscript{27}, as well as pharmaceutical applications (drug delivery; bioreactors)\textsuperscript{28}.

### 1.1.2. Lipid Phases

In aqueous environments, phospholipids form many different mesophases due to the hydrophobic effect. The phospholipid chains are non-polar and hydrophobic, whereas the headgroups are either charged or zwitterionic and therefore are hydrophilic. The ability of a molecule to have both hydrophilic and hydrophobic components is known as amphiphilicity. The amphiphilic nature of the lipids allows them to arrange themselves in a geometry that minimises the exposure of their hydrophobic chains\textsuperscript{29}.

Lipid geometry can be classified into three main types (0, I, II). Type 0 lipids are cylindrical in nature with equal sized head and chain groups; type I lipids are inverted conical and have smaller chains then head groups; and type II lipids are conical and have chains which take up more space than their headgroup (Figure 1.3). The phases these lipids form are thermodynamically driven by factors such as temperature, pressure and concentration, however they are grouped as either bilayer or non-bilayer lipids depending on their preferred curvature. Type 0 lipids form flat, planar bilayers; types I and II lipids are non-bilayer lipids and form curved structures such as micelles and hexagonal phases (Table 1.1). Despite the classification of lipids with their tendency towards curvature, mixtures of lipids might not have enough curvature to elicit a phase change as bilayers tend to have the most minimised exposure of their hydrophobic areas.

![Figure 1.3 - Types of lipid, Type I (left), Type 0 (middle) and Type II (right), Image taken from Shearman, 2006\textsuperscript{30}](image)

### 1.1.3. Curvature in Membranes

To define the curvature of a lipid geometry, two perpendicular curvature terms are used, $c_1$ and $c_2$. These curvature terms are derived from the radii of curvature of a membrane surface where $c_1 = 1/R_1$ and $c_2 = 1/R_2$ (Figure 1.4). These curvature terms must be defined as positive for either the headgroups curving away or towards the aqueous environment as there is no set standards for this.
To attain the curvature of a monolayer comprised of a single lipid type in suspension, each lipid must have the space that it takes up in the monolayer defined, this is known as the specific packing parameter. The specific packing parameter of a lipid, $S$, is described as the ratio between the volume of the hydrophobic hydrocarbon chains to the optimal headgroup volume.

$$S = \frac{V}{a_0 l_c}$$ (1.1)

$S$ is the specific packing parameter, $V$ is the specific volume occupied by the hydrocarbon tails, $a_0$ is the area per lipid molecule, and $l_c$ is the effect length of the tails region. When $S<1$, the lipids have a larger headgroup compared to either only one tail, or very small tails. These $S<1$ lipids are type I lipids, they take a positive curvature with the tails facing inwards and the headgroup facing out into the aqueous media. When $S=1$, both the headgroup and the tails take up approximately the same volume. These $S=1$ lipids are type 0 lipids with very little curvature. When $S>1$ the headgroup is smaller in volume compared to larger, more unsaturated tails. These $S>1$ lipids are type II lipids which form inverted structures with the tails facing out into the aqueous media.

As each different phospholipid has its own specific packing parameter, they also then exhibit a preference for a certain curvature of the monolayers that are formed in solution. The nature of this curvature is described using two separate curvature parameters the mean curvature, $H$, and the Gaussian curvature, $K$.

The mean curvature, $H$, is an average of the two curvature terms, $c_1$ and $c_2$. The mean curvature is defined as positive for type I lipids that curve away from the aqueous media and negative for type II lipids that curve towards the aqueous media. The Gaussian curvature, $K$, is the multiplication of the two curvature terms, $c_1$ and $c_2$. The Gaussian curvature changes along with surface topology; phase changes between parabolic surfaces to hyperbolic surfaces ($K<0$) or ellipsoidal surfaces ($K>0$) induce...
changes in Gaussian curvature\textsuperscript{32}. An example of these curvatures can be seen when a flat monolayer is rolled into a cylinder, the mean curvature can change but the Gaussian curvature will stay at zero as at least one curvature term is still zero. In practise the only way to change the Gaussian curvature is to stretch or compress the membrane itself.

\[ H = \frac{c_1 + c_2}{2} \]
\[ K = c_1 c_2 \]

(1.2)

Table 1.1 – Representations of the different phospholipid phases, Image (left) taken from Mouritsen, 2011\textsuperscript{33} and data taken from several different literature references\textsuperscript{29,34,35}. 

<table>
<thead>
<tr>
<th>Principal Curvature</th>
<th>Principal Curvature</th>
<th>Mean Curvature</th>
<th>Gaussian Curvature</th>
</tr>
</thead>
<tbody>
<tr>
<td>( c_1 )</td>
<td>( c_2 )</td>
<td>( H )</td>
<td>( K )</td>
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</table>
1.1.4. Curvature Elastic Energy

A phospholipid structure determines its preferred curvature, therefore there will be an inherent energy penalty when different curvature lipids are mixed together into a single membrane. This energy penalty is defined as the curvature elastic energy per unit area, $g_c$. By considering the monolayer as a single, infinite elastic surface, the curvature elastic energy can be defined by the Helfrich-Hamiltonian in terms of the mean and Gaussian curvatures \[ g_c = 2k_b(H - H_0)^2 + k_gK \] (1.3)

In the Helfrich-Hamiltonian equation: $K_b$ is the bending modulus (energy required to change the mean curvature or bend the surface), $k_g$ is the Gaussian bending modulus (energy required to deform the monolayer and change the genus of the surface topology) and $H_0$ is the completely relaxed mean membrane curvature (or spontaneous mean curvature of the monolayer).

The spontaneous mean curvature is obtained from the lateral pressure across a monolayer, $\tau(z)$ \cite{30}. The later pressure profile across a monolayer is described as the relationship between lipids; including both the attractive components and repulsive components (Figure 1.5).

![Figure 1.5 - Lateral pressure profile across a lipid membrane, image taken from Shearman, 2006\cite{10}](image)

The attractive components of the membrane result from the interfacial pressure between polar headgroups and non-polar tails. This interfacial pressure arises from the hydrophobic interactions that protect the tails from hydration, effectively decreasing the entropy of the surrounding water. The repulsive interactions are caused by steric or electrostatic repulsions of either the tails or headgroup and depend upon the chemical structure of the lipid, including net charge and saturation \cite{30}.

The lateral pressure at equilibrium will be 0 due to the balance the forces. The spontaneous mean curvature of the monolayer is proportional to the first moment of the lateral pressure which is expected to be negative for lipids with less sterically bulky headgroups. This affirms lipids with specific packing parameters less than 1 tending to form membranes with negative curvature \cite{30}.
The Gauss-Bonnett theorem takes the Gaussian curvature contribution and applied it to the stored curvature elastic energy in the Helfrich-Hamiltonian\textsuperscript{37}.

\[
\int KdA = 4\pi(1 - g) \quad \int k_gKdA = k_g4\pi(1 - g) \quad (1.4)
\]

The Gauss-Bonnett theorem shows that for a membrane with negative Gaussian bending modulus to reduce its curvature elastic energy, it would need to change the genus of its surface topology such as forming spherical micelles from flat monolayers. The Helfrich-Hamiltonian can then be extended from one monolayer into two monolayers ‘sandwiched’ together with the hydrophobic tails facing inwards, a bilayer. The parameters are altered to take into account the bending modes of the two monolayers being coupled together, which can then be used to calculate the bilayer stored curvature elastic energy.

The curvature elastic energy of a bilayer is a manifestation of the energy required to force the membrane into a flat bilayer, away from the net preferred curvature of the two separate monolayers. The curvature elastic energy stored in the bilayer can be relieved by the presence of proteins or other molecules such as cholesterol.

Figure 1.6 shows an example of curvature elastic energy changes. The monolayer has a preferred curvature based on the large hydrophobic tails of some of the lipids. When the monolayer is forced into a flat formation the tails are constrained into a smaller space which increases the curvature elastic energy. When a molecule inserts into the bilayer, it relieves the strain of the hydrophobic chains and therefore relieves the curvature elastic stress. One downside to this insertion is the energy penalty that occurs when the molecule exposes the hydrophobic core to the aqueous media, therefore there can be a balance between the hydrophobic and steric energies. Once the curvature elastic energy exceeds a certain threshold then the membrane tends to bend and phase change away from the flat bilayer.
It is therefore evident that if the concentration of a type II lipid increased in a type 0 lipid membrane, the membrane would favour curvature towards the aqueous phase and would have an increase in curvature elastic energy. This increase in curvature elastic energy can affect many different properties including protein insertion kinetics.

1.1.5. Phospholipid Phase Behaviour

The bilayer phase, also known as lamellar phase, is the phase that most plasma membrane phospholipids undertake at room temperatures\(^{39}\). In a bilayer the hydrophobic tails are facing each other to create a hydrophobic pocket, however the tails are still able to undergo hindered long-axis rotation\(^{40}\). This rotation is controlled by the curvature elastic stress in the membrane, as well as the thickness of the hydrophobic pocket between each leaflet of the bilayer. The free rotation of lipid tails, as well as the ability of lipids to diffuse, affect which type of lamellar phase the bilayer can take.

While there are many lamellar phases, there are 2 biologically relevant ones (Figure 1.7). In the liquid-crystalline phase, L\(_\alpha\), the lipids are free to rotate and diffuse within each leaflet. In comparison the gel lamellar phase, L\(_\beta\), is much more ordered and the lipids are virtually immobile within the bilayer\(^{41}\). The transitional ripple phase, P, allows lipids some vertical movement which creates a ripple-like motion, these have only been isolated with specific lipid mixtures and buffers\(^{42,43}\). Cellular membranes are majority L\(_\alpha\) phase with lipids free from movement constraints\(^{44}\). Although evidence has shown that the cellular membrane can undergo phase changes when other molecules are involved, and these
phase changes can actually be necessary for cellular function, for example the proteins involved in membrane fusion.

\[
L_\alpha \quad L_\beta
\]

Figure 1.7 - Diagram of the \(L_\alpha\) and \(L_\beta\) lamellar phases. In the \(L_\alpha\) phase the lipids are free to diffuse and rotate within each leaflet, whereas \(L_\beta\) phase is more ordered and the lipids are virtually immobile. Image taken from Dowhan, 1997.

1.1.6. Asymmetry

The cell membrane is not simply homogeneous and diffuse. The majority of the lipids in the cell membrane are tightly controlled, with large compositional asymmetry between the two leaflets of the bilayer. Cell membranes have many different lipids, and while asymmetry is not absolute, there are clear trends in the class of lipids existing within solely one leaflet.

\[
\begin{align*}
\% \text{ of total phospholipid} & \\
\text{total PL} & \quad \text{Sph} & \quad \text{PC} & \quad \text{PE} & \quad \text{PS} & \quad \text{PI} & \\
\end{align*}
\]

Figure 1.8 - Graph showing the relative distributions of a number of different lipid types across the two leaflets of a erythrocyte membrane. To define the abbreviations, PL is phospholipid, Sph is sphingomyelin, PC is phosphocholine, PE is phosphoethanolamine, PS is phosphoserine and PI is phosphoinositol. Graph taken from Verkleij, 2000.

As an example, erythrocyte cell membranes tend towards the inner leaflet containing more aminophospholipids and the outer leaflet tending to contain more choline lipids. Evidence also shows that the asymmetry level in the cell membrane is much greater than that of vesicles or membrane-bound organelles.

Bilayer asymmetry can be broken down into two groups, head group asymmetry and tail asymmetry, although these are not mutually exclusive. Head group asymmetry is caused by properties such as
charge or size density, and occurs mostly in the inner leaflet which requires different levels of fluidity to the outer leaflet\(^5\). Tail asymmetry is caused by lipid tails being different lengths or saturation levels between the leaflets. Tail asymmetry mostly occurs in vesicles and membrane-bound organelles which are much smaller and so significant strain can be relieved by having different length tails in leaflets\(^5\).

The diffusing rate of lipids crossing between leaflets, known as lipid flip-flop, is a contested issue in membrane biophysics. While sum-frequency vibrational spectroscopy (SFVS) has shown a very quick rate\(^{51,52}\), many experimental techniques have shown a flip-flop rate occurring over hours, or even days\(^4\). The diffusion of lipids across the bilayer is difficult as the energy barrier for a polar headgroup travelling through the hydrophobic interior is very high; this diffusion barrier for flip-flop has been shown to change with headgroup structure and lipid composition.

In nature, the rate of lipid translocation is controlled through the use of three different enzyme subgroups (Figure 1.9). Using ATP, flippases and floppases change conformation and transport lipids from one leaflet to the other\(^4\). While floppases are less characterised due to inefficiencies in isolation techniques, flippases have shown specificity towards phophatidylserine (PS) and phosphatidic acid (PA) lipids\(^5\). Scramblases use the flow of calcium ions to transport lipids between both leaflets; because evidence has shown that scramblases show no particular specificity to any lipid type, it is hypothesised that they equalise asymmetry in the cell membrane\(^5\).

Another mechanism for asymmetry propagation involves the interactions of the lipids in the inner leaflet of the cell membrane with the underlying actin cytoskeleton. Evidence shows that these cytoskeleton interactions work alongside enzymes in generating and maintaining asymmetry in the cell membrane\(^5\).

The cell uses a large amount of energy in maintaining asymmetry in the membrane, implicating asymmetry as an important cellular process\(^4\). PS is usually found in the inner leaflet of the cell.
membrane, but a family of cellular scramblases are triggered by a build-up of PS within the outer leaflet which in turn starts a apoptotic signalling cascade\textsuperscript{56}. Evidence also shows that older and damaged cells have a much larger amount of PS in the outer leaflet as a cellular aging signal for apoptosis, PS build-up also plays a further role in phagocytosis signalling\textsuperscript{60}.

Asymmetry has been implicated in a multitude of other cellular functions, however asymmetry is very difficult to generate and maintain in artificial bilayers and so it’s difficult to determine how it can affect drug interactions\textsuperscript{57–60}. Asymmetry can affect the phase behaviour and curvature of the membrane, which can in turn affect the gating of certain proteins such as mechanosensitive protein channels\textsuperscript{61}. The mechanosensitive channel of large conductance, MscL, is the focus of this thesis, and the majority of evidence has shown a link between asymmetry and the channel gating, so asymmetry will be important to consider throughout\textsuperscript{62}.

1.2. Proteins

Proteins are the second most populous constituent of the cell membrane. Proteins are bulky biological molecules consisting of one or many separate strands of amino acids folded into a specific shape by intramolecular bonds. Proteins have a large range of cellular functions, ranging from enzymes which catalyse reactions and make molecules, to transporting molecules across the cell membrane. While the majority of proteins are free in the cytosol, many are bound to the cell membrane and membrane-bound organelles. Of these membrane bound proteins there are 3 major subgroups: integral polytopic proteins (proteins that span the entire bilayer, both leaflets) that have roles as pores, channels and active transporters, integral monotopic proteins (proteins that bind to just one leaflet of the bilayer) that act as receptors for other molecules or are simply membrane bound enzymes, and peripheral proteins (are only temporarily bound to the membrane) that are often involved in cell signalling pathways\textsuperscript{63}.

Protein structure is divided into 4 categories: primary (the specific sequence of amino acids), secondary (regular repeating structures such as alpha helices), tertiary (intermolecular forces holding a specific spatial arrangement), and quaternary (multiple proteins and small molecules bound into an active complex).

A protein’s specific structure is governed by many intermolecular forces which are sensitive to environmental factors such as temperature, pH, ion gradients, charge density and even mechanical effects. These environmental factors cause a change in the intermolecular forces and therefore change the structure of the protein. While a large change in structure can cause complete denaturing
and loss of function, some proteins actually act as detectors for these environmental factors and changes in protein conformation form part of body homeostasis\textsuperscript{63}.

The two major protein functions are to catalyse reactions by reducing the activation energy (enzymes), and to recognise specific molecules and changes in environmental factors (receptors, transporters and channels). Enzymes bind strongly to specific molecules, orienting their spatial position and creating bond strain with intermolecular forces, these effects reduce the activation energy of a reaction\textsuperscript{63}. Receptors bind to specific molecules which cause changes in protein conformation which trigger many effects and signalling cascades. Channels allow for the passage of molecules across the cell membrane and are divided into two sub-groups. Non-specific channels act as a simple hole in the membrane for any molecule that can fit, these channels are usually either very temporary or toxic to the cells as they disrupt the fine balance of molecules that the cell maintains. Some non-specific channels have open and closed conformations depending on certain environmental or allosteric factors, to control when molecules can pass through. Active transport channels are a specific shape to certain molecules and change conformation once binding has occurred to move the molecule into, or out of, the cell. These large-scale conformation changes from active transport channels require energy, often in the form of ATP\textsuperscript{63}.

\subsection*{1.2.1. Peripheral Membrane Proteins}
Peripheral membrane proteins are defined as proteins that bind to one of the leaflets of the bilayer, but also reside in solution. Peripheral proteins have number of functions related to either altering membrane proteins or signal transduction\textsuperscript{27}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{diagram.png}
\caption{Diagram representing all the different binding modes of peripheral membrane proteins. Image adapted from Hames, 2005\textsuperscript{64}.}
\end{figure}

There are 4 binding main modes of peripheral membrane proteins with a lipid bilayer\textsuperscript{64} shown in Figure 1.10. Number 1 refers to interactions of an amphipathic alpha helix in parallel with the bilayer; number 2 refers to interactions of hydrophobic sections of the protein with the hydrophobic core of the lipid...
bilayer; number 3 refers to a covalently bound lipid that inserts into the bilayer, this is known as lipiddation; and number 4 refers to electrostatic or ionic interactions of membrane lipids with corresponding sections on the protein, which is often mediated through ions such as calcium or other small polar molecules. These peripheral interactions with the membrane are reversible and been shown to regulate and control cell signalling networks within multicellular organisms, alongside a number of other cellular events.65

Peripheral proteins can alter the structure of the membrane through enzymatic hydrolysis of the lipid composition or through altering local curvature by binding and inserting into the membrane.66 As an example, the chaperonin-containing TCP-1 (CCT) protein initially binds into a strained membrane in order to relieve stored curvature elastic stress, as it inserts between headgroups and gives the hydrocarbon tails a larger space relieving specific tail stress.67 It then has the additional activity of increasing the local proportion of type II lipids compared to type 0 lipids: this in turn further relieves elastic stress in the membrane. Once elastic stress has been reduced in the membrane, the CCT protein-membrane binding becomes less energetically favourable, CCT dissociates and the membrane goes back to equilibrium, the cycle then starts again. There are also examples of proteins that have the opposite effect, of building up type I lipid concentrations over type 0 and causing an increase in the elastic stress build-up of the membrane.68,69

![Figure 1.11 - Characterisation of phospholipases, each dotted line is where the enzyme hydrolyses on the phospholipid.](image)

One group of membrane altering peripheral proteins is the Phospholipases, a family of enzymes that catalyse the hydrolysis of the phospholipid bilayer in several different specifically molecular bonds shown above Figure 1.11. One major sub-class is the phospholipase A2 (PLA2), an enzyme found in several animal venoms such as bees and cobras, but also found in the mammalian pancreas. In this project the secretary phospholipase A2 (sPLA2) will be used in order to gate the mechanosensitive protein (see section 1.2.2.). sPLA2 is a calcium-dependant peripheral membrane enzyme that inserts into either leaflet of the bilayer and catalyses the cleavage of the sn-2 fatty acyl bond on the
phospholipid to give one lyso-phospholipid (a phospholipid with only one acyl chain) and leave a free fatty acid chain.

sPLA$_2$ has been shown to be overexpressed in certain types of cancer$^{70}$. It has also been shown to play a role in the physical states of natural liposomes and has also be shown to be completely inactive at low calcium concentrations indicating that the binding mode may involve electrostatic interactions through the ion$^{71}$.

sPLA$_2$ has been shown to have interesting binding and catalysis kinetics shown in Figure 1.12$^{27}$. In this scheme the first step is a second order diffusion limited step to the membrane, then a first order binding of the enzyme to the bilayer (K$_b$). The dissociation of the enzyme is extremely slow and the enzyme stays bound to the bilayer constantly moving around catalysing the hydrolysis of phospholipids, colloquially called the ‘scooting’ mechanism$^{27,72}$.

![Figure 1.12](image_url)

Figure 1.12 - Schematic for the binding and catalysis of sPLA$_2$, everything within the boxed area happens on while the enzyme is bound to the membrane: E is sPLA$_2$, S is the phospholipid substrate and P is the lyso-phospholipid product.

The binding of sPLA$_2$ to a membrane made of 1,2-ditetradecyl-glycero-sn-3-phosphomethanol (DTPM), an anionic phospholipid, was studied using stopped-flow experiments$^{27}$. The rate constant for the binding of the enzyme to the membrane was 5 orders of magnitude higher than the dissociation rate constant indicating a very high affinity for negatively charged membranes. This also indicates that the rate of hydrolysis is not limited by the binding or dissociation rate constants and so the binding and dissociation are not involved in the steady state catalytic cycle$^{27}$.

The same experiment was carried out on a membrane made of dimyristoyl glycero-sn-3-phosphocholine (DMPC), a zwitterionic phospholipid. In this case the dissociation constant was measured to be 10 orders of magnitude higher than for the purely anionic phospholipid DTPM. This indicates that sPLA$_2$ has a binding affinity that depends on lipid charge$^{27}$. The reaction curves for this experiment showed a latency period which changes with thermotropic and isothermal phase transition characteristics of the bilayer. This kind of kinetics is indicative of the auto-catalytic of nature of sPLA$_2$ by its own build-up of asymmetry and membrane curvature$^{27}$.
Another interesting part of the kinetics of sPLA$_2$ is that it displays lag-burst kinetics\textsuperscript{73}. Lag-burst kinetics are described as having a step-function of the rate constants for catalysis, such as having an initially fast reaction, followed by a sudden reduction in speed that varies in time and is then followed by the rate going back up to the initial speed. The time frame of the lag-burst affected by a number of properties including enzyme concentration, substrate concentration, temperature and pressure\textsuperscript{74}. There are two proposed explanations for the lag-burst kinetics: 1) they are the result of the build-up of the hydrolysis products of sPLA$_2$ into ordered domains which form boundaries that sPLA$_2$ cannot cross, dissociation and re-association would need to occur before it can continue hydrolysis; 2) sPLA$_2$ requires a certain concentration of anionic lipids to function effectively, it functions slowly at first producing lyso-phospholipids and fatty acids, the fatty acids produced get deprotonated faster than other lipids (pKa $\approx$ 7.7) and the anionic lipids build up until a threshold is reached and then there is a large increase in sPLA$_2$ function.

The ability to tune and control the rate of sPLA$_2$ hydrolysis using membrane composition is very important in controlling protein-membrane-protein cell signalling pathways. The lag-burst kinetics should also be considered in the rate of MscL activity in this project as the lipids that will be used are similar in structure to those used in the experiments.

1.2.2. Protein Membrane Interactions

As transmembrane proteins sit within bilayer, it stands that if the properties of the membrane change, that the properties of the protein can also change. Physical properties that have been shown to affect protein properties include stored curvature elastic stress\textsuperscript{75}, surface charge density\textsuperscript{76}, curvature\textsuperscript{77}, and bilayer asymmetry\textsuperscript{78}. Additionally, the protein properties that have been shown to change with membrane properties include stability\textsuperscript{79}, folding\textsuperscript{80}, and activity such as opening, closing or changing conformation\textsuperscript{81}. Given the nature of these interactions almost all membrane proteins display some form of mechanical sensitivity, however transmembrane proteins that are significantly affected membrane properties are grouped into the term “mechanosensitive”. An example of mechanosensitivity can be seen in Figure 1.13.
While mechanosensitive channels have been documented in several species of bacteria, archaea and eukaryta, they display a large diversity in their specificity, conductivity and voltage dependencies. By converting mechanical forces into physiological changes within the cell, mechanosensitive channels are able to important roles such as in touch or hearing in mammals, or turgor control in bacteria.

In this thesis the mechanosensitive protein reconstituted into model membranes was the mechanosensitive channel of large conductance (MscL).

### 1.2.3. Mechanosensitive Channel of Large Conductance (MscL) from E. Coli

The Mechanosensitive Channel of Large Conductance (MscL) from *E. Coli* is a well-studied pentameric integral membrane protein. The function of this membrane protein is to form a large, non-selective channel for osmolites when the cell is under osmotic stress, thus preventing the cell from bursting. The MscL has one of the largest conformational changes known in natural, wild type membrane proteins, with an open pore size of 30-40Å. The MscL monomer has 136 amino acids, the large majority of which are non-polar, with a molecular mass of 17kDa. Each monomer is predominantly α-helical with two transmembrane domains, cytoplasmic N and C terminals and a central periplasmic domain. This is further described in the Review of MscL studies section.
Figure 1.14 - Structure of MscL protein, composed of 5 monomers, labelled in 5 different colours, which are arranged into a barrel-like structure. Image taken from Steinbacher, 2007.

Literature evidence shows that naturally MscL opens in response to tension build-up in the local membrane and is involved in the osmoregulation of the cell. When the cell expands due to osmotic pressure, the membrane tension increases and MscL gates. Using various spectroscopic techniques on the structure of MscL (Figure 1.14), it was found that when tension was applied to a bilayer containing the protein, the barrel-like structure enlarged and stretched in the S1-TM1 region, which in turn opened the pore in the protein and allowed molecules to flow through. Later assays proposed three different mechanisms of gating shown in Figure 1.15.

In Figure 1.15a, MscL gates in response to the membrane ‘stretching’ as the cell swells out due to osmotic pressure. In Figure 1.15b, MscL is tethered to either the cytoskeleton or the extracellular matrix, as the cell swells due to osmotic pressure the membrane would expand but the tether would remain in place forcing the protein to open. In Figure 1.15c, another membrane bound protein is involved which is tethered to the either the cytoskeleton or the extracellular matrix, this extraneous protein acts as a signalling molecule for MscL. The authors state that the most likely mechanism is actually a mixture of the different mechanisms, as MscL is known to be tightly regulated to prevent large channels opening without extreme circumstances.
Figure 1.15 - Schematic of proposed mechanisms of mechanosensitive channels responding to changes in membrane tension. 
a) Ion channels gate upon a change in forces (horizontal arrows) within the lipid bilayer; these forces include bilayer tension or curvature. b) The gating of mechanosensitive channels found in sensory cells have proposed links to extracellular and/or cytoskeletal proteins. Changes in tension on either of these links transmit a force to the mechanosensitive channel. c) Another proposed opening mechanism relies on a signalling intermediate which transmits a gating signal to the ion channel through a hypothetical mechanism. Image taken from Lumpkin, 2007.

1.3. Cell Communication and Signalling

Generally, for two cells to communicate, proteins within cells need to have interactions with other proteins or with small molecules. Protein-protein and protein-molecule interactions are controlled by specific shape fitting of domains and often involve a chemical reaction; examples include interactions of transmitter molecules such as steroids or hormones with cell receptors. These interactions are called cell receptor-transmitter type cell signalling. This type of protein interaction allows two or
more cells to signal each other to perform certain tasks such as regulating the production of a certain protein or molecule, and can even command the cell to perform apoptosis, the process of cell death.

Recently, it has also been shown that cell signalling can occur via a process such as a protein modifying the lipid bilayer, which is then sensed by mechanosensitive proteins which alter in response. These type of interactions are called protein-membrane-protein communication networks and have been shown in large eukaryotes where mechanosensitive protein networks have been found to control physiological responses such as the somatic and auditory senses, while in smaller prokaryotes they have been shown to control ion concentration in osmoregulation and also in chemotaxis.

Whilst direct protein-protein or protein-molecule interactions are very specific and often have recognition or allosteric sites which will only activate in response to precise interactions, the mechanisms of protein-membrane-protein communication allows for much more freedom in terms of the types of interactions required. Therefore, a mechanosensitive channel will respond to physical changes in the bilayer regardless of what caused the physical change. For instance, a mechanosensitive protein that is activated by sensing local asymmetry could be activated by lipases, scrambalases, flippases or floppases.

There have been a number of mechanosensitive proteins isolated and characterised, and a number of membrane changes via proteins have also been characterised, however there is not much data on protein-membrane-protein interactions and the mechanisms of action are poorly understood.

1.4. Review of MscL Studies

Whilst some of these studies are not relevant to the thesis work, the purpose of this section is to outline the current scope of research concerning the MscL and mechanosensitive channels in general. Studies of MscL that are directly relevant are outlined in detail within the relevant chapter.

MscL is one of the most well-studied of the mechanosensitive channel proteins. The structure of the MscL family has been determined by crystallography and spectroscopy but the crystal structure of the MscL from E. Coli has yet to be determined. The structure of the closed, intermediate and open conformations of the MscL are shown in Figure 1.16.
Figure 1.16 – The MscL family of proteins have had many crystal structures observed. This cartoon shows the conformations that the MscL can take, each helix is shown as a cylinder with one of the monomers coloured blue. a) Extracellular view looking down on the MscL structure, the helices that form the channel structure are visible. The crystal structure of the MscL from *Mycobacterium tuberculosis* (TbMscL, top left) revealed the closed state of the channel. The open structure was spectroscopically derived from the MscL from *Escherichia coli* (EcoMscL, top right). The crystal structure of the intermediate ‘pre-expanded’ phase was isolated from the tetrameric truncated MscL from *Staphylococcus aureus*, shown as a pentamer for clarity (Δ26 SaMscL, top middle). While the pre-expanded state is expanded, the pore is not open. b) Side views of the MscL reveal that as expansion and gating occurs, the pore tilts towards the plane of the membrane becoming flatter. The helices maintain the flatter orientation as the channel expands into the open state. Image taken from Vasquez & Perozo, Nature, 2009.

There is a very high degree of sequence identity between homologs of the MscL family, although there are some reasonable differences in gating and functionality. The MscL is usually homopentameric, with the exception of staphylococcal MscL which exists as a homotetramer. Each MscL monomer consists of two transmembrane domains which have two alpha helices, TM1 and TM2. The N terminus of the monomer is cytoplasmic and the TM1 helix forms the inner part of the channel. The two transmembrane domains are linked by a periplasmic loop and the TM2 helix forms the exterior of the channel. The gating of the MscL is caused by a large reorientation of the two transmembrane helices (Figure 1.16) although the precise trigger of the MscL gating is unclear. What is clear, is that the MscL responds to mechanical changes in the forces within the bilayer which can be regulated by lipid-protein interactions.
1.4.1. Computational Studies

The crystal structure of the closed state of the MscL in *Mycobacterium tuberculosis* has provided a large amount of information for use in dynamical and conformational computational models\(^{98,99}\).

The mechanism for gating was modelled in three-dimensions for both the open and closed states, as well as many of the intermediate transition states. Using transition state models of time resolved recordings showed three different sub-conductance states, of which only the first was mechano-dependant (Figure 1.17).

![Figure 1.17 - Schematic of the three sub-conductance levels proposed in the gating of MscL. The outer portion is postulated to be the mechano-sensor, and expands substantially before the inner channel opens. The central channel portion is postulated to consist of five components (for the pentamer) which are connected to the outer portion by mechano-sensing “strings”. The transition between the closed resting state (C), and the closed expanded state (CE), is elastic and electrically silent. The central channel first opens to a sub-conducting state (S1), when the expansion of the outer portion places enough stress on the “strings” to cause one of the channel components to pull away from the closed portion. The mechano-dependency of the transition from CE to S1 suggests a further increase to the in-plane area. The channel is then proposed to go through two more sub-conductance states (S2 & S3) of the same type before reaching the fully open conformation (O). The latter transitions (S2 -> O) have been shown to have little mechano-dependency suggesting very little change in the outer portion. Schematic adapted from Sukharev, Biophys J, 2001\(^{98}\).](image)

The computational study showed a five stage sequence for the gating of the MscL which were later correlated with experimental conductance, kinetics and mechanosensitivity results\(^{98}\).

1) Membrane tension induces an iris-like expansion of the transmembrane helices. TM1 and TM2 radially tilt away from the pore’s axis.
2) The outer helix, TM2, and the periplasmic loop that links the two helices flattens and moves into the transmembrane space.
3) The linker between the cytoplasmic N terminus and the TM1 helix becomes stretched.
4) Tension in the N terminus – TM1 linker reaches a threshold and pulls apart the N terminus bundle.
5) As the pore opens each monomer TM1 helix docks onto the outer portion of the channel.

A different computational study of the MscL used normal mode analysis to look at the mechano-induced iris-like first step of the MscL gating mechanism\(^{99}\). Normal mode analysis is a form of computational study that looks at the motions of macromolecules and simplifies the computation, which allows for longer timescales for the computation. At longer timescales, it’s possible to look at
the first step in greater depth and within different areas of each monomer. The conclusion of the analysis shows that the initial step included a compression of the transmembrane helices and a twisting motion around the axis of each helix.

While computational models are useful in elucidating mechanisms in the MscL activity, because of the assumptions used it’s difficult to get fine detail analysis or information on the kinetics, especially with respect to how it interacts with the lipids in the bilayer.

1.4.2. Mutagenesis studies

In protein research, mutagenesis is the study of how mutations in the amino-acid primary structure of the protein can affect its folding and overall structure, and therefore how it affects protein activity. The MscL has been the focus of many mutagenesis studies validating which regions of the protein are essential for function, and enabling ligand gating and protein isolation functions.

Ligand gating involves a glycine in the 22-position mutation to a cysteine, G22C, increasing the hydrophobicity of the MscL pore and increasing the tension threshold for gating. Upon reaction with a cysteine reactive compound, the MscL pore decreases in hydrophobicity and gates. Ligand gating is important in assessing MscL active reconstitution in control experiments and can provide a basis for kinetic gating experiments in new model systems100.

To measure the amount of the MscL used in this thesis a phenylalanine in the 93-position is replaced with a tryptophan, F93W. This allows for the protein concentration to be characterised by tryptophan absorption. A group of histidines, a His tag, is also added onto a terminus of each monomer unit to allow for purification by metal binding.

One of the most important large-scale high-throughput mutagenesis studies of the MscL used random mutations throughout the entire amino acid chain, and paired them with a functional assay101. Each functional screen characterised how each mutation affected bacterial growth, as well as changing the osmotic down shock of the channel for the bacterium. The mutations have two major categories; gain of function (GOF), mutations that cause the channel to open spontaneously or reduce the membrane tension threshold for gating and loss of function (LOF), mutations that cause an increase in the membrane tension threshold or completely removing gating entirely. Two of five GOF mutations were found in the TM2 helix, suggesting functional significance and supporting computational mechanisms. Most of the LOF mutations were concentrated on the N-terminus – TM1 link, supporting computational evidence for this part of the gating mechanism. Interestingly some of the LOF
mutations were concentrated around areas of the protein near to the headgroups of the outer leaflet of the lipid bilayer, suggesting a key role for lipids in modulating the functionality of the MscL.

While mutagenesis studies do not provide detailed information beyond changes to functionality, they are an extremely important step in determining areas of importance for structure and function, and direct future studies and assays.

1.4.3. Electrophysiological studies

Electrophysiological methods are widely used in the study of membrane proteins, one recent type of electrophysiological study, relying on droplet interface bilayers, is expanded on in detail in Chapter 5. Electrophysiological studies grant the ability to study the behaviour of single protein channels and directly quantify environmental effects on functionality. While there are many types of electrophysiological studies, a large amount of studies on the MscL are undertaken using a technique called a patch clamp.

Patch clamping is performed by the formation of a high resistance seal on a very small patch of membrane with a minimal number of protein channels, and recording electrical activity. In the case of mechanosensitive channels, a pressure can be applied on the seal, and the activity of the protein gating can be measured. An example of the results of a patch clamp study on mechanosensitive channels MscS and MscL is seen in Figure 1.18.

Figure 1.18 – A patch clamp experiment showing current activity, against the pressure applied to a membrane containing five MscS channels and one MscL channel. Currents were recorded within an inside-out excised membrane patch at a holding potential of +20mV. A single opening of MscS (MscS O₁) has a respective pressure sensitivity for gating and the current amplitude of a single-channel can be used to calculate channel conductance using Ohm's Law. Image adapted from Booth, Nat. Rev. Microbiology, 2007\textsuperscript{102}. 

\textsuperscript{102}
Patch clamps have three main configurations\textsuperscript{103}:

- **Cell attached**: the seal between the pipette and the membrane is caused by a small applied pressure.
- **Inside out**: the cell attached model is followed, but the membrane patch is torn from the cell after the seal is created.
- **Whole cell**: the intracellular space is continuous with the internal pipette, so the pipette solution is the inside of the vesicle.

One example of a patch clamp study on MscL, examined the effect of membrane tension and lipid headgroups on the activity of MscL in a patch of membrane consisting of pure DOPC\textsuperscript{104}. The pressure required for channel gating of the MscL was measured on different membrane patches. The pressure induced a curvature in the membrane patch, and both the pressure and curvature were measured and converted into membrane tension using the Laplace Law\textsuperscript{105}. It was found that the MscL could not be reliably gated with pressure, or curvature alone, and that membrane tension gave consistent gating between each patch.

The same set of MscL experiments also investigated the effect of lipids with negatively charged headgroups on the membrane tension required for MscL gating. While it is known that negatively charged lipids are required for successful reconstitution of the MscL, it was postulated that the membrane tension required for gating would change with addition of charged lipids, however no distinct changes were seen. Increased proportions of the type I lipid DOPE were also investigated and was found to cause distinct changes in tension, providing further evidence that the MscL is susceptible to biophysical changes in lateral pressure.

A different patch clamp experiment described the effects of asymmetrical membrane tension on two mechanosensitive channels, MscS and MscL\textsuperscript{106}. The asymmetrical membrane tension was generated on an excised membrane patch by allowing the inner leaflet to relax while the outer leaflet is in contact with the glass pipette (Figure 1.19).
It was shown that both MscS and MscL show an adaptation in behaviour based on the asymmetric tension, indicated by a reversible current decline under sustained pressure\textsuperscript{106}. Given that MscS and MscL are structurally unrelated, this behaviour suggests that the biophysical properties of the membrane are causing the behaviour rather than any specific protein interaction. This adaptive behaviour was not present when the membrane tension was isotropically distributed. While this work was not completely conclusive, patch clamp studies have been very useful in investigating MscL activity deeply, and chapter 5 builds upon this.

1.4.4. Fluorescence studies
A large bulk of this thesis relies upon fluorescent techniques, outlined in Chapter 3. While fluorescent experiments often only provide bulk properties, they also mostly don’t disturb the membrane in the way that patch clamp often does. Fluorescence can be used in assays as a measure of kinetics\textsuperscript{107}, or used to directly label the protein itself\textsuperscript{108,109}. By exploiting the environmentally fluorescent Tryptophan amino acid (trp) it is possible to get a measure of protein concentration, hydrophobic thickness, and even binding affinities for the surrounding lipid membrane.

1.5. Summary
While many of the properties of the protein channel used in this thesis, MscL, have been outlined, there is still a lot of information and kinetic data that has not been confirmed. The field of mechanosensitive protein channel research, especially within artificial membranes, is relatively new and unexplored. It is hoped that the work in this thesis helps to build upon that knowledge base.
Chapter 2

Expression and Purification of MscL

2. Overview

Membrane proteins are known for being difficult to work with; studying them often involves complex procedures to purify them in model membranes while maintaining functionality. MscL is one of the most well-established gated ion-channels\textsuperscript{110}. MscL was chosen partly because of its well-established expression and purification protocols, but also because it is known to be gated by a range of different membrane properties including tension and asymmetry\textsuperscript{62,110}. By reconstituting MscL into model membrane systems, and gating it with respect to membrane properties, many biophysical membrane properties can be determined, quantified, and controlled. It is hoped that assays involving MscL could be translated to other ion channels and model membrane systems.

This chapter details the transformation of \textit{E. Coli} competent cells with a plasmid for the mutant form of MscL used in all following experiments. This MscL mutant has a Glycine residue at the 22-position substituted for a Cysteine, G22C, and a Phenylalanine at the 93-position was substituted for a Tryptophan, F93W. The G22C mutation grants the channel a higher activation threshold in terms of its mechanosensitivity, while also increasing reactivity to Cysteine reactive molecules. The F93W mutation allows for quantification via Tryptophan absorption. Motivation for using this mutant is explained in greater detail in the \textit{Introduction} chapter.

This chapter then details the expression and purification of the MscL from the newly transformed \textit{E. Coli}, as well as changes that were made to the established protocols. After the purification, the MscL protein is then evaluated for purity via Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) and concentration quantified, while the main activity testing will be detailed in chapter 3.
2.1. Introduction
Integral membrane proteins make up approximately 30% of all cellular protein types, despite this membrane proteins remain the least studied of all-natural proteins due to the difficulty in purifying them in their native forms. Studying membrane proteins in isolation in vitro must mimic the membrane properties of their in vivo environment as close as possible, or you risk changing its native behaviour.

Due to the relatively low quantities of membrane protein in vivo, it is common practise to overexpress and purify them in their functional form. Attempts have been made to use In-Vitro Transcription Translation (IVTT) to produce cell-free membrane proteins, these use isolated cellular protein production enzymes in order to produce proteins outside of cellular conditions. However the majority of current IVTT proteins are extremely simple, monomeric protein pores, whereas MscL is far more complicated.

Here the relevant theory and techniques for this chapter are detailed, more information about the overall science can be found in the Introduction chapter.

2.2. Techniques

2.2.1. Transformation of Competent Cells
Competent cells are defined as bacterial cells that can incorporate foreign DNA from the environment. The cell wall and cytoplasmic membrane are made porous enough that DNA is able to pass through, either through active translocation or passive diffusion. Competent cells are extremely useful in producing proteins in large quantities; the foreign DNA is incorporated into the cell and the protein is overexpressed (see Protein Expression and Purification).

Competent cells can either be naturally competent, as seen in bacterial strains such as Streptococcus pneumonia, or be given artificial competence. Natural competence is highly regulated, the uptake of foreign DNA only occurs at certain times in the cell cycle or within certain populations. The foreign DNA can then be degraded by cytoplasmic nucleases or it could recombine with the cellular chromosome. While natural competence is an important process in transformation, it is only efficient for linear molecules such as chromosomal DNA.

Artificial competence is induced via chemical treatment and heat shock, this process is usually undertaken while the cells are in the exponential growth phase, also known as log phase (Figure 2.2). Cells are made competent much easier while undergoing very rapid growth, then any other stage
of growth. For instance *E. Coli* cells are made competent though a process that uses calcium chloride and heat shock\(^\text{114}\).

### 2.2.2. Protein Expression and Purification

To produce suitable amounts of a protein for study, proteins are overexpressed in, and extracted from cells\(^\text{115}\). These proteins are then included in a wide range of studies including crystallisation studies to determine structure\(^\text{116}\), folding studies\(^\text{117}\), or lipid interaction studies\(^\text{118}\). In order to produce these proteins, the cells must be genetically modified.

![Figure 2.1 - Schematic of expression vector creation, expression vector transfection into a cell, and protein overexpression. The red section indicates the promoter sequence and the blue section indicates the protein coding sequence. Adapted from in-group poster presentation.](image1)

To genetically modify a cell, first a plasmid expression vector must be chosen\(^\text{111}\). These vectors include a highly active promoter sequence and usually also includes a region with confers resistance to a specific antibiotic. The DNA sequence which codes for the protein is inserted, directly after the promoter sequence, into the plasmid using restriction and ligation enzymes which are specific to a nucleotide sequence\(^\text{111}\). The plasmid expression vector is then transfected into competent cells and grown in large numbers, and an antibiotic is added which kills every cell which doesn’t have the antibiotic resistant plasmid. Generally, cells will prevent the expression of foreign proteins and so most vectors are designed to delay protein synthesis until a chemical trigger \(^\text{119}\). A schematic of this process has been shown in Figure 2.1.

For the work described in this thesis, MscL expression was triggered by the addition of Isopropyl-β-D-1-Thiogalactopyranoside (IPTG). IPTG binds to the lac operon repressor and inhibits it, enabling protein expression. However, in the case of MscL, this expression is toxic to the cells and therefore IPTG was only added after the log growth phase\(^7\).
The growth rate of a bacterial culture is not constant, as shown in Figure 2.2. The first phase of a bacterial growth is the lag phase, growth is very slow due to the low number of dividing cells. The second phase is the log phase or exponential phase, the growth of the bacteria increases exponentially before eventually reaching a saturation level. The third phase is the stationary phase, almost all the nutrients in the system are used up and the cells are dividing at the same rate as they die. The final phase is the death phase, all the nutrients in the system are used within bacterial cells, the bacteria still divide but the death rate is faster than the growth rate. As cells die some nutrients are released back into the medium and so the rate of the death phase is not exponential. The length of each phase depends upon many factors including temperature, speed of rotation, and the type of cell being grown.

To measure the growth of cells over time an Optical Density reading is taken at 600nm (OD600). As bacterial cells grow they become more turbid, their turbidity scatters light which lowers the amount of light that reaches a detector.

### 2.2.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

One dimensional Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) is a common technique used to separate proteins by size. The protein sample is prepared by mixing it with a sample buffer containing many components, but the most important of these is β-mercaptoethanol and SDS. The β-mercaptoethanol breaks the disulphide bonds in the protein’s structure and the SDS denatures the protein. Sometimes to fully denature the protein, the mixture must be heated or reduced before loading into the gel, however this has not been documented as being required for MscL. The SDS forms a negatively charged SDS: protein complex, these complexes are rod-like structures with a length which is roughly proportional to the molecular weight of the protein. If the
protein itself is charged, those charges are balanced out by electrostatic interactions with the sample buffer. All SDS: protein complexes have similar charge densities, this allows separation based on size alone, with smaller molecular weight proteins moving through the porous medium faster. The speed at which proteins move down the gel is controlled by the pore size of the gel; the pore size is controlled by the amount of acrylamide per unit volume and the degree of cross linkage. As cross linkage increases, pore size decreases, and the speed at which proteins travel down the gel slows\textsuperscript{120}.  

**2.3. Materials**

The materials and methods sections are separate to allow for justification for components. Relevant concentrations for compounds are found in the methods sections.

### 2.3.1. Transformation of BL21 (DE3) E. Coli

BL21 (DE3) competent *E. Coli* (C2527) was purchased from New England BioLabs Inc. The BL21 (DE3) strain is the standard cell type for the MscL expression protocol and was chosen for its high expression levels. The *E. Coli* was transformed with a pET28a kanamycin resistant plasmid which contained the mutant form of MscL (G22C F93W) with a His tag. The plasmid was extracted from glycerol stocks for MscL expression given as a gift by Professor Paula Booth at Kings College London using a QIAGEN QIAprep Spin Miniprep Kit. This MscL mutant is known to react with [2-(Trimethylammonium) ethyl] Methane Thiosulfonate Bromide (MTSET)\textsuperscript{100}, a cysteine reactive compound.

![Figure 2.3 - Chemical structure of MTSET.](image)

### 2.3.2. MscL Expression

Agar plates were prepared using Luria Bertani (LB) Miller Agar and both the small and large cultures were grown in LB Miller broth. Each was inoculated with 30µg/ml Kanamycin sulphate from *Streptomyces kanamyceticus* purchased from Sigma Aldrich. All LB agar and broth were autoclaved before use. The incubator used for the majority of expressions was a New Brunswick Scientific Innova 44 incubator shaker series. The expression was induced using Isopropyl-β-D-1-Thiogalactopyranoside (IPTG) purchased from VWR International Ltd. The OD absorbance reader was a Jenway 7300 Spectrophotometer. The cells were centrifuged down using a Beckman Coulter J6-M1 centrifuge and
Beckman Coulter 1L flasks. The complete EDTA free Protease Inhibitor tablets, as well as all other non-specified materials, were purchased from Sigma Aldrich.

2.3.3. MscL Purification

The purification protocol required Deoxyribonuclease I (benzonase nuclease) from bovine pancreas and n-Dodecyl-β-D-maltoside (DDM) which were purchased from Sigma Aldrich. Phenylmethanesulfonyl fluoride (PMSF) was purchased from VWR International Ltd. Cells were lysed using a Constant Systems Cell Disrupter (TS). The ultracentrifuge used was a Beckman Coulter Optima L-100 XP Ultracentrifuge using either a Beckman Coulter Ti45 or Ti70 rotor.

Membranes were homogenised using a 55ml Homogeniser from Fisher Scientific. The binding step was performed using HisPur TALON Cobalt Resin purchased from Fisher Scientific and eluted in a 5ml Polypropylene Column from Qiagen. The elution was concentrated using an Amicon Ultra-15 100,000 MWCO centrifugal filter unit with an Ultracel-50 membrane from Millipore. Imidazole was removed with a PD-10 desalting column purchased from GE Healthcare. MscL concentration was measured by 280nm UV spectrometry using a ThermoScientific NanoDrop 2000c.

Buffers were made up as listed below:

**Solubilisation buffer:** 20mM HEPES pH 7.2, 100mM KCl, 2% (w/v) DDM

**Dilution buffer:** 20mM HEPES pH 7.2, 100mM KCl

**Wash buffer:** 20mM HEPES pH 7.2, 100 mM KCl, 0.13% DDM, 0.1mM PMSF and 6mM imidazole

**Elution buffer:** 20mM HEPES pH 7.2, 100mM KCl, 0.13% DDM, 0.1mM PMSF, 150mM imidazole

**Equilibration buffer:** 20mM HEPES pH 7.2, 100mM KCl, 0.13% DDM, 0.1mM PMSF

All solutions were pH adjusted using 1M stock solutions of Sodium Hydroxide (NaOH) and Hydrogen Chloride (HCl) purchased from Sigma Aldrich.

2.3.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The SDS PAGE gels were undertaken in pre-cast NuPAGE 12% Bis-Tris Protein Gels using NuPAGE MOPS SDS Running Buffer (20X) purchased from Life Technologies. Pre-cast gels were chosen in order to maintain consistency. The gels consisted of 12% polyacrylamide. Each sample was mixed with NuPAGE™ LDS Sample Buffer (4X) purchased from Life Technologies. The protein ladder used for size
determination of the protein was a Benchmark Pre-stained Protein Standard 6 – 180 kDa purchased from Thermo Fisher Scientific. The Gel container was a XCell SureLock Electrophoresis Cell and the amplifier was a BioRad Power Pac 300. Proteins were stained with Instant Blue coomassie stain from ExpeDeon. The gels were visualised on a Fujifilm LAS-3000 using ImageQuant LAS 4000 software on a half-second exposure time.

2.4. Methods

2.4.1. Transformation of BL21 (DE3) E. Coli
The transformation protocol used is a specific heat shock protocol provided for the New England BioLabs BL21 (DE3) E. Coli cells. A condensed version follows:

Within the tube provided, the competent cells were thawed completely. 5ul of the mixture containing 100ng of the extracted MscL plasmid was added to the mixture and the cells were carefully mixed without vortexing. The mixture was placed on ice for 30 minutes. The mixture was then carefully placed in a water bath at exactly 42°C for exactly 10 seconds before being placed back on ice for 5 minutes. 950 µl of room temperature SOC outgrowth medium was added into the mixture. This mixture was placed in a water bath at 37°C for 60 minutes before vigorous mixing.

LB agar selection plates containing 30µg/ml of kanamycin were pre-warmed to 37°C. The cells were mixed thoroughly, and several serial dilutions were made. These serial dilutions were streaked onto the selection plates and allowed to incubate overnight at 37°C.

2.4.2. MscL Expression
This summary of steps to express and purify MscL are a modified protocol of Blount et al. and modified again from the supporting information of Charalambous et al. Explanation of, and justification for, changes made to the protocol are expanded in the Results and Discussion section of this chapter. The protocol was undertaken in either 3 or 6 litre preparations depending on need for MscL. All buffers listed are for 6L preps and halved in amount for 3L except for directly after the litre cultures are centrifuged, as the minimum volume for the cell disrupter is 50ml.

The expression was either undertaken directly after transformation and selection plating, or the glycerol stock of the MscL transformed E. Coli was streaked and incubated overnight on LB plates
containing 30µg/ml kanamycin at 37°C. One colony was picked and incubated in 100ml of LB broth overnight at 37°C and 250 rpm.

The culture was re-seeded 1:100 into 1L fresh LB broth containing 30µg/ml kanamycin and incubated at 37°C and 250rpm to approximately the maximum of the exponential phase ($OD_{600} = 1$). Protein production was induced by adding 0.5mM IPTG and incubating at 37°C and 250rpm for 90 minutes, or until the cells have fully stopped growing. OD recordings were taken every 30 minutes to plot the growth both pre- and post-induction. The OD growth plot is discussed further in Results and Discussions. The cells were collected by centrifugation at 5000rpm at 4°C for 30 minutes. The pellet was then re-suspended in 50ml PBS supplemented with EDTA-free protease inhibitors (1 tablet per 50ml). This cell suspension was then frozen overnight at -20°C.

2.4.3. MscL Purification

The cell suspension was then thawed at 4°C with rotation. Once thawed a trace amount of benzonase was added to the suspension (1µl per 50ml), and was incubated for 20 minutes at 4°C with rotation.

The cells were then lysed by 2 passages through a cell disrupter at 25kPSI. The cell membranes were isolated by ultracentrifugation at 38,000rpm at 4°C for 60 minutes. Membranes were solubilised in 50ml solubilisation buffer (HEPES, KCl and 2% DDM) and a EDTA-free protease inhibitor was added. Membranes were homogenised and incubated at 4°C overnight with rotation.

Insoluble material was removed by ultracentrifugation at 38,000rpm at 4°C for 60 minutes. The DDM-solubilised protein was diluted in dilution buffer (HEPES and KCl) to a final DDM concentration of 1% and was His-tag batch-bound to 4ml TALON cobalt metal affinity resin (pre-washed) for 90 minutes at 4°C with rotation. The bound protein resin was centrifuged at 1000rpm at 4°C for 5 minutes to pellet the resin, the supernatant was removed and 40ml wash buffer (HEPES, KCl, 0.13% DDM, PMSF and 6mM imidazole) was added. The suspension was then incubated at 4°C at 10 minutes with rotation to remove non-specifically bound protein. The suspension was centrifuged again at 1000rpm at 4°C for 5 minutes to pellet the resin, and the supernatant was removed.

The next steps were all undertaken in a 4°C cold room to ensure temperature stability. The resin was re-suspended in 10ml of wash buffer (HEPES, KCl, 0.13% DDM, PMSF and 6mM imidazole) and transferred to a gravity-flow column. The resin settled out of suspension without letting it dry out completely. The DDM-solubilised MscL was eluted from the resin in 15ml Elution Buffer (HEPES, KCl, 0.13% DDM, PMSF, 150mM imidazole) and concentrated using a centrifugal concentrator down to approximately 2ml.
The imidazole was removed by buffer exchange using a desalting column which had been equilibrated in equilibration buffer. After the imidazole had been removed, the MscL was concentrated further, aliquoted, snap frozen in liquid nitrogen and stored at -80°C. The concentration was calculated using a Nanodrop absorbance at 280nm, calculations are shown in Results and Discussion.

2.4.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)
Throughout the expression and purification, samples were taken to track the concentration of the MscL through each stage. The samples were prepared by mixing 1µl of the sample, 2.5µl of the sample buffer, and 6.5µl of water. The pre-cast gel was loaded into the gel container and the container was filled with SDS running buffer. 10µl of the prepared samples and the protein ladder were loaded into the wells in the gel, the gel was then switched to 200V and the gel was left to run for 50 minutes. The gel was stained for 1 hour and was then visualised under white light.

2.5. Results and Discussion
The MscL was expressed from E. Coli and purified via metal affinity using known established protocols. The concentration of the MscL was measured by Tryptophan absorption. The purity of the MscL was evaluated by SDS PAGE gel electrophoresis. Changes were made to established protocol to improve yield and activity.

2.5.1. MscL Expression and Purification
The growth of the E. Coli cells was measured using the optical density at 600nm (OD600). Measurements were taken at regular intervals and the growth of the cells was plotted as a function of time. A sigmoidal curve was fitted to the cell growth using Origin 2016. To compare the effects of the MscL production, a comparison was run between cells after IPTG addition, and cells left to grow without induction. In each case where the cells were induced, the cells stopped growing due to the overexpression of the MscL. A comparison of growth rates is shown in Figure 2.4.
2.5.2. SDS Page

The stages of MscL purification was followed via SDS PAGE run at 200V. For each stage, a small sample was taken and prepared. The samples were pipetted into the gel lanes and compared to a molecular weight marker to determine any stages where MscL was lost, and the final concentrated MscL purity. The gel lanes were loaded as listed in Figure 2.5.
Figure 2.5 shows a large band of protein present which is indicative of the MscL monomer at 17kDa. The ‘smiling’ effect, where the sides of a gel appear to curve upwards, causes the protein band in Figure 2.5 to appear higher than 17kDa. As the MscL band is very concentrated, there are quite a few impurities that can be seen, however most of these bands are most likely due to the various oligomeric states of MscL. While MscL mostly exists as a monomer, or pentamer in its active form, there is also a semi-stable trimeric form which is seen as around 51kDa on Figure 2.5.

The large amount bands in the DDM-solubilised protein fraction is an example of the efficacy of the metal affinity binding purification, as the non-specifically bound proteins simply flowed through. It seems that there is a reasonable amount of MscL at 17kDa in the washed off non-specifically bound protein, this is due to the strength of the His-tag–cobalt binding allowing for some MscL to be washed off. However, the amount lost is not significant and it’s much better to increase purity than yield. There is also a small band of MscL in the flow-through of the 2nd concentrator, which is indicative of reaching a concentration limit, causing some MscL to leak through the size limit concentrator.
2.5.3. Concentration Calculations of Purified MscL

After concentrating and aliquoting the final MscL sample, it was necessary to obtain the concentration of the MscL in each aliquot. The accepted method for MscL concentration calculation is an absorbance measurement which uses the Beer Lambert Law\textsuperscript{122} to relate the amount of absorption from a sample to the total number of atoms within the path of light. This is defined as:

\[
\text{Absorbance} = A = a(\lambda)bc
\]  \hspace{1cm} (2.1)

Where \(a(\lambda)\) is the molar extinction coefficient (M\textsuperscript{-1} cm\textsuperscript{-1}), \(b\) is the path length of the light passing through the sample (cm) and \(c\) is the concentration of the protein (Molar). The MscL mutant we used had a F93W point mutation and the Tryptophan absorbs at 280nm, the known molar extinction coefficient of the MscL monomer at 280nm is 6990 M\textsuperscript{-1} cm\textsuperscript{-1}. As the Beer Lambert Law is only accurate between absorbances of 0 and 1, a 20-fold dilution of the MscL sample was made. An example spectrum of the absorption of MscL is included (Figure 2.7).
Figure 2.7 – Example absorption spectrum of a purified MscL sample.

With this example the absorbance at 280nm was measured 5 times and the average was 0.752, the measurement was repeated for each preparation of MscL to calculate the exact concentration.

\[
c = \frac{A}{a(\lambda)b} = \frac{0.752}{6990 \times 1} = 1.08 \times 10^{-4} M
\]  

(2.2)

The concentration of the MscL monomer at a 20-fold dilution was 1.08 \times 10^{-4} M. Factoring in the dilution, the concentration of the MscL monomer in the sample was 2.15 \times 10^{-3} M. Working on the assumption that 100% of the MscL is in its active pentamer form, the concentration was 4.30 \times 10^{-4} M. The final volume of the concentrated protein was 200µl and hence the number of moles of the MscL produced was equal to:

\[
Moles = Concentration \times Volume = 8.60 \times 10^{-8} mol
\]  

(2.3)

As the MscL pentamer has a mass of 85kDa, the total mass of protein produced was:

\[
MscL Pentamer Mass = Moles \times Molecular Weight = 7.31 \times 10^{-3} g
\]  

(2.4)

Therefore, for this 3L prep, the yield of the MscL pentamer was approximately 7mg. One of the major drawbacks to determining protein concentration with 280nm absorption is that Imidazole, which is used to elute MscL, also has an absorption at 280nm. While steps are taken to remove the Imidazole...
via buffer exchange, it is possible that residual Imidazole could be affecting concentration calculations. The process was examined in one prep by repeating the buffer exchange process and recalculating MscL concentration; no appreciable change in absorption was measured.

2.5.4. Changes made to protocols

Many changes were made to the protocol for MscL expression and purification. At the beginning of this research, MscL production took place off-site; moving production to an on-site facility allowed for a faster feedback time on yield and purity which meant changes could be attempted without too much disruption.

One of the biggest problems with MscL production is its thermal instability. It must be kept at 4°C throughout many of the steps to prevent denaturing of the proteins structure. Previously this involved keeping many components on ice while changing between steps. This was found to be inefficient at maintaining temperature and so a cold room set to 4°C was accessed and all steps following the cobalt binding step were performed in this cold room. By maintaining temperature, and ensuring limited thermal shock between steps, the yield remained relatively constant however the activity of the protein had a measurable increase (see activity assays in Chapter 3).

The original protocol took place over 4 days, from bacterial growth to purification, and between each day the cells or membrane fraction were frozen at -20 and defrosted the next day. However, this freeze-thaw cycle potentially damages the protein structure and so it’s important to minimise the amount of freeze-thaw cycles that are needed. Therefore, the protocol was made more efficient, and working days were made longer, in order to shorten the protocol down into a 2 to 3-day prep. This decrease in the length of the protocol dramatically increased the activity proving that freeze-thaw cycles can significantly affect protein structure.

Another problem with MscL production is its cellular toxicity. The E. coli must be grown to the log phase before inducing protein expression to avoid cell death and maximise MscL production. However, the longer the cells spend growing, the more cells will die releasing proteases into the medium, lowering the yield and activity of MscL. While the original protocol stated using 230rpm rotation for the incubation, an attempt to grow the cells quicker, and minimise MscL degradation, lead to the use of a different incubator at 250rpm. The faster rotation speed increased the mixing of the medium, essentially reducing the size of the lag phase (Figure 2.8). The MscL produced from this faster growth E. Coli expression had a much larger yield, almost double the expression from the 230rpm growth. Attempts at faster rotation speeds lead to leaking of the medium from the flask and no faster growth was attained.
2.6. Conclusions

Expression of the G22C F93W MscL mutant from *E. coli* and subsequent purification via His-tag cobalt metal affinity resin was successful. The purity was evaluated using SDS PAGE, which showed that the concentrated MscL monomer had very few impurities and didn’t need further purification. The purification protocol for the MscL has been improved in many small areas; this improvement has led to increased yield, purity and activity. The concentration of the MscL was calculated using Tryptophan absorption at 280nm and separated from trace imidazole absorption by performing a second buffer exchange and ensuring that the absorption doesn’t change.

Figure 2.8 - The clear differences between incubators. The old incubator at 230rpm (clear squares) has an elongated lag phase compared to 250rpm (black squares).
Chapter 3

MscL Liposome Assays

3. Overview

This chapter deals with the fluorescence based functional assay for MscL activity. The assay involves the reconstitution of the MscL into extruded liposomes with an encapsulated dye, Calcein. The proteoliposomes were deposited by ultracentrifugation, purifying the calcein loaded liposomes from unencapsulated calcein. The MscL was gated by a chemical activator, the cysteine reactive compound MTSET\textsuperscript{62}. The MscL was also gated by the membrane-mediated activity of an enzyme, the secretory phospholipase A\textsubscript{2}, sPLA\textsubscript{2}. When the MscL gated, the calcein was able to flow through the pore into the surrounding solution. The self-quenching properties of the calcein dye were fundamental to this assay, while encapsulated at high concentration the calcein was quenched, but when flowing out of the liposomes the dye unquenches and a fluorescence increase can be measured. Controls were completed to assure that the MscL was reconstituted in the closed conformation, and the liposomes were not leaking. The controls were characterised by a lack of calcein leakage from the liposomes, meaning any calcein translocation was due to MscL activation. Controls were undertaken using dynamic light scattering to ensure that any fluorescence changes were not due to disruption of the liposome structure.

After initial activity assays were undertaken, further liposome assays were undertaken to find a concentration of MscL that would optimise signal-to-noise, while still allowing parallelisation with the same MscL purification batch. A set of assays with altered membrane compositions were used to measure the effect on both the rate of MscL activity and maximum achieved MscL activity, these are known as lipid sweeps. The first set of lipid sweeps altered the composition of charged lipids in the proteoliposomes from pure DOPC to a DOPC:DOPG 30:70 molar ratio in 10mol\% steps. These compositions were designed to investigate the minimum composition of charged lipids necessary for reasonable MscL activity, this was important for a different model membrane system used in Chapter 4. The second set of lipid sweeps investigated the effects of increasing membrane tension by increasing composition of the curved lipid DOPE in liposomes, from 95:5 DOPC:DOPG molar ratio to 45:50:5 DOPC:DOPE:DOPG. It was found that membrane tension has varying effects on both sPLA\textsubscript{2} and MscL activity and some explanations for these phenomena are given.
3.1. Introduction

Over 80% of pharmacological compounds specifically target membrane proteins\textsuperscript{28}, and investigations into the mechanisms of action of these molecules are often undertaken on model versions of cell membranes. Model membranes allow research into membrane interactions without the complex interactions of the cytoskeleton or the multitude of different lipids found \textit{in vivo}. Model membranes allow the characterisation of specific protein-membrane interactions, at a far higher fidelity, though it is important to understand that some cellular interactions may be critical to protein function\textsuperscript{124,125}.

3.1.1. Bottom Up versus Top Down

Originally, model membrane systems were created by taking normal cells and removing any parts that were interfering with analysis by ‘knocking-out’ those membrane protein genes. This process of removing complexity from living cells is known as the \textit{Top Down} approach. However, the process of removing complexity can lead to cell non-proliferation, and there are many uncharacterised interactions that need to be taken into account\textsuperscript{124,125}.

The other approach to building minimal model systems from basic purified elements is known as the \textit{Bottom Up} approach. This approach takes advantage of the ability of lipids to self-assemble into bilayers and other curved structures to create systems where complexity can be added in using pure components\textsuperscript{126}. This approach can then mimic cellular processes and allows us to create ‘minimal cells’ with functions that can be controlled. In this thesis the bottom up approach is used to create many model membrane systems to study membrane protein interactions with membranes of known lipid compositions.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{flowchart.png}
\caption{Flowchart showing the differences between 'bottom up' and 'top down' approaches.}
\end{figure}
3.1.2. Liposomes

In this chapter the model membranes used are called Liposomes (also known as vesicles). Liposomes are small spherical bilayer membranes which self-assemble to encapsulate components for specific functions, and maintain a separate inner and outer environment. Liposomes have been used for many years, in many functional assays to explore protein and membrane functions, and are very well characterised.

The most common assay involves the reconstitution of a membrane protein into liposomes in an attempt to activate the protein using chemical and physical methods. One example of this assay is loading the liposomes with a self-quenching dye, and gating a protein pore, allowing the dye to be released where it can be detected by fluorescence imaging. This allows for kinetic profiling of protein activity and shows a relationship between the lipids in the liposomes and the reconstituted protein. Liposomes with reconstituted membrane proteins are known as Proteoliposomes, and are form the starting material for many other model membranes.

Due to the amphiphilic nature of lipids, membranes tend to self-assemble upon hydration of dry lipids to minimise their hydrophobic contact with water. When the lipids self-assemble, if they have the right curvature, they immediately form multiple bilayers stacked into a single sphere, these are known as multilamellar vesicles (MLVs). Extra manipulation steps can then transform these multilamellar vesicles into liposomes with a single bilayer of various sizes (Figure 3.2).

The largest size of liposome is giant unilamellar vesicles (GUVs), which are defined as any unilamellar vesicle above 10μm which is close in size to mammalian cells. GUVs are made via an electroformation, microfluidic or phase transfer protocol, explained further in Chapter 7. GUVs are usually quite polydisperse in size, and the process to make them is time-consuming, however their large size makes them easy to study by normal optical microscopy methods.

Smaller liposomes are made by using sonication, freeze-thaw cycles or extrusion to break up the MLVs into smaller monodisperse self-assembled liposomes. As the liposomes decrease in diameter, the curvature of the bilayer increases which in turn increases the geometrically induced membrane tension. As these smaller liposomes have increased membrane tension, they will often merge together to relieve the membrane tension. This merging means that liposomes are generally less stable, and will last for minutes to hours outside of refrigeration, though this is dependent on environment factors such as the lipid composition and temperature.
3.1.3. Membrane Tension and Stored Curvature Elastic Stress

This section details the fundamentals of membrane tension and stored elastic stress, which are needed in the design of assays involving mechanosensitive protein channels such as MscL. This is an extension of the Introduction explanations of curvature elastic energy related to the topics covered in this chapter.
Broadly lipids can be generalised into three main types according to their shape and preferred curvature type 0, type I and type II (Figure 3.3)\(^{30}\). The three distinct shapes are then able to influence the lipid phases formed when they self-assemble into monolayers and bilayers. Generally, type 0 lipids are called ‘bilayer’ lipids, and type I and II are known as ‘non-bilayer’ lipids, as type I lipids tend to form micelles, and type II lipids tend to form inverse systems\(^{30}\).

![Figure 3.3 – Schematic of lipid types demonstrating type 0, type I and type II lipid types.](image)

Cellular membranes are a mixture of lipids of many preferred curvatures. To minimise the hydrophobic exposure of the lipid tails, the bilayer is constrained into a planar orientation. The membrane tension of a bilayer is related to many different forces including curvature elastic energy within the monolayers and is a manifestation of the energy required to force the membrane into a flat bilayer, away from the net preferred curvature of the two separate monolayers. The curvature elastic energy of the monolayer, and therefore the membrane tension, can be altered by including lipids of different types, chain saturation and headgroups\(^{135}\).

The membrane tension stored in the bilayer can be relieved by the presence of proteins or other molecules such as cholesterol\(^{105}\). Figure 1.6 shows an example of membrane tension changes. The monolayer has a preferred curvature based on the large hydrophobic tails of some of the lipids. When the monolayer is forced into a flat formation, the tails are constrained into a smaller space which increases the membrane tension. When a molecule inserts into the bilayer, it relieves the strain of the hydrophobic chains and therefore relieves the tension\(^{105}\). One downside to this insertion is the energy penalty that occurs when the molecule exposes the hydrophobic core to the aqueous media, therefore there can be a balance between the hydrophobic and steric energies. Once the membrane tension exceeds a certain threshold then the membrane tends to bend and phase change away from the flat bilayer\(^{105}\).
It is therefore evident that if the concentration of a type II lipid increased in a type 0 lipid membrane, the membrane would favour curvature towards the aqueous phase and the bilayer would have an increase in membrane tension. This increase in membrane tension can affect many different properties including protein insertion kinetics and membrane protein reconstitution\textsuperscript{136}. In this chapter we exploit the differences between lipids with different preferred curvatures, to comprehensively tune the elastic stress and the membrane tension, in an attempt to measure the effect on MscL gating.

### 3.1.4. Literature MscL Assays

This is an expansion upon the Introduction MscL studies section, with studies focussing on MscL-membrane interactions. The large majority of MscL studies to date, focussed on the structure of protein and the mechanism of gating, however the studies outlined below inform the membrane assay optimised in this, and later, chapters.

**Identification of the ideal membrane thickness for MscL\textsuperscript{108}**

This study identified the hydrophobic thickness of the MscL pentamer in both its closed and open conformation. By combining tryptophan mutagenesis and fluorescence microscopy, along with literature details for lipid interactions with tryptophan residues, it was possible to identify the portions of the protein based in the hydrophobic pocket of the membrane. These results were confirmed by cysteine mutagenesis and fluorescence lifetime measurements. The hydrophobic MscL domains were defined as running from Leu-60 on the periplasmic side of the membrane, to somewhere between residue 91 and 94 on the cytoplasmic side. The hydrophobic thickness, and therefore ideal membrane thickness, was identified as approximately 25Å.
Direct lipid – MscL interactions

This study involved a fluorescence quenching assay in liposomes reconstituted with the G22C mutant of MscL, without the F93W mutation. The assay is similar to the assays mentioned in this chapter as calcein is encapsulated inside the liposomes; the calcein was then released and measured on gating with MTSET. The assay measured how the presence of anionic lipids changed MscL activity, and attempted to show how they can affect reconstitution efficiency. The study showed that the mutation of specific charged residues removed the effect of anionic lipids on the calcein efflux and therefore on MscL activity, providing evidence for a high-binding affinity for charged lipids.

The study also investigated the effects of spontaneous curvature on activity by measuring MscL activity in 50:50 DOPC:DOPA and 50:50 DOPC:DOPE. DOPA is 1,2-dioleoyl-sn-glycero-3-phosphate, and has a very similar spontaneous curvature value to DOPE, except that DOPA is negatively charged. The study found that the MscL activity measured differed and it was concluded that the changes were not due to spontaneous curvature. Zwitterionic effects were also investigated in liposomes of a mixture of DOPC:DOPE:DOPA by varying the DOPC concentration. It was found that MscL activity changed as a function of DOPC concentration, but upon methylation of the DOPC and DOPE headgroups, the correlation between rate and lipid composition was lost.

The study concluded that the specific lipid-protein interactions and hydrogen bonding was the cause of changes to MscL activity, as they could be disrupted by specific point mutations, and did not appear to correlate with changes in spontaneous curvature.

Effect of anionic phospholipids on MscL activity

To follow on from the direct lipid interactions study, the authors used the same fluorescence quenching assay with calcein to measure the effect of anionic lipids on MscL activity with MTSET as the activator. The concentration of DOPG and DOPA were varied with the concentration of DOPC, and the rate and final extent of dye release was measure. The study found that for increasing concentrations of both DOPG and DOPA, the rate and final extent of MscL activity increased up to 50:50 molar ratio, although the trend didn’t seem to follow any pattern.

The study also investigated the polarity of MscL reconstitution, to see if it changed with the change in charged lipid composition. By using adding a fluorescent molecule that requires the presence of free cysteine residues, and knowing that only one side of the protein has a free cysteine residue, it was possible to determine that MscL showed a 50:50 polarity to its reconstitution with no change with charged lipid composition.
Overall, they concluded that MscL activity changes with anionic lipid concentration, and varies more with lipid headgroup structure, further proving their statement that direct lipid interactions take precedence over membrane properties in affecting MscL activity.

**Original MscL – sPLA$_2$ membrane-mediated communication$^{62}$**

The work of Charalambous et al.$^{62}$ was the proof of concept that most of this thesis is based upon and proved that it was possible to actively reconstitute MscL into liposomes, and that MscL opens in response to both a chemical activator, and in response to asymmetry generated by the phospholipase sPLA$_2$. This asymmetry response allowed the two proteins to communicate using the membrane as a mediator for their interaction shown in Figure 3.5. While this research has a number of important implications to our understanding of cell signalling and communication, it has also shown that it is possible to design completely *de novo* protein communication networks between two proteins that don’t need to directly interact. These new protein communication networks can then be exploited and investigated to show how the composition of the membrane can affect the activity.

![Diagram](image)

**Figure 3.5 –** MscL activity measured in a liposome assay composed of 1:1 DOPC:DOPG bilayer using sPLA$_2$ as an activator. The sPLA$_2$ hydrolyses lipids in the sn-2 acyl bond, producing a fatty acid and a lysophospholipid. The membrane asymmetry is then sensed by MscL embedded in the membrane, leading to gating and release of calcein quenched in the liposome. This figure was taken from Charalambous, 2012$^{62}$. 
3.2. Introduction to Techniques

The section outlines the theory for some of the techniques used in this chapter.

3.2.1. Detergent Reconstitution

Purified membrane proteins need to be reconstituted into model membranes. The main method used to reconstitute membrane proteins into liposomes is using a method known as detergent-mediated reconstitution\textsuperscript{123}. In this method, lipid-detergent liposomes are created which are just under the critical micelle concentration (CMC). At this lipid-detergent concentration, the liposomes have many bilayer defects caused by the detergent, however they maintain their liposome structure and do not degrade into micelles\textsuperscript{123}. When detergent solubilised membrane proteins are added to these detergent saturated liposomes, the membrane proteins are able to insert into the bilayer defects in the liposomes reducing their hydrophobic aqueous contact\textsuperscript{123}. The detergent can then removed from the liposomes by the addition of an adsorbent substance, leaving the liposomes with reconstituted membrane proteins\textsuperscript{123}.

3.2.2. Fluorescence

Fluorescence techniques rely on the ability of a molecule to absorb electromagnetic radiation in the form of a photon or photons. This photon causes an electron in the molecule to become excited from the ground state, up to an excited state. This excited electron then decays back down to the ground state by emitting a photon. The photon that is emitted is at a longer wavelength, and therefore a lower energy, than the photon that it absorbs. The photon emitted is at a lower energy due to the vibration of bonds and release of heat, this process is called Stokes shift\textsuperscript{138}. The exception to this is two-photon excitation, but that will not be explored in this thesis\textsuperscript{138}.

\begin{align*}
\text{Excitation} & \quad S_0 + h\nu_{ex} \rightarrow S_1 \quad (3.1) \\
\text{Fluorescence (emission)} & \quad S_1 \rightarrow S_0 + h\nu_{em} + \text{heat} \quad (3.2)
\end{align*}

Equation 3.1 - Equations for the excitation and emission of an electron in a fluorophore, $S_0$ is the ground state and $S_1$ is the excited state.

In this thesis, the main fluorescent dye of use is calcein, a fluorescein complex with an excitation and emission at 495nm and 515nm respectively\textsuperscript{128}. Calcein is useful a fluorescent dye as it displays self-quenching properties at very low concentrations, and also displays calcium dependence. The biggest downside to Calcein is its pH sensitivity, due in part to the large number of acid groups surrounding the fluorophore centre (Figure 3.6).
3.2.3. Dynamic Light Scattering (DLS)

In order to assure that liposome size, and therefore liposome curvature, isn’t affecting the MscL activity results, the liposome size must be monitored in all conditions of the assay. To monitor the size of the liposome a technique called Dynamic Light Scattering (DLS) is used. DLS enables to sizing of spherical particles in solution across a large size distribution, between 1nm and 1μm. DLS displays the population distribution of each particle size, indicating the monodispersity of the population\(^1\). DLS measures the intensity of scattered light from a beam irradiating a suspension of particles in solution\(^2\). The particles that are measured must be significantly smaller than the wavelength of light used, and it is assumed that the particles scatter light elastically.

Extruded liposome populations should have a single normal distribution, though smaller micelles and larger aggregates may be seen dependant on the liposome stability. This was especially important after MscL reconstitution as the detergent level must be monitored to see how it affects liposome stability.

3.3. Materials

This section detains the materials used in each liposome assay and control used in this chapter.

3.3.1. Preparation of Liposomes

All lipids mentioned were purchased from Avanti Polar Lipids. The lipids were purchased as a lyophilised powder with a purity of >99%. The lyophilised lipids were stored at -20°C until needed. The lipids used were 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (DOPG), 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (LysoPC) and 1,2-di-O-(9Z-octadecenyl)-sn-glycero-3-phosphocholine (Diether PC). When needed, the lipids were dried on a Thermoscientific MicroModulyo Lyophiliser, then dissolved in chloroform purchased from VWR. The lipid samples were

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Figure 3.6 - Structure of the fluorescent dye, Calcein.
extruded using an Avanti Mini Extruder using polycarbonate membranes and filter supports purchased from Avanti Polar Lipids.

Figure 3.7 – Chemical structures for the lipids obtained from Avanti Polar Lipids.

3.3.2. MscL Reconstitutions and Activity Assays

The detergent n-Octyl-β-D-Glucopyranoside (OG) and surfactant 4-(1,1,3,3-Tetramethylbutyl) phenyl-polyethylene glycol (Triton X-100) were purchased from Sigma Aldrich. The [2-(Trimethyl ammonium) ethyl] Methane Thiosulphonate Bromide (MTSET) was purchased from VWR. The OG and MTSET were stored at -20°C until required. Biobeads SM2 with mesh size 20-50 were purchased from BioRad and calcein was purchased from Sigma Aldrich. Liposome-calcein separation was performed using a Beckman Coulter Optima L-100 XP Ultracentrifuge using either a Beckman Coulter Ti45 rotor. Where mentioned liposome-calcein separation was performed using G50 Sephadex (medium) purchased from GE Healthcare along with polypropylene columns with a 5ml bed volume purchased from Fisher Scientific. Dynamic Light Scattering was undertaken using a Beckman Coulter Delsa Nano C particle analyser.

The components for the buffers used are listed below. Unless previously mentioned, all buffer components were purchased from Sigma Aldrich.

- **Reconstitution Buffer**: 100mM KCl, 20mM HEPES, 40mM OG, 50mM Calcein, pH 7.2.
- **Centrifuge Buffer**: 100mM KCl, 20mM HEPES, pH 7.2.
- **Centrifugal Pellet Resuspension Buffer**: 100mM KCl, 20mM HEPES, 0.5M Sucrose, pH 7.2.

All solutions were pH adjusted using 1M stock solutions of Sodium Hydroxide (NaOH) and Hydrogen Chloride (HCl) purchased from Sigma Aldrich.
3.4. Methods

This section outlines the protocols required to produce liposomes, reconstitute MscL and undertake the calcein release assay.

3.4.1. Preparation of Liposomes

The lipid mixtures were weighed in a molar ratio, dissolved in chloroform, and gently mixed to ensure all lipids were dissolved. The chloroform was evaporated under a steady stream of nitrogen, and the resultant lipid film was placed in the lyophiliser overnight, or for a minimum of 2 hours before hydration. Lipid films were hydrated in the reconstitution buffer stated in the Materials section at 12.7mM, unless stated otherwise. Lipid-detergent resuspensions were freeze-thawed 4 times using liquid nitrogen and a hot-air gun, mixtures were vortexed before each freeze stage. Once freeze-thawed, the lipid-detergent mixtures were extruded through a 100nm filter 11 times, to produce mixed lipid-detergent unilamellar liposomes.

3.4.2. MscL Reconstitution

For each mixed lipid-detergent liposome sample prepared as described, 3 independent aliquots of 100mg of Bio-Beads SM2 were prepared and allowed to equilibrate for 1 hour in detergent-free reconstitution buffer. Purified MscL was added to prepared mixed lipid-detergent liposomes at a lipid-protein ratio of 1:10,000, and was allowed to equilibrate for 1 hour on rollers in a cold room at 4°C. In order to remove the detergent, the MscL reconstituted liposomes were incubated with 100mg of equilibrated Bio-Beads for 1 hour, on rollers, in a 4°C cold room. After 1 hour, the 100mg of Bio-beads were replaced and the process was repeated twice more. After 3 hours of Bio-Bead incubation, the Bio-Beads were removed and the MscL reconstituted liposomes were stored at 4°C until used. For MscL free controls, an equivalent amount of DDM was added to the mixed lipid-detergent liposomes, and the same detergent removal process was followed.
3.4.3. Ultracentrifugation Purification Method

The MscL reconstituted liposomes were diluted 140-fold in centrifuge buffer stated in the Materials section. Ultracentrifugation at 130,000 x g, at 4°C, for 1 hour, yielded a liposome pellet. The supernatant, which contained unencapsulated calcein, was removed and the pellet was resuspended in 0.5ml centrifugal pellet resuspension buffer stated in the Materials section. The purified liposomes were then stored at 4°C until further use.

3.4.4. MscL Activity Assay

For well-plate assays, the resuspended liposomes were diluted at a ratio of 1:50 with resuspension buffer. The diluted liposomes were then loaded into a 96-wellplate purchased from Sigma Aldrich. The fluorescence assay was then undertaken on a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA, slit setting 5, 5) using the kinetics mode. The ideal excitation and emission for calcein was used, 494nm and 517nm respectively. Background readings were taken for 5 minutes prior to the addition of the activation mechanism. To activate the MscL, 1µl of 100nM PLA₂ or 1µl of 300mM MTSET was added to each well. The wells were gently mixed and recordings were taken for 60 minutes, or longer if needed. At the end of the assay 1µl of 0.1% Triton X-100 was added to each well and gently mixed. The detergent breaks open every liposome giving a maximum fluorescence of all encapsulated calcein.

For cuvette assays, the same process was followed as the well-plate assay except that each volume was scaled up by a factor of 10 in order to fit in the 2ml cuvettes purchased from VWR. Measurements were taken on a Horiba Jobin Yvon FluoroMax-4 Spectrofluorometer.

The lipid compositions used for the lipid sweeps, given in molar ratios, are: DOPC:DOPG (100:0, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50) and DOPC:DOPE:DOPG (95:0:5, 85:10:5, 75:20:5, 65:30:5, 55:40:5, 45:50:5).
An example of a typical MscL activity assay is shown in Figure 3.9. The percentage fluorescence change is used to allow for direct comparison. Unless otherwise stated, each lipid composition was performed using a minimum of 3 independent lipid mixtures, reconstitutions and assays for each lipid composition. Unless otherwise stated, the mean of the results was plotted, and the error bars were one standard deviation. An example of the well plate assay set-up is shown in Figure 3.10.
3.4.5. Control Experiments

Various controls were undertaken to establish that any fluorescence increase is due solely to MscL activity. The size of the liposomes was measured using Dynamic Light Scattering (DLS) before and after MscL reconstitution in order to ascertain that the reconstitution process doesn’t change the size of the liposomes. To make sure that PLA$_2$ and MTSET were not causing dye leakage, they were tested on liposomes that had an equivalent amount of DDM but without reconstituted MscL. To make sure MscL was natively in a closed conformation, a leakage assay was undertaken with the addition of an equivalent amount of buffer without any activator. To ensure that PLA$_2$ activates MscL through membrane interactions, rather than directly, MscL reconstituted into diether-PC liposomes were used. Diether-PC is not a substrate of PLA$_2$, as PLA$_2$ cannot hydrolyse the diether linkage, so PLA$_2$ should not be able to activate MscL.

3.5. Results and Discussion

In this section the results of MscL activity assays are detailed. First, a number of calibrations and controls are shown, followed by the initial activity assays and then lipid sweeps.

3.5.1. Calcein Dye Characterisation

The calcein release assay relies upon the autoquenching properties of calcein at high concentrations, with increased fluorescence as concentration decreases. Therefore, it’s important to calibrate at what concentration the autoquenching properties start to manifest.
As Figure 3.11 shows, the maximum calcein fluorescence in this buffer is displayed at around 0.5mM. As the concentration increases above this point, it starts to display autoquenching behaviour, and the fluorescence decreases. In the following experiments, the concentration of calcein used is 50mM which is well into the autoquenching region of the graph. It is therefore expected that the calcein will display limited fluorescence while encapsulated in the liposomes, and any fluorescence changes will be solely due to changes in effective concentration.

3.5.2. Liposomes Size Controls

Each MscL experiment detailed used 100nm extruded liposomes, however the liposomes are used several hours after extrusion. It was therefore important to ensure that the monodispersity of the liposome size did not significantly decrease over time. Dynamic light scattering was used at regular intervals over 24 hours to measure the size of particles within a liposome sample. The results are shown in Figure 3.12.
As is clear from Figure 3.12, the monodispersity of the liposome solution did reduce by a marginal amount over 24 hours, however, the peak amplitude remains at 100nm. The decrease in monodispersity is explained by liposomes merging together into larger nanoparticles, though the precise mechanism of liposome merging is not investigated further in this thesis.

**How MscL Reconstitution Affects Liposome Size**

Each MscL reconstitution contained the detergent OG, the OG was then subsequently removed using Biobeads. Due to the nature of detergents, it is important to verify that the reconstitution process does not affect the liposome size, or disrupt the liposomes in any other way. Therefore, dynamic light scattering measurements were taken before and after MscL reconstitution at 50:50 molar ratio DOPC:DOPG. Not all lipid compositions were scanned for size, it was assumed that if this reconstitution was stable, that all others would be as well. The results are shown in Figure 3.13.
Figure 3.13 – Dynamic light scattering results showing the effects of MsCl reconstitution on the size and monodispersity of 100nm liposomes. Each data point is an average of 3 individual samples, error bars are one standard deviation.

These DLS results show that the reconstitution process shows a very small decrease in the monodispersity, as displayed by the increase in the size range and decrease in peak amplitude, however the size is still very well defined.

**How sPLA\textsubscript{2} Lipid Hydrolysis Affects Liposome Size**

The enzyme sPLA\textsubscript{2} hydrolyses the ester linkage in lipids to create a lysolipid and a free fatty acid\textsuperscript{27,141}. It is therefore not unreasonable to expect the action of sPLA\textsubscript{2} to affect the size of liposomes, as the lipids preferred curvature state changes. Dynamic light scattering measurements were taken before, and after 30 and 60 minutes of sPLA\textsubscript{2} activity for both 50:50 DOPC:DOPG and pure diether-PC liposomes. The results are shown in Figure 3.14.
Figure 3.14 – Dynamic light scattering results showing the effects of sPLA$_2$ on stability and size of 100nm extruded liposomes of composition (a) 50:50 DOPC:DOPG (b) Pure diether-PC. Each data point is an average of 3 individual samples, error bars are one standard deviation.

As these results have shown, the activity of sPLA$_2$ on the 50:50 DOPC:DOPG liposomes down shifts the peak size slightly which is likely the result of the liposomes becoming more deformable and smaller.
These 50:50 DOPC:DOPG results also show a small change in size range over time likely due to liposomes destabilising and merging together into larger liposomes. The diether-PC liposomes barely changed at all over time, this is because ether-linked lipids are not a substrate of sPLA₂ and therefore the enzyme does not hydrolyse the membrane or change any membrane properties.

3.5.3. Control Measurements

The first control experiment was to ensure that sPLA₂ and MTSET do not cause any calcein release without the presence of MscL. These were undertaken by following the same liposome reconstitution procedure with equivalent DDM but without MscL. These liposomes were then tested in the presence of equivalent MTSET and sPLA₂ for fluorescence changes, and max fluorescence was measured after addition of triton-X to ensure the liposomes were present. Controls were undertaken for a range of lipid compositions to ensure that the membrane composition doesn’t affect the behaviour in this case.

Figure 3.15 – Changes in absolute fluorescence at 517nm in the absence of MscL on addition of MTSET and sPLA₂. a) 100:0 DOPC, b) 75:25 DOPC:DOPE, c) 75:25 DOPC:DOPG, d) 50:50 DOPC:DOPG. MTSET and sPLA₂ were added at 10 minutes, and triton-X was added at 90 minutes. For all lipid compositions the addition of MTSET or sPLA₂ did not significantly change fluorescence. All lipid compositions were tested 3 times for the same liposome sample, the points represent the mean, and the error bars represent standard deviation.
As is clear from Figure 3.15, the addition of MTSET and sPLA$_2$ doesn’t directly cause the release of the calcein cargo. Some fluorescence increase is seen in the 75:25 DOPC:DOPE control upon addition of sPLA$_2$ and this is likely due to the preferred curvature of DOPE and the sPLA$_2$ lysophospholipid product both acting to destabilise the liposome and cause some leakage, however, the fluorescence change is minimal compared to MscL activity. Any decrease in fluorescence is either due to a small amount of photobleaching or evaporation.

A further control undertaken was on liposomes composed of pure di-ether linked DOPC. As sPLA$_2$ hydrolyses the ester link in phospholipids, and not the ether link, when proteo-liposomes composed of di-ether PC were used in the assay, the MTSET addition showed MscL activity but sPLA$_2$ displayed no effect on MscL gating. Thus proving that sPLA$_2$ had no direct effect on MscL causing gating, but rather the membrane was required as a mediator for their interactions.

### 3.5.4. Initial MscL Activity Assay

The first assay that was undertaken was an initial MscL activity assay which was repeated every time a new MscL batch was grown and purified. These initial MscL activity assays were a variation on the first MscL activity assays from Charalambous et al.$^{62}$ In Charalambous experiments, a protein to lipid ratio of 1000:1 was used, however this was found to have too much activity and in future initial MscL tests and all assays, a ratio of 10,000:1 was used. This ratio reduced the amount of protein used, allowed for further rationing, but still provided detailed activity. See the section “Effects of Protein to Lipid ratio on Activity” later in this chapter for more information.

As the lipid composition, protein reconstitution and purification by ultracentrifugation, can change the background and maximum fluorescence, it can be difficult to direct compare results when using absolute fluorescence intensity. Instead, the percentage dye release was used to allow for direct comparison of results and produce more reliable results.

$$\% \text{ dye release} = \frac{\text{fluorescence}_t - \text{fluorescence}_{t=0}}{\text{fluorescence}_{max} - \text{fluorescence}_{t=0}} \times 100$$

(3.3)

The equation above calculates the % dye release using the first fluorescence measurement as time zero (t=0) even though in the case of control experiments this means that photobleaching can cause negative fluorescence change. 100% dye release was calculated as the maximum fluorescence (max) after detergent addition minus the fluorescence at time zero.
The initial MscL activity assays are undertaken in 100nm liposomes of 50:50 DOPC:DOPG composition using MTSET as the activator. The DOPG is negatively charged and the MscL is known to require some level of charged lipid to maximise its reconstitution efficiency and activity. DOPC and DOPG make liposomes with a flat preferred curvature ensuring there would display no curvature effects. The assay is repeated with 3 separate reconstitutions to assure that the activity is reliably measured. The same initial MscL activity assay was repeated for each new MscL batch so that the activity could be directly compared, and allowing for a faster feedback time on any changes to the MscL growth and purification protocol. An example of the results of an initial MscL activity assay is shown in Figure 3.16.

Figure 3.16 – Example of an MscL initial activity assay at 10,000:1 lipid to protein ratio at 50:50 DOPC:DOPG. The activator was added at 10 minutes and the Triton-X was added at 185 minutes. Each data point is an average of 3 samples from the same reconstitution, error bars are one standard deviation.
3.5.5. Data Fitting

To obtain rate constants and enable comparison during lipid sweeps, each fluorescent assay was fitted with an exponential decay using a function inbuilt to Origin. The fit provides a rate constant for dye release, but also provides the plateau point of dye release which is a function of the proportion of active MscL reconstitutions. An example of the data fitting process is shown in Figure 3.17.

![Graph showing data fitting process](image)

Figure 3.17 – Example of Origin fitting data for an MscL activity assay in 50:50 DOPC:DOPG. In this exponential decay equation, A1 is the plateau point and t1 is the rate constant.

While this fit was used for all following composition sweeps, it is noted that in certain assays with sPLA2 as the activator there was a ‘hump’ in the activity profile, which could be indicative of the lag-burst kinetics observed in sPLA2 activity explained in the *Introduction*73. However, as these results were not reproducible, they were not investigated further.
3.5.6. Effects of Protein to Lipid ratio on Activity
Part of the optimisation process for MscL activity assays was deciding how much protein was needed to get reliable results. If more protein was used the signal-to-noise ratio would go down, in this case ‘noise’ was classed as liposomes that did not have any active MscL reconstituted. However, using more protein meant that less experiments could be run from the same MscL expression and purification batch, as these batches only produced a limited amount of protein. A balance needed to be found so that reliable results for any MscL activity assay could come from the same MscL purification batch. A set of calcein release assays were undertaken in 50:50 DOPC:DOPG liposomes with different lipid-to-protein ratios from 100,000:1 to 250:1, along with a DDM control. 300mM MTSET was used as the activator and the plateau point was plotted, shown in Figure 3.18. These assays were undertaken in cuvettes rather than a well plate, as the cuvette reader was slightly more accurate.

![Figure 3.18](image)

Figure 3.18 – A plot of the plateau point of dye released against the lipid to protein ratio. The plateau point of the assay is taken prior to detergent addition, the liposomes were composed of 50:50 DOPC:DOPG, and MscL activated by 300mM MTSET. Protein-free controls are included to enable comparison. Each data point is an average of 3 samples from the same reconstitution, error bars are one standard deviation.
These results show that below 10,000:1 the activity of MscL is barely distinguishable from control. Between 10,000:1 and 500:1, the activity increases non-linearly with a maximum displayed at around 50% activity. The activity does not increase linearly as the reconstitution of each MscL pentamer can increase the likelihood of other MscL pentamers inserting into the same liposome\textsuperscript{142}. As the lipid-to-protein ratio decreases, there is more active reconstituted MscL in the liposome population, leading to more fluorescence leakage through MscL.

However, while at 250:1 the percentage dye release increases dramatically, when looking at the absolute fluorescence values, the total amount of calcein in the system decreases. This is likely due to the large amount of protein destabilising some of the liposomes and reducing the maximum fluorescence of the system.

From each of these liposomal reconstitutions, while a decreased lipid-protein ratio caused a decrease in signal-to-noise, it takes a lot more protein to parallelise experiments with the same protein purification batch. Therefore, for all further liposome experiments a lipid-protein ration of 10,000:1 was chosen, as the activity remained above background but allowed for many more experiments.

### 3.5.7. Charged Lipid Composition Sweeps

The final results of the lipid sweeps examining the effects of charge on MscL activity are presented here. The calcein release assay was undertaken on charged lipid membrane compositions, and MscL activity was measured. Data was obtained with both MTSET and sPLA\textsubscript{2} as the activator.
Figure 3.19 - MscL activity assay results for lipid composition sweeps as a function of DOPG added to DOPC. a) DOPC:DOPG sweep with MTSET as the activator, b) DOPC:DOPG sweep with sPLA2 as the activator. A larger rate constant indicates a slower MscL activity. Each data point is an average of 3 separate liposome samples, and the error bars are one standard deviation.
The results in Figure 3.19 show that with both MTSET and sPLA₂ as activator, as the concentration of DOPG increases, the rate of MscL activity also increases up to 50:50 molar ratio DOPC:DOPG. The MTSET results correlate well with the anionic phospholipid experiments mentioned in the introduction to this chapter. As the amount of charge in the membrane increases, the MscL rate of gating increases, either a result of more active proteins being reconstituted or because the charged lipids are directly interacting with the protein and increasing the probability of it opening.

The sPLA₂ results are slightly more interesting as the rate increases beyond that of the chemical activator MTSET, which should be difficult given that the membrane hydrolysis is additional step in the gating of MscL. One explanation for the increase in rate could be because both MscL and sPLA₂ have shown a charged-lipid preference in the literature. As both proteins are in favourable environments, it is easier for gating to occur and therefore the rate increases.

It is noted that MscL is not stable at room temperature, and the fluorimetry work is conducted at room temperature, therefore it is possible that some MscL channels are not functional over the entire time of the assay.

### 3.5.8. Curved Lipid Composition Sweeps

Lipid sweeps were also undertaken examining the effects of membrane tension on MscL activity. The calcein release assay was undertaken on liposomes with increasing composition of the spontaneously curved lipid DOPE. Each assay included a small amount of DOPG as MscL has shown a requirement for some charged lipid to maximise signal-to-noise. Data was obtained with both MTSET and sPLA₂ as the activator. It should be noted that 45:50:5 DOPC:DOPE:DOPG did not form liposomes, and the assay failed, so no results are shown above this ratio. Above 50% DOPE the lipids go into the hexagonal phase and are not suitable for encapsulation of calcein, or detergent reconstitution of MscL. It is noted that variations in temperature, caused large variations in rate, and therefore the error could be larger than normal.
Figure 3.20 – MscL activity assay results for lipid composition sweeps as a function of DOPE added to DOPC with a constant 5% DOPG. a) DOPE sweep with MTSET as the activator, b) DOPE sweep with sPLA₂ as the activator. Each data point is an average of 3 separate liposome samples, and the error bars are one standard deviation.
From Figure 3.20a it is clear that increasing concentrations of DOPE, causes a decrease in MscL activity with MTSET as the activator. These results correlate well with direct lipid-MscL experiments mentioned in the introduction to this chapter\textsuperscript{107}. Either the increase in stored curvature elastic stress, effects the amount of MTSET that is required for full opening of the MscL channel, as there are 5 possible cysteine bonds for MTSET to bind to in the pentamer each increasing the probability of gating; or the stored curvature elastic stress in the membrane is lowering the probability of functional reconstitution of MscL into the membrane, again demanding an assay which separates reconstitution activity from MscL activity.

The results from Figure 3.20b have conversely shown that the rate of MscL activity, with sPLA\textsubscript{2} as the activator, seems to slowly increase with DOPE concentration, up to 65:30:5 molar ratio DOPC:DOPE:DOPG where activity seems to level out. As DOPE concentration increases in the membrane the stored curvature elastic stress and membrane tension also increase. The binding of sPLA\textsubscript{2} into the membrane relieves the membrane tension and therefore is energetically more favourable in membranes with higher membrane tension. sPLA\textsubscript{2} relieves tension both through insertion and reduction of hydration of the hydrophobic tails, and through the hydrolysis of lipid tails, as lyso-lipids have the opposite spontaneous curvature. Above 65:30:5 DOPC:DOPE:DOPG the rate stops increasing, this is likely because the effect of membrane tension on MscL outweighs, the increase in binding efficiency of sPLA\textsubscript{2}.

\textbf{3.6. Conclusion}

In conclusion, this chapter has shown the first investigation on the effect of the membrane composition on MscL activity using a liposomal quenched fluorescence assay. Many controls were undertaken to ensure that only MscL activity was being measured, and there were no destabilising effects causing the leakage of calcein from the liposomes. The original assay where MscL was reconstituted into 100nm liposomes composed of 50:50 molar ratio DOPC:DOPG was recreated, and a fluorescence increasing was measured indicating functional MscL reconstitution. The amount of MscL reconstituted into the liposomes was then optimised in order to ensure a good signal-to-noise ratio while maintaining a large enough supply of protein from the same purification batch, thus maximising reproducibility. The MscL assay was performed in liposomes ranging from 100:0 DOPC:DOPG to 50:50 DOPC:DOPG and from 95:0:5 DOPC:DOPE:DOPG to 55:40:5 DOPC:DOPE:DOPG. These phospholipids were chosen for their matching tail length, and their different charge and spontaneous curvature properties. The aim of these assays was to measure the effect of changing membrane properties on MscL rate of activity and MscL maximum activity. The data was successfully
collected and fitted with an exponential decay from which the rate constant and maximum MscL activity could be deduced.

It was found that increasing DOPG content in the membrane, increased the MscL activity with both MTSET and sPLA$_2$ as the activator. It was also found that sPLA$_2$-MscL activity was faster than that of MTSET as DOPG content increased, likely due to both proteins having a charged-lipid preference. As DOPE content increased, the activity of MscL with MTSET decreased due to a membrane tension effect. However, the activity of MscL with sPLA$_2$ increased with DOPE content, likely due to sPLA$_2$ relieving the membrane tension caused by the increasing content of the spontaneously curved DOPE. sPLA$_2$ relieves the tension by both hydrolysing the membrane forming the type I lyso-lipids, and through insertion and preventing hydrophobic interactions with the lipid tails.

It is important in all of these results to note that the correlation between fundamental membrane properties and membrane protein activity, does not fully prove a causation pattern. There may be some complex direct lipid-protein interactions that are controlling activity beyond the effects of charge, curvature or membrane tension. However, the techniques needed to investigate these direct lipid-protein interactions will not be expanded further in this thesis.

While it is true that the composition of charged and curved lipid in the liposomes affects the activity of MscL in complex ways, one of the questions raised is, what is the difference between protein reconstitution efficiency, and an increase in protein functional activity? This is one of the focuses of the next chapters which takes the liposome assays mentioned into another model membrane system, the Droplet Interface Bilayer.
Chapter 4

Translation of MscL assay in Droplet Interface Bilayers

4. Overview

This chapter deals with the translation of the MscL liposome assay described in Chapter 3 into a different model membrane system, the droplet interface bilayer (DIB). The MscL reconstituted liposomes are injected into an oil, at the oil-water interface a monolayer of lipids forms. By bringing two of these monolayer coated droplets together, the oil is displaced between the monolayers and a bilayer forms at the interface. In the DIB assay, one droplet contains quenched calcein and reconstituted MscL, and the other droplet contains no fluorescence and an MscL activator. The assay functions similar to the liposome assay, the MscL reconstitutes into the DIB, when the MscL enters the open conformation it forms a channel between the two droplets. The calcein diffuses through the open conformation of MscL and the fluorescence increase can be measured using fluorescence microscopy.

While this chapter details the process of translating the liposome assay into DIBs, there were many controls and experiments needed to optimise the DIBs in terms of stability in order to measure MscL activity over relevant timescales. These controls included an investigation into the effect of glass coatings on DIB stability. The final portions of this chapter detail the active reconstitution of MscL into the DIB format, followed by a set of lipid sweeps comparable to those in chapter 3 with both MTSET and sPLA₂ as the activators, in order to ascertain whether the same kinetic profiles can be obtained in both liposomes and DIBs. These experiments were necessary to move into networked protein interactions and rheological activation of MscL in later chapters.

Many parts of this chapter formed part of a published collaboration with Hanna Barriga, a postdoctoral research associate in our research group. The published article detailed the first translation of the MscL liposome assay into DIBs made of pure DOPC, and provided a proof of concept for the study of MscL activity in DIBs. Where relevant I have mentioned what work was published in the article, and have separated assays completed specifically for the article.
4.1. Introduction

There are a number of drawbacks to using liposomes as a model membrane to study protein kinetics. They are very useful in showing bulk kinetics but it can be difficult to distinguish between specific protein kinetics. Due to being quite small, they can possess their own inherent curvature, which can then affect protein kinetics\(^\text{36}\). Also, it is impossible to network multiple proteins together, or get any kind of complex kinetic profiles.

Droplet Interface Bilayers (DIBs) are an ideal miniaturised planar bilayer to improve on liposome limitations. DIBs are much more stable than liposomes, often showing stability for days or weeks depending on formation and supporting techniques\(^\text{143}\). They are sturdy enough to be manipulated once formed allowing for proteins and other molecules to be injected into one droplet while maintaining bilayer integrity\(^\text{144}\). They can be formed, pulled apart, and then remade easily, allowing for analysis of the contents of each droplet separately, even after DIB formation\(^\text{143}\). Also, while they have a use as stable planar bilayers for single protein channel recordings, they can also be used in droplet networks, arrays and in the study of bilayer asymmetry\(^\text{143}\). Detailed here is the methods of DIB formation, as well as a review of some of the current membrane protein studies in DIBs.

4.1.1. Formation of Droplet Interface Bilayers (DIBs)

DIBs are formed when two monolayer coated aqueous droplets come together in oil. At the interface between the two monolayers, the oil is displaced, and a bilayer is formed Figure 4.2\(^\text{143,145}\). There are two methods to form the monolayer coated aqueous droplets known as lipid-in and lipid-out\(^\text{146}\) seen in Figure 4.1. In the lipid-in method, the lipids are dissolved in the aqueous phase in the form of liposomes, the liposomes fuse at the oil-water interface and the lipids form a monolayer around the outside. In the lipid-out method, the lipids are dissolved in the oil phase, and diffuse to the oil-water interface to form the monolayer.
While each DIB forming method has advantages and disadvantages, the *lipid-in* method has one key advantage, the ability to form asymmetric bilayers. By injecting two aqueous droplets containing liposomes of different lipid compositions, the monolayers around the outside of the droplets would contain different lipids, and therefore the resultant DIB would be asymmetric (Figure 4.2). While asymmetric bilayers can be produced in theory, the flip-flop rates of lipids in a bilayer could limit the stability and longevity of the asymmetry\(^{147}\).
4.1.2. Proteins in DIBs

As DIBs are stable planar bilayer systems, it is possible to reconstitute transmembrane proteins into them, allowing deeper characterisation of behaviour and kinetics then in bulk interactions in liposomes. Unlike systems such as patch clamps, the DIBs are not distorted by applied pressures and so the membrane environment is more of a mimic of their natural planar environments.

Membrane proteins are generally incorporated into DIBs by reconstituting the protein into the liposomes that is used to form the droplet in the lipid-in method. When the protein containing liposomes fuse with the oil-water interface, so will the proteins. When the DIB forms, the proteins will preferentially move into the bilayer to minimise any contact with the oil. While self-insertion of proteins into DIBs is energetically favourable, the folding and activity of the protein cannot be guaranteed without looking at the membrane environment\textsuperscript{146}. As most transmembrane proteins will only function in one direction, there is a lot of literature work looking at the preferential insertion of proteins into the bilayer in only one orientation. This orientation control has been achieved through various conditions such as pH, voltage differences, buffer differences and the inclusion of chaperone proteins\textsuperscript{143,148}. It is noted that in almost all cases, the insertion of the membrane protein into the DIB after DIB formation, rather than at the same time, led to a significantly lower reconstitution efficiency\textsuperscript{149}.

4.1.3. Formation of DIB Networks

DIBs do not have to be constrained to just two droplets, bilayers can form in any direction in 3D space as long as any two monolayers are able to be brought into contact\textsuperscript{144,150}. Arrays and networks of DIBs can be formed with micromanipulators or droplet printers, and have been shown to possess collective properties unable to be produced in just two droplets\textsuperscript{150}. One example of DIB network behaviour came from work that showed that a four-droplet network linked by the membrane protein alpha Hemolysin (αHL) could act like a full wave rectifier converting alternating current into direct current. The wave rectification could occur under positive and negative potentials, and over a range of ionic strengths, which was an improvement on previous attempts at biological diodes which were limited by ionic strength. As the work showed an oriented insertion of αHL, they were able to create both current limiters and half wave rectifiers too. A schematic for this full wave rectifier is seen in Figure 4.3.
Figure 4.3 – Full-wave rectification in a four-droplet network connected by αHL pores, a) a model of a bridge rectifier made of semiconductor diodes, b) the DIB bridge rectifier with each αHL acting as a diode, image adapted from Maglia, 2009.

4.2. Review of Membrane Protein DIB Studies

A number of membrane proteins have been actively reconstituted into DIBs including Kcv, a potassium ion channel, and Bacteriorhodopsin, a photon driven proton pump. These proteins are simple in structure, and are usually reconstituted into membranes made of artificial lipids such as DPhPC. Here are two examples of membrane proteins reconstituted into DIBs, and a discussion on the results of their studies.

**Light-activated communication in synthetic tissues**

In-vitro transcription and translation systems (IVTT) has become a target for several different model membrane assays. The process of producing proteins from DNA components allow artificial cells to produce proteins to provide important functionality. This study encapsulated a light activated IVTT system which produced a fluorescent tag alpha hemolysin (αHL) pore inside a DIB. One of the droplets contained the IVTT system, the other was empty, when the two droplets were brought together to form a DIB no background fluorescence was measured. Upon radiating the DIB with UV light, the IVTT promoter was activated, producing the fluorescent αHL which was measured by an increased in the fluorescence intensity. As the αHL is a membrane protein, it favoured being reconstituted into the DIB and this was measured by an increased fluorescence intensity at the interface between the two droplets, also proving the formation of the DIB. The study also proved that the αHL reconstituted in its correctly folded form as an electrophysiological study proved that current could flow between each side of the DIB. In conclusion, they were able to produce a membrane protein through light activated IVTT and prove that it reconstituted into DIBs using both fluorescence and electrophysiology.
Membrane Perfusion of Hydrophobic Substances Around Channels in the Contact Bubble Bilayer

Cellular membranes contain hundreds of lipids and proteins that diffuse restlessly, yet the lipid composition of the membrane is known to steadily change. In model membrane systems the membrane composition is often completely static and therefore this study produced a method for changing membrane constituents in a DIB system. Their system relied on the fact that the hydrophobic exterior of the DIB, and the bulk oil phase, are completely contiguous. By injecting the hydrophobic, cytotoxic peptide, Polytheonamide B, into the oil phase, the protein was able to move into the bilayer hydrophobic space and incorporate into the DIB. The incorporated protein acted as a protein pore and its reconstitution was proven by both fluorescent and electrophysiological readings. Next the study introduced a different membrane protein to the DIB, Nystatin. Nystatin is an ion channel that gates in response to sterol constituents in the membrane. When no sterols were in the DIB, there was no Nystatin activity, however steady injection of cholesterol into the oil phase, lead to steady increase in Nystatin activity, again proving that the membrane constituents can be changed via membrane perfusion. The study concluded that this membrane perfusion route could allow for the study of the effects of many hydrophobic molecules during continuous recordings of channels across the DIB.
4.3. Introduction to Techniques

The section outlines the theory for some of the techniques used in this chapter.

4.3.1. Microfluidics

Microfluidic channels are micron sized fluid channels made of glass, silicon or other polymeric materials\textsuperscript{153}. They are used to handle precise amounts of liquids at less than microliters volumes, which is especially useful when dealing with precious materials, or need precision fluid dynamics. In the field of model membranes, microfluidics is mostly used to generate micron sized liposomes and water-in-oil droplets with very specific lipid compositions. Microfluidic generation can be used to generate specific asymmetric bilayers by creating a monolayer coated droplet which then passed through into another lipid mixture, coating the outside and generating an asymmetric bilayer\textsuperscript{154,155}. By constraining the droplets into a microfluidic channel of a specific size, the DIB be produced with the same size bilayer increasing the reproducibility of the assay with respect to amount of reconstituted protein.

Microfluidic devices can be used to produce DIB networks with specific architectures too, including three dimensional arrays\textsuperscript{156}. A microfluidic assembly of several channels of water-in-oil droplets, each one producing droplets of a different lipid composition, was used to create a 3D array of droplets in a specific network. A schematic for this assembly can be seen in Figure 4.6. This device can produce a 3D network with over 20 different droplet sizes and compositions.
4.3.2. Fluorescence Microscopy

Microscopy magnifies the view of a sample by passing light through the sample and a number of lenses into a viewer or detector \(^{156}\). The simplest form of microscopy is brightfield microscopy, which illuminates the sample with incandescent light, which simply contrasts the specimen and the surrounding light allowing visualisation of general macroscopic structures. In DIBs brightfield microscopy is used to monitor droplet size, droplet shape, bilayer area, and to see the moment that the DIB has formed. When the DIB has formed, the DIB appears as a bright shadow at the interface between the two monolayer coated droplets, which is signalling a change in refractive index. An example of brightfield microscopy is seen in Figure 4.7.

Fluorescence microscopy follows the same photon absorption and emission principals as the fluorimeter studies from Chapter 3. The only difference is that the fluorescence is visualised and monitored by the microscopy detector, rather than receiving absolute fluorescence values as a read-out. Fluorescence microscopy allows real-time local detection of fluorescence changes and
movement. The amount of protein channels reconstituted into the bilayer can be controlled to a much finer detail than bulk measurements in liposomes, and therefore greater detail can be achieved in protein kinetic profiles.

4.4. Materials
This section details the materials used in the MscL reconstituted DIB assays and controls used in this chapter. Parts of the materials section are directly adapted from the our published work on the functional reconstitution of MscL into DIBs1.

4.4.1. Casting of PDMS wells and channels
PDMS was cast using a Sylgard 184 Silicone elastomer kit purchased from Dow Corning. PDMS was stuck to a glass slide aided by a Diener Plasma Cleaning Oven.

For the channels used in published work1, the PDMS was cast around a needle of diameter 0.8mm and length 120mm purchased from B. Braun Melsungen AG. The PDMS channel was stuck to a glass slide aided by a Diener Plasma Cleaning Oven. The channel and wells were filled with hexadecane purchased from Sigma Aldrich.

4.4.2. Surface Functionalisation of Glass Slides
Potassium hydroxide (KOH), sodium bicarbonate, and (3-Aminopropyl)triethoxysilane (APTES) solution were purchased from Sigma Aldrich. Acetone, acetic acid and methanol (MeOH) were purchased from VWR. All water used was MilliQ water supplied in-house. Surfaces were functionalised with poly(ethylene glycol) methyl ether - N-hydroxy succinimide (mPEG-NHS), N-hydroxy succinimide – poly(ethylene glycol) methyl ether – biotin (NHS-PEG-biotin), NHS-PEG-NHS, Neutravidin purchased from Laysan Bio. PDMS surface coating used a Sylgard 184 Silicone elastomer kit purchased from Dow Corning. Functionalised slides were kept in Sterilin boxes at 4°C with a silica gel packet to prevent hydration, both boxes and gel packets were purchased from Sigma Aldrich.

4.4.3. Preparation of Liposomes
All lipids mentioned were purchased from Avanti Polar Lipids. The lipids were purchased as a lyophilised powder with a purity of >99%. The lyophilised lipids were stored at -20°C until needed. The lipids used were 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DOPG), and 1,2-di-O-(9Z-octadecenyl)-sn-glycero-3-phosphocholine (Diether PC). When needed, the lipids were dried on a Thermoscientific MicroModulyo Lyophiliser, then dissolved in chloroform purchased from VWR. The lipid samples were extruded using an Avanti Mini Extruder using polycarbonate membranes and filter supports purchased from Avanti Polar Lipids.
4.4.4. MscL Reconstitution & DIB assay

The detergent n-Octyl-β-D-Glucopyranoside (OG) was purchased from Sigma Aldrich. The [2-(Trimethyl ammonium) ethyl] Methane Thiosulfonate Bromide (MTSET) was purchased from VWR. The secretory Phospholipase A2, sPLA₂ isolated from honey bee venom (Apis mellifera) was purchased as a lyophilized powder from Sigma Aldrich. The OG, MTSET and sPLA₂ were stored at -20°C until required. Biobeads SM2 with mesh size 20-50 were purchased from BioRad and calcein was purchased from Sigma Aldrich.

Two separate buffers were used in these assays, one reconstitution buffer for the MscL containing droplet and one non-fluorescent buffer used for the other half of the DIB. The components for the buffers used are listed below. Unless previously mentioned, all buffer components were purchased from Sigma Aldrich.

- **Reconstitution Buffer**: 100mM KCl, 20mM HEPES, 40mM OG, 50mM Calcein, pH 7.2.
- **Non-fluorescence droplet buffer**: 100mM KCl, 20mM HEPES, 0.5M Sucrose, pH 7.2.

All solutions were pH adjusted using 1M stock solutions of Sodium Hydroxide (NaOH) and Hydrogen Chloride (HCl) purchased from Sigma Aldrich.

4.5. Methods

This section outlines the protocols required to produce the DIB assays used in this chapter. Parts of the methods section are directly adapted from the our published work on the functional reconstitution of MscL into DIBs¹.

4.5.1. Casting of PDMS wells and channels

To make the PDMS, the base and curing agent were combined at a 10:1 ratio, then degassed in a vacuum chamber to remove as many air bubbles as possible. Surface air bubbles were removed by a gentle flow of air, and the PDMS was placed on a flat heating block at 60°C overnight to set. To produce the larger wells, a hole was cut into the PDMS and the PDMS was stuck onto glass slides.

For the channels used in published work¹, the PDMS was cast around a needle of diameter 0.8mm. Once the PDMS was set, the needle was removed, leaving a channel of approximately 0.8mm. To create the oil wells, a 0.1-10μl pipette tip purchased from VWR was inserted into each end of the channel. A schematic of this channel setup is seen in Figure 4.8.
Figure 4.8 – Schematic of the PDMS channel used for the published MscL DIB assay. Channel width was 0.8mm and each end contained a 0.1-10μl pipette tip. The channel and tips were filled with hexadecane.

4.5.2. Surface Functionalisation of Glass Slides
The glass slides were first thoroughly cleaned by a protocol developed in-house. The glass slides were placed in a glass container and rinsed with MilliQ water 3 times. Then the glass slides were rinsed with 1M KOH 3 times. The container was then filled with 1M KOH and sonicated for 20 minutes. The slides were then rinsed with MilliQ water 3 times. The container was then filled with MilliQ water and sonicated for 5 minutes. The slides were then rinsed with acetone 3 times. The container was then filled with acetone and sonicated for 15 minutes. The slides were then rinsed with MilliQ water 3 times. The slides were then rinsed with 1M KOH 3 times. The container was then filled with 1M KOH and sonicated for 20 minutes. The slides were then rinsed with MilliQ water 3 times. Finally, the glass slides were dried under nitrogen.

The clean glass slides were then functionalised by a protocol developed in-house. The glass slide container was cleaned and thoroughly dried, and the clean glass slides were placed into the container. The APTES solution was removed from the -20°C freezer and allowed to thaw to room temperature in the dark. The APTES solution was placed into the glass slide container, and the glass slides were incubated with the APTES solution for 10 minutes. The container with APTES solution was sonicated for 1 minute and glass slides were incubated for a further 10 minutes. The APTES functionalised glass slides were finally rinsed with methanol 3 times, followed by MilliQ water 3 times, and dried under nitrogen.

For PEGylated slides, 5-15mg of NHS-PEG-biotin was placed in a 1.5ml Eppendorf tube with 50mg of mPEG. Then 400μl of 1mM sodium bicarbonate solution was added to the Eppendorf and mixed thoroughly. An APTES functionalised slide was placed on top of PDMS pillars in a Sterilin box with a small square of wet tissue. 80μl of the PEG solution was pipetted onto the APTES functionalised slide, and sandwiched between another APTES functionalised slide, ensuring even coverage with no air bubbles present. The slide sandwich was then incubated in the dark for 2-3 hours. The PEGylated slides were then separated, rinsed thoroughly with MilliQ water, and stored in a dry Sterilin box with a silica
gel packet at 4°C until use. For di-NHS PEGylation, the same process is carried out as for PEG biotin, but 5-15mg of NHS-PEG-NHS was used instead of NHS-PEG-biotin.

For Neutravidin slides, 80μl of a 0.1 mg/ml solution of Neutravidin was pipetted onto a dry PEGylated slide, and sandwiched between another PEGylated slide, ensuring even coverage with no air bubbles present. The slide sandwich was then incubated in a humid container for 1 hour in the dark. The slides were then separated, rinsed thoroughly with MilliQ water, and stored in a dry Sterilin box with a silica gel packet at 4°C until use.

For PDMS coated slides, the PDMS was spin-coated at 3,000RPM for 30 seconds, and cured on a hot plate at 100°C for 10 minutes to create a thin layer on conventional microscopy slides.

4.5.3. Preparation of Liposomes

The lipid mixtures were weighed in a molar ratio, dissolved in chloroform, and gently mixed to ensure all lipids were dissolved. The chloroform was evaporated under a steady stream of nitrogen, and the resultant lipid film was placed in the lyophiliser overnight, or for a minimum of 2 hours before hydration. Dry lipid films were hydrated in the reconstitution buffer or non-fluorescent droplet buffer listed in the Materials section at 12.7mM, unless stated otherwise. Each sample was freeze-thawed 4 times using liquid nitrogen and a hot-air gun, samples were vortexed before each freeze stage to ensure homogenisation. The lipid samples were extruded through a 100nm membrane 11 times, to produce monodisperse unilamellar liposomes.

4.5.4. MscL Reconstitution

For each mixed lipid-detergent liposome sample prepared as described, 3 independent aliquots of 100mg of Bio-Beads SM2 were prepared and allowed to equilibrate for 1 hour in detergent-free reconstitution buffer. Purified MscL was added to prepared mixed lipid-detergent liposomes at a lipid-protein ratio of 1:10,000, unless otherwise stated, and was allowed to equilibrate for 1 hour on rollers in a cold room at 4°C. In order to remove the detergent, the MscL reconstituted liposomes were incubated with 100mg of equilibrated Bio-Beads for 1 hour, on rollers, in a 4°C cold room. After 1 hour, the 100mg of Bio-beads were replaced and the process was repeated twice more. After 3 hours of Bio-Bead incubation, the Bio-Beads were removed and the MscL reconstituted liposomes were stored at 4°C until used. For MscL free controls, an equivalent amount of DDM was added to the mixed lipid-detergent liposomes, and the same detergent removal process was followed.
4.5.5. DIB assay

All DIBs in this chapter were formed using the *lipid-in* method. With open hexadecane wells, a 0.5μl droplet of both the MscL reconstituted liposomes and 0.5μl droplet of the non-fluorescent liposomes containing 300mM MTSET, or various concentrations of sPLA₂, were injected into different parts of a hexadecane filled well. The droplets were left to equilibrate in the oil for 5 minutes to allow time for monolayer formation around the outside of the droplet. The droplets were brought together and a DIB was allowed to form. Each DIB was visualised by a fluorescence inverted microscope (Nikon Eclipse TE 2000-E) with a mercury lamp (Olympus) and a QICAM camera (QImaging Media Cybernetics UK). Fluorescence excitation was achieved using a FITC dichroic filter (460-490nm excitation, Chroma Technology Corp). The software used to control the microscope was built in-house and programmed in LabView. All images were taken with a 4x magnification.

For the channels used in published work¹, a 0.5μl droplet of MscL reconstituted liposomes was injected into one end of the channel. Another 0.5μl droplet of the non-fluorescent liposomes containing 300mM MTSET were injected into the other end of the channel. The droplets were left to equilibrate in the oil for 5 minutes to allow time for monolayer formation around the outside of the droplet. The droplets were brought together by gentle flow of hexadecane and a DIB was allowed to form. The DIBs were visualised using the same microscope system and settings as above.

4.5.6. Results Analysis

All microscopy images obtained during DIB assays were analysed using ImageJ software version 1.8.0.112. The width of the DIB was measured in pixels and converted to absolute length using a stage graticule slide. Fluorescence image analysis was achieved using image integration to quantify intensity changes. The image integration outputs from a specified area and so for each image integrated the region of integration was plotted smaller than the droplet measured to exclude all background pixels. For each set of results the integration area was kept constant, which proved difficult if the droplet moved or changed size. An example of image integration can be found in Figure 4.9. Data obtained was plotted and analysed using Origin.
This section details the results for the translation of the MscL Liposome assay from chapter 3 into the droplet interface bilayer (DIB) format. This section starts with a section with a focus on DIB stability, and the methods that were used to improve stability. Following this is a detailed analysis of the use of coated glass slides to improve DIB stability. Then there is a section on the controls assays undertaken followed by the published results of the translation of the MscL assay into DIBs. Then some further assays are described including lipid sweeps and comparisons with the lipid sweep results in chapter 3.

Figure 4.10 shows an example of typical DIB formation between two monolayer coated aqueous droplets made of pure DOPC. The right-hand droplet contains calcein, which makes it dark in the brightfield, and fluoresce in the fluorescence microscopy image. When the droplets are originally placed into the oil, they are left for 5 minutes for monolayer formation to occur then they are brought slowly together. When the two droplets are brought close enough, a DIB assembles, this is when T=0.
is measured, and the DIB formation and any activity is monitored over time. Additionally, the DIB size, and droplet size are measured over time to ensure no changes that might affect results.

Figure 4.10 – Example of DIB formation between two aqueous droplets containing pure DOPC liposomes. The droplet on the right contains calcein, and the droplet on the left contains an osmotically balanced amount of sucrose. a, c, e, g, i) brightfield images of the DIB forming over 8 minutes. b, d, f, h, j) fluorescence images of DIB formation and showing no calcein permeation. Scale bar is 500µm.
The DIB formation can be clearly seen by the white shadows that form at the interface between the two droplets, which is a sign of a change in refractive index. This change is shown in the zoomed in images seen in Figure 4.11.

![Figure 4.11](image.png)

Figure 4.11 – Zoomed images showing the precise differences upon DIB formation. As the DIB assembles, the refractive index of the bilayer increases, and the DIB can be seen as a bright part in the microscopy images.

One of the biggest problems that DIBs have as a model membrane system, is their limited stability\textsuperscript{159}. The two monolayer coated droplets have a drive to form a DIB at the interface between the two droplets, however sometimes the two droplets coalesce into one droplet, this process is called \textit{merging} in the literature\textsuperscript{143}. When a droplet containing quenched calcein merges with a non-fluorescent droplet, the calcein concentration effectively halves meaning the calcein is no longer quenched. This merging therefore can be seen as both a change in shape of the droplets in the brightfield image, and an increase in fluorescence. This merging behaviour is something that many stability assays in this chapter attempt to solve. An example of merging can be seen in Figure 4.12.
4.6.1. DIB Stability

It was noted that most literature sources that claim that DIBs are a stable model membrane system use artificial, zwitterionic lipids such as DPhPC\textsuperscript{143,144,156}. These artificial lipids can create DIBs which are stable for many hours, or even days. However, when the composition of the lipids is changed to naturally occurring lipids, with charge, and intrinsic curvature, the results are very different. Droplet merging becomes much more frequent as the composition of charged and curved lipids increases within the monolayer due to a change in the bilayer mechanics and energetics as the DIB forms. In order to maintain DIB stability long enough to get MscL activity, and especially for any lipid sweep assays, the stability had to be drastically improved.

4.6.2. Coated Slides

One factor in the stability of DIBs is the ability of each droplet to adhere to glass surfaces as a result of intermolecular interactions and surface roughness, this interaction is known as wetting\textsuperscript{160}. Wetting is determined by a balance of adhesive and cohesive forces, as well as the roughness of the surface. When an aqueous droplet in oil is placed on a glass surface, it wets on the surface and deforms into a more hemi-spherical shape with more of the droplet in contact with the glass. This wetting is seen as a decrease in the contact angle between the droplet and surface compared to a perfectly spherical.
droplet. When a droplet is wetted, it is visualised in brightfield microscopy as a lighter patch, with non-symmetrical sides indicating where the droplet and the glass are in contact. An example of wetting and non-wetting droplets is seen in Figure 4.13. While there are many literature references to how wetting occurs, and how to change it, there are no literature references to how droplet wetting affects DIB stability and assays were carried out to investigate it.

![Diagram of droplet wetting and non-wetting](image)

Figure 4.13 – Cartoon and brightfield microscopy examples of droplet wetting and not wetting. a) the droplets are placed on glass and have clear deformations shown by non-symmetric light patches. b) the droplets have been placed on PDMS coated glass slides and aren’t wetting due to changes in interactions between the droplet and the surface. As shown the contact angle, $\theta$, of the wetted droplet is smaller than for a spherical droplet.

In each assay that was undertaken on glass slides a number of interesting phenomena were observed. As the composition of charged lipid increased in the droplet, the amount of wetting increased to the point where it was not possible to move droplets that were above 40% DOPG due to intense wetting. It is proposed that the increased charge in the droplet monolayer increases electrostatic intermolecular interactions with the glass. Also, with an increased amount of wetting, there seemed to be a decrease in the size of the DIB that formed between two wetted droplets. The decreased DIB size is proposed to be purely due to the inability for the two droplets to move due to interactions between the droplet and the glass. Finally, DIBs formed between wetted droplets seemed to be less stable than droplets which were not wetting. It is proposed that the merging becomes energetically more favourable when the wetting is stronger, as merging increases the size of the overall droplet, increasing the surface area contact between the merged droplet and the glass.

It was proposed that functionalising or coating the glass slide in a thin layer of transparent, hydrophobic material would decrease the wetting of the droplets and therefore improve DIB stability. Therefore, a range of functionalised or coated glass slides were produced and assays were carried out to test how they improved wetting, and how they improved DIB stability. The surface coatings tested can be seen in Figure 4.14.
It was originally proposed that amination of the silicon bonds in the glass using APTES solution, followed by functionalisation, would be the best method to prevent wetting. The functionalised slide would be much rougher than laboratory grade glass, the material would still be transparent, and it is possible to functionalise the slide with hydrophobic molecules\textsuperscript{161}. Therefore, a systematic functionalisation of glass slides was carried out, each functionalisation had PEGylation as a base. A spin-coated PDMS glass coating was also tried as coating was significantly easier than direct functionalisation, and so the glass slides were spin-coated in PDMS to provide hydrophobicity while still being transparent. An example of the slides effect on wetting using 80:20 DOPC:DOPG liposomes can be seen in Figure 4.15.
Figure 4.15 – Example brightfield microscopy images of the same batch of 80:20 DOPC:DOPG droplets, 10mg/ml, on different functionalised and coated glass slides. Wetting is seen as a light asymmetric patch in the centre, and the edges of the droplet become less defined as the droplet stops being spherical.

The results for the functionalised slides were not promising, and in fact for all of them wetting seemed to increase in comparison to glass. The increased wetting on these slides, meant that it was impossible to move the wetted droplets and therefore impossible to form DIBs. Each of the functionalised slides were intended to prevent wetting through hydrophobic interactions, however it is proposed that the PEG backbone for each of the functionalisation molecules interact with the monolayer coated droplets and increases the wetting. PEG is known to attractively interact with lipid monolayers and bilayers\textsuperscript{162},
and because this interaction brings the droplets closer to the glass surface it increases wetting and therefore decreases stability. However, the spin-coated PDMS slides seemed to drastically reduce wetting and increase DIB stability, it is proposed that the rough hydrophobic nature of the polymer decreases the intermolecular forces between the surface and the droplet and therefore the droplet keeps its spherical shape. The PDMS coated slides were then taken forward to investigate the limitations of their effect on wetting with respect to the composition of charged lipid. These results are seen in Figure 4.16.

Figure 4.16 – Example brightfield microscopy images to show that as the amount of charged lipid in the droplet increases, it’s wettability increases on glass surfaces, however the PDMS coating seems to stop the wetting entirely.
The results for the compositional sweep of charged lipid on different surfaces reinforced the results that showed that as the composition of charged lipid increases, the degree of wetting on the droplets on glass also increases. But it also has shown that up to 60:40 DOPC:DOPG, PDMS coated glass slides completely negated the wetting effect and allowed stable DIB formation for up to 30 minutes. Above 50% DOPG on PDMS, while the wetting was still negated, stable DIBs would not be formed. It is proposed that above 50% DOPG, electrostatic interactions between the monolayers override the general stability of the DIB and therefore DIBs cannot form for long periods.

While these results proved promising for increasing the stability of DIBs, there were still several limitations to PDMS coated slides. Their general lifespan was short, as they got scratched and it was difficult to fully clean the coated slides. However, due to their ease of creation, when the PDMS coated slides got damaged, they were simply replaced. Also, it was more difficult to create a tight seal between the microfluidic channel or well and the PDMS coated glass slides, compared to the very tight seal that is created between plasma bonded PDMS and glass. The inability to form a tight seal, meant that the volume of oil within the channel or well would not be constant, which would also change the shape of the droplets, therefore if the oil leaked out the device was replaced.

All DIB assays in this chapter from this section onwards were performed on PDMS coated glass slides.

4.6.3. Lipid Concentration

One of the considerations for DIB stability was concerning whether the entire droplet was coated in a continuous monolayer and if there was enough lipid in the membrane. This was especially concerning for assays involving sPLA₂ as the hydrolysis of lipids would overtime deplete the amount of lipid in the membrane. Therefore, a calculation was carried out to see how many lipids it would take to cover the surface area of the aqueous droplet entirely. By assuming that the droplet consists of a perfect sphere, it’s possible to calculate the surface area and divide it by the surface area of a lipid, to find how many lipids it would take it to fill the entire area. The assumptions that the droplet was spherical, and that lipids have a circular cross-sectional area and are tightly packed, would introduce a large amount of error, but the calculation was only intended to give an estimate. A much larger simulation would be required to model more accurately. Another assumption is that all the lipid in the droplet is contained within the monolayer, which is false as at least some of the lipids will be stored in stable liposomes within the droplet. The equations used for the estimation have been shown below.

\[ \text{Droplet surface area } (A_D) = 4\pi r^2 \]  
(4.1)

\[ \text{Number of lipid molecules in monolayer } (N_{tot}) = \frac{A_D}{A_L} \]  
(4.2)
The lipid area (A_L) has been approximated by several literature sources\textsuperscript{163,164}, and while it varies with many different factors, most agree that for DOPC the lipid area is approximately 0.71\text{nm}^2. Also using the relationship between the volume of a sphere and its radius, it’s possible to calculate the radius of the droplet. Therefore, based on the 500nl volume of the droplet the radius is approximately 500\text{µm}.

<table>
<thead>
<tr>
<th>Droplet Surface Area (nm(^2))</th>
<th>Number of lipids in monolayer, (N_{tot})</th>
<th>Moles of lipids in monolayer (mol)</th>
<th>Molar concentration required for monolayer (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3.04 \times 10^{12})</td>
<td>(4.29 \times 10^{12})</td>
<td>(7.13 \times 10^{-12})</td>
<td>(1.43 \times 10^{-02})</td>
</tr>
</tbody>
</table>

Table 2 – Calculation of the amount of lipid required to coat the entire outside of the droplet.

As the lipids in the droplet are at 3.4\text{mM}, the calculations show that the amount of lipid that is required to coat the entire outside of the droplet is 2 orders of magnitude less than the amount of lipid within the droplet. Therefore, the amount of lipid used in the assays is proposed to be adequate. Though it is noted that this assumes that the diffusion time of the lipids in the droplet is negligible.

4.6.4. Control Assays
The control assays undertaken for the translation of the MscL fluorescence release assay into DIBs are presented in this section. The first control was intended to show that the size of the DIB was constant between assays, especially for the lipid sweep assays where the composition of the DIB changes. It was proposed that as long as the concentration of MscL remained constant, and the DIB size also remained constant, any changes in MscL activity would be solely due to lipid composition. Figure 4.17 shows how the size of the DIB varies between droplets of different lipid compositions over time.
Figure 4.17 – Graph showing the variation of DIB size between droplets of different lipid compositions. (a) Brightfield microscopy image of a DIB formed between two pure DOPC droplets in oil, (b) Brightfield microscopy image of a DIB formed between two 80:20 DOPC:DOPG droplets in oil, (c) Plot of the variation in DIB width over time. Both scale bars are 500μm.
The graph in Figure 4.17 shows that the composition of the droplets does not seem to affect DIB size. However, there does seem to be quite a large amount of error, which is attributed to the pipette error when using droplets of small size. Another aspect of the graph is showing that after the initial DIB formation there appears to be a period where the DIB size increases over a period of 5-10 minutes after which it plateaus and the DIB size remains constant. The DIBs in this control assay were all stable for up to an hour, however the stability of the DIB was not assured in every assay and only the assays that were stable for long enough were analysed and included in future data sets.

After proving that the DIB size was relatively constant, the second control that was needed for the assays was to ensure that there was no leakage of dye across the DIB. The controls in the chapter 3 liposome assays have shown that the liposomes do not leak without the presence of both MscL and an activator, therefore the same controls were undertaken in DIBs to ensure the change in model membrane system hasn’t affected the leakage of the dye. The results of MscL and activator free controls can be seen in Figure 4.18. In all control cases the darker droplet contained calcein, and the clear droplet contained an osmotically balanced amount of sucrose in a dye-free environment. All DIBs were measured over the same timescales as other assays, 1 hour, and the DIB size was kept constant. The results show that in the absence of both MscL and MTSET, the DIB does not allow calcein leakage. If there were calcein leakage, then it would be expected that the dye-free droplet would increase in fluorescence signal.
Figure 4.18 – Controls in the absence of both MscL and MTSET. (a) Brightfield microscopy image of a DIB formed between two pure DOPC droplets in oil, (b) Fluorescence microscopy image of a DIB formed between two pure DOPC droplets in oil, (c) Plot of the variation in fluorescence over time. Scale bars are 500μm. Each data point is an average of three DIBs, error bars are present but smaller than the data points.
Figure 4.18 shows that the DIBs are stable over the lifespan of the assay even when the contents of each droplet are different. The results also show that calcein is unable to diffuse across the DIB in the lifetime of the assay showing two distinct populations, the fluorescent droplet and the non-fluorescent droplet. There is a slight decrease in the fluorescence of the fluorescent droplet which is attributed to a photobleaching effect, and any fluctuations in measurements are likely due to droplet movement. In order to assure that neither the closed conformation of MscL or MTSET are causing leakage of calcein across the DIB, two further control assays were carried out. The two controls were in the presence of the closed conformation of MscL but without MTSET, and with MTSET but without reconstituted MscL. These controls were clear, and showed that without both MscL and MTSET, calcein could not diffuse into the second droplet. The results for these two controls are shown in Figure 4.19.
Figure 4.19 – a) Controls including MscL and without MTSET, b) Controls including MTSET and without MscL. Each side has (i) one Brightfield microscopy image of a DIB formed between two pure DOPC droplets in oil, and (ii) one fluorescence microscopy image of a DIB formed between two pure DOPC droplets in oil, (c) Plot of the variation in fluorescence in the non-fluorescent droplet over time. Scale bars are 500μm. Error bars are present but smaller than data points.
In conclusion, the controls mentioned in this section have shown that the DIB model membrane system is robust and stable, and that calcein cannot diffuse across the DIB in the presence of either MscL or MTSET alone.

4.6.5. Results for the translation of the MscL calcein release assay into DIBs

In this section is detailed the results of the published successful translation of the MscL calcein release assay into DIBs, as well as the results that were not included in the published figures. The initial results of the translation assay are shown in Figure 4.20. In this assay the calcein in the dye containing droplet is at 0.5mM rather than 50mM. By using a lower concentration of dye, the dye is not quenched, therefore the results show a fluorescence decrease in the dye containing droplet and a fluorescence increase in the non-fluorescent droplet. Both droplets were integrated using ImageJ and the mean fluorescence for each droplet was plotted over time.

![Figure 4.20](image)

This result was then repeated several times, but in all the following assays the droplet containing dye was quenched at 50mM. Therefore, the initial droplet was dark in fluorescence images, and both droplets increase in intensity over time as calcein translocates from the quenched droplet into the non-fluorescent droplet. The reason this was carried out, was to allow for direct comparison with the liposome assays. Select raw data images have been included to show what both brightfield and fluorescence images of the DIB look like before analysis. The results of these repeats can be seen in Figure 4.21.
Figure 4.21 – MscL DIB assays made of 100% DOPC with quenched calcein at 50mM, the non-fluorescent droplet contained 300mM MTSET. (a) Brightfield image of the DIB at t=10 minutes. (b) – (d) Fluorescence images of the DIB at different times during the assay. (e) A graph showing the intensity increase of both sides of the DIB, each data point is averaged over 3 DIB assays from the same reconstitution, the error bars are 1 standard deviation. Scale bars are 500μm.
These assays are clearly showing that fluorescence can diffuse across the DIB in the presence of both MscL and MTSET, compared to the controls which showed no diffusion. These results mean that calcein from the first droplet is diffusing through the gated MscL channel into the second droplet, causing fluorescence changes.

Of note in the results of Figure 4.20 and Figure 4.21 is that the droplets do not have the same mean fluorescence at the end of the experiment. Given that the dye is purely moving along a concentration gradient, this is slightly unexpected, and while photobleaching is something that needs to be taken into account, that cannot fully explain the droplets not equilibrating, as both droplets should photobleach equally. One explanation for the result is that the MscL channels have closed after initially opening, which could be supported by the 10 minute half-life displayed by MTSET\textsuperscript{165}, as MTSET degrades MscL could close again. However, we used MTSET in excess to account for its short half-life and so if it did close due to MTSET degradation, another non-degraded molecule should replace it within the allosteric site and gate MscL again. Another possible explanation is that the system simply cannot equilibrate in the lifetime of the assay, therefore the rate of dye flow across the DIB through MscL was calculated. The results of this calculation are shown in the next section of this chapter.

### 4.6.6. DIB Diffusion Calculations

In order to estimate the time taken for the two droplets to equilibrate in terms of concentration of calcein, it is first important to calculate the diffusion coefficient of calcein. According to the Stokes-Einstein (SE) relation\textsuperscript{166}, the diffusion coefficient, $D$, is inversionally proportional to the viscosity, $\eta$, and the molecule radius, $a$. The Stokes-Einstein equation is shown below, $K_b$ is the Boltzmann constant and $T$ is temperature in Kelvin.

$$D = \frac{K_b T}{6 \pi \eta a} \quad (4.3)$$

Using the stokes radius of calcein from literature, 0.7nm\textsuperscript{167}, and simplifying the viscosity to that of pure water, 0.001Pa.s, the diffusion coefficient of calcein in DI water can be estimated as $D = 3.11777 \times 10^{-10} \text{ m}^2\text{s}^{-1}$.

By assuming that the free energy of transfer for channels larger then 1nm in diameter is negligible\textsuperscript{168}, it is then possible to calculate the change in concentration of each droplet using the diffusion coefficient using Fick’s First Law of Diffusion. In this calculation the diffusion is calculated assuming that calcein is unable to translocate across the DIB and that there is only one MscL channel acting as a cylinder with a diameter of 3nm and channel thickness of 10nm which are taken from x-ray crystallography and modelling data\textsuperscript{96,98}. Taking a simple approximation using Fick’s First Law:
\[ J = -D \nabla C \]  

(4.4)

Where \( J \) is the diffusion flux. To simplify for a single dimension system, it is possible to say:

\[
\frac{dC_1}{dt} = -\frac{dC_2}{dt} = -\frac{DA}{V\delta} (C_1 - C_2)
\]  

(4.5)

Where \( A \) is the cross-sectional area of the channel, \( \delta \) is the thickness of the channel and \( V \) is the volume of one of the droplets in the DIB assuming that the DIB in entirely symmetrical. Mole balancing the two droplets, where \( M_1 \) is the moles in droplet 1 and \( M_2 \) is the moles in droplet 2, gives:

\[ M_1 + M_2 = M_T \]  

(4.6)

\[ C_2 = \frac{M_T}{V} - C_1 \]  

(4.7)

Where \( M_T \) is the total moles in the two droplets. Substituting:

\[
\frac{dC_1}{dt} = -\frac{DA}{V\delta} \left( 2C_1 - \frac{M_T}{V} \right)
\]  

(4.8)

The solution with a constant value \( x \) is:

\[ C_1 = x e^{-\frac{2DA}{V\delta} t} + \frac{M_T}{2V} \]  

(4.9)

At \( t=0 \), before the DIB has formed:

\[ C_1(t = 0) = x + \frac{M_T}{2V} \]  

(4.10)

\[ x = C_1(t = 0) - \frac{M_T}{2V} \]  

(4.11)

Since the total moles can be described as:

\[ M_T = C_1(t = 0) \times V \]  

(4.12)

The final equation to calculate the change in \( C_1 \) over time is:

\[ C_1 = \frac{C_1(t = 0)}{2} \left( e^{-\frac{2DA}{V\delta} t} + 1 \right) \]  

(4.13)

Using the cross-sectional area and thickness of one MscL channel, the volume of the droplet, and the time from DIB formation, it is then possible to calculate an approximation of how quickly the calcein flows across the DIB over time. The results of this approximation are shown in Figure 4.22.
Figure 4.22 – Graph showing the results of a Fick’s first law calculation of the change in concentration of calcein in the second droplet of a DIB assuming calcein cannot diffuse across the DIB, and assuming one open MscL pore of 3nm diameter and 10nm thickness.

Figure 4.22 shows that in the time frame of the assay the concentration of calcein in the second droplet is still orders of magnitude smaller than the first droplet. While this approximation supports the results that show that the concentration of calcein in the two droplets of the DIB doesn’t equilibrate, it should be noted that this approximation only assumes that there is exactly one MscL channel in the DIB, which is unlikely given the concentration of protein used.

4.6.7. Lipid Composition Sweeps with MTSET

As the MscL calcein release assay was successfully transferred into the DIB model membrane system, it must now be shown to be as versatile in terms of possible assays. Therefore, to take the MscL DIB assay further, it was necessary to show that the lipid composition of the DIB affected the MscL activity.

**Charged Lipid Composition Sweeps**

The first lipid composition sweep that was proposed was to examine the effect of charge on MscL activity in DIBs. The MscL DIB calcein release assay was undertaken on charged lipid membrane compositions containing DOPG, and MscL activity was measured. All data was obtained with MTSET as the activator. The maximum amount of DOPG measured was 30% due to instability caused by the charge in the DIB.
Figure 4.23 – MscL DIB assay results for charged lipid sweeps. a)-b) 70:30 DOPC:DOPG brightfield microscopy image, c)-d) 70:30 DOPC:DOPG fluorescence microscopy image, e) graph to show the fluorescence increase in the non-fluorescent droplet, t=0 is measured as the data point before the first fluorescence increase is measured. The data points are an average of 3 DIB assays from the same reconstitution, the error bars are one standard deviation. In each case, the droplet fluorescence is measured on ImageJ using an area which was the same size. Scale bars are 500μm.
The results in Figure 4.23 show that with MTSET as the activator, as the composition of DOPG increases in the membrane, the rate of MscL activity also increases up to 70:30 molar ratio DOPC:DOPG, agreeing well with literature\textsuperscript{137}. As the amount of charge in the membrane increases, the MscL rate of gating increases, either a result of more active proteins being reconstituted or because the charged lipids are directly interacting with the protein and increasing the probability of it opening\textsuperscript{137}. In the time frame of the assay the rate of MscL activity did plateau for every composition, indicating either the MscL degrading at room temperature, or MTSET degrading and MscL closing as a response. However, with increasing membrane charge there was an increase in the maximum fluorescence transfer, which could indicate a greater proportion of MscL reconstitution which is active over time, or it could be a by-product of faster calcein translocation before the same amount of degradation occurs. Again, it is difficult to separate rate of calcein translocation and MscL reconstitution efficiency.

One note that was made during this assay, was that with increasing DOPG composition, there was an increase in the amount of wetting. While this wetting is not known in literature to affect membrane protein activity in the DIB, as there are many interactions that take place in wetted lipids it is plausible that the wetting can cause a localised ordering of the lipids in the droplet and that ordering could mean that the lipids in the DIB itself are not homogenous and therefore could affect the repeatability of the assay. A comparison of DIBs and Liposomes is carried out in the next section.

**Curved Lipid Composition Sweeps**

The other lipid composition sweep that was proposed was to see the effect of increasing curvature elastic stress on the MscL activity in DIBs. The MscL DIB calcein release assay was carried out with lipid membranes containing different amounts of DOPE and 5% DOPG, MscL activity was then measured. As the amount of DOPE in the membrane increases, so does the stored curvature elastic stress caused by forced the lipid into a planar bilayer. All data was obtained with MTSET as the activator. The maximum amount of DOPE measured was 20% due to instability in the DIB.
Figure 4.24 - MscL DIB assay results for curved lipid sweeps. a) 75:20:5 DOPC:DOPE:DOPG brightfield microscopy image, b) – d) 75:20:5 DOPC:DOPE:DOPG fluorescence microscopy images at different time points, e) graph to show the fluorescence increase in the non-fluorescent droplet, t=0 is measured as the data point before the first fluorescence increase is measured. The data points are an average of 3 DIB assays from the same reconstitution, the error bars are one standard deviation. In each case, the droplet fluorescence is measured on ImageJ using an area which was the same size. Scale bars are 500μm.
The results in Figure 4.24 do show a trend implying that increased DOPE composition, decreases MscL activity, agreeing with literature sources which have also shown that membranes with increased curvature stress, decrease MscL activity\textsuperscript{107}, however there is a very large amount of error. The increase in stored curvature elastic stress caused by the increase in DOPE composition, either decreases the reconstitution efficiency or decreases the probability of gating, therefore decreasing the rate of fluorescence transfer across MscL. The increase in error can be attributed to the fact that the lipids in the monolayer are free to diffuse, and therefore when the DIB forms the DOPE can diffuse into the monolayer rather than the bilayer to reduce the elastic stress caused by having a curved lipid in the bilayer.

Of note in this assay, the higher compositions of DOPE are dramatically more unstable, with a much higher frequency of merging events. Only results that lasted longer than 30 minutes were included in these results. This decrease in stability is likely due to an increase in membrane tension causing a decrease in the stability of the bilayer.

While these results are promising and can show that the DIB model membrane system is reliable in different lipid compositions, it is still difficult to separate the effect of lipid composition on reconstitution efficiency and on MscL activity. It is now necessary to compare the two model membrane systems directly to see if the trends are reproducible between the two systems.

4.6.8. Comparison of Liposomes and DIBs

In chapter 3, lipid composition sweeps were carried out to determine the effect of changing membrane properties on MscL activity. To determine whether the DIB system is effective as a model membrane for membrane protein studies, it was necessary to directly compare the results from the lipid sweeps in the two systems. The difficulty of this comparison was that it was difficult to get a maximum fluorescence for the entire system, and therefore it was difficult to get a percentage change in fluorescence except by comparing to the calcein containing droplet which was also changing over time. The direct comparison was still attempted, though the results were treated qualitatively. The rate of calcein translocation across MscL in DIBs was plotted, and an exponential decay was fitted using Origin in the same manner as the rate results in Chapter 3. Then plotting the rate constant from the two model membrane systems, against the composition of lipids allows a direct comparison of their reproducibility. It is noted that the liposomes were more stable towards charged and curved lipids, and therefore only the compositions that were stable in DIBs were compared.
Charged Lipid Composition Sweeps

The results from the comparison between the liposome and DIB charged lipid composition sweeps are presented here. The rate of calcein translocation across MscL was plotted onto one graph in order to make a direct comparison. For all the following data points the MscL activator used was MTSET.

![Figure 4.25](image)

**Figure 4.25** – MscL activity rates for charged lipid sweeps for both MscL reconstituted liposome and DIB model membranes. Each liposome data point is an average of 3 separate liposome samples, and the error bars are one standard deviation. Each DIB data point is an average of 3 DIBs from the same reconstitution, and the error bars are one standard deviation.

The results in Figure 4.25 show that the same trends can be seen in both the liposome and DIB assays, as the composition of DOPG increases, so does the rate of MscL activity. Also, the two model membrane systems seem to correlate quite well in terms of trend, however it is noted that the rate that is being measured is for a different number of MscL proteins reconstituted in a different sized bilayer. The liposome system has a much larger surface area of bilayers overall than the single DIB and so it can be difficult to relate lipid composition to rate of calcein transfer without knowing the exact amount of active protein in the bilayers. Attempts at estimating the amount of MscL reconstituted into each system, did not produce an accurate assessment, and did not account for the differences between the systems seen in Figure 4.25.

Another difference between the two model membrane systems is that in liposomes the diffusion boundary is much smaller than in the DIBs, a calcein molecule has to diffuse across a maximum of
100nm in liposomes whereas in DIBs a molecule has to diffuse across a maximum of 500μm which can significantly change the rates of calcein translocation.

**Spontaneous Curvature Lipid Composition Sweeps**

The results from the comparison between the liposome and DIB spontaneous curvature composition sweeps are presented here. The rate of calcein translocation across MscL was plotted onto one graph in order to make a direct comparison. For all the following data points the MscL activator used was MTSET.

Figure 4.26 – MscL activity rates for curved lipid sweeps for both MscL reconstituted liposome and DIB model membranes. Each liposome data point is an average of 3 separate liposome samples, and the error bars are one standard deviation. Each DIB data point is an average of 3 DIBs from the same reconstitution, and the error bars are one standard deviation.

The results in Figure 4.26 show that similar trends can be seen in both the liposome and DIB assays, as the composition of DOPE increases, the rate of MscL activity decreases. However, there is a very large discrepancy between the two model membrane systems, not only are the rates very different, but the trend in the decrease in rate as DOPE concentration increases, is also very different. The rate of MscL activity decreases much faster in the liposome system than in the DIB system, implying that the DOPE is either effecting the two model membrane systems differently, or more likely is implying that the DOPE is not homogenously mixing into the DIB due to energetic unfavorability compared to
the droplet monolayer. So, the MscL is most likely reconstituted into a DIB with a composition that is not the same as the lipid composition in the bulk droplet.

4.6.9. sPLA₂ controls

While the proof-of-concept results for the reconstitution of MscL into a DIB calcein release assay showed MscL activity when MTSET was used as the activator, the next stage of the translation from liposome into DIB system was to show protein-membrane-protein interactions. In liposomes, when sPLA₂ was added, there was no significant change in the stability of the liposomes as shown by DLS, and controls without the presence of MscL. However, DIBs are an inherently unstable system with certain lipid compositions and so further controls were carried out to see the effect of sPLA₂ on DIBs without the presence of MscL before continuing with the MscL DIB calcein release assay.

The first control that was carried out was to investigate the effect of sPLA₂ concentration on DIB stability. The original sPLA₂ concentration used was optimised for the liposome system, and therefore a set of assays needed to be carried out to determine what level of sPLA₂ would show optimal MscL activity while also optimising the stability of the DIB in the presence of the enzyme. By performing a serial dilution of sPLA₂, in one order of magnitude steps between a final droplet concentration of 100pM and 100aM, it was possible to determine the level of sPLA₂ which can be used without destabilising the DIB in a 100% DOPC DIB. Some example brightfield microscopy images from this serial dilution stability assay, as well as a graph displaying the DIB lifetime with each concentration of sPLA₂, are found in Figure 4.27.

It should be noted there are two methods used to introduce sPLA₂ into a DIB system. Either the sPLA₂ is included in the droplet with the liposomes when it is initially injected into the oil phase, or the sPLA₂ is injected into one of the droplets of the DIB after initial formation of the DIB. If the sPLA₂ is included in the droplet before injection into the oil, the process is much easier to carry out and therefore quicker. However, the sPLA₂ has time to hydrolyse lipids while the droplets equilibrate to coat the entire monolayer before DIB formation, and therefore there may already be some lyso-lipids in the DIB before MscL can reconstitute. Conversely, injecting sPLA₂ into the droplet after DIB formation means the hydrolysis can be measured directly from the start. However, it was difficult to inject into a droplet after DIB formation without causing a merging event, which made measuring the system difficult.

For all following control assays, the sPLA₂ was included into the droplet before injection into the oil, and the droplets were equilibrated for exactly 5 minutes before DIB formation. The reason for this was because it was found that the injection of even small amounts of buffer into the droplet after DIB formation caused MscL activity from the pressure changes of the needle going into the droplet.
The results in Figure 4.27 show that the sPLA₂ completely destabilised the DIB up until 100fM where the first semi-stable DIB is recorded, though only for a few minutes. The reason for the destabilisation of the DIB is that the sPLA₂ is hydrolysing the lipids in the DIB leaving lyso-lipids and free fatty acids, which energetically destabilises the DIB and makes merging more favourable. As the concentration of sPLA₂ goes down the rate of hydrolysis starts to compete with the rate of lipid replacement as the lyso-lipids diffuse out of the bilayer and complete lipids diffuse back in, and so the DIB is more stable. It could also be true that the sPLA₂ does not favourably bind to the monolayer due to interactions with...
the oil once it is inserted, therefore there may be a higher effective concentration of sPLA$_2$ bound to the DIB than initially injected in.

After the initial stability assays, it was then necessary to prove that the sPLA$_2$ did not cause dye leakage across the membrane without the presence of MscL. Therefore, the calcein release assay was carried out after following the reconstitution protocol with an equivalent amount of MscL free DDM, and tested with 1fM sPLA$_2$ in the non-fluorescent droplet which was the highest concentration that was stable consistently for the timescale of the assay.

Figure 4.28 – a) example brightfield microscopy images of a DIB of 100% DOPC with 1fM sPLA$_2$ in the non-fluorescent droplet, b) – c) example fluorescence microscopy images of DIBs of 100% DOPC with 1fM sPLA$_2$ in the non-fluorescent droplet at different time frames, d) graph showing the change in fluorescence of the non-fluorescent droplet over time. Each data point is an average of 3 DIB assays from the same MscL free reconstitution, error bars are present but smaller than the data points. Scale bars are 500μm.
The results in Figure 4.28 show that while higher concentrations of sPLA₂ can destabilise the DIB to cause merging, it doesn’t cause leakage of calcein as the level of fluorescence in the droplet doesn’t change over time. The result correlates well with the liposome control assays with sPLA₂, showing that the hydrolysis of the bilayer doesn’t cause pores to form or calcein to leak out.

It should be noted that while three sets of stable DIBs were achieved in the time frame of the assay, and the results are shown in the above graph, there were 2 other assays that destabilised and merged that were not included in the results. The sPLA₂, even at the very low concentration used, still destabilises the DIB, however a balance needs to be maintained to ensure that there is enough sPLA₂ activity to cause MscL gating. The next section covers assays that explore the relationship between sPLA₂ and MscL in DIBs, and how that relationship differs from liposome assays.

### 4.6.10. MscL DIB calcein release assay with sPLA₂

To fully translate the MscL activity assay into DIBs, the MscL channels need to show mechanosensitivity in the DIB system. If the behaviour displayed in liposomes is mirrored in DIBs, the calcein should translocate across the open MscL due to a build-up of the lyso-lipid product of sPLA₂ hydrolysis. To investigate the interaction, a DIB was set-up composed of 100% DOPC, one droplet contained MscL reconstituted liposomes and quenched calcein, the other droplet contained was non-fluorescent and sPLA₂ was added to the non-fluorescent droplet immediately before injection into the oil. In the first assay, the sPLA₂ concentration was kept constant at 100aM, while 1fM sPLA₂ was the upper limit of concentration that still allowed for stable DIB formation, it was not possible to get a DIB stable in the time frame of the assay in the presence of MscL. The results of the first assay are found in Figure 4.29.
Figure 4.29 – a) Brightfield microscopy images of a DIB of 100% DOPC with 100aM sPLA2 in the non-fluorescent droplet b) – d) Example fluorescence microscopy images of DIBs composed of 100% DOPC, reconstituted MscL and 100aM sPLA2, e) graph showing the change in fluorescence for both the quenched calcein droplet, and the non-fluorescent droplet over time. Each data point is an average of 3 DIB assays from the same MscL reconstitution, and the error bars are one standard deviation. Scale bars are 500μm.
The results in Figure 4.29 show that it is possible to get MscL-sPLA2 communication in DIBs, as demonstrated by the increase in fluorescence in both droplets as calcein translocates across the gated MscL channel. There is a much lower activity of MscL with sPLA2 then with MTSET, which could be attributed to the very low concentration of sPLA2 used, meaning the hydrolysis of lipids in the DIB is competing with lipid diffusion and very few MscL channels are opening. The low activity could also be caused by the sPLA2 mode of action which means the binding of sPLA2 to the bilayer is much stronger than the dissociation, so much of the sPLA2 could be bound to free liposomes in the droplet. There is also a large amount of error in the system, which is attributed to the ability of sPLA2 to destabilise the bilayer. Furthermore, the result doesn’t show the characteristic increase in fluorescence in the non-fluorescent droplet before plateuing due to MscL deactivation which implies that in tests with MTSET, the plateau is caused solely by MTSET deactivation.

It should be noted again that the 3 DIB results in the graph in Figure 4.29 were 3 DIBs that were stable for the entire length of the timescale of the assay, however many more DIBs merged than were able to be measured. Therefore, an investigation was run to see how low a concentration of sPLA2 could be used that would give MscL activity while reliably producing stable DIBs. Too high and the DIB becomes unstable, but too low and the activity of sPLA2 wouldn’t be able to compete with lipid replacement in the DIB and the membrane wouldn’t reach a threshold asymmetry level to gate MscL. Therefore, the same MscL DIB calcein release assay was carried out with a range of concentrations of sPLA2 and the results of this assay are shown in Figure 4.30.

![Figure 4.30 – Graph showing change in fluorescence for the non-fluorescent droplet in MscL DIB calcein release assays composed of 100% DOPC with a range of sPLA2 concentrations. Each data point is an average of 3 DIB assays from the same MscL reconstitution, and the error bars are one standard deviation.](image)
The results in Figure 4.30 show that the optimal sPLA₂ concentration that maximises both MscL activity, and DIB stability, is between 100aM and 10aM. Assays that attempted to investigate this further by using sPLA₂ concentrations between these values were not conclusive, likely because of the ‘scooting’ mechanism of action\(^{27}\) that states that the dissociation of sPLA₂ from the bilayer is very slow compared to the binding step. There are many bilayers in each droplet, the DIB is one of them but there are also likely to be many liposomes still present in each droplet, therefore there is no assurance that when the DIB forms there is any free sPLA₂ in the system compared to the amount bound to the liposome bilayers. At such low concentrations of sPLA₂ where there are only around 100,000 sPLA₂ enzymes in the droplet, it’s difficult to control whether the sPLA₂ binds to the DIB rather than the liposomes.

After proving that sPLA₂ – MscL communication had been demonstrated in the DIB system, it was then necessary to investigate how changing lipid composition in the MscL DIB calcein release assay would affect the protein-membrane-protein interaction. However, it proved to be extremely difficult to create stable DIBs that contained 10% or more DOPG or DOPE. For DOPG, sPLA₂ displays affinity for negatively charged membranes\(^{27}\), and therefore the increased activity likely destabilised the membrane even more than in pure DOPC membranes. For DOPE, the increased membrane tension in the system due to the curved lipid introduction likely increases sPLA₂ activity correlating well with the results seen in chapter 3, and therefore increases the destabilisation effect. An attempt was made to reduce sPLA₂ concentration and measure the effect of investigate lipid composition, however almost all results from this attempt either showed no MscL activity or DIB merging.

**4.7. Conclusions**

In conclusion this chapter has shown the first ever translation of the MscL liposome calcein release assay into the DIB format, producing a whole new model membrane that can be used to study the effects of membrane properties on MscL activity. These investigations first proved that the DIBs were stable for long periods of time, even under the stress of MscL gating and in the presence of sPLA₂ hydrolysing the membrane. It was shown that dye did not flow across the DIB in the absence of MscL, MTSET or sPLA₂. Only when both MscL and the activator were present in the system did fluorescence transfer occur and a proof of concept was later published. Then the DIB system was compared to the liposome system, and while the same trends were seen with MTSET as the activator in the DOPG lipid sweeps, the DOPE lipid sweeps were less conclusive implying that the DIB is not as homogenous as first believed. sPLA₂ lipid sweeps did not appear to be possible in this setup due to stability issues.
These proof of concept experiments provide a new platform with which to study membrane protein activity. There is also potential to use this system to network multiple bilayers together by placing monolayer coated droplets in a row to create multiple DIBs. Attempts were made to create these using this calcein release assay and those results are shown in Figure 4.31. However, what was found was that the very small concentration gradient between the second droplet and the third, and subsequently the forth, meant it was very difficult to gather any useful information. It could however provide information if the flow of ions were to be used, as is the case in an electrophysiology setup.

![Figure 4.31 – Brightfield and fluorescence microscopy images of attempts to network multiple MscL reconstituted DIBs together with MTSET as the activator. Fluorescence can be seen transferring into one droplet, but not the third.](image)

The results in the chapter have clearly shown that it’s possible to reconstitute MscL into a DIB based calcein release assay, it was now important to start looking more heavily into the problems that the system faced. Similar to the liposome system, it was difficult to separate reconstitution efficiency from a change in MscL activity rate, because an increase in fluorescence transfer across the DIB could indicate faster or easier MscL opening, or more MscL channels reconstituted. It was also difficult to network the MscL activity across multiple DIBs possible due to the very low concentration gradient of calcein across the network, or because of MscL degradation. Finally, it was difficult to get sPLA₂ – MscL activity in DIBs, and impossible to register the effects of lipid composition of this protein-membrane-protein communication because of the instability caused by sPLA₂ activity.

The next chapter deals with taking this DIB assay further into an electrophysiological setting, to perform single-channel analysis, attempt to get more information about reconstitution efficiency and network multiple bilayers together.
Chapter 5

Electrophysiological MscL assay in Droplet Interface Bilayers

5. Overview

The previous chapter laid out the proof-of-concept assays that showed that it was possible to actively reconstitute MscL into a DIB and measure its activity. However, the dye release assay had a number of drawbacks when it came to networking multiple DIBs together, due to the concentration gradient of the dye across multiple droplets. The calcein release assay also could not distinguish between active reconstitution efficiency and changes in MscL gating rates as the amount of MscL in the DIB could not be controlled. In order to resolve these issues, it was decided to try an electrophysiological assay instead of a dye release assay, as single channels could be resolved individually, and there would not be any dye concentration gradients to take into account.

To conduct electrophysiological assays, DIBs were set-up using a similar technique as the previous assay with monolayer coated water-in-oil droplets, however into the droplets are inserted two silver / silver-chloride (Ag/AgCl) electrodes. When a voltage is passed between the electrodes a current cannot flow as the DIB is not permeable to ionic current, however when a protein channel is reconstituted into the DIB and gated, current can flow across the channel and the amount of current that can flow is related to the structure of the channel.

This chapter details the process of measuring MscL activity in an electrophysiological assay. Firstly, a number of controls and assays were set-up to ensure no current could transfer when MscL wasn’t present in the system, and to ensure that the DIB remained the same size throughout. Following controls, a set of assays were completed to prove that MscL activity could be measured with MTSET as an activator and to analyse the concentration of MscL needed to get single channel activity in a pure DOPC system. After achieving single channel activity in a pure DOPC system, the lipid concentration was varied to investigate how the membrane would affect how many active MscL channels were reconstituted into the DIB, and see if it was possible to change MscL gating rates. Then many assays were carried out to achieve networking of multiple bilayers using MscL reconstituted in each DIB. Finally attempts were made to achieve MscL activity with sPLA$_2$ as the activator.

Parts of this chapter formed a published article detailing the first successful networking of multiple DIBs together linked by a gated ion channel$^1$. Where relevant it has been mentioned work which assays were published, and assays completed specifically for the article are specified.
5.1. Introduction
Electrophysiology is a field of biophysics which is concerned with either inherent electrical properties, or using electrical techniques to study, biological components\textsuperscript{169}. While the first recorded use of electrophysiology dates back to the 18\textsuperscript{th} century when bioelectricity was discovered\textsuperscript{170}, it has evolved into a technique that is used in many different disciplines. One of the most influential examples of electrophysiology use in literature was in the experiments that showed that the movement of ions were responsible for changes in membrane potential in the neuronal action potential, and that the permeability of the membrane to Na\textsuperscript{+} ions changes over time\textsuperscript{171,172}. The invention of the voltage clamp lead to significant advancements in the measurement of ionic currents under membrane potential\textsuperscript{173-175}. The setup consisted of an intracellular electrode, a follower circuit used to measure the membrane potential, a feedback amplifier correcting any difference between the recorded voltage and the preset value, and finally the second extracellular electrode.

Detailed here is the theoretical background behind electrophysiological studies, supported by a review of MscL electrophysiological studies in literature.

5.2. Theoretical Background
An open ion channel can allow the passage of an estimated 10,000,000 ions per second, dependant on the diameter of the channel\textsuperscript{175}. The free movement of charge in a conducting medium can be described as an electrical current, one ampere is equal to a fixed flow of charges at a rate of one coulomb per second. In the case of monovalent salt, one mole of the salt contains one mole of cations and one mole of anions. Multiplying the number of electrons in a mole of a substance by the elementary charge of an electron, is known as the Faraday constant (F). With monovalent anions the charge is -F and for divalent cations the charge is 2F. The faraday constant then allows Ohms law to be applicable to ion channel studies, where the ionic current through the channel is then equal to the conductance of the channel multiplied by the voltage difference\textsuperscript{175}. As well, the cell membrane comprises of an insulating lipid bilayer separating two aqueous compartments, and it is possible to equate the structure to a capacitor. These structures give the ability to model the cellular membrane and channel proteins into approximate circuit diagrams\textsuperscript{175}. An example circuit diagram for a model membrane is seen in Figure 5-1, reflecting both the membrane capacitance and the ion channel conductance.
Figure 5-1 – A model membrane circuit. The circuit is an electrical representation of a cell membrane with an ion channel reconstituted into it. The membrane acts as a capacitor ($C_M$), the ion channel acts a resistor ($g_k$), and the battery depicts the membrane potential ($E_k$). The figure is adapted from Hille, 2001.

The overall conductance of the system is displayed by the product of the voltage gradient across the membrane caused by ions generating an electromotive force (EMF), and the conductance of the ion channel is displayed by a resistor. The circuit in Figure 5-1 allows the current-voltage relationship to be represented. Therefore, electrophysiological recordings can be obtained by putting electrodes on either side of the bilayer and measuring the membrane potential, or in the case of this chapter transmembrane current.

While transmembrane current has been historically measured using Hg/HgCl or calomel electrodes, in this chapter a more practical electrode has been used. In this chapter the electrode used is the silver/silver chloride (Ag/AgCl), this electrode is a standard non-polarised electrode, exhibiting no double layer capacitance. The electrode requires a solution containing chloride ions, such as KCl, where the Cl atoms in the AgCl crust are exchanged like metal atoms when in contact with a salt of that metal. This behaviour effectively allows the Ag/AgCl electrode to behave like a chlorine electrode in a solution of chloride ions.

### 5.3. Electrophysiological Methods

There are many different electrophysiological methods that are employed in literature. Outlined below are some of the main methods used to study compatible model membrane systems.
5.3.1. Patch Clamp

The most common method of electrophysiological measurement used in literature is known as the Patch Clamp\textsuperscript{175}. While it has mostly been used in literature to study cells, there are many different configurations with model membranes that have been used in literature\textsuperscript{177}. A high resistance seal is formed by carefully pressing a glass micropipette with a small tip diameter, typically 1μm, against the surface of a bilayer, and applying suction. This seal allows the membrane ‘patch’ to be electrically isolated from the rest of the membrane\textsuperscript{169}. This method is illustrated in Figure 5-2.

![Patch Clamp Diagram]

**Figure 5-2** – A figure outlining the patch-clamp method for obtaining measurements of protein channels. In this setup the cell is suspended in a solution and a glass micropipette with a 3μm opening is placed onto the cell using a micromanipulator. A suction is applied and a giga Ohm seal is created, electrically isolating the channels in the membrane patch from the rest of the cell. An amplifier connected to Ag/AgCl electrodes inserted into the micropipette allow measurements of changes in transmembrane current. This figure was adapted from Dunlop, 2008\textsuperscript{169}. 
Any protein channels inserted into the membrane patch can then be studied in the membrane patch, or the patch can be removed from the cell using suction techniques allowing access to both sides of the membrane. In either case, an Ag/AgCl electrode inserted into the pipette and a second Ag/AgCl electrode is connected to ground inserted into the solution. Voltage is then maintained across the cell membrane and maintained using an amplifier which contains a voltage clamp feedback circuit. The clamp then measures transmembrane current caused by channels opening and closing within the isolated membrane patch.

While patch clamp measurements allow for the study of single channel measurements, they often suffer from low-throughput and experimental difficulties\(^{178}\). The requirement for each cell, or membrane, to be prepared individually limits the amount of data that can be measured over time. Automated patch clamp platforms have been created using lab-on-a-chip methods, though these have also had some limitations\(^{177}\). Other practical limitations include that it is difficult to control the lipid composition of the patch; the method depends on the protein channel being incorporated into the membrane patch; the method cannot separate the inner and outer membranes of cells that have two membranes; and the creation of membrane patches can change the mechanics and curvature of the membrane itself due to the suction force required to isolate the patch.

### 5.3.2. Planar Lipid Bilayers

One model membrane system known as a black lipid or bilayer lipid membrane (BLM), is a model membrane system that allows ion channel electrophysiology without requiring micromanipulators, nor the maintenance of a membrane patch for recordings\(^{179}\). By assembling an artificial lipid bilayer from a predetermined mixture of phospholipids, measurements of the membrane can be achieved using a voltage-clamp similar to those described in the patch clamp method. The most established method for forming these planar lipid bilayers essentially ‘paint’ the lipid bilayer across a pre-formed aperture\(^{179}\). The Montal Mueller method for BLM formation is shown in Figure 5-3.

![Figure 5-3 – The Montal Mueller method for forming planar lipid bilayers. a) an aperture is engineered made from lipophilic Teflon, each side contains buffer with a monolayer of lipids deposited on top, b) – c) by adding buffer to each side allows a monolayer to form over the Teflon aperture, d) as the second monolayer forms over the aperture, a lipid bilayer forms. Inserting electrodes in each side allow transmembrane currents to be measured using a voltage-clamp.](image-url)
The Montal Mueller method shown in Figure 5-3 is known as the ‘painting’ method of BLM formation, and allows for the formation of planar lipid bilayers across a pre-formed apertures\textsuperscript{179}. The Montal Mueller method comprises of two separate chambers filled with buffer and an Ag/AgCl electrode. The buffer is coated in a lipid monolayer by applying lipids in a volatile solvent such as chloroform, and the chloroform is evaporated off. Between the chambers is a small aperture made from a hydrophobic material such as Teflon. By raising the buffer level in each chamber, a monolayer of lipids forms across the aperture, and when both chambers are filled a bilayer forms between the monolayers in the same way as it does in DIB formation. Channel proteins can then be supplied and incorporated into the BLM in a similar method to DIB formation, and single channel recordings can be taken\textsuperscript{180}. While these BLM systems are stable for up to one hour, they have a number of limitations. The main limitation that these systems face is that the apertures are difficult to engineer, and are often extremely small, meaning it can be difficult to study short lived proteins in them.

5.3.3. Using Droplets

An adaptation of the BLM system used monolayer coated droplets and hydrogels to effectively replace the two chambers of the Montal Mueller method\textsuperscript{181}. One method is the free-standing Droplet Hydrogel Bilayer (DHB) which forms by depositing a monolayer coated aqueous droplet in oil on top of a lipid solution covering an aperture pressed onto a hydrogel. An example of this technique has shown that single channel recordings of channel proteins can be measured, using a BK ryanodine receptor channel and nicotinic receptor as reconstitution examples\textsuperscript{182}. By rolling a droplet across a hydrogel in oil using a micromanipulator, it was possible to get direct membrane channel recordings\textsuperscript{183}. A schematic for this is shown in Figure 5-4. The same group then showed that it was possible to form DHBs without an aperture, allowing a much easier generation of model bilayers, and allowing higher throughput by creating multiple DHBs in any one assay\textsuperscript{184}. 
The concept of using lipid monolayer coated aqueous droplets in oil to form bilayers, DIBs, was covered in chapter 4. Therefore, in a similar manner to the DHBs, by putting electrodes onto either side of the DIB, protein channels can be measured using a voltage-clamp. Electrophysiology in DIBs has been demonstrated in principle in several different environments. One use of the DIB system analysed the construction of bionetworks of nanolitre monolayer coated droplets, proving that multiple bilayers existed between each droplet using electrophysiological methods\textsuperscript{144,147}. The system was then shown to be capable of measuring reconstituted channels shown by the potassium channel Kcv and the use of known blockers\textsuperscript{185}. This system then allowed high throughput screening of Kcv blockers by moving the one of the droplets between droplets containing different blockers.

In another report of non-invasive electrophysiology measurement techniques, a DIB was formed but one or both of the droplets were pierced with an agar-coated Ag/AgCl electrode\textsuperscript{186}. The agar coating of the electrode allowed the electrode to penetrate the inside of the droplet without disturbing the monolayer coating, this is due to interactions electrostatic interactions between the agar and the lipid headgroups. Bilayers formed between droplets manipulated with internal Ag/AgCl electrodes have been shown to be stable for more than 20 hours\textsuperscript{187}. DIBs pierced with agar-coated Ag/AgCl electrodes is the chosen method of electrophysiological recordings in this chapter, due to the ease of formation and stability.
5.3.4. Review of Electrophysiological Membrane Protein studies

While chapter 4 detailed the first ever reconstitution of MscL into DIBs published 4 years ago, there have been a few MscL-DIB articles published since that publication using electrophysiological methods. Here are two examples of articles including the reconstitution of MscL into DIBs.

**Bilayer Formation and Activation of MscL Channels in Response to Harmonic Mechanical Stimuli\(^{188}\)**

Mechanosensitive protein channels are known to open in response to mechanical stimuli, however assays to test exactly how these mechanical stimuli affect MscL activity are difficult to carry out. The following assay used a micropipette-based method to form a MscL reconstituted DIB along with Ag/AgCl electrodes to measure the incorporated channels. Monolayer coated aqueous droplets were anchored into two opposing (coaxially positioned) borosilicate glass micropipettes, as demonstrated by Figure 5-5. The droplets were brought into contact and a DIB formed between them, MscL was then reconstituted using the same method as Chapter 4 by including MscL reconstituted liposomes into one of the aqueous droplets. By having one of the micropipettes connected to a harmonic piezoelectric actuator allowed the group to provide an oscillatory mechanical stimuli to the DIB. By analysing the shapes of each droplet during the deformation phase of the harmonic movement, it was possible to estimate the tension created at the interface. By then measuring the exact moment of MscL activity, it was possible to estimate the tension requirements of MscL activity. Furthermore, the group showed that the system enabled a tighter control of DIB size and tension, and proved that the DIB system is versatile when it comes to methods of study.

![Diagram of micropipette scheme](image)

*Figure 5-5 – The micropipette scheme used to form the DIB, with MscL incorporated into the bilayer. The micropipettes are able to move horizontally allowing the deformation of the DIB. Image taken from Najem, 2015\(^{188}\).*
Activation of MscL by mechanical stimulation of supported Droplet-Hydrogel bilayers

Droplet-hydrogel bilayers (DHB) are often used as a stable medium for studying mechanical effects, it’s relatively easy to modify the droplet while maintaining tight control of the hydrogel surface. In this assay, the group again attempts to probe the effect of mechanical stimuli on MscL activity. The assay involves a MscL reconstituted DHB containing a nanoinjector and an agar coated Ag/AgCl electrode. The MscL gating was recorded as different volumes of buffers were injected into the droplet. As nanolitre injections of buffer were carried out, the droplet swelled out and induced a quantifiable DHB tension which then could be related to MscL activity. These results are seen in Figure 5-6, which shows that MscL activity scales with how much buffer was injected, implying a change in the amount of tension induced as the amount of buffer injected increased. While the results showed that the tension did cause MscL activity, it also showed that the tension equilibrated over time after the initial injection showing a decrease in MscL activity over time. However, the author does not take into account that the MscL activity decrease might have been caused by MscL deactivation due to degredation.

Figure 5-6 – a) Schematic showing the droplet-hydrogel bilayer system including MscL reconstitution from MscL containing liposomes. A nanoinjector with pulled glass pipette is inserted into the droplet and nanolitre volumes of buffer are injected in to cause expansion of the droplet, and stretching of the membrane. By stretching the membrane, asymmetric tension is created and the channel opens in response, b) a transmission microscopy image of the DHB showing the characteristic bilayer shade of a DHB, c) current traces recorded at +30 mV for a DHB containing MscL-G22S, upon injection of different volume of buffer, d) KcsA reconstituted DHB measured upon injection of buffer into the droplet, as KcsA isn’t mechanosensitive the purpose was to show that injection didn’t cause poration. Image taken from Rosholm, 2017.
5.4. Materials

This section details the materials used in MscL DIB electrophysiological assays and controls used in this chapter.

5.4.1. Casting of PDMS wells and slides

PDMS was cast using a Sylgard 184 Silicone elastomer kit purchased from Dow Corning. PDMS was stuck to a glass slide aided by a Diener Plasma Cleaning Oven.

5.4.2. Preparation of Liposomes

All lipids mentioned were purchased from Avanti Polar Lipids. The lipids were purchased as a lyophilised powder with a purity of >99%. The lyophilised lipids were stored at -20°C until needed. The lipids used were 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (DOPG), and 1,2-di-O-(9Z-octadecenyl)-sn-glycero-3-phosphocholine (Diether PC). When needed, the lipids were dried on a Thermoscientific MicroModulyo Lyophiliser, then dissolved in chloroform purchased from VWR. The lipid samples were extruded using an Avanti Mini Extruder using polycarbonate membranes and filter supports purchased from Avanti Polar Lipids.

5.4.3. MscL Reconstitution & DIB assay

The detergent n-Octyl-ß-D-Glucopyranoside (OG) was purchased from Sigma Aldrich. The [2-(Trimethyl ammonium) ethyl] Methane Thiosulfonate Bromide (MTSET) was purchased from VWR. The secretory Phospholipase A2, sPLA₂ isolated from honey bee venom (Apis mellifera) was purchased as a lyophilized powder from Sigma Aldrich. The OG, MTSET and sPLA₂ were stored at -20°C until required. Biobeads SM2 with mesh size 20-50 were purchased from BioRad. Powdered Agar was purchased from Sigma Aldrich. Silver wire was purchased from Goodfellow.

Two separate buffers were used in these assays, one reconstitution buffer for the MscL containing droplet and one buffer used for the other half of the DIB. The components for the buffers used are listed below. Unless previously mentioned, all buffer components were purchased from Sigma Aldrich.

- **Reconstitution Buffer**: 100mM KCl, 20mM HEPES, 40mM OG, pH 7.2.
- **MscL-free droplet buffer**: 100mM KCl, 20mM HEPES, pH 7.2.

All solutions were pH adjusted using 1M stock solutions of Sodium Hydroxide (NaOH) and Hydrogen Chloride (HCl) purchased from Sigma Aldrich.
5.5. Methods

This section outlines the protocols required to produce the DIB assays used in this chapter.

5.5.1. Casting of PDMS wells and slides

To make the PDMS, the base and curing agent were combined at a 10:1 ratio, then degassed in a vacuum chamber to remove as many air bubbles as possible. Surface air bubbles were removed by a gentle flow of air. To make the wells, the PDMS was placed on a flat heating block at 60°C overnight to set and a hole was cut into the PDMS before being stuck onto glass slides. To make the slides, the PDMS was spin-coated at 3,000RPM for 30 seconds, and cured on a hot plate at 100°C for 10 minutes to create a thin layer on conventional microscopy slides.

5.5.2. Preparation of Liposomes

The lipid mixtures were weighed in a molar ratio, dissolved in chloroform, and gently mixed to ensure all lipids were dissolved. The chloroform was evaporated under a steady stream of nitrogen, and the resultant lipid film was placed in the lyphiliser overnight, or for a minimum of 2 hours before hydration. Dry lipid films were hydrated in the reconstitution buffer or MscL-free droplet buffer listed in the Materials section at 12.7mM, unless stated otherwise. Each sample was freeze-thawed 4 times using liquid nitrogen and a hot-air gun, samples were vortexed before each freeze stage to ensure homogenisation. The lipid samples were extruded through a 100nm membrane 11 times, to produce monodisperse unilamellar liposomes.

5.5.3. MscL Reconstitution

For each mixed lipid-detergent liposome sample prepared as described, 3 independent aliquots of 100mg of Bio-Beads SM2 were prepared and allowed to equilibrate for 1 hour in detergent-free reconstitution buffer. Purified MscL was added to prepared mixed lipid-detergent liposomes at a lipid-protein ratio of 1:100,000, unless otherwise stated, and was allowed to equilibrate for 1 hour on rollers in a cold room at 4°C. In order to remove the detergent, the MscL reconstituted liposomes were incubated with 100mg of equilibrated Bio-Beads for 1 hour, on rollers, in a 4°C cold room. After 1 hour, the 100mg of Bio-beads were replaced and the process was repeated twice more. After 3 hours of Bio-Bead incubation, the Bio-Beads were removed and the MscL reconstituted liposomes were stored at 4°C until used. For MscL free controls, an equivalent amount of DDM was added to the mixed lipid-detergent liposomes, and the same detergent removal process was followed.

5.5.4. Silver/Silver Chloride Electrodes

Ag/AgCl electrodes were made by electroplating in 0.1M HCl. Two even lengths of silver wire were cut and soldered onto an electrical circuit and immersed into HCl. It was ensured that both wires were lowered into the HCl at the same depth, and that they were not touching each other. A platinum
counter electrode was inserted into the HCl and a 9V battery was attached. For wire that was 250μm thick the wires were left for exactly 30 seconds, and for wire that was 500μm the wires were left for exactly 60 seconds. The current of the circuit was limited by including a 10k resistor. As the circuit was completed, the Ag wires darkened due to the precipitation of insoluble AgCl salt on the surface of the wire, while hydrogen gas emerged at the platinum electrode.

5.5.5. DIB Electrophysiological Assay

5% agar (w/v) in DI water was coated onto Ag/AgCl electrodes and washed with buffer. A 1.5μl droplet of the MscL reconstituted liposomes and a 1.5μl droplet of the MscL-free liposomes containing 300mM MTSET, or various concentrations of sPLA₂, were injected onto agar-coated Ag/AgCl electrodes. The droplets on the electrodes were lowered into the hexadecane filled well using micromanipulators (ThorLabs), and the droplets were left to equilibrate in the oil for 5 minutes to allow time for monolayer formation around the outside of the droplet. For the droplet networks, an additional droplet of equal size was dispensed into the oil between the two electrodes. Each droplet was brought together by micromanipulators and DIBs formed between each droplet connected. At the end of each measurement the droplets were separated, the droplets were taken to the edge of the well, and the electrodes were withdrawn from the oil. The electrodes were then washed with buffer, before the droplets are replaced onto the electrodes for the next assay.

The DIB was visualised by stereo microscope at 2x magnification (Brunel Microscope). Electrophysiology was performed using an Axopatch 200B amplifier (Molecular Devices), an Axon Digidata 1440A digitiser (Molecular Devices), and a CV-203BU headstage (Molecular Devices). The stage was connected to the amplifier using a shielded cable. The amplifier and digitiser were connected to a computer by USB connection. The microscope, stage, and micromanipulators were stored inside of a 500mm x 820mm x 500mm steel faraday cage built ‘in-house’. The whole unit was connected to the ground port of the amplifier, which was grounded using mains. Current measurements were recorded on pClamp software (Molecular Devices) using a holding potential of 100mV, a 5 kHz low-pass 8-pole Bessel filter, at a sampling rate of 50 kHz. Capacitance measurements were obtained by applying a linear voltage ramp under a triangle waveform; 500 Hz frequency, 1mV peak-to-peak, i.e., ±1V/s voltage ramp, allowing 1pA to be interpreted as 1pF. The measured current response can then be interpreted as bilayer capacitance. A schematic of the electrophysiological DIB setup can be found in Figure 5-7.
Figure 5-7 – A schematic representation of the setup for performing electrophysiological DIB measurements. The translation stage and microscope are positioned inside the faraday cage, positioned on top of the PDMS wells. Ag/AgCl electrodes are attached to the translation stage and connected to the head stage via gold-plated wires. A shielded wire connected the head stage to the amplifier, and both the amplifier and digitiser are connected to the computer by USB.

5.5.6. Results Analysis

Microscopy images were taken using a conventional camera attached to the stereo microscope’s optical eye-piece. Electrophysiological recordings were analysed using Clampfit software (Molecular Devices). Data was digitally filtered using a 1 kHz low-pass filter and reduced to a sampling interval of 120 µs. The filter allowed fine detail to be separated from background noise, and all data was filtered with the same digital filter. An example of the difference between the raw data and filtered data is shown in Figure 5-8. The data could then be manipulated to show specific time frames to display the results more accurately. Single channel analysis could also be carried out by an in-built Clampfit programme giving data on the amount of channel opening events that are present in any electrophysiological recording.
5.6. Results and Discussion

This section details the results from the translation of the MscL DIB assay from chapter 4 into electrophysiological measurements. This section starts with control assays undertaken to ensure that the DIB is stable and doesn’t porate under normal conditions. Following this is the results of the initial assays attempting to get single channel activity with MTSET as the activator by varying MscL concentration systematically. Then, lipid sweeps are shown with MTSET as the activator, and

Figure 5-8 – Example of electrophysiological data analysis using Clampfit. a) raw data recording of MscL activity in a DIB before digital filtering, b) the same recording after digital filtering, showing fine detail.
comparisons are made with the lipid sweeps in liposomes in Chapter 3, and the lipid sweeps in DIBs in Chapter 4. Next, the published results of MscL activity measurements across multiple DIBs with MTSET as the activator\(^2\). Finally, the attempts to record electrophysiological results of MscL activity with sPLA\(_2\) are shown and compared to previous results.

There are many different components in an electrophysiological recording, an example of a typical electrophysiological recording is shown in Figure 5-9.

Figure 5-9 – Typical electrophysiological recordings, a) current recording of MscL activity in a 95:5 DOPC:DOPG DIB, b) a zoomed section of the current recording displaying the DIB forming over time and capacitance increasing, c) a zoomed section of the current recording displaying single MscL channel activity, c) an example of what the current recording looks like upon DIB merging, the current trace saturates, d) DIB capacitance changes over time as the DIB forms, increasing capacitance indicating a larger DIB area.
From Figure 5-9 the main component shown is the current trace, when the baseline gets larger it indicates the capacitance of the system increasing. The capacitance traces in Figure 5-9d show that as the DIB area increases, so does the capacitance, because DIB area and capacitance are proportional. Capacitance traces are important in showing that the DIB area is constant in the system. When the current traces jump slightly, as seen in Figure 5-9c, this indicates a pore forming in the bilayer and allowing free transfer of ionic current, this is known as the channel conductance. Pores in the bilayer are often caused by channel proteins, and protein channels have the same opening size, therefore the same gated protein channel would have the same channel conductance. When the current jumps to saturation, seen in Figure 5-9b, this indicates that the DIB has merged and the bilayer is no longer creating a barrier for ionic current, so current freely transfers. Large thin lines that appear in the current trace are attributed to mechanical noise in the system, likely caused by vibrations of the local environment. This mechanical noise is very difficult to filter out fully, though steps are taken throughout this chapter to minimise it.

As noted, the capacitance of a bilayer system, and the bilayer area are proportional. This proportionality can be described in the same manner as a parallel plate capacitor as follows, assuming a constant voltage$^{181,190}$:

$$C_m = \varepsilon_0 \varepsilon \frac{A}{D} \quad (5.1)$$

$C_m$ is the membrane capacitance in Farads (F), $\varepsilon_0$ is the vacuum permittivity, $\varepsilon$ is the dielectric constant of the bilayer, $A$ is the bilayer area, and $D$ is the bilayer thickness. Given that the lipids used all have the same tail length, and similar dielectric constants, it can be assumed that everything apart from the capacitance and bilayer area are fixed therefore allowing a direct approximation of the bilayer area from capacitance measurements. In general, for DOPC, DOPE, DOPG bilayers that have a perfectly circular bilayer, 100pF is the equivalent of 130μm bilayer diameter. It is noted that the bilayer is unlikely to be circular due to droplet wetting, therefore increasing the error.

It is also commonplace to estimate the bilayer area from the capacitance using the specific capacitance of the lipids. In this case, the current is equal to the change in charge over time which can be described at the change in voltage over time multiplied by the capacitance.

$$i = \frac{dq}{dt} = \frac{dV}{dt} \ast C \quad (5.2)$$

In this situation, a constant voltage is being applied and current is being measured, therefore the change in voltage over time is one. This means that the current and the capacitance are equal.
It is noted that capacitance can also be used to show bilayer defects and bilayer morphology using artefacts from each capacitance peak, however those will not be covered in this thesis.

### 5.6.1. Control Assays

The control assays undertaken for electrophysiological recordings of MscL activity are presented in this section. The first control was a stability assay, intending to show that the DIB has a constant stable size under the voltage clamp of the system. A 100% DOPC DIB was recorded for 30 minutes, and the capacitance of the bilayer was measured every 5 minutes while the current was measured for entire duration. The results from this initial control are shown in Figure 5-10.

![Figure 5-10](image)

Figure 5-10 – The results from initial electrophysiological controls of 95:5 DOPC:DOPG DIBs. a) – c) capacitance traces at different time frames during the course of the assay, e) current trace from the entire length of the assay. The assay was repeated 3 times, one result is shown above.

The results in Figure 5-10 show that at time 0 there is negligible background capacitance in the system, and the DIB has not fully formed yet. As the DIB assembles the baseline current rises-in-line with the increase in bilayer capacitance, which is proven by the capacitance trace at 5 minutes. The background current and the capacitance are almost exactly the same level 30 minutes after initial current recordings, indicating that after initial DIB formation the DIB area is completely stable, and no natural pores form in the time frame of the assay. With a capacitance of 3000pF, the diameter of this average bilayer is 3.9mm, which corresponds well with stereo microscopy images taken.

After proving DIB size and stability, the second control undertaken was to ensure that there was no bilayer poration without the presence of both MscL and MTSET. Therefore, the same DIB electrophysiological setup was undertaken except that in the first, liposomes with an equivalent MscL-free DDM reconstitution were in one droplet and 300mM MTSET was added to the other, and in the second, MscL reconstituted liposomes were added to one droplet, and the other droplet just
contained buffer and liposomes. The results for these controls are seen in Figure 5-11. It is noted that in this case, the amount of MscL reconstituted was at 100,000:1 lipid to protein ratio, the reason for the use of this ratio is explained in the next section, *Effects of MscL Concentration*.

![Figure 5-11](image)

Figure 5-11 – The results from electrophysiological controls of 95:5 DOPC:DOPG DIBs, ai – aii) MscL-free DIB with MTSET in one droplet, bi – bii) MscL reconstituted DIB at 100,000:1 lipid to protein, without the presence of MTSET. Both controls shown include one capacitance trace to ensure bilayer size reproducibility. The assay was repeated 3 times, one result is shown above.

The results in Figure 5-11 show that the DIB is stable over the lifetime of the assay in the presence of either MscL or MTSET. The control also shows that no poration occurs without the presence of both MTSET and MscL combined as the current doesn’t increase or decrease from baseline current, showing that MscL is closed in the DIB and that both the channel and activator are required to see channels forming in the membrane.

In conclusion, the controls mentioned show that the DIB electrophysiology setup is robust and stable, and that ionic current cannot transfer across the impermeable bilayer in the presence of MscL or MTSET alone.

### 5.6.2. Effects of MscL Concentration

While the setup was robust, many results were obtained that showed too much MscL activity, especially when using the same lipid : protein ratio used in the fluorescent DIB assay from *chapter 4*. Therefore, a number of assays were setup to examine the effect of changing the MscL concentration on the electrophysiological recording, with the aim of getting the minimal amount of MscL activity. By achieving the minimal amount of MscL activity, it would be possible to discern smaller changes to both activity and reconstitution efficiency upon changing the membrane properties. Select results of the investigation into MscL concentration are found in Figure 5-12.
Figure 5.12 – Figure representing the effect of MscL concentration on electrophysiological recordings taken in 95:5 DOPC:DOPG DIBs. a) Control current recording for two droplets in presence of MTSET, but without the presence of MscL, b) current trace from 50,000:1 lipid to protein ratio, c) current trace from 100,000:1 lipid to protein ratio, d) above: a selection of focussed sections from the 100,000:1 lipid to protein ratio current trace, below: histograms plotted of each focussed section. A stereo microscopy image is included for reference, the droplet containing MTSET is digitally coloured yellow, the scale bar is 1.5mm. The assay was repeated 3 times, one result is shown above. This figure was adapted with permission from Haylock et al, 2018.2
The results from the systematic investigation into MscL concentration, a selection of which are shown in Figure 5-12, clearly show that in the presence of MscL and MTSET, MscL activity can be attained compared to controls. The results also show that as MscL concentration decreases, the frequency of gating events also decreases in the current trace. This may seem obvious; however, it does show that the proportion of MscL reconstituted into the liposomes in the DIB, directly correlates with the proportion of MscL reconstituted into the DIB. Furthermore, the results of this systematic investigation have shown that at a lipid to protein ratio of 100,000:1, it was possible to start seeing individual MscL channels opening and closing, indicated by characteristic jumps in the focused current traces.

It is noted that in these traces MscL opening corresponds to an opening of approximately 30-40pA which doesn’t specifically match the opening events found in literature\textsuperscript{188,189,191}, though these literature gating currents are from different MscL mutants which could affect channel conductance.

**5.6.3. Lipid Composition Sweeps with MTSET**

As MscL activity was successfully measured in an electrophysiological setup, and a MscL concentration which displayed single channel behaviour was found, it was proposed to investigate the effects of the lipid composition on MscL gating. To investigate lipid composition, the MscL gating event frequency, and the point at which the first gating event was found, was measured in DIBs of different lipid compositions.

The MscL gating event frequency will be proportional to reconstitution efficiency, as the amount of MscL added to liposomes during reconstitution is kept constant, so any changes in the number of active channels will be purely due to lipid composition. The MscL gating event frequency can be determined by single channel analysis using an in-built function in the Clampfit software. The single channel analysis can be subjective as the software essentially needs to be taught what current jumps to look for in each trace, and only the user can select these. However, care was taken to ensure that the results of each single channel analysis was repeatable.
The point of the first gating event was used as a marker for MscL activity. It was proposed that if MscL was more active due to membrane conditions then it would gate earlier. The point of first gating was taken as the first time a full opening event, ≈30-40pA, was obtained. Smaller openings were recorded and it was proposed these signified metastable sub-conductance conformations where the protein isn’t fully open, and therefore these were not taken into account.

**Charged Lipid Composition Sweeps**

The first lipid composition sweep that was proposed was to examine the effect of charge on MscL activity in electrophysiological DIBs. The MscL electrophysiology assay was undertaken on DIBs composed of the charged lipid DOPG, and MscL activity was measured with MTSET as the activator. In these assays the lipid-to-protein ratio was 100,000:1. The maximum amount of DOPG measured was 20% due to instability caused by charge in the DIB, above 20% the droplets merged before the 30-minute time frame of the assay. The effect of charged lipids on DIB stability was more pronounced in these electrophysiological assays, meaning the DIBs are more unstable at higher DOPG compositions compared to the fluorescence assay. It was proposed that the voltage clamp caused a destabilisation effect on the electrostatic interactions in charged membranes. A selection of the electrophysiological recordings for the charged lipid sweep can be found in Figure 5-13, along with a graphical representation of the point of first gating and MscL gating frequency.
The results of the charged lipid sweep show that as the composition of charged lipid increases, the MscL gating frequency increases and the point of first gating occurs earlier in the time frame of the assay. These results correlate well with the results of chapter 3 and 4 and literature results\textsuperscript{137}, showing that membrane charge affects MscL activity. Also, each gating event current measured has a similar opening current of between 30-40 pA showing that the MscL full opening is conserved between DIBs of different compositions. However, these results can suggest one of two things, either that both MscL
activity and MscL reconstitution efficiency are increased with increased membrane charge, or that MscL is able to open and close more frequently as a result of increased membrane charge.

It is noted that the difference in MscL gating frequency between compositions is extremely large. While MscL gating frequency increased as a result of increased membrane charge, the results from this take into account each current jump up and down. If the MscL gating event was open for 6 seconds, the software would count this as one gating event, however, if in those 6 seconds MscL opened and closed several times, the software would count this as several events in the same unit time. Purely from visual analysis of the current traces, it would appear that with increasing membrane charge, MscL does seem to be more active, but it also closes more frequently possibly implying some complex specific lipid-protein interactions which have yet to be explored in literature.

**Spontaneous Curvature Lipid Composition Sweeps**

The second lipid composition sweep that was proposed was the examine the effect of spontaneous curvature on MscL activity in electrophysiological DIBs. The MscL electrophysiological assay was undertaken on DIBs composed of varying amounts of the curved lipid DOPE and 5% DOPG, and MscL activity was measured with MTSET as the activator. In these assays the lipid-to-protein ratio was 100,000:1. The maximum amount of DOPE measured was 20% due to instability in the DIB. A selection of the electrophysiological recordings for the curved lipid sweep can be found in Figure 5-14.

![Figure 5-14](image)

The results of the curved lipid sweep are not as clear as the results from charged lipid sweep. Firstly, the background current with DIBs containing DOPE, seems to be increasing, implying that the DIB is forming small pores over time allowing ionic current through. This background current increase is repeatable, though it doesn’t seem to be present in MscL free controls with the same composition, implying a direct interaction between the DOPE and MscL in the bilayer. Secondly the increase in DOPE
increases the noise in the system significantly, the noise could either be the DOPE composition causing metastable sub-conductance states to open allowing a very small amount of ionic current through, or the DOPE is interacting directly with the protein and causing micropores to open. Finally, the point of first MscL gating and the MscL gating event frequency both decrease with increased DOPE concentration, agreeing with literature results of MscL activity in high curvature environments\textsuperscript{107}.

5.6.4. Comparison of Liposomes, DIBs and Electrophysiology

In \textit{chapter 3} and \textit{chapter 4}, lipid composition sweeps were carried out to determine the effect of changing membrane properties on MscL activity in different model membrane systems. \textit{Chapter 4} then compared the two model membrane systems, showing that the trends could be replicated, though there were large differences which could be attributed to changes mechanical environment between the two model membranes. To determine the effectiveness of electrophysiology in membrane protein studies, it was necessary to compare this system to both the fluorescent DIB studies and the liposome studies. However, as the results from the electrophysiological assays did not provide the data to show a rate of translocation, only the basic trends could be discussed.

Charged Lipid Sweeps

The same trends can be seen in liposomes, fluorescent DIBs and electrophysiological DIBs, increasing membrane charge causes increased displayed MscL activity. However, what is made clear from the electrophysiological data, is that it appears both MscL activity and MscL reconstitution efficiency are both contributing to the increase in activity in these assays. The charged lipid membranes are providing a more stable electrostatic environment for MscL to reconstitute into liposomes, agreeing with literature reports\textsuperscript{137}, and that in turn means more MscL reconstitutes into the DIB. Furthermore, the charged membranes are increasing the rate of MscL gating, implying that either direct lipid interactions between the DOPG and MscL are allowing it gate quicker, or that the charged membrane is ‘priming’ the protein to open meaning less MTSET is required to gate then in non-charged membranes. Either way more ‘in-depth’ analysis of the membrane is required to study these effects in more detail.
**Curved Lipid Sweeps**

The results from DOPE sweeps between liposomes and DIBs, clearly shows that increasing DOPE concentration causes a decrease in displayed MscL activity. The electrophysiological data does provide some evidence to suggest that increasing DOPE concentration, reduces MscL reconstitution efficiency in liposomes. This efficiency loss is due to an increase in curvature elastic stress in the liposomes before reconstitution which in turn affects either the rate of MscL reconstitution, or how active the MscL is once it is reconstituted, possibly even affecting the folding of the protein. However, the results in the electrophysiological setup, shown in Figure 5-14, disagrees with the fluorescent DIB data obtained in chapter 4. The electrophysiological data seems to imply that the DOPE causes an active decrease in MscL reconstitution and MscL activity, whereas the fluorescent DIB data implies a far less significant impact of the curved lipid. In the electrophysiology setup a small amount of poration can be measured over time, proposed to be caused by DOPE-MscL direct interactions causing pores to form as the controls show no poration. Therefore, a proposed explanation for the difference between the fluorescent assay DIBs and the electrophysiological DIBs is that the calcein translocation occurs across both open MscL channels and through these small defects in the membrane caused by direct DOPE-MscL interactions.

5.6.5. MscL Mediated DIB network Interactions

One significant advantage of the DIB model membrane is that the system is not limited to two droplets, in the fluorescent assay this networking was difficult to obtain due to concentration gradients across multiple droplets, however the electrophysiological setup is not limited the concentration gradient of a fluorescent dye.

DIBs have been assembled in 2D and 3D networks in literature, mostly facilitated by soluble pore-forming proteins such as alpha-hemolysin (αHL) which self-inserts into the membrane and doesn’t have an open and closed state, it just forms a basic hole in the membrane. These αHL networks have been shown to possess collective properties by utilising mutated forms, allowing the assembly of biobatteries and current rectifiers, and more recently exploiting a T7 RNA polymerase coupled with a cell-free expression of αHL to show light sensing networks. Yet despite advances in network properties, they still remain tethered to a pore which is constantly open, also αHL presents another functional drawback of a small channel size. Therefore to impart greater functionality a channel capable of opening and closing in response to stimuli must be used.

This section details the first published attempts to gain networked interactions between multiple DIBs using a gated ion channel as a mediator for the network of interactions. A schematic for these MscL-mediated networked interactions is shown in Figure 5-15.
Figure 5-15 shows a three droplet DIB network with MscL reconstituted into each bilayer. Aqueous droplets containing MscL reconstituted liposomes are positioned into contact in an oil well and DIBs form between each droplet in the same manner as two droplet DIB formation. MscL reconstitutes into each bilayer and is initially present in the closed state. The chemical activator MTSET is present in the first droplet, it activates MscL in the first bilayer and diffuses into the second droplet through the open MscL channel where it activates MscL in the bilayer between the second and third droplet. Channel activity is measured in the same way as DIB measurements from the previous sections though electrical measurements via agar coated Ag/AgCl electrodes present in each terminal droplet. In this electrophysiological setup there must be an unbroken link between the two electrodes to allow ionic current to transfer, therefore MscL channels in both bilayers must be gated to measure the characteristic current jumps.

The first attempt at networking MscL containing DIBs was used to show that it was possible to have two channels open at the same time, and get current measurements for them. Therefore, the first assay had 95:5 DOPC:DOPG MscL containing liposomes in each of the side droplets, allowing MscL to reconstitute into each bilayer, and 300mM MTSET was included in the middle droplet MscL-free liposomes. It is noted that initial assays with 100,000:1 lipid to protein did not achieve any current measurements and so the MscL concentration was doubled to 50,000:1 lipid to protein ratio to allow for the fact that the assay needs two MscL channels active at the same time. A selection of results from the assay with MTSET in the middle droplet can be seen in Figure 5-16.
The results for including MTSET in the middle droplet, shown in Figure 5-16, clearly shows MscL activity by characteristic jumps in the current trace as ionic current passes through MscL channels in each DIB of the network. A significant decrease in background current was observed for these three droplet assays compared to the two-droplet system, most likely due to the reduction in bilayer capacitance owing to two capacitors connected in series. This hypothesis was proven by showing that in the microscopy images the DIB was approximately 4mm, but the capacitance traces showed half the normal capacitance of a two-droplet system. Also observed is that the MscL gating currents are approximately 30pA, in agreement with the MscL gating currents observed in the two droplet systems, this is explained by the current output being the same for one resistor and two resistors in series.

Another observation noted was that the current trace appears to be very smooth, with no sharp 10-20 pA peaks associated with MscL sub-conductance or MscL reconstitution. This is not unexpected, as in order to measure sub-conductance states and other membrane associated noises both MscL
channels in each membrane would need to be meta-stable gated at the same time, which is extremely unlikely.

5.6.6. Ion channel mediated communication in a DIB network

The next assay intended to show communication between the two bilayers, and therefore the assay was repeated but with MTSET in the first droplet as shown in Figure 5-15, while 50,000:1 MscL liposomes were in the second and third droplet. Under these conditions MTSET is accessible to the MscL in the first DIB but must diffuse through at least one open channel to trigger the opening of MscL in the second bilayer. A selection of result from the assay with MTSET in the first droplet can be seen in Figure 5-17.

![Diagram of DIB network with MTSET in first droplet and MscL in second and third droplets.](image-url)

The results for including MTSET in the first droplet, shown in Figure 5-17, were found to initially have very brief, less than 0.1 seconds, gating events of approximately 20pA shown by the first two focussed...
sections of Figure 5-17c & d. These smaller gating events were associated with one full open channel in the first DIB, and a sub-conducting channel in the second DIB, likely due to very low MTSET concentrations in the second droplet. The open channel in the first DIB would allow an ionic current of about 30pA but would be entirely limited by how much ionic current could pass through the subconducting channel. Later in the time frame of the assay a gating current of 30pA was held open for several minutes prior to closing, it is proposed this indicates a full gating event of MscL in both DIBs at the same time. It is likely that the delay in full opening of MscL channels in both membranes is owed to the time taken for MTSET to diffuse across a channel in one bilayer and into the next droplet, as lipid bilayers are known to be impermeable to MTSET. 

5.6.7. MTSET Diffusion Calculation

In order to check our hypothesis about the time taken for MTSET to transfer between droplets, a calculation was carried out on the diffusion of MTSET using an adaptation Fick’s first law using the same equation used for the diffusion of calcein in chapter 4. The diffusion coefficient of MTSET, found in literature, and the larger droplet size was then used to graphically display the diffusion of MTSET through a single MscL channel over time. The results from this calculation are seen in Figure 5-18.

![Graph showing the results of a Fick’s first law calculation of the change in MTSET concentration in the second droplet of a DIB assuming MTSET cannot diffuse across the DIB, and assuming only one MscL channel of 3nm diameter and 10nm thickness.](image-url)
The graph in Figure 5-18 shows that in the time frame of the assay, the concentration of MTSET in the second droplet is orders of magnitude smaller than in the first droplet. This approximation supports the results showing a large delay before parallel MscL gating events can be measured, though only up to 5 MTSET molecules are necessary to bind for MscL gating\textsuperscript{197,198}. It is noted that this adaptation of Fick’s first law just shows the change in concentration across the bilayer, and doesn’t take into account the time taken for MTSET to diffuse from the first DIB, across the middle droplet, to the second DIB. However, it is proposed that the diffusion across the channel would be the rate limiting step in the diffusion of the molecule.

In summary, this section has shown that it is the first successful DIB network showing communication purely through ion channel gating events. While a chemical stimuli was used in these networks, the same MscL activation could be achieved by many other factors. The networks were shown to be flexible, though limited by MTSET diffusion. If another activator were used that did not require chemical concentration gradients, such as light, then the DIB networks could be extended in 3D to almost infinite size and geometry.

### 5.6.8. sPLA\textsubscript{2} Controls

While MTSET – MscL results are necessary as a proof-of-concept, and provide a number of interesting results analysing MscL activity, the next stage was to prove protein-membrane-protein communication in an electrophysiological setup. The basis for sPLA\textsubscript{2} work in DIBs was shown in chapter 4, including an assay on how much sPLA\textsubscript{2} is necessary to both activate MscL and not cause instability in the DIB. Nevertheless, a control assay was carried out to see if sPLA\textsubscript{2} had a porating effect on DIB without the presence of MscL that could be present in this electrophysiological DIB setup but not present in the fluorescent DIB setup. It was proposed that the insertion of sPLA\textsubscript{2} into the bilayer could cause micro-pores to form as it ‘pushes’ lipids in order to insert, pores that allowed ionic current to pass through but not calcein translocation. A control was setup with two droplets, one containing 100aM sPLA\textsubscript{2} and the other containing MscL-free DMM reconstituted liposomes. The results for this control can be seen in Figure 5-19.
Figure 5-19 – Results from a MscL-free control electrophysiological assay in DIBs composed of 95:5 DOPC:DOPG, one droplet containing 100aM sPLA₂. a) a current trace for the entire length of the assay, b) – c) capacitance traces at 5 minutes and 30 minutes into the assay. The assay was repeated 3 times, one result is shown above.

The control results in Figure 5-19 show that sPLA₂ alone cannot cause poration in the membrane as shown by not allowing any ionic current through. Also, the capacitance traces show that sPLA₂ does not significantly change bilayer area. It should be noted that even at this low sPLA₂ concentration, a small number of assays resulted in a merged in the time frame of the assay as sPLA₂ destabilises the membrane.

5.6.9. MscL DIB Electrophysiology assay with sPLA₂

The sPLA₂ controls showed no current leakage without the presence of MscL. This section covers the interactions between MscL and sPLA₂ measured through electrophysiological recordings. In this assay, the MscL should open due to mechanical affects caused by the hydrolysis of lipids in the DIB into lyso-lipids. To investigate this fully a two-droplet electrophysiological DIB was setup with 100,000:1 lipid-to-protein ratio liposomes, with 100aM aPLA₂ was included in the second droplet. The results of this assay are found in Figure 5-20.
The MscL-sPLA$_2$ results clearly show that it is possible to achieve MscL-sPLA$_2$ communication in the electrophysiological DIB setup, shown by the characteristic conductance in recorded ionic current. The gating events have an ionic current of 40pA, in agreement with the gating events with MTSET as the activator. It is noted that in this two-droplet system, there is very little noise in MscL channel gating, sub-conductance states are very rarely seen. It is proposed that the activation of sPLA$_2$ does not go through meta-stable states and either resides in an open or closed conformation, unlike MTSET activation which requires 1 to 5 molecules to allosterically bind, often going through meta-stable gating states and increasing system noise. It is noted again that the 3 results obtained in this MscL-sPLA$_2$ electrophysiological assay were 3 results that were stable for the time frame of the entire assay and many more DIBs merged before the end of the recording and were not taken into account.

After proving that it was possible to attain MscL-sPLA$_2$ communication in the electrophysiological setup, attempts were made to investigate the effect changing lipid composition would have on the protein-membrane-protein communication. However, it proved extremely difficult to create stable DIB assays with both MscL and sPLA$_2$ for any membranes with more than 10% DOPG or DOPE, likely due to sPLA$_2$ having an increased affinity for negatively charged and curved membranes$^{27}$. Attempting to reduce sPLA$_2$ concentration either destabilised the membrane or no-activity was shown at all in the time frame of the assay.
Attempts at gaining sPLA$_2$ activation of MscL in three droplet networks were also attempted but no activity was ever measured. It is proposed that sPLA$_2$ cannot sterically fit through the MscL channel and cannot activate the MscL channel in the second DIB, therefore no ionic current could be measured.

5.7. Conclusions

In conclusion this chapter has successfully shown that MscL activity and reconstitution efficiency can be studied using an electrophysiological assay, and that the effects of membrane properties can be investigated. First proving that the DIB is stable to ionic current under control conditions, even in the presence of sPLA$_2$ hydrolysing the membrane. MscL gating was displayed in the presence of both MTSET and sPLA$_2$, as characteristic ionic currents were recorded under many different conditions. It has been shown that increasing membrane charge affects both MscL activity and MscL reconstitution efficiency. Attempts were also made to investigate curvature in MscL activity, however the results disagreed with the results shown in chapter 4 showing a marked decrease in MscL activity with increasing curvature, whereas in the fluorescence assay the calcein translocation barely changed. It has been proposed that DOPE and MscL have a direct interaction that may cause poration of the membrane allowing calcein translocation, but seen in the electrophysiological assays as a leakage current. sPLA$_2$ lipid sweeps were not possible in this electrophysiological DIB setup due to extreme stability issues.

This chapter has shown the first synthetic bilayer network capable of communicating purely through the gated ion channel, MscL. Using electrical measurements have been used to shown interplay between adjacent membranes, showing that the chemical activator, MTSET, can trigger MscL to open and grant access through the MscL channel to activate MscL in a neighbouring membrane. This work shows a significant advancement in the development of artificial tissues that are able to replicate cell-cell interactions found in biological systems. Both the functionisation of the bilayers, and the interplay between them, could have a profound impact on many areas of model membrane and membrane protein studies, from smart drug-delivery systems to more advanced artificial cells.

The next chapter deals with one of the many applications that can be used by the work achieved in studying MscL activity in DIBs, by attempting to show rheological activation of MscL in a custom designed and built device capable of generating computationally measurable shear force.
Chapter 6

Rheology

6. Overview

While previous chapters in this thesis have been more experimental in nature, studying MscL in different model membranes and attempting to control activity, there are also several applications for the advances in membrane protein reconstitution and their methods of study. One of these application areas is in the subject of rheology. Rheology is broadly an area of research concerned with the flow of liquids, in a medical context its main focus is on blood flow and how the flow of blood affects vessel walls and the components of the blood themselves. Given the mechanosensitive nature of MscL it was proposed that it should be possible to gate MscL by altering the rheological properties of the aqueous phase inside each droplet in a DIB.

A device was designed and built to apply shear stress into a DIB constrained within PDMS wells. The device was originally intended to be used in the measurement of a phenomenon known as the ‘unstirred water layer’ which affects diffusion gradients across bilayers. The device was modelled using a COMSOL Multiphysics set of calculations, and it was theorised that the shear stress generated by the device could be used for more than aiding in the diffusion of membrane permeable molecules.

In this chapter, a short theoretical introduction is given to the area of shear stress. Then, an introduction is given to the device and how it generates shear force upon a DIB. The materials and methods used in the creation of the device are given. Following this are controls showing that MscL can be incorporated into DIBs in the device, and that MscL is active within the device itself. Finally, the results of some of the attempts to gain MscL activity through shear force alone are given, including an attempt to parallelise the DIB assay to gain data in a high throughput manner.

The results from these assays show the opposite of what was originally thought. It was originally thought shear force would cause mechanical gating of MscL, but it was found that shear force actively closes the MscL channel, leading to a complete rethink of MscL structure and function.

The device design, build and test formed part of a publication on generating measurable shear force within DIBs and measuring the effect on diffusion of a membrane permeable dye³. Part of the work from this chapter formed sections of the publication and, where relevant, it has been noted which parts were adapted from publication.
6.1. Introduction

Rheology is a field of study concerned with the flow of materials with both non-Newtonian liquid and solid properties, which undergo plastic deformation. In medical research, the field of rheology involves many of the studies related to blood hemodynamics and coagulation, especially with relation to blood borne diseases. Blood rheology depends upon computer simulations for much of the literature data\textsuperscript{199,200}, as there are very few methods for studying the effects of small currents and movements, especially given the highly complex nature of blood and its components\textsuperscript{200,201}. Devices that can accurately measure microcurrents in blood, especially the shear force generated by fast flowing blood in capillaries, do not yet exist.

Detailed here is a short background behind shear force, followed by an introduction to the rheological DIB (rheo-DIB) device and the computational models used to show how it generates shear force within a DIB system.

6.1.1. Background

Shear stress is defined as the tangential force per unit area exerted by a solid boundary on a fluid in motion, it is also exerted by a fluid in motion on a solid boundary\textsuperscript{202,203}. Shear stresses are proportional to the contact area and the surface forces present at the boundary, therefore shear stresses are measured in N/m\textsuperscript{2} or Pa. While there is a normal force (dF\textsubscript{n}) exerted by a liquid in motion on a solid boundary, the shear forces are specifically perpendicular to the contact area (dF\textsubscript{s}) and are a result of a liquid in motion passing over a solid boundary. An example of these two forces can be found in Figure 6.1.

![Figure 6.1](image)

Figure 6.1 – Schematic showing the two different surface forces exerted by the environment through direct contact. The surface stress can be broken down into a normal force (dF\textsubscript{n}) and a component which is tangential to the contact area called shear force (dF\textsubscript{s}). Figure taken from Katritsis, 2007\textsuperscript{202}. 
Shear forces have been shown to play a role in many different biological areas. Shear forces have been used to induce membrane mechanical properties in a static lipid membrane, and have been shown to be affected by the fluidity of the lipids in the membrane\(^2\). As well as affecting the membrane properties themselves, shear forces have been shown to be involved in the regulation and activity of certain membrane proteins\(^3\). The shear forces present affect proteins that extend out from the membrane more than proteins that sit ‘flush’ with the membrane itself, as the shear forces are divided over a 2D area in a flat membrane, but over a 3D area with a projected molecule\(^4\). As shear forces can affect membranes and membrane bound proteins it has been implicated in a number of different disease states including atherosclerosis, as the build-up of plaques affects the turbidity of blood flow\(^5\). Blood cells have also been shown to be damaged by shear stress, indicating that the shear of the blood doesn’t just affect the vessel walls but also the contents\(^6\).

As MscL has been considered for blood based pharmaceutical delivery purposes\(^7\), it is important to understand how the shear forces present in the blood itself might affect it’s delivery properties, or be used as the delivery mechanism itself. MscL has shown mechanosensitive properties to mechanical forces directly applied to the membrane\(^8,9\), but it has not yet been shown to be affected by controlled tangential forces. As MscL is a mechanosensitive protein, it stands to reason that shear forces should affect activity, either through affecting the membrane mechanics or the protein itself. However, to date it has been difficult to generate shear forces on membranes within controllable environments, simulating the shear experienced in turbulent blood.

### 6.2. Introduction to Device

While the rheo-DIB device was designed to study the permeability of a membrane permeable dye, and the effect of shearing inside a DIB on diffusion gradients, this chapter has adapted the device to studying the effect of shear stress on an MscL reconstituted DIB. An short introduction to the device, and the computational studies carried out, is outlined in this section.

The spinning disk rheo-DIB device utilises a disk applied above a DIB well to induce a flow in the oil adjacent to the droplets and the DIB. The rate of spinning of the disk is directly proportional to the rate of flow of the oil phase. The oil phase flows over the top of the DIB in the well, which shears the droplets as it passes over them. The shearing oil phase induces a circular flow in the DIB as the two droplets rotate in place along the axis of flow. This circular DIB flow is shown in Figure 6.2a. Figure 6.2b shows a COMSOL model of the induced flow caused by the rotation of the droplets, indicating that the velocity of the induced flow in the droplet varies across the DIB.
Figure 6.2 – a) Schematic of the velocity profile of the RheoDIB device for a 2D COMSOL model of the rheo-DIB chip. A sliding wall flow 1mm above the droplets introduces a shearing phase in the y-axis. The shearing flow induces rotational motion in each droplet of the DIB, rotating in plane with shearing flow. The rotation of the droplets induces flow inside each droplet, inducing circulation and shearing on the DIB membrane surface. b) The COMSOL model result of the flow inside each droplet of the DIB assuming the shearing flow of hexadecane above the droplets. The velocity magnitude inside the droplets show the velocity is highest near the top oil-water interface.

To better illustrate how the device works, a DIB was constructed loaded with 5µm polystyrene latex beads, a method often used in literature to display rheological flow\textsuperscript{208}. Each droplet had approximately $10^6$ particles per droplet, and the DIB was recorded at 50 frames per second under a 100RPM disk speed. A z-projection of all 50 images was constructed and is shown in Figure 6.3b. The spherical shape of the droplets leads to a symmetrical vorticity shown in a schematic in Figure 6.3c. The symmetrical vortexes were then verified using a COMSOL model, and agree well with simulations and experiments\textsuperscript{209,210}. The vortices present in the droplets under an oil shearing flow are capable of generating a measurable amount of shear force upon the membrane. The amount of shear force generated is covered in more detail in the results section of this chapter.
6.3. Materials

This section details the materials used in MscL DIB rheological assays and controls used in this chapter. It is noted that the materials used in generating the DIB itself are identical to those used in the fluorescent DIB assays in chapter 4. Parts of the materials section are directly adapted from published work on rheological DIBs.

6.3.1. Preparation of Liposomes

All lipids mentioned were purchased from Avanti Polar Lipids. The lipids were purchased as a lyophilised powder with a purity of >99%. The lyophilised lipids were stored at -20°C until needed. The lipids used were 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (sodium salt) (DOPG), and 1,2-di-O-(9Z-octadecenyl)-sn-glycero-3-phosphocholine (Diether PC). When needed, the
lipids were dried on a Thermoscientific MicroModulyo Lyophiliser, then dissolved in chloroform purchased from VWR. The lipid samples were extruded using an Avanti Mini Extruder using polycarbonate membranes and filter supports purchased from Avanti Polar Lipids.

6.3.2. MscL Reconstitution & DIB assay
The detergent n-Octyl-β-D-Glucopyranoside (OG) was purchased from Sigma Aldrich. The [2-(Trimethyl ammonium) ethyl] Methane Thiosulfonate Bromide (MTSET) was purchased from VWR. The secretory Phospholipase A2, sPLA₂ isolated from honey bee venom (Apis mellifera) was purchased as a lyophilized powder from Sigma Aldrich. The OG, MTSET and sPLA₂ were stored at -20°C until required. Biobeads SM2 with mesh size 20-50 were purchased from BioRad and calcein was purchased from Sigma Aldrich.

Two separate buffers were used in these assays, one reconstitution buffer for the MscL containing droplet and one non-fluorescent buffer used for the other half of the DIB. The components for the buffers used are listed below. Unless previously mentioned, all buffer components were purchased from Sigma Aldrich.

- **Reconstitution Buffer**: 100mM KCl, 20mM HEPES, 40mM OG, 50mM Calcein, pH 7.2.
- **Non-fluorescence droplet buffer**: 100mM KCl, 20mM HEPES, 0.5M Sucrose, pH 7.2.

All solutions were pH adjusted using 1M stock solutions of Sodium Hydroxide (NaOH) and Hydrogen Chloride (HCl) purchased from Sigma Aldrich.

6.3.3. Rheo-DIB Chip Manufacture
PMMA sheeting was purchased from Weatherall UK and laser cut on a Universal Laser Systems VLS 3.60 platform. The laser cut PMMA layers were solvent bonded using acetone purchased from Sigma Aldrich. For the proof-of-concept device, metal radial ball bearings, 2mm ID, 6mm OD, were purchased from RS Components. The device rotates by means of a brushed DC motor (7.98 watt/15 volt).

6.4. Methods
This section outlines the protocols required to produce the rheological DI assays used in this chapter. It is noted that the methods used in generating the DIB itself are identical to those used in the fluorescent DIB assays in chapter 4. Parts of the methods section are directly adapted from published work on rheological DIBs³.

6.4.1. Preparation of Liposomes
The lipid mixtures were weighed in a molar ratio, dissolved in chloroform, and gently mixed to ensure all lipids were dissolved. The chloroform was evaporated under a steady stream of nitrogen, and the
resultant lipid film was placed in the lyophiliser overnight, or for a minimum of 2 hours before hydration. Dry lipid films were hydrated in the reconstitution buffer or non-fluorescent droplet buffer listed in the Materials section at 12.7mM, unless stated otherwise. Each sample was freeze-thawed 4 times using liquid nitrogen and a hot-air gun, samples were vortexed before each freeze stage to ensure homogenisation. The lipid samples were extruded through a 100nm membrane 11 times, to produce monodisperse unilamellar liposomes.

6.4.2. MscL Reconstitution
For each mixed lipid-detergent liposome sample prepared as described, 3 independent aliquots of 100mg of Bio-Beads SM2 were prepared and allowed to equilibrate for 1 hour in detergent-free reconstitution buffer. Purified MscL was added to prepared mixed lipid-detergent liposomes at a lipid-protein ratio of 1:10,000, unless otherwise stated, and was allowed to equilibrate for 1 hour on rollers in a cold room at 4°C. In order to remove the detergent, the MscL reconstituted liposomes were incubated with 100mg of equilibrated Bio-Beads for 1 hour, on rollers, in a 4°C cold room. After 1 hour, the 100mg of Bio-beads were replaced and the process was repeated twice more. After 3 hours of Bio-Bead incubation, the Bio-Beads were removed and the MscL reconstituted liposomes were stored at 4°C until used. For MscL free controls, an equivalent amount of DDM was added to the mixed lipid-detergent liposomes, and the same detergent removal process was followed.

6.4.3. Rheo-DIB Chip Manufacture
To build the Rheo-DIB devices, the laser cut 1mm thick PMMA layers were solvent bonded with acetone as shown in Figure 6.4. The well plate and base plate were bonded with acetone to form the DIB wells and the spacer plate is added to keep the disk height constant. The well plate has various 1mm diameter DIB wells cut into it, 17mm from the centre of the device. The overflow plate was bonded to the spacer plate to prevent shearing fluid loss. The pin is bonded perpendicular to the base plate which allows for the disk to be set with ball bearing and pulley. The spacer plate has a hole cut in slightly smaller than the spinning disk to allow the disk to rest on the edge of the spacer plate. The overflow plate as a hole cut through slightly larger than the spinning disk to allow room for rotation. For the proof-of-concept device the disk was 6.3cm diameter and included the two ball bearings shown in Figure 6.4. For the high-throughput device, the disk was 13.9cm diameter and was bonded to the belt pulley without the ball bearings as they were not needed to achieve smooth rotation. The disk and device were thoroughly cleaned and dried every time a DIB was replaced.
6.4.4. DIB assay

All DIBs in this chapter were formed using the *lipid-in* method. A 0.5μl droplet of both the MscL reconstituted liposomes and 0.5μl droplet of the non-fluorescent liposomes containing 300mM MTSET, were injected into the hexadecane filled wells in the Rheo-DIB devices. The droplets were left to equilibrate in the oil for 5 minutes to allow time for monolayer formation around the outside of the droplet. The droplets were brought together and a DIB was allowed to form.

For the proof-of-concept Rheo-DIBs, each DIB was visualised by a fluorescence inverted microscope (Nikon Eclipse TE 2000-E) with a mercury lamp (Olympus) and a QICAM camera (QImaging Media Cybernetics UK). Fluorescence excitation was achieved using a FITC dichroic filter (460-490nm excitation, Chroma Technology Corp). The software used to control the microscope was built in house and programmed in LabView. All images were taken with a 4x magnification.

For the high-throughput Rheo-DIBs, the Rheo device was too big for the microscope and so was mounted on an Olympus SZ-STL non-reflective base and imaged with a QImaging Retiga EXi Fast 1394 camera at 400 ms exposure time. The source light was filtered by a Semrock RazorEdge 532 nm short pass filter and the DIBs are illuminated directly from the side (90° from the imaging direction). The fluorescent DIB images were acquired with a 575–625 nm band pass filter. The light source consisted of a fibre optic cable attached to a Stocker Yale Imagelite m20 broad spectrum light source set to maximum output. A diagram for the high throughput Rheo-DIB setup can be seen in Figure 6.5.

Figure 6.4 – Schematic of Rheological-DIB chip assembly. Note this schematic is not to scale. Figure adapted with permission from Barlow, Bolognesi, Haylock et al, 2018.
Figure 6.5 – Schematic for the high-throughput Rheo-DIB setup. a) photograph of the larger rheo-DIB device for high-throughput DIB studies, 10 columns of 8 wells were drilled into the well plate, b) example of a DIB well from the device, c) Schematic of the bespoke kit consisting of an Olympus stereoscope stand with non-reflective base, the device into mounted below a microscope camera fitted with a macro lens and band pass filters (575 - 624 nm). The device is illuminated with a broad spectrum light source where the output is filtered by a short pass filter (532 nm). Uniform illumination is achieved by positioning the fibre optic cable to optimise intensity distribution. Figure adapted with permission from Barlow, Bolognesi, Haylock et al, 2018.

6.4.5. Results Analysis

All microscopy images obtained during DIB assays were analysed using ImageJ software version 1.8.0.112. The width of the DIB was measured in pixels and converted to absolute length using a scale. Fluorescence image analysis was achieved using image integration to quantify intensity changes. The image integration outputs from a specified area and so for each image integrated the region of integration was plotted smaller than the droplet measured to exclude all background pixels. For each set of results the integration area was kept constant, which proved difficult if the droplet moved or changed size. An example of image integration can be found in Figure 4.9. Data obtained was plotted and analysed using Origin.
6.5. Results and Discussion

This section details the results from the translation of the fluorescent DIB assay from chapter 4 into a rheological DIB (rheo-DIB) setup. This section starts with controls assays undertaken to ensure that the DIB is stable, and calcein cannot translocate through rheological force alone. Then the results of proof-of-concept assays looking at MscL activity in rheo-DIBs are provided. Following this are the results of the high-throughput rheological DIB assays.

It is noted that the building of the device used in these results was published³, and where relevant it is noted what assays were adapted for publication.
6.5.1. Control Assays

The control assays undertaken in the rheo-DIB setup are presented in this section. The control was intended to show that DIB was stable under the rheological shear stress environment for the time scale of the assay. It was also intended to show that the membrane shearing force did not induce poration and allow calcein to translocate across the non-permeable membrane. It was proposed that the shearing force could open pores large enough for molecules to translocate across. Therefore, a rheo-DIB was setup without MscL made of 95:5 DOPC:DOPG, and the fluorescence intensity of the non-fluorescent droplet was measured without shearing for 20 minutes, the DIB was put under membrane shearing for 2 hours and fluorescence intensity was measured. Figure 6.7 shows the results of this control assay.

Figure 6.7 – Control assays of RheoDIBs without the presence of MscL. The DIB was formed from 95:5 DOPC:DOPG with 50mM calcein. A 200RPM shearing force was applied for 2 hours. The image contrast has been adjusted for clarity. Each data point is an average of 4 DIBs, the error is one standard deviation. Figure adapted with permission from Barlow, Bolognesi, Haylock et al, 2018³.
The control assays, shown in Figure 6.7, exhibit an initial background fluorescence which shows no appreciable increase in intensity over the first 30 minutes under stagnant conditions. After 2 hours of shearing at 2 hours, no fluorescence intensity increase is apparent. If shear induced poration had occurred, then the fluorescence intensity in the non-fluorescent droplet would have increased. It is noted that the calcein containing droplet did seem to decrease in fluorescence, this is attributed to photobleaching.

It is also noted that the DIB looks like it has moved in the control DIBs shown in Figure 6.7. This DIB movement was common in each of the rheo-DIB assays as the droplets would shift within the DIB wells affecting DIB size. The shear forces generated by the shearing disk on the droplets deformed the droplets, and caused the droplets to move. The movement of the DIB could not be prevented in these assays, however, attempts were made to ensure the droplets fit exactly into the DIB wells to maintain DIB size.

6.5.2. Proof-of-Concept Rheological Assay

In this section is detailed the results from the first proof-of-concept assay carried out to investigate if the shearing forces generated by the rheo-DIB device were capable of gating the MscL channel. These initial assays were undertaken in 95:5 DOPC:DOPG DIBs with 10,000:1 lipid to protein ratio and 50mM calcein. These initial assays are shown in Figure 6.8.
The results in Figure 6.8 show that there was no measurable MscL activity in the time frame of the assay, shown by having no significant increase in the fluorescence intensity over time. This result was disappointing as the literature assays involving the mechanical activation of MscL were often successful\cite{188,189,211}.
In order to check that the shear force upon the membrane is sufficient to open MscL, a 2D COMSOL model was used to calculate the average shear stress across the membrane. The shear stress on the membrane was calculated as a function of the aqueous viscosity and the gradient of the velocity in the z direction. The average shear stress was then found by taking the integral of the shear stress from top to bottom in the z direction. The results of this model are shown in Figure 6.9, a diagram of the variable shear stress along the z direction is also shown to show that the highest stress is present at the top of the membrane and drops to zero closer to the bottom.

Once the average shear stress across the membrane had been modelled, it was then possible to convert shear stress into the force applied to the membrane using the equation below:\(^202\):

\[
\tau = \frac{F}{A}
\]

In this equation, \(\tau\) is the shear stress, \(F\) is the force in newtons, and \(A\) is the membrane area in metres. Using the shear stress modelled at 200RPM, and the area of the DIB calculated from images, it was then possible to calculate the amount of force applied to the membrane. Therefore, at 200RPM, the amount of force applied is 22.4 nN. In literature the amount of force shown to be required for MscL gating is 1.13pN\(^{188}\) which is orders of magnitude below the amount of force generated by this device. Therefore, in this device at 200RPM, there should definitely be enough

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Figure 6.9 – A graph showing average shear stress (Pa) on the DIB membrane as a function of the velocity of the rotating disk. Data is calculated from a 2D COMSOL model. The shear stress on the membrane is an average of the variable shear stress along the z-axis from -0.3mm to 0.3mm. Figure adapted with permission from Barlow, Bolognesi, Haylock et al, 2018\(^3\).
mechanical stimulus to gate MscL, so there must be another reason why MscL activity could not be measured in the rheo-DIB device.

It is noted that the force applied by the vortexes inside each droplet is not applied evenly across the entire DIB, and therefore this equation is limited in scope. However, a much larger simulation would be needed to define exactly how much force is applied to the DIB.

6.5.3. MTSET MscL Activity in the Rheo-DIB Device

It was proposed that the device itself was causing a deactivation of the MscL reconstituted into the DIB and therefore another assay was proposed. In this assay a DIB would be set up with one droplet containing MscL reconstituted at 10,000:1 lipid to protein ratio and 50mM calcein, and the other droplet would contain 300mM MTSET. The DIB would be allowed to form and be left for 10 minutes without the disk spinning, giving time for MTSET to activate reconstituted MscL and calcein to translocate across the DIB. Then the shearing would be activated at 200RPM for 10 minutes and the fluorescence in the second droplet would continue to be measured. If the MscL was still active in the DIB under these conditions then calcein should still be able to translocate across the MTSET activated MscL channel, and a fluorescence increase should be measured. After 10 minutes of shearing, the shearing would be stopped and the fluorescence would continue to be measured to check monitor MscL activity.

Another rheo-DIB assay was also proposed with the same DIB setup, one droplet contained MscL reconstituted at 10,000:1 lipid to protein ratio and 50mM calcein, and the other droplet contained 300mM MTSET. In this assay the DIB would be allowed to form, and the fluorescence of the non-fluorescent droplet would be monitored. In this assay, immediately after DIB formation the shearing would be applied at 200RPM for 20 minutes, then the shearing would be stopped and fluorescence would continue to be measured. The results from these two assays are shown in Figure 6.10.
Figure 6.10 – A graph to show the change in mean fluorescence intensity for the non-fluorescent droplet. The DIB was formed from 95:5 DOPC:DOPG, with Mscl reconstitution at 10,000:1 and 300mM MTSET was in the non-fluorescent droplet. Shearing was achieved by spinning disk at 200RPM. Each data point is an average of 4 DIBs from the same Mscl reconstitution, the error bars are one standard deviation.

The results from Figure 6.10 were quite surprising. These show that the Mscl channel is active within the device, as shown by an increase in fluorescence in the non-fluorescent droplet with MTSET presence and without the rotation of the disk. However, as soon as the shearing force is applied, Mscl activity is entirely stopped shown as no appreciable change in the fluorescence while shearing forces are applied. Almost as soon as the shearing force is stopped, Mscl activity resumes again as shown by an increase in fluorescence for both assays.

This result shows that the act of shearing within the rheo-DIB device actively prevents Mscl activity, the opposite of the result that was expected. It is proposed that the main reason for Mscl deactivation is due to the method of shearing within the device. A COMSOL model of the specific method of shearing is shown in Figure 6.11.
As shown in Figure 6.11, the droplets in the rheo-DIB fully rotate as the shearing disk spins. This rotation means that the two leaflets of the DIB are constantly in antagonistic movement. It is possible that the constant lipid motion within the DIB does not provide a suitable environment for MscL reconstitution and therefore no MscL activity is detected while the shearing takes place.

If this proposal is correct then it should be possible to still get MscL activity under slower shearing speeds, however attempts to gain MscL activity under even the slowest shearing speeds was not possible. It was hoped that by performing the assay in a larger device, allowing a slowing shearing speed, and measuring multiple DIBs at the same time would allow for active MscL activity measurements.

6.5.4. High-Throughput Rheological Assay

A larger rheo-DIB device was designed and built, allowing multiple DIBs to be measured at the same, but also allowing a slower shearing speed to be reached. The large rheo-DIB device, and an example of the amount of DIBs that can be measured at once is given in Figure 6.12.
Once the larger device was setup, the same DIB assays as before was set up made of 95:5 DOPC:DOPG with one droplet containing a MscL reconstitution of 10,000:1 lipid to protein ratio. Then 16 DIBs per assay were setup, DIBs were allowed to form without shearing for 5 minutes. The shearing was then started at 5RPM and increased in 5RPM jumps up to 40RPM. The DIBs were left in each shearing speed for 5 minutes before the shearing speed was increased. The results from this larger rheo-DIB assay are shown in Figure 6.13.
Figure 6.13 – The results from the larger rheo-DIB assay with DIBs composed of 95:5 DOPC:DOPG and containing MscL reconstituted at 10,000:1 lipid to protein ratio. a) a fluorescence microscopy image of the entire field of view for the assay, the image contrast has been adjusted for clarity, b) – c) focused fluorescence microscopy images of DIBs at different time frames, image contrast has been adjusted for clarity, d) a graph showing the average fluorescence of the non-fluorescent droplet for every droplet that did not merge over the time scale of the entire assay, each data point is an average of 10 droplets as 6 of the DIBs had merged before the end, the error is one standard deviation. The scale bar for the whole field of view is 4mm, the scale bar for the focused section is 500μm.
The results in Figure 6.13 show possible MscL activity as the fluorescence intensity in the non-fluorescent droplet shows increase between 15 and 30RPM, however the increase appears to be quite small and the error is very large as many of the DIBs measured did not increase in intensity at all. However, looking at individual DIBs over the time frame of the assay it is possible to say that MscL has been activated by shear force alone, shown in Figure 6.14.

Figure 6.14 – Graph showing the fluorescence increase of the non-fluorescent droplet in a DIB composed of 95:5 DOPC:DOPG and containing MscL reconstituted at 10,000:1 lipid to protein ratio. This is an isolated result of one DIB from the result in Figure 6.13 to better show MscL activity.

The result in Figure 6.13 shows that it’s definitely possible to get MscL activity purely though shear force alone, as the fluorescence intensity of the non-fluorescent droplet increases over time at a shearing speed of 15RPM. Using the equation in mentioned earlier in this chapter, it is possible to estimate the force applied onto the membrane at 15RPM. At 15RPM, the force applied would be approximately 2.8nN which is still orders of magnitude above the force required to gate MscL.

This result is very interesting as it shows that at low shearing speeds MscL activity can be measured, yet at high speeds MscL activity is completely dampened. It is proposed that in this device a balance
needs to be maintained between generating enough force on the membrane, while not causing disruption to the membrane by rotating the droplets too quickly. A more in-depth study into the generation of shear force in a DIB will need to be carried to order to determine the exact cause of MscL deactivation, but the result shows that the rheo-DIB system is effective in the generation of shear force within a DIB.

**6.6. Conclusions**

In conclusion, this chapter has shown one of the many applications for the work of previous chapters. Optimising the conditions for membrane protein reconstitution and activation in different model membranes, with different mechanisms of study, has meant that transferring the setup into different devices was facile. The rheo-DIB device was originally designed for looking at permeability assays for membrane permeable dyes, but the device has been shown to be easily applicable to other situations.

Controls were undertaken to show that the rheological shear force applied by the device does not cause poration of the DIB and no calcein translocates under these conditions. Then, an attempt was made to activate MscL using a shearing speed of 200RPM. At this speed it appeared that MscL activity was completely dampened, and an assay using MTSET as an activator showed that in the absence of the shearing force, MscL activity could be measured. It was proposed that the shearing force itself was causing MscL deactivation as the droplets were rotating and causing an antagonistic friction in the bilayer. By using a slowing rotational speed MscL activity was gained by mechanical activation alone. Future studies will need to take into account a balance between generating enough force on the membrane to active MscL, and not producing an inhospitable environment for membrane protein activity due to droplet rotation.

While this chapter provided a result, which was slightly different to what was expected, it has opened up whole new avenues of research into mechanical stimulation of a model membrane which can be modified and optimised by future generations.
Chapter 7

Future Work

7. Overview

The outlook and direction for the work of each separate chapter has been outlined in their relevant conclusions section, however this chapter intends to show that the field is extremely adaptable and applicable to many areas.

The platform technology, and the ability to translate each assay between devices and methods of study, is not only useful for looking at the activity of MscL but also the assays and technologies can be applied to a range of membrane proteins and activation mechanisms. There are currently a number of projects already underway which are using the results and knowledge gained in the development and optimisation of these assays to study MscL activity with a range of amphipathic proteins. A brief outline of this work is shown in this chapter.

In addition, the work optimising the reconstitution of MscL in different model membranes has led to a series of assays looking at how MscL is oriented in model membrane systems. This work uses oriented synchrotron circular dichroism to investigate how MscL reconstitutes into bilayers with respect to whether it reconstitutes perpendicular to the membrane, or in a bent conformation. The reconstitution orientation is then studied with respect to the pressure of the system. A brief outline of this work is shown in this chapter.

Parts of this chapter are going to eventually form part of published collaborations, though the publications have yet to be finalised. Included is preliminary data on studies which are currently being worked on, showing the ways that the work in this thesis are already being applied to various applications.
7.1. Amphipathic Proteins and MscL

As has been shown in this chapter, it is possible to measure MscL activity with a range of activation mechanisms. It has been shown that changes in membrane mechanics can affect MscL activity, through activation of MscL with sPLA₂. It was proposed then that the same MscL response could be triggered by a range of proteins that cause changes in membrane mechanics. Thus protein-membrane-protein communication should be promiscuous, allowing any two proteins that cause and respond to changes in membrane mechanics regardless of which proteins they are.

Therefore, a set of proteins was found that each displayed amphipathic properties. Amphipathic proteins contain both a hydrophobic component and a hydrophilic component, and these proteins are known to insert their hydrophobic components into the lipid bilayer to minimise aqueous interactions. A schematic for the insertion of a protein into a bilayer is shown in Figure 7.1.

![Figure 7.1](image-url) -- A proposed schematic for the thermodynamic cycle for the partitioning of amphipathic helices at a lipid droplet surface. The amphipathic helix is initially unfolded and soluble, this protein then inserts its hydrophobic residue into a packing defect in the lipid droplet surface (I), the helix then folds separating the aqueous phase from the hydrophobic core (II), high surface pressure then promotes desorption of the helix (III) which eventually unfolds (IV). Figure taken from Prevost, 2018.
This insertion into the bilayer, then causes the lipids to be ‘pushed out’ and membrane tension rises accordingly. It was proposed that this increase in membrane tension due to protein insertion would be enough to cause MscL activity\textsuperscript{212}. Therefore, a set of assays were set up both in liposomes, identical to \textit{chapter 3}, and in electrophysiological DIBs, identical to \textit{chapter 5}. Except that as well as showing MscL activity with MTSET and sPLA\textsubscript{2}, MscL activity would be measured after the addition of a set concentration of an amphipathic protein. The amphipathic proteins that were chosen were α-synuclein\textsuperscript{213,214}, Lysozyme & Amyloid Beta\textsuperscript{215,216}, which all showed amphipathic properties in literature.

Preliminary electrophysiological results shown in Figure 7.2 show that MscL activity can be gained by insertion of the amphipathic α-synuclein. However, the results imply that the gating is not a full channel opening, and imply that the insertion of α-synuclein is causing MscL to consistently enter a meta-stable sub-conductance state. It is proposed that the membrane tension arising from the amphipathic protein insertion is not sufficient enough to overcome the gating threshold for MscL. Though these results are still preliminary, and need to be investigated further.

It should be noted these results are an example of the MscL amphipathic protein assay, and that they were recorded in collaboration with James Hindley. These results will eventually form part of a publication examining the effect of a range of amphipathic proteins on MscL activity.
Figure 7.2 – an example of the electrophysiological results of 95:5 DOPC:DOPG DIBs with MscL reconstituted at 100,000:1 lipid to protein ratio. The left hand side is a focussed section displaying MscL activity for a number of seconds, allowing direct comparison of the activity, the right hand side is a histogram of the activity and the full current trace from the assay. a) a control assay with MscL reconstituted but no activator present, b) MscL activity with MTSET present as a chemical activator, c) MscL activity with sPLA2 present as a membrane mechanics activator, d) MscL activity with α-synuclein present as a membrane mechanics activator, the MscL channel current is lower than the channel current present with other activators, indicating that MscL is not fully opening and ‘flickering’ into a meta-stable sub-conductance state. These results were undertaken in collaboration with James Hindley, and are forming part of a publication on this topic.
7.2. Oriented Synchrotron Circular Dichroism

Circular dichroism (CD) is a technique that uses plane polarised light to examine the structure of proteins in solution\(^{217}\). A plane of polarised light can be thought of as possessing a clockwise (L) and counter-clockwise (R) component. If after being passed through a sample, the L & R components are absorbed to equal extents, then the recombination of the L & R components would regenerate the original plane. However, if the L & R components are absorbed to different extents, then the resulting radiation would possess polarisation. In protein CD studies, a CD signal can arise from many amino acid side chains, however between the wavelengths of 160 and 260, absorption mainly arises from peptide bonds. Each major secondary structure has a characteristic CD spectrum in this far UV region and therefore it’s possible to analyse the composition of helices, sheets and turns that a pure solution phase protein can possess\(^{218}\). An example CD spectrum displaying each secondary structure is shown in Figure 7.3.

![CD Spectrum](image)

Figure 7.3 – An example CD spectrum showing the various secondary structures, the solid line is an α-helix; the long-dashed line is an anti-parallel β sheet; the dotted line is a type-I β-turn; the cross dashed line and short dashed line are irregular structures. Figure taken from Kelly, 2005\(^{218}\).
A number of algorithms then exist which can turn the data from CD spectra into an estimation of a protein’s structure\textsuperscript{219}. By altering the environment, and analysing how the protein structures change with respect to conditions such as pH, temperature and pressure, it is then possible to gain information on protein function. While CD has mostly been used to study soluble proteins, it is possible to study membrane proteins reconstituted into model membranes using the same techniques\textsuperscript{220}. Studying proteins in model membranes and in solution gives information which is averaged in all 3 dimensions, as the protein can exist in any of the 3 axes at any one time. This averaging decreases the signal-to-noise ratio and decreases the amount of structural information that can be gained.

In recent years, developments have been made in using CD to study proteins that are oriented in a direction. This means that the data gained is only averaged in 2 dimensions, removing one of the degrees of freedom and increasing signal-to-noise. This has been achieved by anchoring proteins onto a surface\textsuperscript{221}, or by aligning them with a magnetic field\textsuperscript{220}. It has also been shown that it’s possible to study membrane-active proteins by attaching them into planar lipid bilayers, thus orienting them in plane with the bilayer\textsuperscript{222}. Given the progress made in the optimisation of MscL reconstitution into model membranes, it was proposed that oriented CD of MscL reconstituted into a planar lipid bilayer could give a great deal of structural information.

Therefore, an assay was set up where MscL was reconstituted into planar lipid bilayers at 3000:1 lipid to protein ratio, and studied using CD from a synchrotron radiation source at between 200 and 260nm to see structural properties. The synchrotron radiation source increased signal-to-noise, and the oriented protein allowed even further study of structural changes. Then, once the assay had been set up, the pressure in the system was increased and decreased, this pressure change induced a change in the thickness of the bilayer. It was proposed that this change in thickness in the bilayer, would change the way the protein reconstituted into the planar bilayer, whether it reconstituted perpendicular to the bilayer, or slightly bent, as demonstrated in Figure 7.4.
Preliminary results from the oriented CD can be found in Figure 7.5. The results in Figure 7.5 show that with increasing pressure there is a change in the CD spectrum, with a slight upshifting in the amount of rotation as pressure increases. This indicates that as pressure increases, the bilayer becomes thinner and the protein bends to minimise hydrophobic interactions. The graph in Figure 7.5c shows a linear change in the characteristic α-helix wavelength of 215nm, further indicating that the protein is changing in orientation with increasing pressure.

It should be noted these results are an example of the MscL oriented CD assay, and that they were recorded in collaboration with James Hindley. These results will form part of a publication examining the effect of bilayer thickness on membrane protein structure.
Figure 7.5 – an example of the CD results of a supported lipid bilayer with MscL reconstituted at 3000:1 lipid to protein ratio. The results are normalised at 257-260nm and in this example the pressure is increasing in 500 bar steps. a) the CD spectrum of MscL in a planar lipid bilayer changing as a function of pressure, b) a graph showing an increase in the characteristic α-helix component at 215nm, indicating that the protein is changing its orientation with increasing pressure. These results were undertaken in collaboration with James Hindley, and are forming part of a publication on this topic.
This thesis focuses primarily on the optimisation of the conditions needed to achieve the active reconstitution of the mechanosensitive channel of large conductance (MscL) from *E. Coli* into a new model membrane system, the droplet interface bilayer (DIB). The MscL was successfully expressed and purified from competent *E. Coli*, and its concentration was calculated by tryptophan absorption. A fluorescent liposome based assay was used to ensure the activity of the purified MscL, and then the effect of charge and curvature elastic stress on MscL was investigated using a lipid composition sweep using both a chemical activator and membrane mechanics. It was found that the addition of DOPG or DOPE has surprising effects on the activity of MscL, leading to the conclusion that MscL is sensitive to membrane charge and spontaneous curvature. However, it was unclear whether the lipid composition was affecting MscL activity, or affecting how much active MscL could be reconstituted.

In order to better investigate how fundamental membrane properties effect MscL, and the differences between MscL activity and MscL reconstitution efficiency, the first translation of the liposome MscL activity assay into the droplet interface bilayer (DIB) format has been shown leading to a major proof-of-concept publication. MscL activity was measured using both a chemical activator and through membrane mechanics, and lipid composition sweeps were carried out to further examine the effect of charge and curvature elastic stress on MscL activity. While the results from the DOPG composition sweep agreed well with literature and the results from the liposomal assays, the DOPE composition sweep did not. It is possible that the DIB setup does not provide a homogenous environment for mixtures of lipids due the ability for the lipid to freely diffuse between either the monolayer or the bilayer. This work can then lead to further studies investigating how lipids partition in a DIB setup.

The MscL DIB assay was then translated into an electrophysiological setup to better examine single MscL opening events, and to examine MscL activity in-depth. MscL activity was successfully measured in a voltage-clamp setup, and the attempts were made to examine the effect of lipid composition. The results from all three assays were then compared, and the results have shown that the interactions between MscL and the lipid environment are more complex than they originally seemed. By linking multiple bilayers together a synthetic bilayer network was successfully built, for the first time showing inter-bilayer interactions purely through a gated ion channel, leading to a major publication. This work presents a significant advance towards the development of artificial tissues by showing multiple artificial membranes communicating through a user-defined stimulus. It is hoped this will lead to
further advances in the study of artificial cell-cell interactions and presents a paradigm shifting motif for next generation artificial tissues.

In addition, the work from the previous chapters was then shown to have many applications, by simply translating the MscL DIB assay into different activation mechanisms and devices. It was possible to show mechanical activation of MscL in a shear force generating environment. It has been shown that not only does MscL activate through shear forces generated inside a DIB, but also above a certain point the shear forces deactivate MscL. This work will hopefully lead to a more in-depth study into how shear forces interact with artificial membranes, and lead to studies looking MscL structure under extreme shearing forces.

Finally, it has been shown that the work in the previous chapters has already started to be used in multiple membrane studies. Preliminary results have been shown for the activation of MscL through the insertion of an amphipathic helix causing membrane tension build up. Also, preliminary results have been shown that MscL structure can be analysed in a supported lipid bilayer using synchrotron circular dichroism.

Fundamentally, this thesis has proven that the model membranes used in the study of membrane proteins are very flexible, and with some optimisation, can be used to study complex lipid-protein interactions, and possibly even generate completely artificial protein-protein interactions in membranes with controllable architectures. The work in this thesis has led to a better understanding of how to build truly artificial cells, capable of cross communication controlled purely through a control of membrane composition and architecture.
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