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Investigating Structural Properties of Lipid Bilayers Through the Use of Molecular Rotors

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*“Facts are meaningless. You can use facts to prove anything that’s even remotely true” –
Homer Simpson*

“In this house we obey the laws of thermodynamics” – Homer Simpson

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As the physical boundary separating the cell interior from the outside world, the plasma membrane is arguably one of the most important cellular organelles. Along with the proteins embedded within it, the plasma membrane plays a part in a number of vital roles including intercellular communication, cell motility and homeostasis. Despite this, comparatively little is known about the exact structure of the plasma membrane. The plasma membrane is a lipid bilayer that is widely believed to undergo transient phase separation into domains of different viscosities, which may be responsible for certain membrane properties.

By using molecular rotors (organic fluorophores whose fluorescence properties depend on the viscosity of their surrounding environment) in conjunction with fluorescence lifetime imaging microscopy (FLIM), it is possible to generate a viscosity map of a heterogeneous biological system.

In this thesis, we use molecular rotors to investigate the viscoelastic properties and phase behaviour of a range of lipid bilayer systems. We begin by using molecular rotors based on a boron-dipyrin (BODIPY) core to investigate membrane viscosity within model systems, finding that different lipid phases display different viscosities, and visualising lipid phase separation within giant vesicles. We go on to use BODIPY rotors to image viscosity within the plasma membranes of live *Escherichia coli* bacteria at different temperatures, finding that they display a much higher degree of membrane ordering than previously observed in eukaryotic cells. Next we explore synthetic alternatives to the conventionally used BODIPY rotors in order to address their shortcomings, before using molecular rotors based on a thiophene moiety to determine the viscosity of the plasma membranes of live human cell lines. Our results from these studies suggest that phase-separation may indeed occur within the plasma membrane.

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1. Introduction

1.1 The plasma membrane

As the barrier separating the cell interior from the outside world, the plasma membrane is perhaps the most crucial organelle for maintaining the homeostasis of the cell. The plasma membrane is a complex structure, consisting of a phospholipid bilayer studded with membrane proteins that carry out specific functions and often covered by carbohydrates, and is one of the few common features of all known cells.¹ Membrane proteins have a variety of roles vital to the functioning of the cell, including control of what enters and leaves the cell and communication between cells. As such, membrane proteins are a major drug target, with over 50% of drugs thought to target membrane proteins.² A thorough understanding of the structure of the plasma membrane is therefore paramount in the design of membrane-targeting drugs and in order to better comprehend the everyday functioning of the cell as a whole.

1.1.1 Early models of the plasma membrane

Despite its importance to the functioning of the cell, comparatively little is known about the structure of the plasma membrane when compared with other cellular organelles. At its most basic level the plasma membrane can be thought of as a lipid bilayer that essentially acts as a solvent for membrane proteins. In eukaryotic cells this lipid bilayer consists of a complex mixture of phosphoglycerides, sphingolipids and sterols. Phosphoglycerides and sphingolipids that contain a phosphate group are known as phospholipids, and lipids that contain a carbohydrate group are

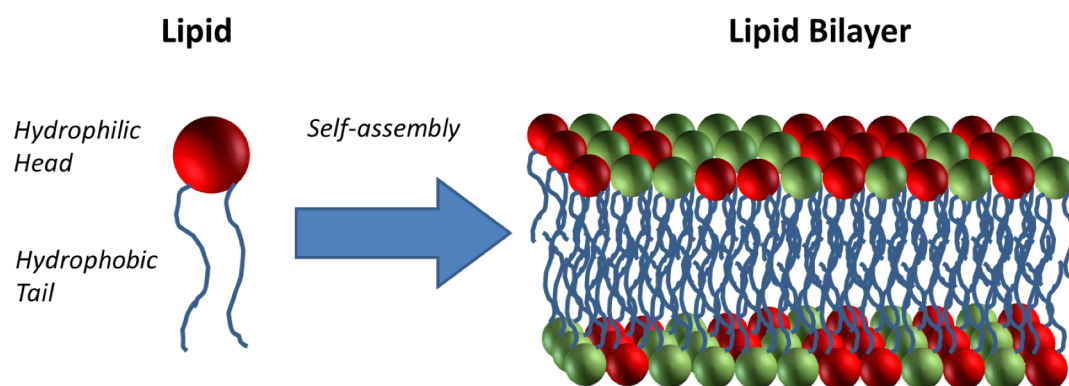


Figure 1-1 Generalised structure of a lipid and a lipid bilayer. Lipids consist of a hydrophilic head group and a hydrophobic tail region, which can self-assemble into a bilayer structure in water, as the head groups interact favourably with the water and the hydrophobic tails try to avoid contact with the water

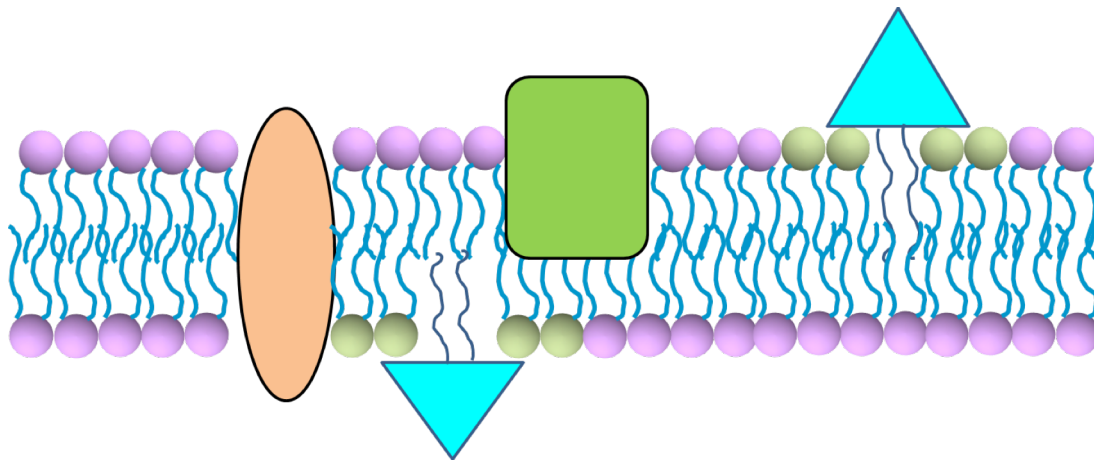


Figure 1-2 The fluid mosaic model.⁸ The lipid bilayer is essentially a disordered solvent containing integral membrane proteins. There is no long range lipid structuring, but certain proteins are able to induce some short range ordering of the bilayer lipids

known as glycolipids.³ A significant feature of phospholipids is that they contain both hydrophilic and hydrophobic segments – they are amphiphilic. The polar and hydrophilic phosphate “head” group will readily associate with water molecules in an aqueous medium, whereas the nonpolar and hydrophobic hydrocarbon “tail” group prefers an environment where water is excluded.³ This leads to the formation of a lipid bilayer in aqueous solution, where the phosphate head groups face out into solution and the hydrophobic tail groups intercalate with each other (Figure 1-1).

Whilst earlier work^{4–6} had established the existence of an elastic boundary layer at the edges of cells two molecules thick,⁷ the first widely accepted model describing the structure of the plasma membrane was the “fluid-mosaic” model described in 1972 by Singer and Nicholson.⁸ This model, which revolutionised scientific thinking at the time, described the plasma membrane as a lipid bilayer that acts as a bulk discontinuous fluid with integral proteins either partially or wholly embedded within it (see Figure 1-2 for a schematic of the fluid-mosaic model). The lipid bilayer was believed to have no overall ordering, essentially acting as a viscous solvent for the membrane proteins, although a small fraction of the lipids were thought to interact specifically with membrane proteins leading to small scale protein aggregation and ordering. This model was built on the experimental observations of the time and helped to explain several previously poorly understood characteristics of the plasma membrane such as lipid asymmetry between leaflets of the bilayer and how proteins incorporate into the plasma membrane. It was however unable to explain certain properties of the plasma membrane, such as the large diversity of lipids within the membrane and the varying, yet characteristic, lipid compositions of different cell types.⁹

The fluid-mosaic model was developed further by Israelachvili in 1977,¹⁰ who incorporated concepts such as membrane folding, pore formation and thickness variations into the model, before

Mouritsen and Bloom developed¹¹ the “mattress model”, which was based on a thermodynamic model for “matching” the length of the hydrophobic region of the lipid bilayer with the hydrophobic regions of membrane proteins. This hydrophobic matching was theorised to lead to lipids organising themselves around proteins of certain hydrophobic lengths in order to prevent a thermodynamically disfavoured exposure of the hydrophobic protein to the aqueous solvent. This could then lead to inhomogeneities within the plasma membrane, such as clustering of membrane proteins, and small scale phase separation of the lipid bilayer into coexisting lipid phases. The idea that the distribution of lipid throughout the plasma membrane may be heterogeneous led to further developments in the understanding of the structure of the plasma membrane.

1.1.2 Phase separation in lipid membranes

Around the time when the fluid-mosaic model was being developed, it was discovered that certain lipid mixtures were capable of separating into coexisting phases *in vitro*.¹² It was hypothesised that a similar phase-separation into coexisting lipid domains may take place within the plasma membrane,¹³ and a number of studies showed evidence that the plasma membrane may contain cholesterol-rich ordered phases that coexist with the fluid phases more conventionally associated with the plasma membrane.¹⁴

Lipid-driven lateral separation into immiscible liquid-ordered (L_o) and liquid-disordered (L_d) phases is currently well-understood within model membrane systems, and can be readily observed in ternary lipid mixtures of saturated and unsaturated lipids with cholesterol.¹⁵ The L_o phase is favoured by high melting point lipids with saturated acyl chains such as sphingolipids mixed with cholesterol, whereas the L_d phase is typically associated with unsaturated lipids, and displays a lower degree of acyl chain

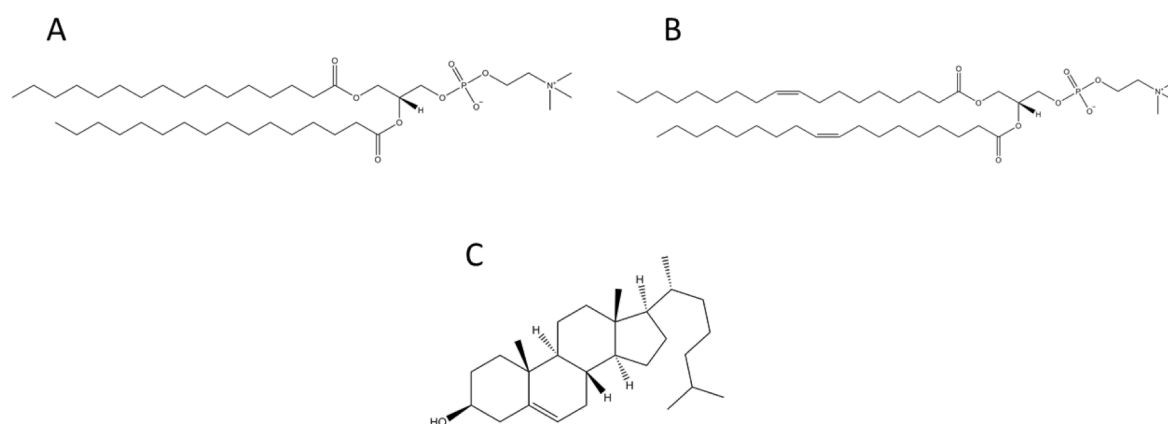


Figure 1-3 Structures of A) dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), a saturated lipid, B) 1,3-bis(*sn*-3'-phosphatidyl)-*sn*-glycero-3-phosphocholine (DOPC), an unsaturated lipid, and C) cholesterol

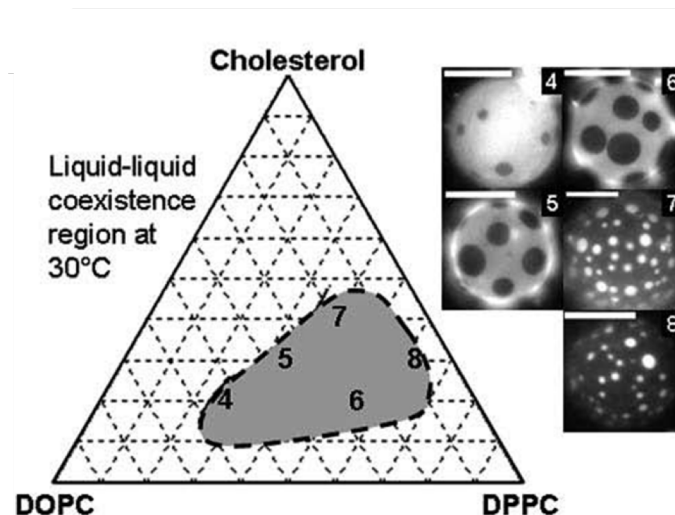


Figure 1-4 Phase diagram for a ternary bilayer of DOPC, DPPC and Cholesterol, showing the region of L_o - L_d phase coexistence at 30 °C. The numbers correspond to fluorescence microscope images of giant unilamellar vesicles (GUVs) of those compositions, which are shown to the right of the phase diagram. GUVs are stained with Texas Red, a fluorescent dye that highlights the L_d phase. Scale bar = 20 μ m (adapted from¹⁵)

ordering (see Figure 1-3 for example structures of saturated and unsaturated lipids, as well as the structure of cholesterol). By varying temperature and lipid composition, a variety of complex patterns can be observed in simple three-component lipid bilayers which can be characterised using a phase diagram, which outlines the different lipid phases that can be observed at a specific temperature depending on the overall composition of the bilayer (see Figure 1-4 for an example of a phase diagram and the coexisting L_o and L_d phases that can arise from it).¹⁶

The exact composition of the coexisting L_o and L_d phases can be determined by using tie-lines within the region of phase coexistence of the phase diagram. At any position along a given tie-line, the L_o phase will have a composition that can be determined by following the tie-line to the boundary between the region of phase coexistence and the region that gives a pure L_o phase, and the composition of the L_d phase can be determined by following the tie-line to the boundary between the region of phase coexistence and the region that gives a pure L_d phase.¹⁷ The tie-lines within the region for L_o - L_d phase coexistence can be followed up to the critical point, the point at which the compositions of the L_o and L_d are identical (see Figure 1-5 for an example phase diagram showing tie-lines and the critical point for L_o - L_d phase coexistence).¹⁸ Interestingly, the lipid composition of the plasma membrane is thought to be close to the critical point, and plasma membrane extracts are known to phase separate into stable coexisting phases at reduced temperature.¹⁹

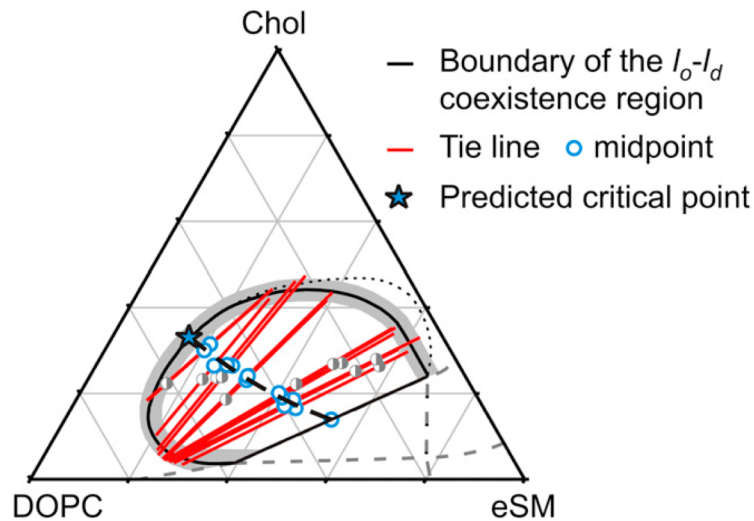


Figure 1-5 Phase diagram for a ternary bilayer consisting of DOPC, cholesterol and egg yolk sphingomyelin, a saturated lipid. Red lines represent the tie-lines within the phase diagram, and the star represents the critical point, the point at which L_o and L_d phases have identical compositions (adapted from¹⁸)

1.1.3 The lipid raft hypothesis

The area of research surrounding lateral lipid organisation within the plasma membrane remained relatively unexplored however, with the lipid bilayer thought to play a purely passive role in the activity of the plasma membrane, until in 1997 Simons and Ikonen proposed the landmark “lipid raft hypothesis” (Figure 1-6).²⁰ The lipid raft hypothesis originally stated that, rather than acting as a passive solvent, the lipids of the plasma membrane phase-separate into stable, coexisting ordered and disordered domains, with the ordered “raft” phases acting as platforms for the transportation of specific proteins and as relay stations in intracellular signalling. The evidence for this was primarily based on the observation that, at low temperatures, plasma membranes are partially insoluble in non-ionic detergents such as Triton X-100, leaving lipids that form a detergent resistant membrane (DRM).²¹ These DRMs were found to be rich in cholesterol and sphingolipids and were therefore believed to exist in the L_o phase,²² and this was taken as evidence of the existence of such phases within the plasma membrane. Certain membrane proteins were found to be enriched in DRMs, which was interpreted as evidence that these proteins associate with lipid rafts within the cell membrane.²³ Perhaps the most widely studied family of proteins believed to reside within lipid rafts in the plasma membrane are glycosphosphatidylinositol-anchored proteins (GPI-APs), which are involved in a range of cellular functions, including adhesion, membrane trafficking, immune system signalling and nutrient uptake.²⁴ GPI-APs are enriched in DRMs and have been found to partition into L_o phases within artificial bilayers,²⁵ and so are believed to be evidence of the active role of lipid rafts in the functioning of the plasma membrane.

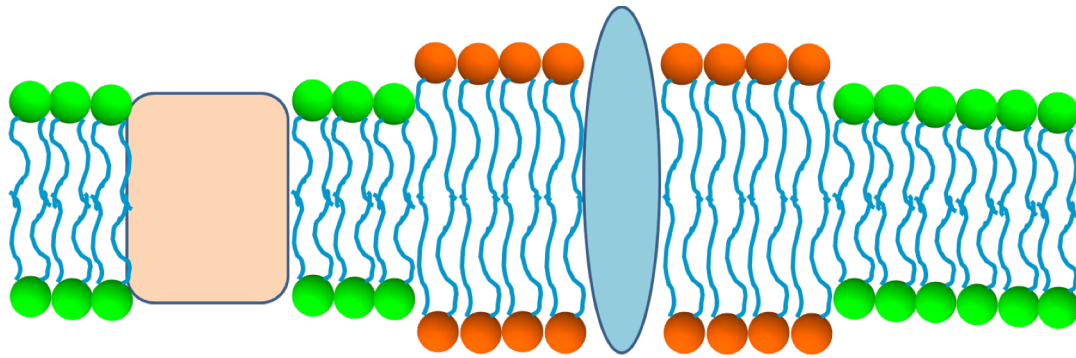


Figure 1-6 The lipid raft hypothesis²⁰ - the lipids of the plasma membrane phase separate into coexisting ordered (raft, red lipids) and disordered (green) domains. The raft domains act as platforms for proteins diffusing around the membrane and play a number of roles in the functioning of the cell. The orange rectangle and blue oval represent proteins that preferentially orientate in different lipid phases

The lipid raft hypothesis brought the lipids of the plasma membrane to the forefront of membrane research and this hypothesis is now widely accepted within biology, with lipid rafts thought to play a role in a wide array of cellular functions and biological events.²⁶ The lipid raft hypothesis is not without controversy however. Proteins are often deemed to be associated with lipid rafts if they are found to be detergent insoluble, although the proportion of the protein that needs to be insoluble in order to associated with lipid rafts has never been defined, and in some cases proteins have been associated with lipid rafts if as little as 10% of that protein remained insoluble upon detergent treatment.²⁷ Recent research has even suggested that GPI-APs, once the cornerstone of research into raft-protein interactions, are not actually associated with ordered lipid phases within the plasma membrane and do not influence their membrane environment over distances beyond their own physical size.²⁸ Troublingly, the use of detergents has even been suggested to induce artifactual changes in protein solubility,^{27,29} raising serious questions over many of the conclusions drawn from DRMs.

Direct detection of lipid rafts within the plasma membranes of live cells has proved elusive however. Conventional fluorescence microscopy in living cells has consistently failed to reveal large scale segregated structures within the plasma membrane, suggesting that any rafts within the plasma membrane must have sizes below the resolution limit of conventional fluorescence microscopy techniques, i.e. below *ca.* 300 nm.⁹ This is in stark contrast to the lipid phase-separation that has been observed in artificial bilayers consisting of cellular plasma membranes extracts.^{30–32} We must however bear in mind that plasma membranes are highly dynamic compared with stable artificial bilayers and also contain high protein concentrations and associate with the cytoskeleton, both of which^{33,34} may influence the phase behaviour of the lipids within the membrane and limit the size of any lipid domains.

Contemporary evidence suggests that the original lipid raft hypothesis may therefore be an oversimplification of the reality of plasma membrane phase behaviour. Due to their small size and potentially transient nature it may be more accurate to describe lipid rafts as “membrane microdomains”, and an updated definition describes lipid rafts as “small (10-200 nm) heterogeneous, highly dynamic, cholesterol and sphingolipid enriched domains that compartmentalise cellular processes”.³⁵

1.1.4 Thermodynamics of lipid bilayer phase separation

The dynamic nature of the plasma membrane and apparent transience of lipid microdomains imply that the plasma membrane of a live cell is not in thermodynamic equilibrium. Lipid domains may therefore form as a purely kinetic process as certain components transiently accumulate in a particular area. This could be the result of an enzymatic action, for example the hydrolysis of phosphatidylinositol-4,5-bisphosphate by phospholipase C to produce a local increase in the concentration of diacylglycerol (DAG), thereby leading to the formation of a DAG domain which then quickly dissipates.³⁶ These types of kinetic domains would have very short lifetimes, on the order of 10 μ s, but may still be useful as transient signals, although they would not persist for long enough to influence the structural organisation of the membrane. For domains to have a significant lifetime, there must also be a degree of thermodynamic stabilisation.³⁶

Under equilibrium conditions (i.e. so kinetic factors aren’t taken into account) lipid domains will form due to favourable and unfavourable interactions of the different components of the membrane. Consider a lipid molecule in a bilayer surrounded by neighbouring lipid species (Figure 1-7). If the lipids are of two different species, lipid A and lipid B, both in the fluid state, then there are three possible lipid-lipid interactions: A-A, A-B and B-B. Only one thermodynamic parameter is required to describe these mutual interactions: the difference between an A-B interaction and the average of the A-A and B-B interaction. To see this, consider the hypothetical “reaction” shown in Figure 1-7, where two pairs of like neighbours are exchanged to produce two pairs of unlike neighbours. The enthalpy change for this reaction per molecule (there are two molecules of each, so we divide by 2) is given by equation 1-1:³⁶

$$\omega_{AB} = h_{AB} - \frac{1}{2}(h_{AA} + h_{BB}) \quad (1-1)$$

where h_{AA} and h_{BB} are the enthalpy changes of interaction between two A or two B lipids, g_{AB} is the enthalpy change of interaction between one A and one B lipid and ω_{AB} is the unlike nearest

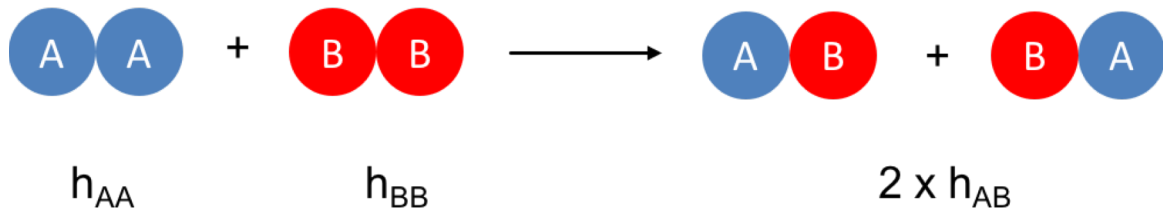


Figure 1-7 The reaction between lipid pairs AA and BB to give 2 AB

neighbour interaction enthalpy, or interaction energy. It is this parameter that determines whether lipids A and B mix well or separate into domains, and the change in Gibbs free energy for lipid exchange (ΔG^0) will be a multiple of ω_{AB} .³⁶

The Gibbs free energy change of mixing is given by equation 1-2:

$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix} \quad (1-2)$$

Where ΔH_{mix} is the enthalpy change of mixing T is the temperature and ΔS_{mix} is the entropy change of mixing. The entropy change of mixing will always be positive and the enthalpy change will vary depending on the interactions between the lipid species. For phase separation to occur, ΔH_{mix} will be positive, meaning that changing temperature can be used to induce mixing/demixing.³⁷ A negative ΔG_{mix} means that mixing of the two species is promoted, whereas a positive ΔG_{mix} means that A and B will phase separate into different lipid domains.

1.2 Viscosity

1.2.1 Biological implications of viscosity

Diffusion-limited processes are ever-present within cell biology and often control the rate of mass transport of reagents through a biological system, thus meaning that the rate of diffusion throughout the cell is intimately connected with the functionality of the cell.³⁸ This is especially true within membrane systems where the rates of diffusion and lipid dynamics control the activity of the membrane.³⁹ The formation of ordered domains that control diffusion around the two-dimensional surface of the membrane is also one of the central facets of the lipid raft hypothesis.²⁰ Diffusion within membranes via Brownian motion can be highly efficient: a lipid is able to rotate about its own axis in under 10 μs , and can explore *ca.* 16 μm^2 per second laterally.⁴⁰ The rate of diffusion is controlled by a number of different parameters of which viscosity is one of the most important, and L_o and L_d phases within model membrane systems are known to have largely different viscosities.⁴¹ A

solid understanding of membrane viscosity is therefore necessary to fully understand the functionality of the membrane, and may also help to shed further light on the lipid raft hypothesis and the nature of the lipid phase behaviour within the plasma membrane.

The viscosity of the plasma membrane influences the activity of membrane proteins, and changes in plasma membrane viscosity have been linked with several diseases, including atherosclerosis, hypercholesterolemia, diabetes and even cancer.⁴² Increased membrane viscosity is believed to contribute to the onset of age-related diabetes by impairing the activity of the hepatic insulin receptor kinase (IRK), leading to insulin resistance.⁴³ Several studies^{44–46} have shown a decreased plasma membrane viscosity within cancer cells, which is believed to be related to a decrease in membrane cholesterol content. As will be discussed later in this thesis, the development of atherosclerosis is believed to be associated with changes in endothelial cell membrane viscosity related to blood flow within the endothelium.^{42,47}

1.2.2 A model to describe viscosity

The viscosity of a fluid is defined as its resistance to shear stress; the greater ability of one fluid to resist shear flow over another fluid is caused by a difference in viscosity between the two fluids. An example of this is the fact that “thick” fluids such as treacle cannot be as easily stirred as “thin” fluids such as water.⁴⁸

Viscosity can be defined in a quantitative manner by considering a hypothetical experimental setup consisting of a fluid in between two wide parallel plates, as shown in Figure 1-8. The bottom plate is fixed in place and the top plate is free to move. Upon application of a force, F , on the top plate, a shearing stress, τ , develops at the plate-fluid interface (equation 1-3):

$$\tau = \frac{F}{A} \quad (1-3)$$

where A is the area of contact between the plate and fluid. If the top plate moves at a velocity of u , and the fluid is thought of as a series of infinitesimally thin layers, then the velocity at which the layers move will vary with distance, y , away from the top plate, with the fluid layer closest to the bottom plate having zero velocity. This leads to a velocity gradient, $\frac{\delta u}{\delta y}$, that is proportional to the shear stress applied to the top plate (equation 1-4):

$$\tau = \eta \frac{\delta u}{\delta y} \quad (1-4)$$

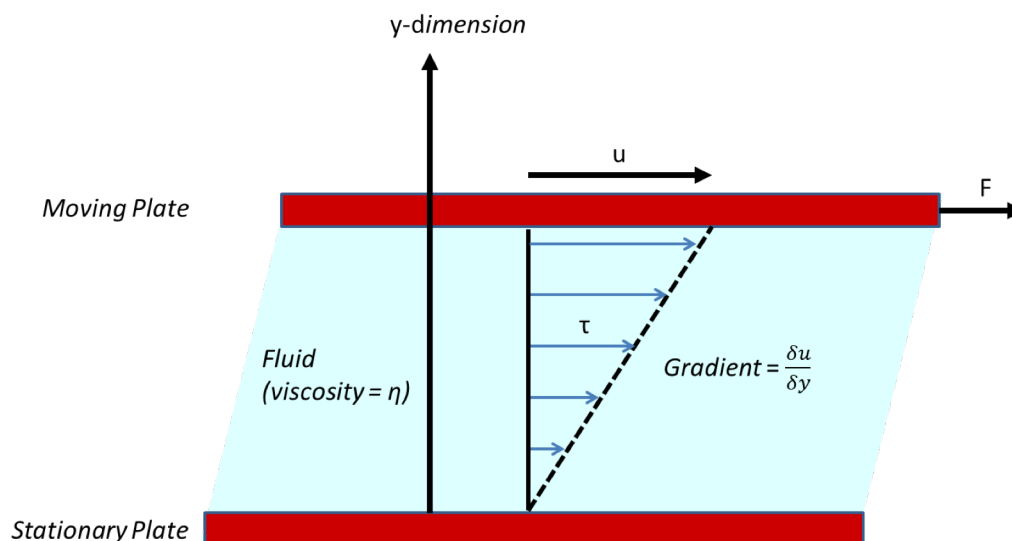


Figure 1-8 Schematic showing the definition of viscosity (adapted from⁴⁹)

The proportionality constant, η , is defined as the viscosity of the fluid. Fluids where the shear stress is linearly related to the velocity gradient are known as “Newtonian fluids”, and comprise the majority of commonly found liquids.⁴⁹

A viscous fluid will limit the movement of a species within it, lowering the rate of diffusion of the species. In terms of a transition from species A to species B, the frictional effects associated with an increase in solvent viscosity will increase the energy barrier of the transition. A diagram of this effect is shown in Figure 1-9, where E_η represents the extra energy associated with moving the solvent molecules to allow the rearrangement of the reacting molecules to form a transition state.³⁹

It is important to note that equilibrium properties of a system are unaffected by viscosity, owing to

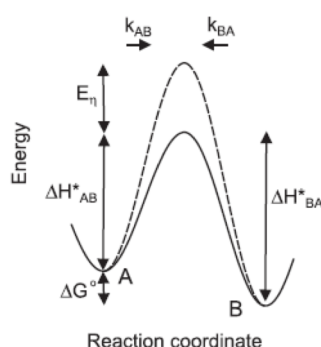


Figure 1-9 The effect of an increase in viscosity on the energy barrier to a chemical reaction, where the solid line represents the intrinsic energy barrier to the reaction and the dashed line shows the same reaction in a viscous environment. Increased viscosity adds an extra energy barrier, E_η , onto the intrinsic energy barrier to both the forwards and backwards reactions, leading to increased enthalpy changes of reaction, ΔH^*_{AB} and ΔH^*_{BA} . Whilst this means that there will be no overall change in the ΔG^0 of the reaction, the rates of both the forwards and backwards reactions, k_{AB} and k_{BA} , will decrease (reproduced from³⁹)

the time-independence of thermodynamic parameters. An increase in solvent viscosity will increase the activation energy of both the forward and back reactions, decreasing k_{AB} and k_{BA} equally. The equilibrium constant, K_{eq} , of the reaction (equation 1-5):

$$K_{eq} = \frac{k_{AB}}{k_{BA}} \quad (1-5)$$

will therefore not be affected by an increase in the viscosity, and, because the free energy change, ΔG° , can be related to the equilibrium constant by equation 1-6

$$\Delta G^\circ = -RT \ln K_{eq} \quad (1-6)$$

ΔG° will also not be affected by the solution viscosity.

The viscosity of a fluid will have a strong influence on any species diffusing through it. The Stokes-Einstein equation can be used to describe the diffusion of a spherical object through a bulk, viscous solution. The size-dependent lateral diffusion coefficient can be determined using equation 1-7:⁵⁰

$$D = \frac{k_B T}{6\pi\eta R} \quad (1-7)$$

Where k_B is the Boltzmann constant, T is the temperature and R is the hydrodynamic radius of the diffusing object.

1.2.3 Viscosity and diffusion within a bilayer

Stokes-Einstein diffusion may not accurately describe diffusion within a membrane, which is a quasi-two-dimensional system. Calculating the two-dimensional equivalent of the Stokes-Einstein diffusion coefficient within a membrane has been a challenging problem and the first attempt at solving it was presented by Saffman and Delbrück in 1975⁵¹, who, by modelling a membrane as a homogeneous thin layer of fluid and a membrane inclusion as a cylinder spanning the membrane, were able to derive an equation linking the diffusion coefficient of the membrane inclusion with the viscosity of the membrane. The Saffman-Debrück equation is shown in Equation 1-8

$$D_{sd} = \frac{k_B T}{4\pi\eta_m h} \left[\ln \left(\frac{2L_{sd}}{a} \right) - \gamma \right] \quad (1-8)$$

Where D_{sd} is the Saffman-Delbrück diffusion coefficient, a is the radius of the membrane inclusion, h is the bilayer thickness, η_m is the membrane viscosity, γ is the Euler-Mascheroni constant ($\gamma \approx 0.577$), and L_{sd} is the Saffman-Delbrück length, given by equation 1-9:

$$L_{sd} = \frac{h\eta_m}{2\eta_f} \quad (1-9)$$

where η_f is the viscosity of the surrounding fluid. Noteworthy in the Saffman-Delbrück approach is the weak dependence of diffusion coefficient on the radius of the membrane inclusion, where $D \propto \ln\left(\frac{1}{a}\right)$. This equation does not however hold in cases where either membrane viscosity is low or the membrane inclusion is large, where the relationship between diffusion coefficient and size of membrane inclusion is more accurately portrayed using a Stokes-Einstein-like model where $D \propto \frac{1}{a}$.⁵²

Additionally, the assumption that the membrane is a homogenous fluid ignores the heterogeneity across the z -axis of the bilayer. There are a variety of lateral stresses acting upon the lipids within a bilayer (Figure 1-10A), including the repulsive electrostatic and steric forces and the attractive forces such as hydrogen bonding and Van der Waals attractions between the individual lipids, which leads to the lateral pressure profile shown in Figure 1-10 (B).⁵³ This changing lateral pressure profile means that the microviscosity experienced by a membrane inclusion will differ depending on its position within the bilayer. This means the Saffman-Debrück model may be unsuitable for small-molecule membrane inclusions. It also suggests that caution must be taken when describing the viscosity of a lipid bilayer, as it will change depending on the depth within the bilayer.

Despite these potential drawbacks, the Saffman-Delbrück model is still a well-established and highly-employed method for relating diffusion coefficient to membrane viscosity.^{52,54,55} Although in recent

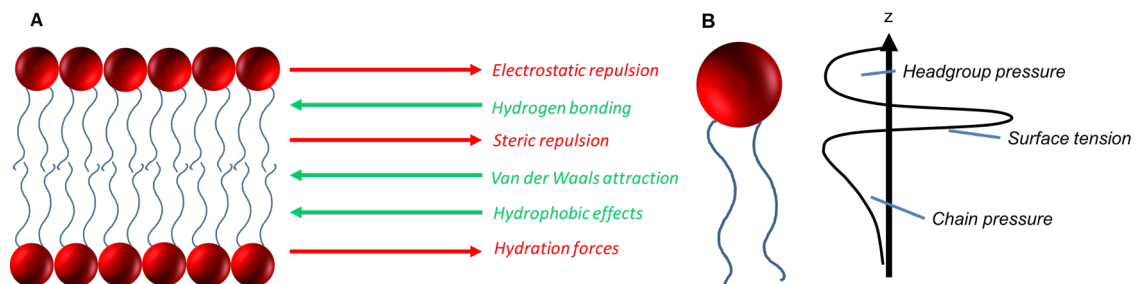


Figure 1-10 (A) The forces acting on the lipid bilayer at varying depths (B) the lateral pressure profile of a lipid within the bilayer (adapted from⁵³)

years more mathematically rigorous and complex models⁵⁵ have arisen for relating diffusion coefficient to membrane viscosity, within the scope of this chapter the Saffman-Delbrück model is an adequate model, and will be used throughout this thesis to relate membrane viscosities to diffusion coefficients.

A viscous fluid will impart a frictional force opposing the movement of any molecule dissolved within it. A simple way of picturing this on the microscale involves imagining the frictional forces that arise from the attractive interactions between solvent molecules. In order for a solute molecule to move through the liquid, it must push some liquid molecules out of the way. The resistance to motion of the solute can be expressed in terms of the viscosity of the liquid and on the microscale can be thought of as the “microviscosity” of the system, which may differ significantly from the bulk viscosity of the system³⁸ depending on intermolecular phenomena such as hydrogen bonding, polarity and molecular radius. The microviscosity experienced by one molecule or species may therefore be different from that experienced by a different species, and should therefore be discussed with a degree of caution. A further drawback to using a single number to describe microviscosity as one would with the bulk of a system is that it assumes isotropic behaviour at the microscale, i.e. that resistance to motion is identical in all directions. A more accurate description of microviscosity could involve describing it as a tensor that is dependent on orientation. This concept, however, is likely beyond the capability of the fluorescence-based techniques discussed throughout this thesis, although it may become an interesting area of discussion in future applications of fluorescence-based techniques for probing microviscosity. A note on terminology – often when discussing microviscosity within membranes, the term “fluidity” will be used, which is the inverse of the viscosity.³⁹

Despite the apparent importance of microviscosity within the cell and the plasma membrane, there are significant challenges associated with measuring it. The mechanical methods that are used to measure macroscale viscosity, such as the capillary viscometer, the falling ball viscometer and the rotational viscometer⁴² cannot be used on the microscale. Mechanical methods for measuring viscosity generally rely on subjecting the fluid to shear forces and measuring the resistance of the fluid to these forces. The high amount of sample fluid required and the slow measurement times make these techniques unsuitable for microscale viscosity measurements, in addition to the fact that these mechanical methods cannot be used on an intact cell. Alternative methods must therefore be used to measure viscosity within the plasma membrane.

1.3 Fluorescence

1.3.1 The process of fluorescence emission

Fluorescence-based techniques have found widespread use in determining viscosity on the microscale, and prior to discussing individual techniques it is useful to describe the general physical phenomenon of fluorescence. Fluorescence is a very well-studied phenomenon that involved molecules absorbing then emitting photons and provides a non-invasive method for studying various environments that is well-suited for use within biology

The phenomenon of fluorescence is perhaps best illustrated through the use of a Jablonski diagram (Figure 1-11),⁵⁶ where the singlet (i.e. the two electrons in the state have opposite spins) ground, first and second electronic energy levels are depicted by S_0 , S_1 and S_2 , respectively. At each electronic energy level, the fluorophore can also exist in a number of vibrational energy levels depicted by 0, 1, 2, etc. Transitions between states are depicted by vertical lines and typically occur on a timeframe of *ca.* 10^{-15} s, a time too short for significant displacement of the nuclei, a concept known as the Franck-Condon principle.

Following absorption of a photon several processes can occur. A fluorescent molecule, or fluorophore, is excited to a higher vibrational energy level of either S_1 or S_2 , before usually rapidly

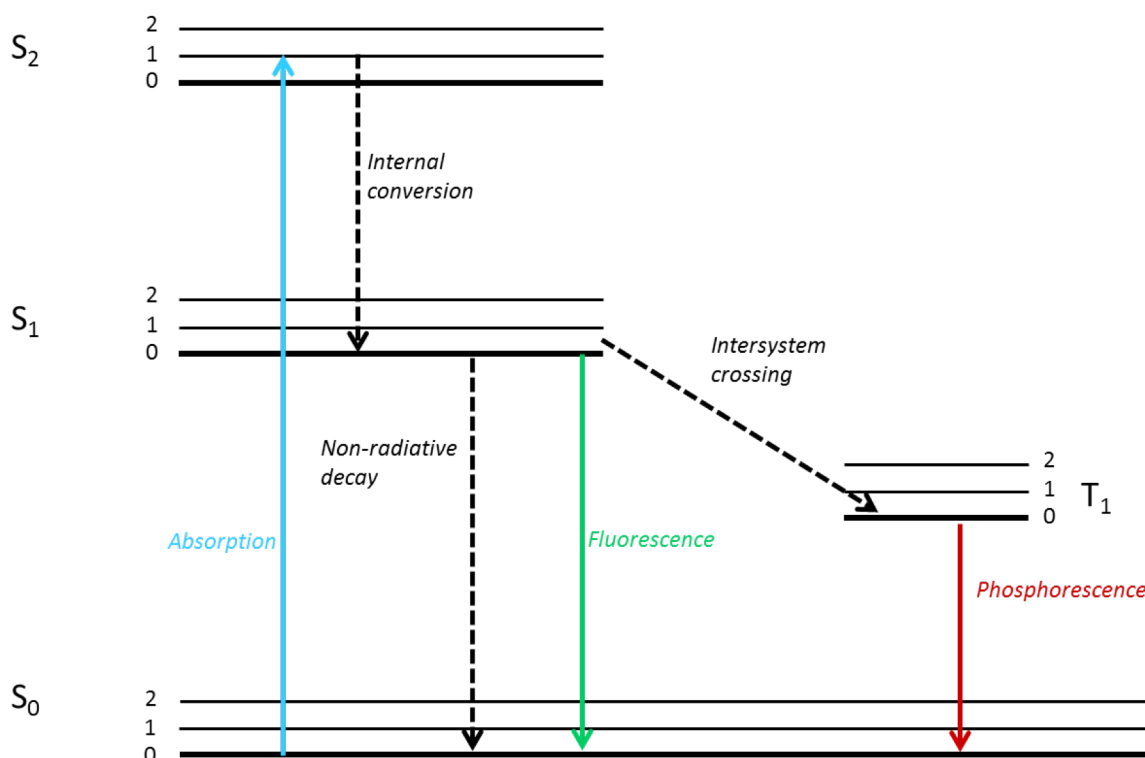


Figure 1-11 A Jablonski diagram (adapted from ⁵⁶)

relaxing to the lowest vibrational state of S_1 in a process known as internal conversion. This process generally occurs within 10^{-12} s and so is usually complete before any photonic emission occurs, meaning that emission generally results from a thermally equilibrated excited state, usually the lowest vibrational state of S_1 . From this state, the molecule can then return to the ground state via emission of a fluorescence photon (radiative decay) or can lose its energy through a non-radiative decay process, typically involving thermal vibrations. This process typically occurs on a timescale of *ca.* 10^{-9} s.

Return to the ground state via radiative or non-radiative decay generally occurs to a higher excited vibrational ground state level, that rapidly (10^{-12} s) reaches thermal equilibrium. A consequence of emission to higher vibrational S_0 states is that the emission spectrum is typically a mirror image of the absorption spectrum of the S_0 - S_1 transition. This is because electronic excitation does not significantly affect nuclear geometry between the ground state and the excited state, meaning that the vibrational structures in the absorption and emission spectra are similar.

Molecules in the S_1 state may also undergo a conversion termed “intersystem crossing” to the first triplet state, T_1 , via spin conversion. The T_1 state can also undergo radiative emission in a process known as phosphorescence. Because the transition from T_1 to the singlet ground state is forbidden, the rate constants for triplet emission are several orders of magnitude smaller than those for fluorescence, and as such this process can take place on timescales ranging from 10^{-3} s to several minutes.

The energy of emission is lower than that of absorption, meaning that fluorescence occurs at longer wavelengths than absorption. This phenomenon is known as the Stokes shift, and is caused by processes such as the internal conversion process to the lowest vibrational state of the S_1 energy level and the tendency of fluorophores to undergo radiative decay to a higher S_0 vibrational level than the ground state. Effects such as solvent relaxation can also affect the Stokes shift, meaning that solution properties such as polarity can affect the emission properties of a given fluorophore. This concept will become important in the discussion of the emission of molecular rotors later on in this chapter and at other points in this thesis.

The ratio between photons emitted as fluorescence by a given fluorophore and photons absorbed is known as the fluorescence quantum yield, and determines how emissive a given fluorophore is. Both the radiative and non-radiative decay processes depopulate the excited state, and so the quantum yield, ϕ_f , can be expressed in terms of the radiative (k_r) and non-radiative (k_{nr}) decay constants (equation 1-10):

$$\phi_f = \frac{k_r}{k_r + k_{nr}} \quad (1-10)$$

Note that the term k_{nr} encompasses all the possible non-radiative decay processes. The quantum yield will therefore be higher if $k_{nr} < k_r$, approaching the maximum value of 1. The energy yield of fluorescence will always be less than 1 due to the energy losses associated with the Stokes shift.

Also important is the fluorescence lifetime, τ_f , of a fluorophore. The fluorescence lifetime describes the average time that the fluorophore spends in the excited state before returning to the ground state. The fluorescence lifetimes of most organic fluorophores are generally in the range of 0.1 – 10 ns, and can be related to the radiative and non-radiative decay constants by equation 1-11:

$$\tau_f = \frac{1}{k_r + k_{nr}} \quad (1-11)$$

The fluorescence lifetime can therefore be related to the quantum yield using equation 1-12:

$$\phi_f = \tau_f k_r \quad (1-12)$$

In a fluorescence experiment, we observe the intensity of the fluorescence, I . At a given time the intensity, $I(t)$ can be related to the initial intensity I_0 using the fluorescence lifetime (equation 1-13):

$$I(t) = I_0 e^{-t/\tau_f} \quad (1-13)$$

where t is time. Fluorescence lifetime is a random process, so few excited state molecules emit their photons at precisely their fluorescence lifetime. For a single exponential decay 63% of the excited state molecules will have decayed prior to time $t = \tau$, and 37% will decay at $t > \tau$. This is because when $t = \tau$, $I(t) = I_0 \times e^{-1} \approx I_0 \times 0.37$. In the case of intensity decays such as the one described in equation 1-13 where the fluorophore only has one fluorescence decay (i.e. the decay is monoexponential), analysis of decay times is fairly straightforward. However it is often the case that fluorescent samples will display more than one decay time (i.e. the decay is multiexponential). A multiexponential decay can be related to fluorescence intensity using equation 1-14:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i} \quad (1-14)$$

Where τ_i now represents the different lifetime components of the decay, and α_i represents the pre-exponential factor and determines the weighting of each lifetime component.

Multiexponential decays are much harder to process than ones that are monoexponential as decays with different lifetime components can be almost indistinguishable from each other.⁵⁷ This means

that for analysis of multiexponential fluorescence decays, much higher signal-to-noise ratios are required and accuracy of the fitting process may be impaired.

1.3.2 Methods of determining the fluorescence lifetime

The fluorescence lifetime of a fluorophore can be a very useful parameter to investigate, as it can be used to give information about the immediate environment of the fluorophore and is generally independent of fluorophore concentration, unlike intensity-based measurements.³⁸ This concept will be discussed further in section 1.4.3, and will outline the bulk of the work in this thesis.

The most common method for determining the fluorescence lifetime of a fluorophore is the use of time-correlated single photon counting (TCSPC).⁵⁶ TCSPC uses a pulsed excitation source such as a femtosecond laser or a pulsed laser diode (PLD) to excite a fluorescent sample, causing it to emit fluorescence photons. The TCSPC detector then detects the first photon that hits the detector, recording the time taken between excitation and detection in a histogram, which represents the waveform of the fluorescence decay (see Figure 1-12 for a diagram illustrating the principle of TCSPC).⁵⁸

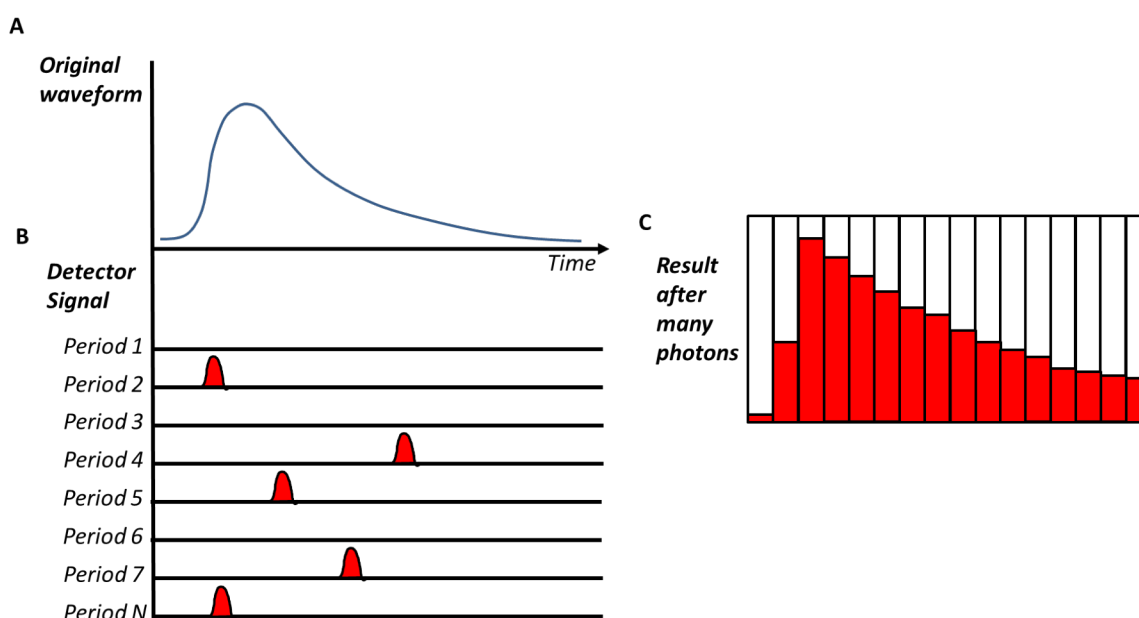


Figure 1-12 Principle of TCSPC, the original waveform (A) is reproduced by the TCSPC software by measuring the time taken for the first photon to arrive from a fluorescent sample (B), then putting it into histogram of number of photons against arrival time. After N photons have been detected the histogram resembles the original waveform and can be used to determine the fluorescence lifetime (C) (adapted from ⁵⁸)

The experiment starts with the excitation pulse that excites the sample.⁵⁶ This pulse sends a signal to a constant fraction discriminator (CFD), which accurately measures the arrival time of the pulse. This signal is transferred to a time-to-amplitude converter (TAC) which generates a voltage ramp, causing a voltage to build up linearly with time, essentially acting as a highly sensitive timer. When the detector detects the second pulse of light from the first photon of fluorescence, the CFD sends a signal to stop the voltage ramp. The TAC now contains a voltage proportional to the time delay between the excitation and emission signals. This voltage is then amplified and converted into a numerical value using an analogue-to-digital converter (ADC). By repeating this process several thousand times, the TCSPC is able to build up a histogram of the fluorescence decay which can then be fitted using a monoexponential or multiexponential model to give an estimate of the fluorescence lifetime of the fluorophore.

Whilst this outlines the basic process of TCSPC in “forward” mode, the majority of TCSPC measurements are performed in “reverse” mode. The process is essentially the same as described in the preceding paragraph, except that the emission pulse is used to start the TAC and the excitation pulse is used to stop it. Reverse mode is used because of the high repetition rate of modern pulsed-light sources. The TAC must be reset in between each individual measurement, which takes a finite amount of time, meaning that the TAC can be constantly in reset mode if the start signals arrive too rapidly. Due to the way a TCSPC experiment is typically set up, emission signals only occur in approximately 1 in every 100 excitation pulses, much less frequently than excitation pulses. These emission pulses are used to start the TAC in reverse mode and the next laser pulse is used to stop the TAC to allow more efficient measurement of the overall fluorescence decay.

TCSPC can be combined with fluorescence microscopy to give a technique known as fluorescence lifetime imaging microscopy (FLIM). FLIM can be used to generate a map of fluorescence lifetimes across a sample and is thus a powerful technique in quantitative fluorescence microscopy.⁵⁹ FLIM is generally coupled with confocal laser scanning microscopy in order to generate a pixel-by-pixel image of a sample, where each pixel contains a fluorescence decay as derived by TCSPC.

In addition to the fact that fluorescence lifetime is generally independent of fluorophore concentration, which means that the measurement is generally unaffected by variations in fluorophore concentration, illumination intensity, light pathlength, scatter or photobleaching, the use of FLIM has several advantages. FLIM can robustly probe the local environment of a fluorophore without disturbing it and it is also able to experimentally distinguish spectrally similar probes, providing they have a different fluorescence lifetime. Perhaps the most important advantage of FLIM however is its ability to quantitatively map fluorescence properties across a sample. There are,

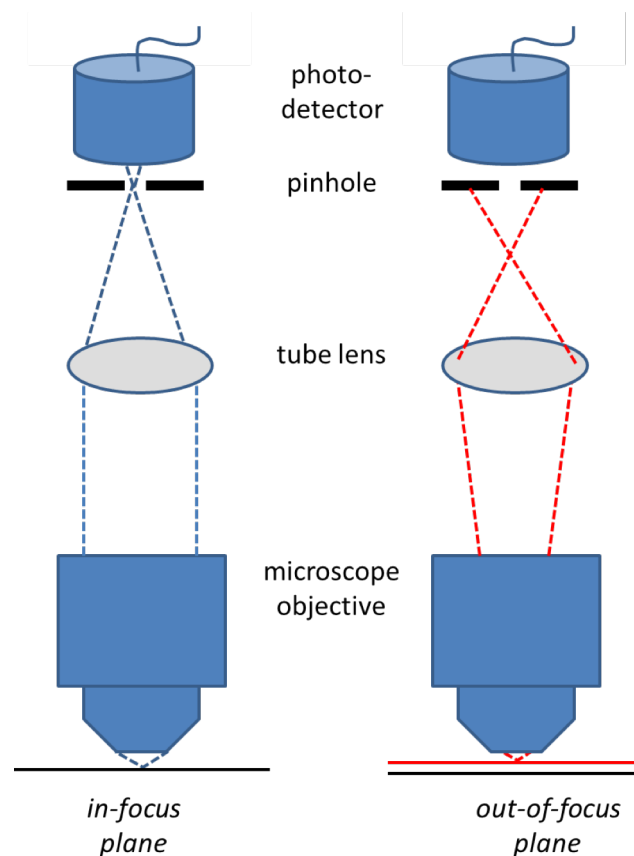


Figure 1-13 Schematic of a confocal microscope. Light coming from the focal plane goes through the pinhole and onto the photo-detector, but out-of-focus light is blocked by the pinhole (adapted from⁶⁰)

however, certain caveats to the use of FLIM, such as the complex and expensive equipment that is often required for a FLIM setup and the fact that fluorescence lifetime data can be very complex to interpret, especially when several fluorescent species are present, as may be the case with biological autofluorescence. Additionally, the interpretation of what specific changes in fluorescence lifetime mean for the surrounding environment of the fluorophore may not be straightforward.⁵⁹

1.3.3 Methods of performing FLIM

FLIM is often performed in conjugation with confocal laser scanning microscopy. Confocal microscopy differs from conventional microscopy in the fact that it uses a pinhole to increase the depth resolution of the microscope. In a conventional wide-field fluorescence microscope the entire sample is illuminated, meaning that out-of-focus planes will contribute to the overall image, reducing the resolution of the image. In confocal microscopy, the depth resolution is improved by using a pinhole in front of the detector to block out any out-of-focus light, meaning that the only contribution to the microscope image comes from the in-focus region (Figure 1-13).⁶⁰ Confocal microscopy will often use a laser as an excitation source, rather than a lamp. The pinhole results in a

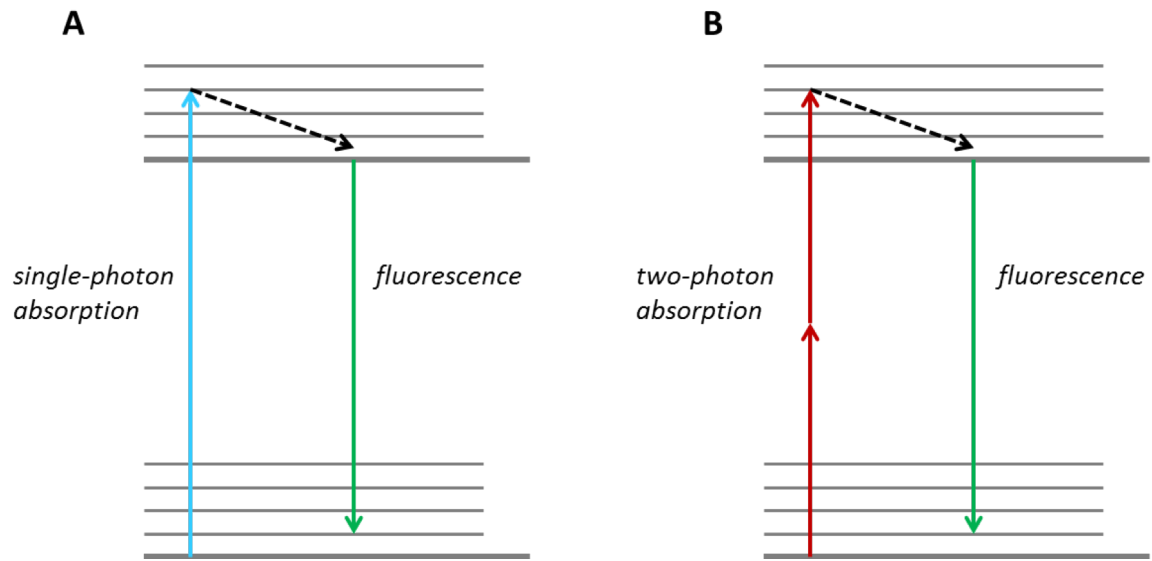


Figure 1-14 Simplified energy diagram outlining the processes of (A) single-photon and (B) two-photon absorption. In two-photon absorption, the same electronic transition is accomplished by absorption of two photons of half the energy required for single-photon absorption (adapted from⁶⁰)

large amount of lost signal, so a laser is used as a more intense excitation source and the small focal point of the laser also contributes to the increased resolution. The laser can then be scanned over the region of interest to generate an image of the sample, a method known as confocal laser scanning microscopy.

Another method that can be used to increase the resolution of a microscope is the use of two-photon absorption microscopy.⁶⁰ Two-photon absorption is based on the principle that a fluorophore can be excited by the simultaneous absorption of two photons, so the fluorophore is excited to an energy state with twice the energy of the incident photons (Figure 1-14). For example, if a fluorophore has a single-photon absorption maximum at 400 nm, two-photon absorption will allow excitation using radiation with a wavelength of 800 nm. After excitation the fluorophore will rapidly relax to its lowest vibrational state within the excited state, meaning that the emission spectrum is identical for both single- and two-photon excitation. The two-photon absorption spectrum is typically slightly blue-shifted from the expected spectrum based on the single-photon absorption spectrum, and the probability of two-photon absorption is influenced by the two-photon cross section of the fluorophore.⁶¹

The probability of two-photon absorption scales quadratically with the excitation intensity (equation 1-15):

$$I_{TPA} \propto \sigma_{TPA} \cdot (I_{exc})^2 \quad (1-15)$$

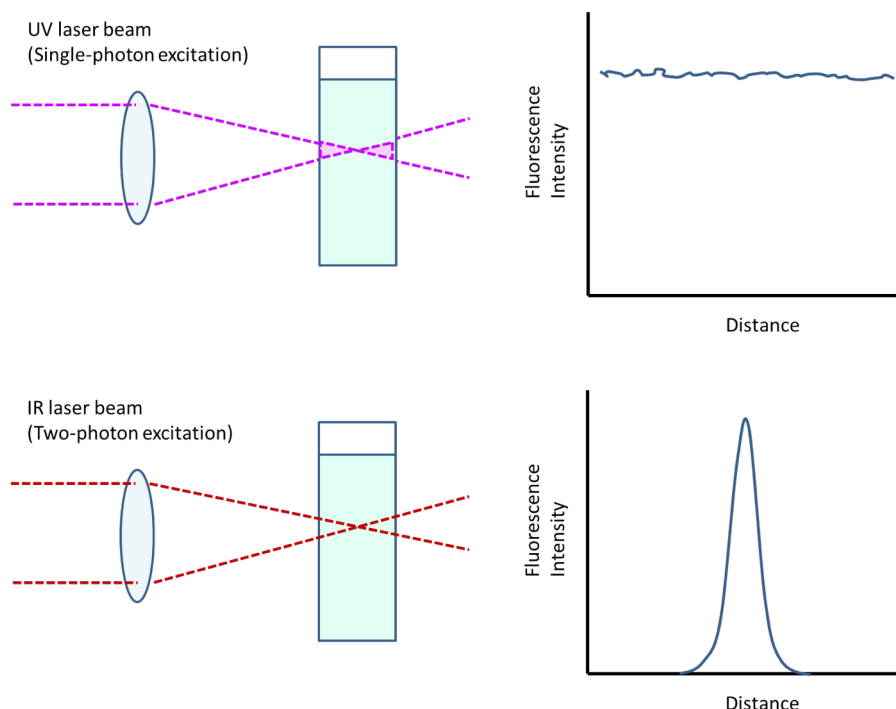


Figure 1-15 Schematic comparison of one- and two-photon excitation, highlighting the reduction in cross section associated with two-photon excitation (adapted from⁵⁶)

where I_{TPA} represents the detected two-photon induced fluorescence, σ_{TPA} is the two-photon absorption cross section and I_{exc} is the excitation intensity. This means that a twofold increase in excitation intensity yields a fourfold increase in two-photon absorption fluorescence. Because of the square dependence on excitation energy, absorption only takes place in regions where the excitation intensity is high enough – a very small focal region (Figure 1-15). This means that fluorophores are only excited near the focal plane of the microscope objective, resulting in a very high resolution and meaning that two-photon microscopy does not need a pinhole like confocal microscopy. Two-photon excitation also has a larger penetration depth into biological tissue due to its longer wavelengths, avoids out-of-focus photobleaching as excitation only takes place in the focal region, and significantly reduces excitation light scattering, as scattering scales with approximately λ^{-4} . Both confocal laser scanning microscopy and two-photon microscopy can be used with a pulsed femtosecond laser, allowing them to be used for TCSPC FLIM.

1.3.4 Fluorescence-based methods for determining viscosity and diffusion within the bilayer

A range of different fluorescence-based techniques have previously been used to investigate viscosity within lipid bilayer systems, typically by monitoring the translational diffusion coefficient of the fluorophore within the bilayer.

In addition to providing information about the viscosity of the bilayer, an investigation into the diffusive properties of a species within the plasma membrane can provide information about the structural organisation of the membrane. In heterogeneous plasma membranes, the diffusion coefficient, D , may change depending on the size and the timescale of the measurement, potentially owing to transient confinement of the diffusing species within lipid rafts.⁶² This concept was first employed in 1987 by Yechiel and Edidin,⁶³ who investigated the rate of diffusion of a fluorescent lipid probe within fibroblast plasma membrane. They found that the measured diffusion coefficient decreased as the measurement area increased, and interpreted this as evidence that the probe was transiently trapped in protein-rich lipid domains *ca.* 1 μm in size, an observation predating the lipid raft hypothesis by 10 years. By observing both local and global diffusion of species within the plasma membrane it is therefore possible to build a more detailed model of the plasma membrane in order to better understand the underlying physical concepts (see Figure 1-16 for an illustration of the link between diffusion and plasma membrane organisation).⁶²

Fluorescence recovery after photobleaching (FRAP) has been extensively used to map rates of diffusion within the plasma membrane and artificial bilayers. In a FRAP experiment, a laser is focused on a region of interest within a membrane stained by a given fluorophore, causing all the dye molecules within this region to photobleach. The laser intensity is then decreased, allowing continuous monitoring of the emission intensity from this region of the membrane. As fluorophore molecules diffuse back into this region of interest, the emission intensity increases, and the rate of fluorescence recovery is then used to calculate the diffusion coefficient of the fluorophore within the membrane (see Figure 1-17 for a schematic illustrating this).⁵⁶ Whilst this technique does not probe viscosity directly, the diffusion coefficient of the species within the membrane can be used to give a qualitative estimate of the viscosity, or it can be converted into an actual viscosity value using the Saffman-Delbrück approach outlined earlier or another more computationally intensive approach, such as that outlined by Petrov and Schwille.⁵⁵

FRAP has previously been used to investigate the lipid raft hypothesis. If rafts are small immobile domains of high viscosity, then raft-favouring proteins would have low diffusion coefficient.⁶² In

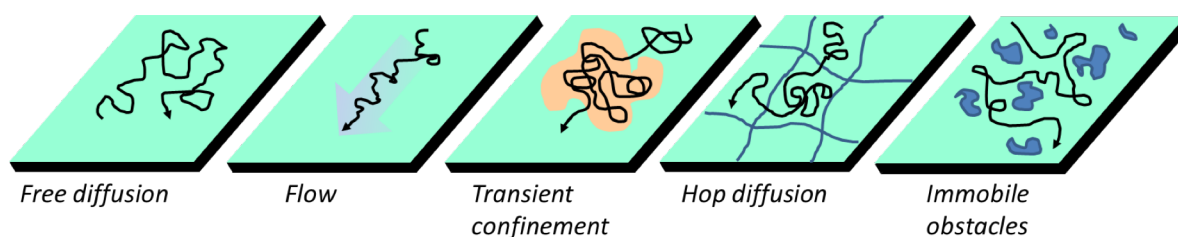


Figure 1-16 Schematic of the various different modes of diffusion of species within the plasma membrane. Transient confinement is often taken as evidence of lipid phase separation within the plasma membrane (adapted from⁶²)

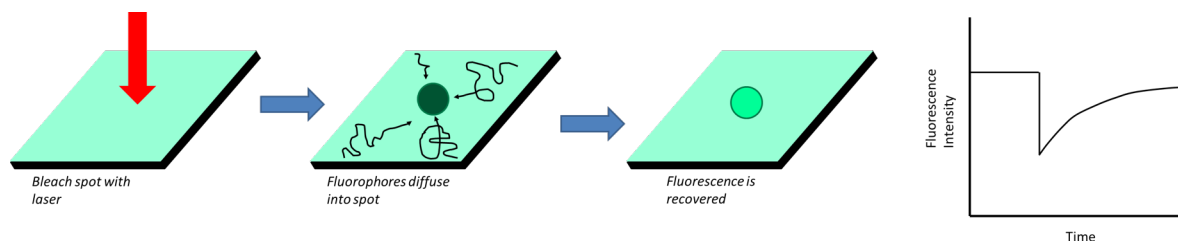


Figure 1-17 The principle of FRAP. A spot is photobleached using a laser. Fluorescent molecules then diffuse into this bleached region causing the fluorescence intensity to increase again. the rate of fluorescence recovery is related to the diffusion kinetics of the system⁶²

2004 Kenworthy *et al*⁶⁴ used FRAP to monitor the diffusion coefficients of several putative raft and non-raft proteins within the cell membrane, finding that raft proteins were able to diffuse freely over large ($> 4 \mu\text{m}$) distances with a large variation in their diffusion coefficients. They interpreted this as evidence that association with lipid rafts is not a dominant factor in determining protein mobility, although it could also be interpreted as evidence that plasma membrane lipids do not in fact form long-lived ordered domains around proteins.

Goodwin *et al*⁶⁵ used FRAP to investigate the effects of plasma membrane viscosity of the diffusion of fluorescently-tagged Ras proteins (Ras is a small membrane-bound GTPase protein known to display exceptionally rapid diffusion through the plasma membrane), with their results suggesting that Ras is able to sense membrane viscosity, potentially allowing for a unique regulation of protein regulation and localisation. In 2014, Nenninger *et al*⁶⁶ used FRAP to investigate the viscosity of the plasma membrane of live *Escherichia coli* cells, finding that viscosity is strongly temperature dependent.

The main limitation of FRAP is that it is essentially a bulk or ensemble measurement, and therefore struggles to identify sub-populations that diffuse differently. To this end, it may be better to use techniques with single molecule sensitivity to investigate viscosity and diffusion within the plasma membrane.⁶²

Fluorescence correlation spectroscopy (FCS) is another fluorescence-based technique that has been extensively⁶⁷ used to investigate viscosity and structure within the plasma membrane. FCS is generally performed using confocal microscopy to detect fluorescent molecules that pass through a small detection volume. The rate of fluorescent molecules passing through the detection volume is then used to calculate the diffusion coefficient of the fluorophore (Figure 1-18).⁶⁸ FCS is a very sensitive and increasingly popular technique, which despite the relative complexity of the data analysis, is becoming increasingly popular in the field of membrane biophysics.

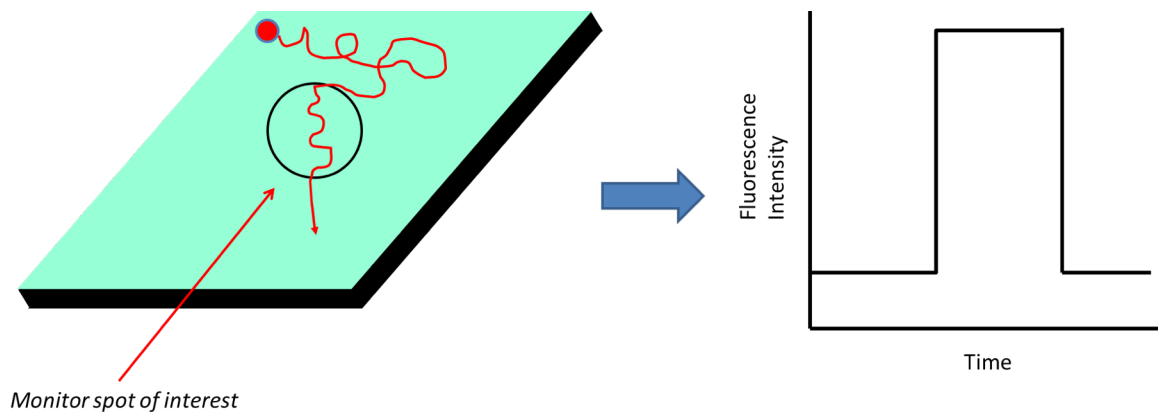


Figure 1-18 The principle of FCS. The fluorescence intensity of a region of interest is monitored. When a fluorophore diffuses through this spot, the observed intensity increases. This allows us to determine the rate of diffusion through the medium and to determine the diffusion coefficient of the fluorophore.⁶²

FCS has been used to show different large difference in diffusion coefficients between model L_o and L_d phases, owing to the increased viscosity of the L_o phase.⁶⁹ In 2004, Bacia *et al*⁷⁰ used FCS to measure the diffusion coefficients of L_o and L_d targeting fluorescent probes in giant unilamellar vesicles (GUVs) displaying L_o - L_d phase separation and in the plasma membranes of live cells. They observed large differences in the diffusion coefficients of the L_o and the L_d targeting probes in both systems, which they interpreted as evidence of phase separation within the plasma membrane but could also be caused by a number of other factors, such as attractive interactions with mobile species within the plasma membrane.⁷¹

FCS studies⁷²⁻⁷⁴ on proteins and lipid raft markers within the plasma membrane have revealed heterogeneous diffusion profiles at a sub-micron scale, which has been interpreted as evidence of the existence of lipid microdomains. It is worth bearing in mind however that this is by no means definitive proof of L_o - L_d phase separation, and there are other factors that could lead to this heterogeneous diffusion, such as transient binding to species within the plasma membrane.

One of the main advantages of FCS is that it can be combined with super-resolution fluorescence techniques, such as stimulated emission depletion (STED), thereby allowing it to bypass the resolution limit of *ca.* 300 nm associated with traditional fluorescence microscopy techniques. This theoretically allows it to directly probe lipid rafts, which are thought to be 10-200 nm in size.³⁵ FCS has previously been combined with STED to investigate the diffusion dynamics of fluorescently tagged lipids, with experiments suggesting that sphingolipids are transiently confined within cholesterol-dependent domains on scales below 20 nm, whereas phosphoglycerolipids aren't. This may be evidence of a transient phase separation into L_o and L_d microdomains within the plasma membrane,⁷⁵ with the size of these domains limited by interactions with the cytoskeleton,⁷⁶

consistent with the current understanding of the plasma membrane.³⁵ Recent evidence⁷⁷ using fluorescent lipids with improved phase-partitioning properties has however suggested homogenous diffusion, regardless of the preference for L_o or L_d phases. This suggests that there is no microscale phase separation within the plasma membrane, and that the transient trapping of the fluorescent sphingolipids that was observed earlier was caused by alternative interactions.

A similar fluorescence-based method of determining diffusion within a membrane is the use of single particle tracking (SPT) whereby individual particles such as fluorescent quantum dots are labelled, successively imaged, tracked and the trajectories analysed. If only a few, well separated molecules are fluorescent, then their location can be pinpointed to a high resolution. The main advantage of this method is that it allows different modes of diffusion of the same molecule to be tracked, allowing the investigation of transient trapping of individual molecules, potentially within L_o phases within the membrane.⁶² Much like FCS experiments however, the results have been inconclusive in probing the existence of lipid domains within the plasma membrane. Certain proteins and membrane inclusions show transient trapping, which may be evidence of lipid phase separation but also of binding to specific receptors causing anomalous diffusion. Anomalous diffusion may also be due to the cytoskeleton creating a “picket-fence” model of the membrane,⁷⁸ where the whole membrane is compartmentalised into 30-250 nm sized compartments causing anomalous “hop-like” diffusion,⁷⁹ where proteins and lipids are slowed down as they hop from one compartment to another, but display free diffusion within these compartments.⁶²

Fluorescence anisotropy studies provide a method to directly determine the viscosity of a medium, rather than indirectly via a diffusion coefficient, and carry the advantage that theoretically any dye can be used for fluorescence anisotropy rather than the specific dyes often required for other techniques.³⁸ The concept of fluorescence anisotropy is based upon the idea that a given fluorophore molecule will only absorb and emit radiation of a certain polarisation. Therefore by controlling the polarisation of the excitation light and monitoring the polarisation of the emission, the rate that the fluorophores rotate and tumble in solution can be determined.⁵⁶ In a viscous, rigid environment, the fluorophore will not be able to rotate in the excited state before emitting a fluorescence photon, meaning that the overall emission will be largely constant in polarisation. By contrast, in a nonviscous media, where the fluorophore molecules are free to tumble more rapidly, allowing them to rapidly change their orientation before emission of a photon, the polarisation of the emission will be much less uniform. Anisotropy measurements have been used to determine the viscosity of a number of biological environments, such as the cytoplasm^{80,81} and different organelles within the cell interior.⁸²

However, without complex experimental setups these techniques are generally restricted to single point measurements and struggle to provide detailed spatial or temporal resolution, preventing the generation of a map of viscosities across a heterogeneous sample.

1.4 Molecular rotors

An alternative fluorescence-based technique for determining membrane viscosity is the use of molecular rotors, which are small organic fluorophores whose fluorescence quantum yield and lifetime are directly related to the viscosity of the surrounding medium. Upon excitation, a molecular rotor can either undergo radiative decay (fluorescence) or decay via a non-radiative pathway which typically involves an intramolecular rotation, hence the name “molecular rotor”. The rate of this intramolecular rotation is directly affected by friction imparted on it by the surrounding environment and an increased solution viscosity will therefore reduce the rate of non-radiative decay. The competition which subsequently arises between the radiative decay, which is typically unaffected by solution viscosity, and non-radiative decay then leads to the rotor displaying viscosity-sensitive fluorescence properties.³⁸ Viscosity can therefore be measured by detecting changes in the fluorescence properties of the molecular rotor.³⁸

1.4.1 The first molecular rotors

The concept of viscosity dependent fluorescence is not a new one. The use of molecular fluorophores with a non-radiative decay pathway involving an intramolecular rotation as a method of probing viscosity was first postulated in 1956 by Oster and Nishijima⁸³ who reported the effects of solution viscosity on the fluorescence of auramine O, a substituted diphenylmethane dye (Figure 1-19). They hypothesised that, in addition to the typical non-radiative decay mechanisms of excited state internal conversion, this dye also displayed a non-radiative decay pathway involving intramolecular rotation of the excited state fluorophore. By calculating the quantum yield of this dye in glycerol and viscous glycerol/water mixtures at a range of temperatures, they showed a linear correlation between the $1/\text{quantum yield}$ and temperature/viscosity.

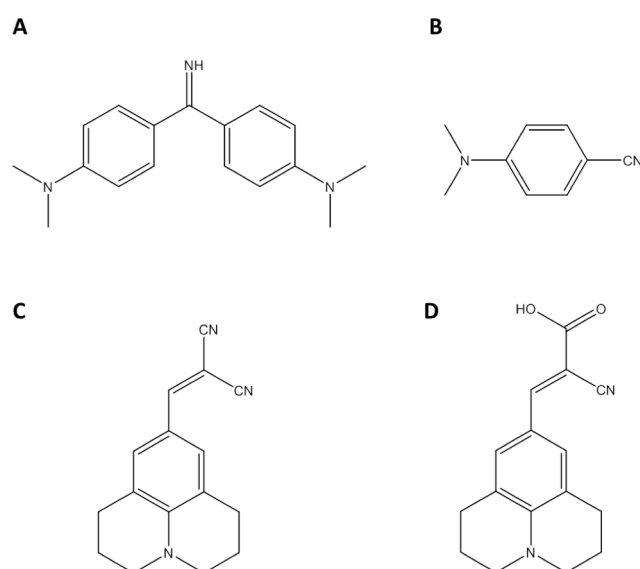


Figure 1-19 Structures of four well-studied molecular rotors, (A) auramine O, (B) DMABN, (C) CCVJ, (D) DCVJ

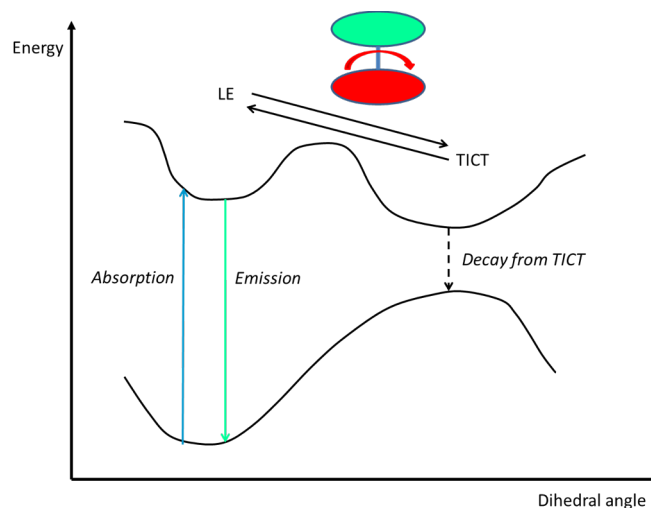


Figure 1-20 Energy diagram for the excitation and emission of a TICT molecular rotor. Absorption of a photon elevated the molecule to the lowest excited (LE) state in a planar configuration, where the dihedral angle of intramolecular rotation is close to zero. From this state, the molecule can either undergo radiative decay via emission of a photon, or it can undergo an intramolecular rotation to a twisted (TICT) state. From this twisted state, the molecule can relax back to the excited state, either non-radiatively or by emission of a lower energy photon than from the LE state, resulting in a red-shifted emission (adapted from⁸⁵)

The first reported class of molecular rotors were those that display a twisted intramolecular charge transfer (TICT) excited state.³⁸ These fluorophores can undergo a twisting motion in the excited state leading to the formation of a dark (TICT) state that allows non-radiative relaxation. The chemical structure of a TICT rotor contains an electron donor unit conjugated to an electron acceptor unit (D- π -A motif). Following photoexcitation this motif is able to relax either by fluorescence emission or by an internal non-radiative molecular rotation that occurs round the σ -bonds that connect the π -electron system of the donor and acceptor.⁸⁴ This leads to the viscosity sensitivity of the fluorescence emission of TICT rotors, as the rate of the rotation pathway leading to this non-radiative TICT state is directly influenced by viscosity of the surrounding media, so an increased viscosity means the TICT rotor is more likely to remain in the emissive state, increasing the fluorescence quantum yield and lifetime (see Figure 1-20 for a sketch of an energy diagram displaying this process).⁸⁵

One of the first developed and best studied TICT molecules is 1,4-dimethylamino benzonitrile (DMABN, Figure 1-19). The TICT state of DMABN was characterised by vibrational spectroscopy,⁸⁶ where the S_1 - S_0 energy gap in the twisted state was found to be large enough to allow emission of a photon when the molecule relaxes from the TICT to the ground state. This results in a second distinct emission band, red-shifted from the first excited state fluorescence. In solutions of high viscosity, the formation of the TICT state is partially inhibited, causing emission from the excited state to be favoured. This means that the ratio of TICT emission intensity compared with excited state emission intensity will decrease with increasing viscosity,⁸⁷ which could potentially be

used as a way of monitoring solution viscosity. The emission profile of DMABN is strongly influenced by solvent polarity however, with the excited state emission being strongly quenched by solvents of increased polarity. The strength of this polarity sensitivity and the fact that polarity and viscosity influences on the emission spectrum cannot be separated suggests that DMABN is not suitable as a viscosity sensor for biological systems, where polarity may not be known and cannot be easily controlled.⁸⁷ Insensitivity to polarity is an important feature in the design of a successful molecular rotor, and will be discussed at several points in this thesis.

Figure 1-19 also shows the structure of two other commonly used molecular rotors, 9-(dicyanovinyl)-julolidine (DCVJ) and 9-(2-carboxy-2cyanovinyl)-julolidine (CCVJ). The mechanism for the viscosity sensitivity of these dyes is much less well understood than for DMABN however, and there are two different proposed mechanisms for the non-radiative decay pathway currently in circulation.⁸⁸ Internal conversion effected by isomerisation around the double bond has been supported by computational^{89,90} and experimental⁹¹ studies although non-radiative decay via a TICT state is also widely accepted.⁴² These julolidine rotors are significantly less sensitive to solvent polarity than DMABN⁸⁷ and so have more potential for use in imaging viscosity within a biological environment. Figure 1-21 shows overlaid emission spectra of DCVJ in solutions of varying viscosity. The emission intensity of the dye increases with solution viscosity, consistent with the hypothesis that it is a molecular rotor. These rotors have found a number of applications in monitoring changes in viscosity in different environments, such as probing the kinetics of mast cell degranulation⁹² and following the polymerisation of tubulin within cells^{93,94}. By attaching a julolidine rotor to the hydrophobic lipid tail, Haidekker *et al* were able to synthesise a molecular rotor that displayed an increased localisation within the membrane of an endothelial cell line.⁹⁵ In their seminal 2000 study, Haidekker *et al*⁴⁷ used DCVJ to investigate the effects of shear stress on the plasma membrane viscosity, finding that an

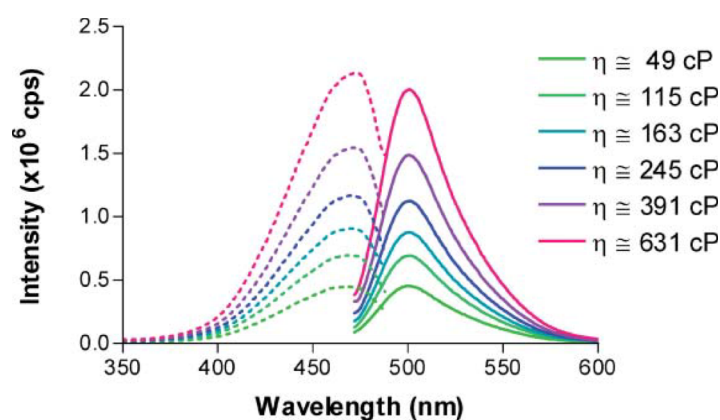


Figure 1-21 Emission (solid lines) and excitation (dashed lines) spectra of a TICT molecular rotor in solutions of varying viscosity. Increasing solution viscosity causes an increase in emission intensity (reproduced from⁴²)

increase in fluid shear stress leads to a reduction in plasma membrane viscosity.

These julolidine rotors do have disadvantages associated with their use however. The absorption and emission profiles of these rotors are within the 350-500 nm region, meaning they are not well suited to biological environments where short wavelength visible radiation displays poor depth penetration and can cause toxicity.⁹⁶ It has also been suggested that DCVJ and CCVJ respond to the porosity of the surrounding environment rather than the actual viscosity. Furthermore, all of the experiments discussed above are based on determination of changes in fluorescence intensity, which suffers from uncertainties associated with unknown concentrations of the rotors, and struggles to provide a quantitative assessment of the viscosity of the environment.

The relationship between fluorescence quantum yield of a molecular rotor and the viscosity of the surrounding media was first investigated by Förster and Hoffmann,⁹⁷ who in 1971 developed their eponymous equation (equation 1-16):

$$\Phi_f = z\eta^\alpha \quad (1-16)$$

where Φ_f is the fluorescence quantum yield, η is the viscosity of the surrounding solution, and α and z are constants unique to a specific molecular rotor. As the theory of Förster and Hoffmann was further developed, the constant α was determined to typically take on a value between 1/3 and 2/3.^{98,99} Note that the Förster Hoffmann equation will only apply within a limited range of intermediate viscosities, as Φ_f becomes solvent independent at low viscosities as the barrier to intramolecular rotation becomes insignificant, and also at very high viscosities, where the radiative decay rate will be significantly higher than the non-radiative decay rate meaning that Φ_f will only have a weak dependence on changing viscosity.

By taking logarithms of both sides of the Förster Hoffmann equation, we obtain the following equation (1-17):

$$\log(\Phi_f) = \alpha \log(\eta) + \log(z) \quad (1-17)$$

A plot of $\log(\Phi_f)$ against $\log(\eta)$ should therefore give a straight line of slope α . This allows us to draw a calibration plot, whereby the quantum yield of the molecular rotor can be related to the solution viscosity. This therefore allows us to determine the viscosity of an unknown solution based on the quantum yield of the molecular rotor.

Whilst this may be a useful method of determining the viscosity of a bulk homogeneous solution, it is not usually applicable to imaging applications, due to the difficulty associated with determining

the local concentration and absorption of a molecular rotor in a heterogeneous system. It is therefore impossible to determine whether changes in the fluorescence intensity are caused by changes in viscosity or local fluorophore concentration and is thus impossible to accurately determine the quantum yield.³⁸ Alternative methods must therefore be developed in order to allow the accurate quantification of viscosity within a heterogeneous environment.

1.4.2 Ratiometric molecular rotors

A potential means of achieving this is by synthetically tethering a molecular rotor to another fluorophore whose quantum yield is unaffected by solution viscosity, allowing the quantum yield of the rotor to be determined by comparison with the other fluorophore. This concept was first developed in 1993 by Luby-Phelps *et al*,¹⁰⁰ who developed a ratiometric method for determining solvent viscosity. The system consisted of a viscosity sensitive Cy3 dye and a viscosity insensitive Cy5 dye, both linked to inert carrier molecules. By comparing the emission intensities of the two dyes, they were able to investigate the viscosity of the cytoplasm, finding it was similar to that of bulk water.

This concept was later refined by Haidekker *et al*,¹⁰¹ who synthetically attached a coumarin-based reference dye to the TICT rotor DCVJ. The emission peak of the reference dye did not change with solution viscosity, whereas the emission intensity of the DCVJ rotor did. They hypothesised that this would allow them to bypass the difficulties associated with determining the quantum yield in a heterogeneous environment, terming this new type of viscosity probe a “ratiometric molecular rotor” (Figure 1-22). This work was built upon by Nipper *et al*,¹⁰² who used a similar ratiometric molecular rotor to probe viscosity in large unilamellar vesicles (LUVs) composed of the saturated lipid DLPC, finding a bilayer viscosity of 61 cP. More recently, ratiometric molecular rotors have been

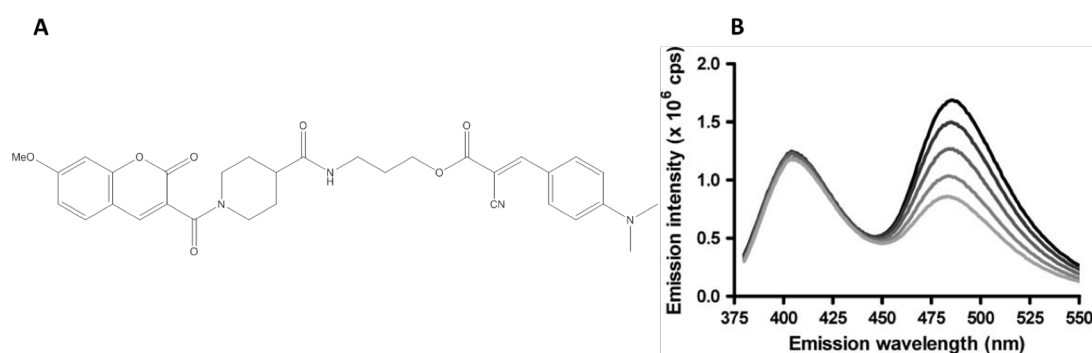


Figure 1-22 Structure (A) and emission in solutions of varying viscosity (B) of a ratiometric molecular rotor. The emission of the reference fluorophore is unaffected by solution viscosity, whereas the emission of the molecular rotor increased with solution viscosity. This allows an accurate assessment of the change in quantum yield, allowing viscosity to be assessed quantitatively (Figure 1-22B reproduced from⁴²)

used to investigate viscosity within the mitochondria,^{103,104} the nucleus¹⁰⁵ and the cytoplasm.¹⁰⁶

Conjugated porphyrin dimers have also been reported to act as ratiometric molecular rotors. In 2009, Kuimova *et al*¹⁰⁷ used a porphyrin dimer that was also capable of singlet oxygen generation to measure the change in viscosity upon photoinduced cell death, finding that there is a sudden increase in the local viscosity around the rotor upon cell death. These porphyrin dimers were later used by Vyšniauskas *et al*¹⁰⁸ to determine viscosity within a lipid monolayer, and to also simultaneously image temperature and viscosity within methanol/glycerol solutions.¹⁰⁹

Whilst the ratiometric method can provide an accurate way to determine the viscosity within a heterogeneous biological environment, it does still have some drawbacks, namely the potential difficulty associated with the synthesis of two covalently-linked fluorescent dyes, and the possibility of artefacts resulting from environmentally induced shifts in the fluorescence or absorption spectra or binding of the probe. Additionally, the ratiometric method makes it difficult to identify autofluorescence from a biological sample.¹⁰⁸

1.4.3 Fluorescence lifetime-based molecular rotors

An alternative approach to using molecular rotors to investigate viscosity within a heterogeneous environment where local dye concentration is unknown is the investigation of the fluorescence lifetime of the molecular rotor, which also increases with the viscosity of the surrounding environment. Unlike emission intensity, the fluorescence lifetime of a probe is concentration independent, assuming no self-quenching or aggregation of the dye, and so by monitoring the fluorescence lifetime of a molecular rotor the viscosity can be determined without any prior knowledge of dye concentration.³⁸ The signal-to-noise ratio in a lifetime imaging/TCSPC experiment will however be affected by fluorescence intensity. This is therefore influenced by fluorophore concentration, meaning that fluorescence intensity in a lifetime experiment should ideally be as high as possible, whilst also taking into account the possibility of aggregation induced quenching, fluorophore toxicity, etc. By substituting equation 1-12 into equation 1-16, we obtain a derivative of the Förster-Hoffmann equation that relates fluorescence lifetime, τ_f , to viscosity, η (equations 1-18 and 1-19):³⁸

$$\tau_f = \frac{z\eta^\alpha}{k_r} \quad (1-18)$$

$$\log(\tau_f) = \log\left(\frac{z}{k_r}\right) + \alpha \log(\eta) \quad (1-19)$$

A lifetime-viscosity calibration plot of $\log(\tau_f)$ as a function of $\log(\eta)$ will therefore give a straight line with slope α . The values of α determined from both quantum yield and lifetime measurements should be identical, and can be used to test the accuracy of lifetime measurements. By using FLIM, an image of the fluorescence lifetimes across a sample can be obtained which can then be converted to a plot of the viscosities across the sample using the calibration plot obtained from equation 1-19. Note that in order to be suitable for lifetime imaging, a molecular rotor should have a high α value, meaning that its lifetime shows a large viscosity dependence, and its fluorescence quantum yield should be high enough to allow rapid signal accumulation.³⁸ The fluorescence lifetime of the molecular rotor should also be long enough to allow easy determination using conventional FLIM, i.e. above *ca.* 100 ps. Some molecular rotors, such as the cyanine based rotors CCVJ and DCVJ that were discussed earlier, have been shown to have very low fluorescence lifetimes,⁸² preventing them from being used as lifetime-based molecular rotors using contemporary FLIM techniques.

The FLIM method has been used alongside several different molecular rotors in order to probe viscosity with a number of different systems. Hosny *et al*¹¹⁰ used FLIM along with the cyanine-based molecular rotor Cy3 to investigate viscosity changes in aerosols upon changes in the relative humidity and ozonolysis reaction, and later¹¹¹ used the same rotor to investigate viscosity changes within aerosol particles upon chemical aging of the particles. In 2014, Jiang *et al*¹¹² reported a cyanine-based molecular rotor capable of both ratiometric and lifetime-based viscosity imaging, using it to probe viscosity within the mitochondria, reporting a mitochondrial viscosity of 12 cP. This value was contradicted by the work of Battisti *et al*,¹¹³ who, using FLIM of a TICT rotor, reported a mitochondrial viscosity of *ca.* 200 cP. Cyanine-based molecular rotors have also been used alongside FLIM to study the viscosity changes associated with the aggregation of proteins in formation of amyloid fibrils,¹¹⁴ and have been used with genetic tagging to allow the targeting of specific proteins within cells.¹¹⁵

1.4.4 BODIPY-based molecular rotors

Molecular rotors based on a boron-dipyrin (BODIPY) core have garnered increasing interest in recent years,³⁸ owing to their high quantum yields and fluorescence lifetimes in comparison with other molecular rotors,⁸² in addition to their chemical stability and relative ease of synthesis and modification.^{116,117} Their intense, narrow emission profiles within the biologically amenable spectral range of 500-700 nm suggests that BODIPY based molecular rotors would lend themselves well to

imaging viscosity within biological systems.¹¹⁸ The generalised structure of a *meso*-phenyl substituted BODIPY dye is shown in Figure 1-23.

meso-substituted BODIPY dyes were first synthesised by Wagner and Lindsey¹¹⁹, who later¹²⁰ noted the comparably low quantum yields of these dyes in conventional solvents compared with other similar BODIPY dyes without the phenyl substitution.¹²¹ Their initial density functional theory (DFT) studies suggested that this effect was caused by rotation of the phenyl group,¹²² which they later¹²⁰ confirmed by synthesising a range of these dyes with and without internal steric hindrance to rotation of the phenyl ring, showing that this low quantum yield is only displayed when the phenyl ring is free to rotate.

They then calculated the relative energies of the ground and excited states of the phenyl-substituted BODIPY molecule as a function of phenyl ring rotation, finding a ground state barrier to rotation of approximately 50 kJmol⁻¹, with the lowest energy conformation being one in which the phenyl ring is twisted to approximately 55° relative to the planar BODIPY structure. In the excited state however, they found the lowest energy to be a planar conformation with a dihedral angle of 180°, with an associated barrier to rotation of 25 kJmol⁻¹. When the phenyl ring takes a dihedral angle of 180°, repulsions between the hydrogen atoms on both groups force the BODIPY group to pucker. This puckered conformation is accompanied by an increased electron delocalisation, which can efficiently decay back to the ground state via nonradiative processes, causing the molecule to display reduced fluorescence (Figure 1-24).

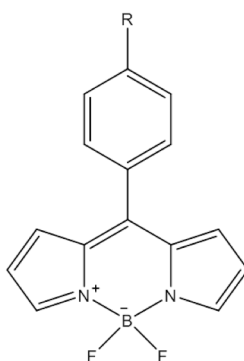


Figure 1-23 Generalised structure of a *meso*-phenyl substituted BODIPY rotor

In 2008, Alamiry *et al*¹²³ as well as Kuimova *et al*¹²⁴ hypothesised that rotation of this phenyl group could be impaired by increasing the viscosity of the surrounding environment, which would lead to an increase in quantum yield, i.e. that this type of BODIPY molecule would act as a molecular rotor. To demonstrate this, they synthesised a prototype with two remote BODIPY rotor groups linked by a long flexible chain (ROBOD, Figure 1-25), and determined fluorescence quantum yield as a function of solution viscosity. They found that the quantum yield of the dye increased with increasing solution viscosity, with a linear correlation on a double logarithmic scale, in agreement with the Förster-Hoffmann equation.⁹⁷ They performed calculations to determine potential energy profiles for the excited and ground states of their BODIPY dye, confirming the mechanism of nonradiative decay suggested earlier by Kee *et al*,¹²⁰ and concluded that BODIPY based molecular rotors had strong potential for use as viscosity sensors.

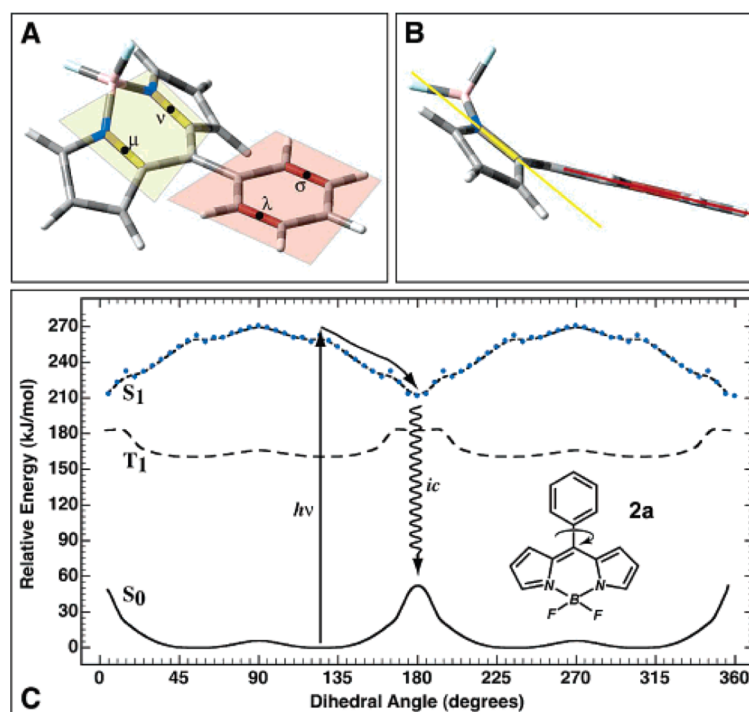


Figure 1-24 The ground state, first excited triplet state and first excited singlet state of a phenyl-substituted BODIPY molecule as a function of phenyl ring rotation (C). In the ground state, the lowest energy conformation of the molecule is one with a dihedral angle of approximately 55°. A dihedral angle of 180° increases electron delocalisation and causes a puckering as shown in panels A and B (Adapted from¹²⁰).

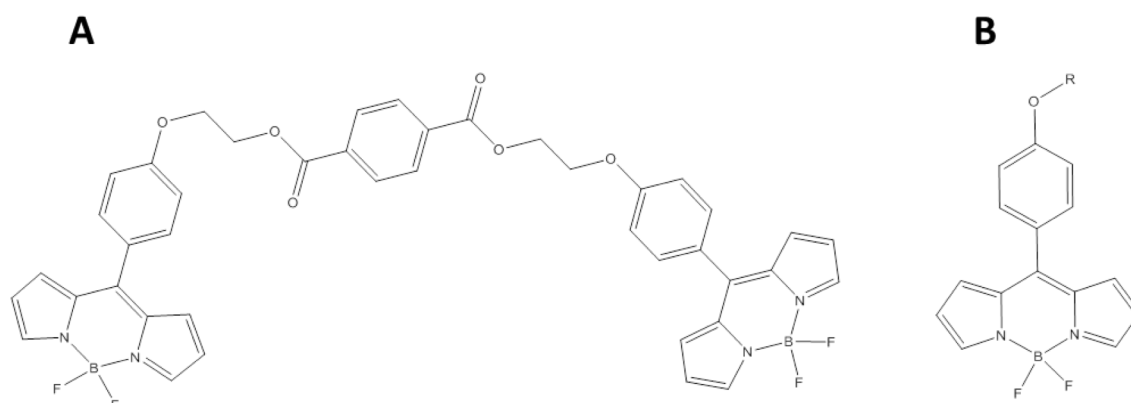


Figure 1-25 Structures of (A) ROBOD¹²³ and (B) BODIPY C12 (R = C₁₂H₂₅)¹²⁴

BODIPY based molecular rotors have since found application in a wide variety of areas such as determining microviscosity within microbubbles,¹²⁵ polymer solutions¹²⁶, sensing pressure¹²⁷ and sensing temperature.¹²⁸ The relative ease of synthetic modification has led to novel molecular rotors based on dimers¹²⁹ and trimers of BODIPY,¹³⁰ in addition to BODIPY-ferrocene dyads.¹³¹

BODIPY based molecular rotors were first applied to the imaging of viscosity in biological environments by Kuimova *et al* in 2008¹²⁴, who used a BODIPY rotor with a 12 carbon chain attached to the phenyl group (BODIPY C12, Figure 1-25) in conjunction with FLIM to image viscosity within the interior of live SK-OV-3 human ovarian carcinoma cells (Figure 1-26). By first “calibrating” the rotor by determining its fluorescence lifetime in methanol/glycerol solutions of known viscosities, they were able to use the lifetime of BODIPY C12 to predict viscosity values. They went on to incubate SK-OV-3 cells with BODIPY C12, finding that the dye internalised to give a punctate (i.e. studded with small dots) staining within the cytoplasm, which they attributed to BODIPY C12 staining the endocytotic vesicles. By comparing BODIPY C12 lifetime with their calibration graph, they calculated

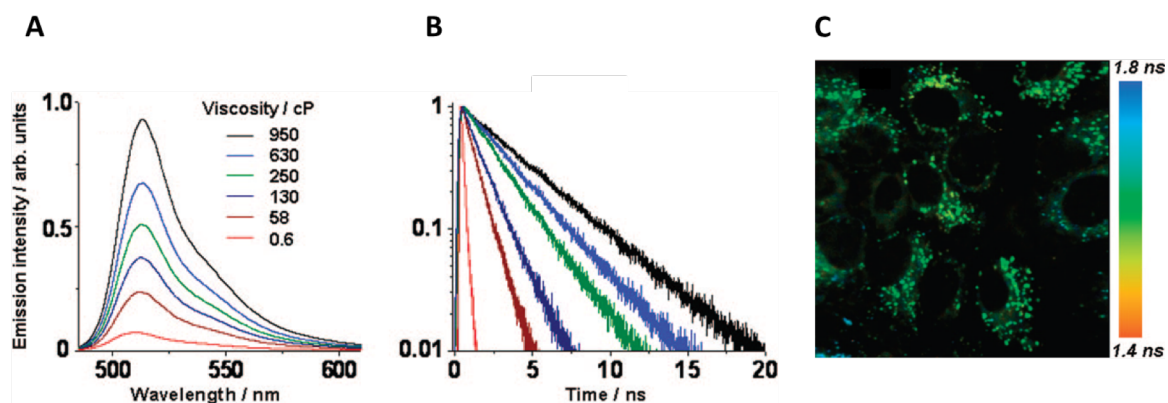


Figure 1-26 (A) overlaid emission spectra of BODIPY C12 in solutions of varying viscosity, (B) fluorescence decay traces of BODIPY C12 in solutions of differing viscosity (C) lifetime image of SK-OV cells stained with BODIPY C12 (adapted from ¹²⁴)

the intracellular viscosity, finding the cells to have an average intracellular viscosity of 140 ± 40 cP. To ensure that this viscosity value was not simply due to the probe binding to intracellular species, causing a restriction in rotation, they performed time-resolved fluorescence anisotropy of the cells as an alternative means of calculating the intracellular viscosity. They calculated a value of approximately 80 cP, which is on the same order of magnitude as the viscosity calculated through FLIM, suggesting that no binding is taking place.

They further developed this work in 2009¹³² by performing FLIM of BODIPY C12 and a similar rotor probe with a farnesyl group attached to the BODIPY phenyl group within SK-OV-3 carcinoma cells. Both of the rotors were once again found to target intracellular domains, showing two distinct regions with viscosity values of 160 ± 20 and 260 ± 40 cP.

In recent years there has been an addition focus on developing synthetically modified BODIPY rotors for targeting individual areas within the cell. In 2013 Yang *et al*¹⁰³ developed a ratiometric BODIPY probe that targeted the mitochondria of HeLa cells, which were found to show a viscosity of approximately 62 cP, which increased to approximately 110 cP upon mitochondrial apoptosis. Ferri *et al* recently¹³³ synthesised a BODIPY rotor that was shown to bind to double stranded DNA, in addition to staining the cytoplasm. Dziuba *et al*¹³⁴ synthesised a BODIPY rotor probe linked to nucleotide, in order to target DNA and investigate changes in the DNA microenvironment. Interactions between DNA incorporating this probe and DNA binding proteins and lipids caused changes in the BODIPY lifetime in the range of 0.5-2.2 ns.

In 2013, Lopez-Duarte *et al*¹³⁵ developed a molecular rotor with a doubly charged carbon chain attached to the phenyl ring which was shown to target the plasma membranes of SK-OV-3 carcinoma cells, reporting an average membrane viscosity of 270 cP. This rotor represents a significant development in the design of BODIPY molecular rotors, as previous membrane soluble dyes have been found to rapidly endocytose,^{124,132} preventing them from being used for investigating the viscoelastic properties of the plasma membrane, and potentially shedding some light on the lipid ordering and heterogeneity within the plasma membrane. This doubly charged BODIPY has not yet been used to give information about lipid ordering or heterogeneity within the plasma membrane, however.

This thesis will focus on the use of molecular rotors to investigate viscosity within lipid bilayer systems, beginning with artificial lipid bilayers as models for L_o - L_d phase separation before moving onto to the investigation of lipid ordering within the plasma membranes of live cells. The work will generally focus on the use of BODIPY rotors, before exploring the use of thiophene-based dyes as

molecular rotors. We first investigate the difference in viscosity between the L_o and L_d phase in model systems, and attempt to verify the molecular rotor derived viscosity by comparison with FCS and computational techniques. We then use molecular rotors to investigate plasma membrane viscosity within *Escherichia coli* bacteria and the effects of temperature on this membrane viscosity. We subsequently move onto exploring synthetic alternatives to our currently available BODIPY rotors, before investigating the feasibility of using thiophene-based dyes as molecular rotors for investigating membrane viscosity. We use these molecular rotors to determine viscosity within the plasma membranes of live cells, with the aim of investigating the phase behaviour of the lipids within the plasma membrane in order to shed further light on the lipid raft hypothesis.

2. Methodology

2.1.1 Overview of materials used

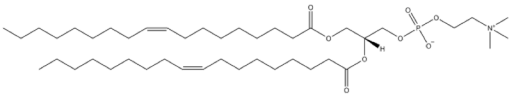
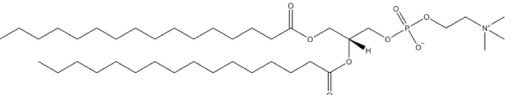
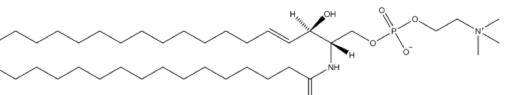
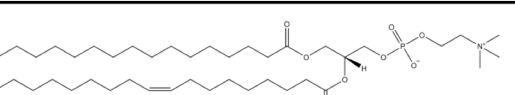
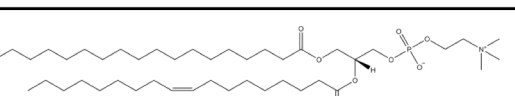
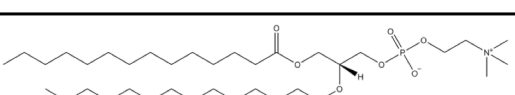
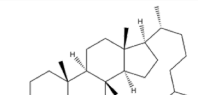
Spectrophotometric grade solvents were used for all spectroscopy studies. The glycerol used in the calibrations described in Chapters 3 and 5 were obtained from Sigma Aldrich (Cambridge, UK). Castor oil and fluorescein were obtained from Acros Organics. For synthetic work, commercial reagents were used as supplied with no further purification. All lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama), and the structures and full names of all the lipids used in this thesis is shown in Table 2-1. Note that this table gives the structure of 16:0 sphingomyelin as a representative structure for egg yolk sphingomyelin, which makes up 84% of its total lipid composition. The rest of the lipid profile of egg yolk sphingomyelin is 6% 18:0, 2% 20:0, 4% 22:0 and 4% 24:0 sphingomyelin.¹³⁶ *E. coli* total lipid extracts were also obtained from Avanti Polar Lipids, and were extracts of *E. coli* B (ATCC 11303) grown in Kornberg Minimal media at 37 °C and taken at ¾ log growth phase. Total *E. coli* lipid extract is a chloroform:methanol extract of the bacteria. This extract is then partitioned against deionised water and the chloroform phase is concentrated. The lipid profile of this extract is 57.5 wt% phosphatidylethanolamine (PE), 15.1 wt% phosphatidylglycerol (PG), 9.8 wt% cardiolipin (CA) and 17.6 wt% unknown.¹³⁷

All lipid bilayer studies used a maximum rotor concentration of 0.5 mol% (1:200 rotor:lipid) to prevent dye aggregation^{41,125} and to avoid significant disruption of the bilayer structure. Viscosities of the methanol/glycerol solutions used for calibrations in Chapters 3 and 5 were determined previously in our group using a Stabinger 300 SVM viscometer (Anton Paar) with 1% accuracy.^{41,135} The castor oil viscosities discussed in Chapter 3 were determined by Craig Young (Department of Chemistry, Imperial College London) using an AR 2000EX rheometer (TA Instruments, New Castle, Delaware)

2.1.2 Synthesis of molecular rotor dyes

BODIPY-C10 was synthesised in our group by Yilei Wu (Department of Chemistry, Imperial College London) according to the procedure outlined in the literature and was used as received.¹³² BODIPY-C++ and BODIPY-Chol were synthesised in our group by Dr Ismael López-Duarte (Department of Chemistry, Imperial College London) via synthetic procedures outlined elsewhere, and were used as received.^{135,138} The planar BODIPY dye BODIPY-alkene was synthesised in the group of Dr Gonzalo

Table 2-1 Names and structures of the lipids used throughout this thesis

	1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)
	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)
	Egg yolk sphingomyelin (EYSM) – representative structure
	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)
	1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC)
	1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)
	Cholesterol

Cosa (McGill University, Canada) as described in the literature¹³⁹ and kindly donated to us. The thiophene rotor described in Chapter 6 was synthesised in our group by Dr Ismael López-Duarte according to the procedure outlined in the literature.¹⁴⁰

The planar BODIPY dye PlanB was prepared in three steps, according to the procedure outlined by Zhu *et al.*¹⁴¹ Intermediates **1** and **2** and PlanB were synthesised as follows.

Synthesis of 1 (8-[(acetyloxy)methyl]-2,2-difluoro-4,6,10,12-tetramethyl-1λ5,3-diaza-2-boratricyclo[7.3.0.0^{3,7}]dodeca-1(12),4,6,8,10-pentaen-1-ylum-2-uide) (Figure 2-1)

2,4-Dimethylpyrrole (1.10 ml, 10.6 mmol) was dissolved in CH₂Cl₂ (100 ml), and acetoxyacetyl chloride (0.53 ml, 5 mmol) was added. The reaction was stirred under argon overnight at room temperature, before being cooled to 0 °C in an ice bath. Triethylamine (2.79 ml, 20 mmol) and then BF₃·OEt₂ (2.47 ml, 20 mmol) were next added dropwise and the mixture was stirred at 0 °C for 3 hours. The solvent was then removed under reduced pressure, and the crude product was purified by column chromatography (silica, hexane/CH₂Cl₂ = 1:1 v/v) and washed with pentane to obtain orange crystals (152 mg, 12% yield). ¹H NMR of **1** (400 MHz, CDCl₃): δ 6.11 (s, 2H, pyrrole-H), 5.32 (s, 2H, CH₂), 2.56 (s, 6H, CH₃), 2.38 (s, 6H, CH₃) 2.16 (s, 3H COCH₃). ¹³C NMR (400 MHz, CDCl₃): δ 170.6,

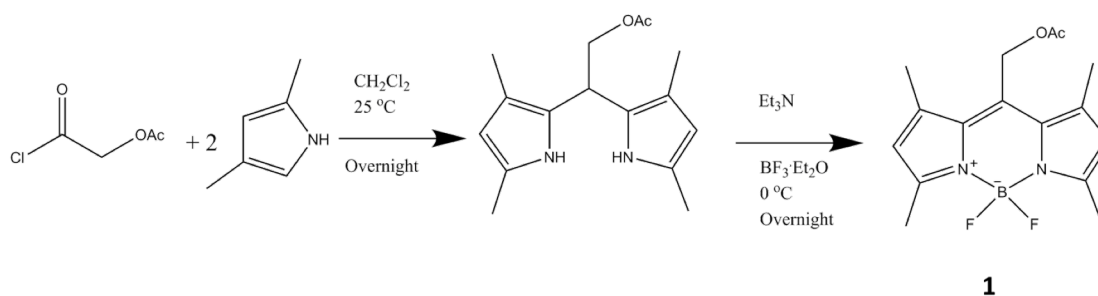


Figure 2-1 Reaction scheme describing the synthesis of **1**

156.6, 141.5, 133.3, 132.7, 122.3, 57.9, 20.6, 15.6, 14.7. **IR** 2957, 1741, 1553, 1508, 1371, 1307, 1190, 1074, 1024, 975, 835, 811, 719 cm^{-1} .

Synthesis of 2 (2,2-difluoro-8-(hydroxymethyl)-4,6,10,12-tetramethyl-1 λ 5,3-diaza-2-boratricyclo[7.3.0.0^{3,7}]dodeca-1(12),4,6,8,10-pentaen-1-ylum-2-uide) (Figure 2-2)

Compound **1** (152 mg, 0.473 mmol) was dissolved in CH_2Cl_2 (5 ml) and MeOH (10 ml) and $\text{NaOH}_{(\text{aq})}$ (0.1 mol dm^{-3} , 2 ml) were added. This reaction mixture was stirred at room temperature for 4 hours under argon. The solvent was then removed under reduced pressure, and the residue was redissolved in CH_2Cl_2 , washed with brine, dried over Na_2SO_4 , filtered and evaporated. The crude product was purified by column chromatography (silica, hexane/ethyl acetate = 3:1 v/v) to obtain an orange powder (31 mg, 24 % yield). **¹H NMR** of **2** (400 MHz, d_6 -DMSO): δ 6.24 (s, 2H, pyrrole-H) δ 5.56 (t, J = 5 Hz, 1H, OH), 4.73 (d, J = 5 Hz, 2H, CH_2), 2.50 (s, 6H, CH_3), 2.43 (s, 6H, CH_3). **¹³C NMR** (400 MHz, d_6 -DMSO) δ 154.9, 142.5, 141.4, 132.2 122.0, 54.6, 15.6, 14.7. **IR** 3551, 2925, 1552, 1508, 1474, 1305, 1199, 1027, 967, 812, 719 cm^{-1} .

Synthesis of PlanB (2,2-difluoro-8-formyl-4,6,10,12-tetramethyl-1 λ 5,3-diaza-2-boratricyclo[7.3.0.0^{3,7}]dodeca-1(12),4,6,8,10-pentaen-1-ylum-2-uide) (Figure 2-3)

Compound **2** (31 mg, 0.113 mmol) was dissolved in CH_2Cl_2 and pyridinium chlorochromate (PCC, 48.7 mg, 0.226 mmol) followed by MgSO_4 (54.3 mg, 0.451 mmol) were added. The reaction mixture was stirred at room temperature under argon overnight, filtered, evaporated and purified by column

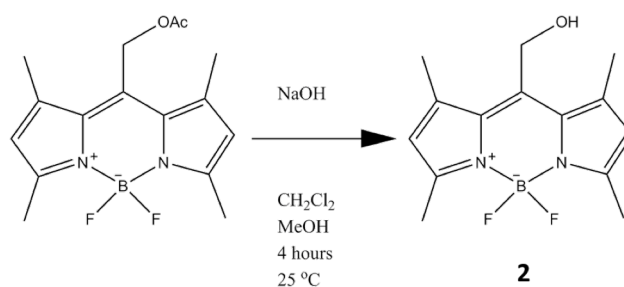


Figure 2-2 Reaction scheme describing the synthesis of **2**

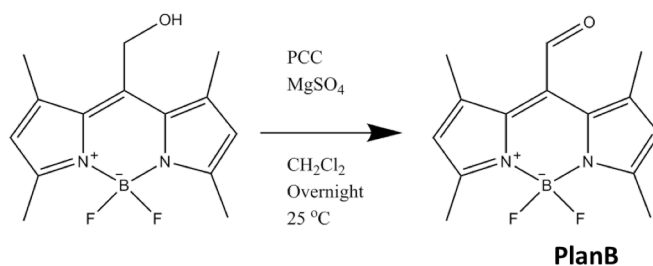


Figure 2-3 Reaction scheme describing the synthesis of PlanB

chromatography (silica, hexane/CH₂Cl₂ = 3:2 v/v) to obtain a purple powder (5.5 mg, 18 % yield). ¹H NMR of PlanB (400 MHz, CDCl₃): δ 10.60 (s, 1H, CHO), 6.11 (s, 2H, pyrrole-H), 2.57 (s, 6H, CH₃), 2.16 (s, 6H, CH₃). ¹³C NMR (400 MHz, CDCl₃): δ 193.0, 158.5, 141.5, 136.0, 128.8, 121.8, 29.7, 15.4, 14.8. IR 2919, 2850, 1710, 1549, 1511, 1308, 1189, 1156, 1068, 977, 808 cm⁻¹.

¹H and ¹³C NMR spectral data are consistent with those described in the literature.¹⁴¹

2.1.3 Preparation of large unilamellar vesicles (LUVs)

Large unilamellar vesicles (LUVs) were prepared according to the gas extrusion technique.¹⁴² A solution of the appropriate lipid or lipid mixture and fluorescent dye was prepared in chloroform, which was subsequently evaporated off under nitrogen. This was then dried under vacuum for at least two hours to remove any remaining solvent, yielding a thin film of lipid and dye. Multilamellar vesicles (MLVs) were then prepared by hydrating this film with enough water to give a *ca.* 1 mmol dm⁻³ solution of lipid, then vortexing for 1 minute above the gel transition temperature of the lipid. This was then extruded with N₂ gas at a pressure of 6-8 bar 10 times through a polycarbonate

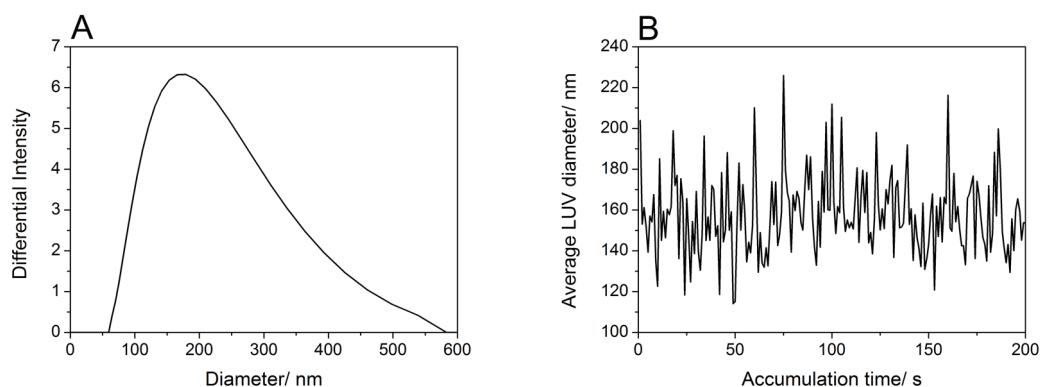


Figure 2-4 DLS studies to determine the average size of DOPC LUVs stained with BODIPY-C10 as produced by gas extrusion. (A) intensity distribution of the DOPC LUVs, (B) average diameter as determined by DLS over 200 seconds

membrane with a pore diameter of 200 nm using a LIPEX extruder (Northern Lipids Inc.), again ensuring that the mixture was kept above the gel transition temperature of the lipid. The average size of the LUVs was determined by dynamic light scattering (DLS) which was performed by William Pitchford (Department of Chemistry, Imperial College London), using a Delsa Nano C particle sizer (Beckman Coulter, Inc.). The average particle was determined to be 179 nm by performing DLS over a period of 200 s (Figure 2-4).

2.1.4 Preparation of giant unilamellar vesicles (GUVs)

Giant unilamellar vesicles (GUVs) were prepared using the electroformation method (Figure 2-5).¹⁴³ A solution of 1 mg ml^{-1} lipid or lipid mixture and fluorescent dye was prepared in chloroform, and added dropwise (*ca.* 30 μl) onto a clean conductive indium tin oxide (ITO) coated glass slide (Sigma-Aldrich) and spread using a glass coverslip to give a thin film of lipid and dye. This was then dried under vacuum for 60 minutes. A polydimethyl siloxane (PDMS) spacer of thickness 2 mm was placed over the lipid film and a second ITO coated slide was then placed on top of the spacer, creating a sealed chamber. The chamber was then filled with 0.1 mol dm^{-3} aqueous sucrose solution with a syringe. A function generator (TG315, Aim-TTi) was then used to apply an AC voltage of 1.0 V and 10 Hz to the chamber for 90 minutes, ensuring that the chamber was held above the gel transition temperature of the lipids being using. To allow observation using phase contrast microscopy, the GUVs formed in this way were suspended in a $0.125 \text{ mol dm}^{-3}$ aqueous solution of glucose.

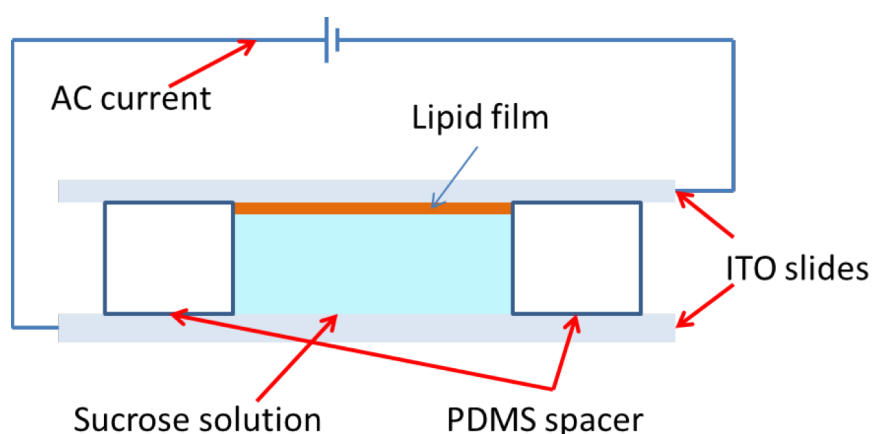


Figure 2-5 Schematic of the electroformation method

2.1.5 Cell culture and preparation for imaging

Cell imaging experiments were performed on a range of cell lines, specified in the individual Chapters. SK-OV-3 and neuroblastoma cell lines were obtained from the European Collection of Cell Cultures (ECCAC), HeLa cells were obtained from Shanas Choudhury (Department of Bioengineering, Imperial College London) and the HCT-116, NIH 3T3 and BE cell lines were obtained from Doug Wylie (Department of Chemistry, Imperial College London). Cells were cultured at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) % penicillin-streptavidin (PS), 1% L-glutamine (LG), and were split when confluence reached *ca.* 70%.

For imaging, live cells suspended in a solution of trypsin (1 ml) were quenched with media (5 ml, DMEM, 10% FBS, 1% PS, 1% LG) and 10 µl of this solution was placed into a haemocytometer and counted under magnification. Enough of this solution was added to each well of a borosilicate chamber slide (Lab-Tek, Nunc) to give 4×10^4 cells per well. The wells were then incubated overnight in DMEM prior to microscopy studies to ensure confluence. For imaging with BODIPY-C10, the cells were incubated in DMEM containing 1-10 µmol dm⁻³ of BODIPY-C10 for 1 hour before imaging. For imaging with BODIPY-C++ or thiophene rotor, ice cold phenol red-free Hank's balanced salt solution (HBSS) without magnesium or calcium was used to induce selective staining of the plasma membrane.¹³⁵ Well chambers loaded with cells were first placed on ice, before the culture media was replaced with cold HBSS containing 1-10 µmol dm⁻³ of BODIPY-C++ or thiophene rotor and the chamber was placed on the microscope stage for imaging at room temperature (22 °C)

The red blood cells used in the experiments described in Chapter 7 were obtained from donors by Marion Koch and Katherine Wright (Department of Life Sciences, Imperial College London).

2.1.6 *Escherichia coli* culture and preparation for imaging

Escherichia coli (*E. coli*) bacteria were grown by Dr Jacek Mika (Katholieke Universiteit Leuven, Belgium) for the experiments described in Chapter 4. Briefly, the *E. coli* strain MG1655 was grown under the conditions described elsewhere.¹⁴⁴ Spheroplasts were produced by Dr Jacek Mika by a technique described elsewhere.¹⁴⁵ The cells were labelled in culture using a stock solution of BODIPY-C10 in DMSO for 2 hours to give a final concentration of 0.2 µmol dm⁻³ for live cells or 0.008 µmol dm⁻³ for spheroplasts. The cells were then centrifuged and the media was replaced, in order to reduce any background fluorescence, and the resulting cell suspension was added to a microscopy well chamber (Lab-Tak) coated with poly-L-lysine. For measurements performed at 37 °C, the chambers and cell suspensions were pre-equilibrated at 37 °C before imaging.

2.2 Measurements

2.2.1 Absorption spectroscopy

In all cases, quartz cuvettes were used for absorption measurements, and wherever possible, absorption at the wavelength of interest was kept below 0.1 to prevent effects such as reabsorption.¹⁴⁶ All absorption spectra were measured using an Agilent UV-Vis 8453 spectrometer (see Figure 2-6 for a schematic of the spectrometer),¹⁴⁷ with deuterium (190-800 nm) and tungsten (370-1100 nm) lamps as radiation sources. The source lens receives radiation from the lamps, collimating it and sending it towards the sample compartment. For measurements, the shutter opens, allowing radiation to reach the sample. In between measurements, the shutter is closed in order to limit the exposure of the sample to radiation from the two lamps. After passing through the sample, the radiation then enters the spectrograph, which processes the radiation in order to determine the levels of absorption. The main components of the spectrograph are the lens, the slit, the grating and the photodiode array with front-end electronics. The spectrograph lens refocuses the radiation after it has passed through the sample. The slit is a narrow aperture in a plate located at the focus of the spectrograph lens exactly the size of one of the photodiodes. By limiting the size of the incoming beam of radiation the slit ensures that each band of wavelengths is projected onto only the appropriate photodiode after diffraction through the grating. The grating acts in a similar way to a prism, separating the beam of light into its constituent wavelengths, and reflecting this as a spectrum. The grating causes the beam of collimated radiation to diffract, dispersing the different wavelengths of the radiation onto the photo diode array, allowing simultaneous access to information across all wavelengths. The diode array is a series of 1024 individual photodiodes covering a wavelength range of 190-1100 nm, meaning that the array has a sampling interval of *ca.* 0.9 nm. The spectrum of radiation obtained from a sample is then subtracted from a blank spectrum taken with no sample present in order to generate the absorption spectrum of the sample.

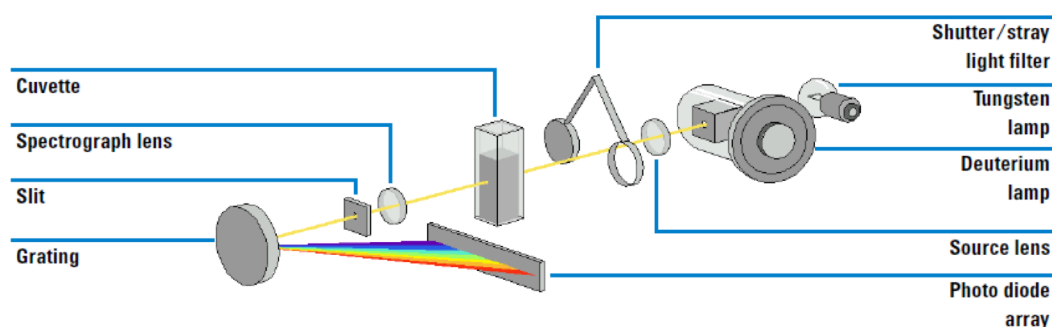


Figure 2-6 Schematic showing the design of the Agilent UV-Vis 8453 spectrometer (reproduced from¹⁴⁷)

2.2.2 Fluorescence spectroscopy

With the exception of the high pressure fluorometer discussed in Chapter 7, all bulk fluorescence measurements were performed using a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer with a 150 W xenon arc-lamp as a source of continuous radiation (see Figure 2-7 for a schematic of the fluorometer).¹⁴⁸ Radiation from the xenon arc-lamp (1) is collected by a diamond-turned elliptical mirror, and then focused on the entrance slit of the excitation monochromator (2). The Fluoromax-4 fluorometer uses Czerny-Turner type monochromators (Figure 2-8).¹⁴⁹

In a Czerny-Turner monochromator, continuous wavelength radiation arrives through an entrance slit and hits a curved mirror which collimates the radiation. This then hits a diffraction grating, dispersing the radiation into a full spectrum. This radiation then hits another curved mirror, which refocuses it onto an exit slit, which blocks all wavelengths except for those that go through the slit. The wavelengths of radiation that are focused through the slit are controlled by rotating the diffraction grating.

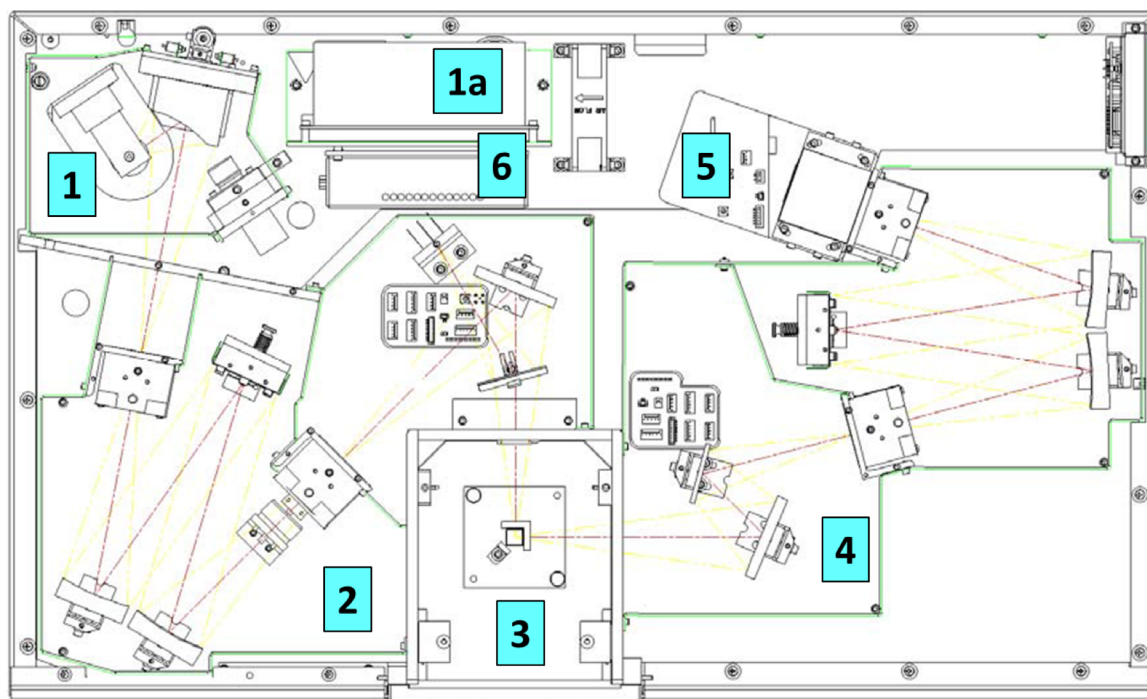


Figure 2-7 Schematic showing the design of the Horiba Jobin Yvon Fluoromax-4 spectrofluorometer (adapted from the user manual¹⁴⁸) 1 – xenon arc-lamp and lamp housing, 1a – xenon-lamp power supply), 2 – excitation monochromator, 3 – sample compartment, 4 – monochromator, 5 – signal detector (photomultiplier tube and housing), 6 – reference detector (photodiode and current-acquisition module). This then connects to the host computer, where emission spectra are processed using FluorEssence software.

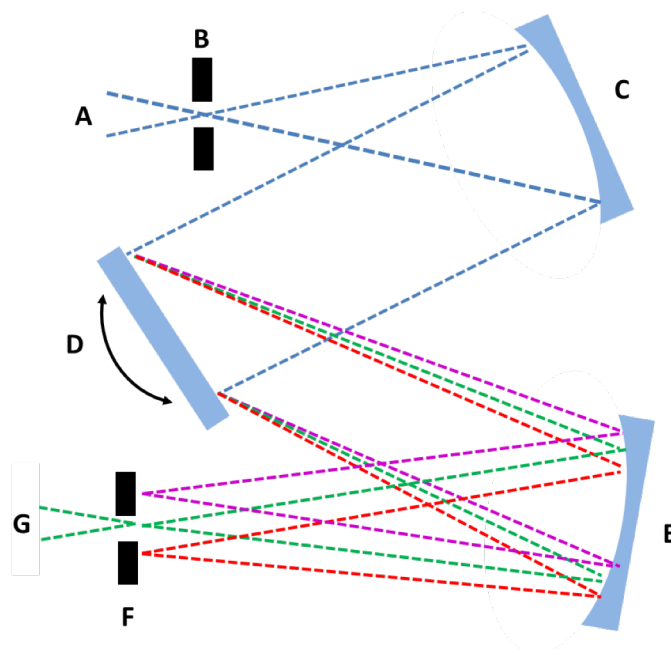


Figure 2-8 Schematic of a Czerny-Turner monochromator. The incoming beam of radiation (A) passes through a pinhole (B), hits a curved mirror (C), collimating the radiation. This then hits a diffraction grating (D), dispersing the radiation. This then hits another curved mirror (E), reflecting it towards a pinhole (F). Radiation of a specific wavelength travels through the pinhole (G), which is controlled by the angle of the diffraction grating.¹⁴⁹

This monochromatic radiation is then directed into the sample chamber (3) via a toroidal mirror.¹⁴⁸ About 8% of this excitation light is split off, using a beam-splitter, to the reference photodiode. Fluorescence from the samples is then collected and directed towards the emission monochromator (4). This radiation is then directed to the emission detector (5), which is a photomultiplier tube (PMT) that increases the signal detected from the emission of the sample. This emission detector measures emission intensity at each wavelength over a given wavelength range in comparison with the reference intensity, in order to generate an emission spectrum. Both the reference and emission detectors have correction-factor files run for them, to correct for wavelength dependencies of each optical component.

The temperature of the sample chamber within the fluorometer was controlled using a Peltier thermostated cuvette holder (F3004, Jobin-Yvon, Horiba), and temperatures were allowed to vary within 0.1 °C of the set temperature and left to equilibrate for 5 minutes.

Emission spectra were acquired using FluorEssence software (Horiba), and then analysed and manipulated using Origin 8.0 (OriginLab Corporation) software.

For quantum yield calculations, the comparative method of Williams *et al*¹⁵⁰ was used, whereby the quantum yield of a dye is determined by comparison with a known standard. As quantum yield is a

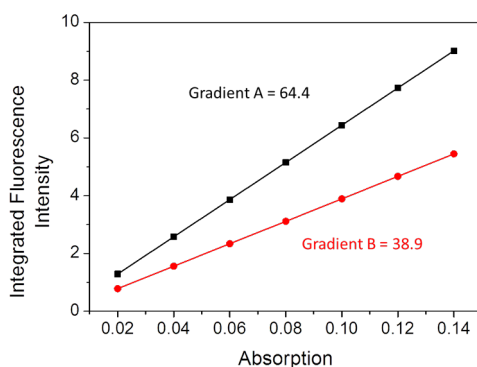


Figure 2-9 Linear plots of absorption against integrated fluorescence intensity. The quantum yield for each sample is proportional to the gradient

measure of the ratio of photons absorbed to photons emitted, if two fluorophores have the same absorbance at a specific wavelength, i.e. they are absorbing the same number of photons, the quantum yield of one dye can be determined by comparing their emission spectra. Therefore, the unknown quantum yield can be determined by comparing the ratios of the integrated fluorescence intensities.

It is difficult to ensure that the reference and the dye have identical absorptions, and it may therefore be more practical to generate a plot of integrated fluorescence intensity against absorption, which should give a linear plot (Figure 2-9). The gradients of these plots can then be compared in order to give the quantum yield of the dye, using equation 2-1:

$$\Phi_x = \Phi_{ST} \left(\frac{Grad_x}{Grad_{ST}} \right) \left(\frac{\eta_x^2}{\eta_{ST}^2} \right) \quad (2-1)$$

Where the subscripts ST and X denote standard and unknown dye, respectively, Φ is the fluorescence quantum yield, $Grad$ is the gradient from the plot of integrated fluorescence intensity against absorbance and η is the refractive index of the solvent. Quantum yield calculations in this thesis use fluorescein in $0.1 \text{ mol dm}^{-3} \text{ NaOH}_{(aq)}$ ($\Phi = 0.95$)⁵⁶ as a standard.

2.2.3 Time-correlated single photon counting (TCSPC)

All lifetime measurements on bulk samples were performed using a Horiba Jobin-Yvon DeltaFlex TCSPC system in reverse mode, as described in Chapter 1, using a pulsed NanoLED (Horiba) source at 404 or 467 nm. Figure 2-10 shows the architecture of a typical TCSPC device in reverse mode.⁵⁸ All TCSPC measurements gave 10,000 counts in the peak channel, and were collected within a 100 ns time window with 1064-4096 channels used. For temperature-controlled measurements, a Lauda

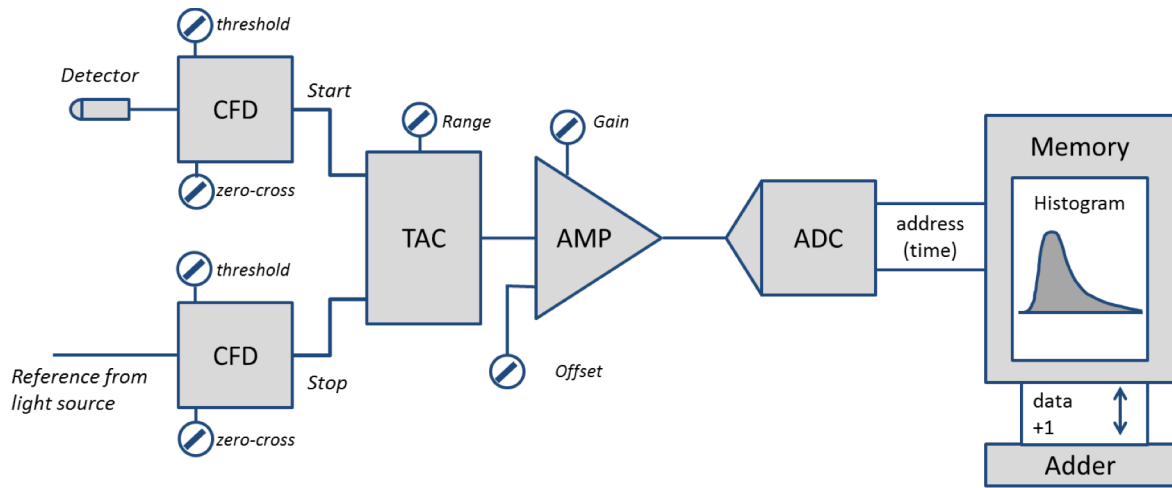


Figure 2-10 Reverse mode configuration of a TCSPC setup (adapted from the Becker and Hickl TCSPC handbook). The detector senses the first photon from the fluorescent sample, which links to a constant fraction discriminator (CFD), which sends a signal to the time to amplitude converter (TAC). A second CFD is used to obtain a timing reference pulse from the light source, in this case a pulsed NanoLED. In reverse mode, the TAC is started when a fluorescence photon is detected and stopped with the next reference pulse from the light source, increasing the time efficiency over conventional “forward mode” TCSPC. The TAC output voltage is sent through a “biased amplifier” (AMP), which has variable gain and a variable offset and is used to select a smaller time window within the full-scale conversion range of the TAC. The amplified TAC signal is fed to the analogue-to-digital converter (ADC), which monitors the arrival time of each photon. The photon arrival times are fed into one of a number of time channels, and a histogram of this data is then used to replicate the overall fluorescence decay.⁵⁸

Ecoline Star Edition circulating pump was used to heat the sample holder, to 1 °C accuracy.

Instrument response functions (IRFs) were recorded on this system using colloidal silica (Ludox, Sigma-Aldrich) suspended in water.

DAS6 software (Horiba) was used for fitting the decays. Decays were established as biexponential if a monoexponential fit for the data gave a χ^2 value of 2 or above and if fitting with a biexponential function improved the quality of the fit (as indicated by a lower χ^2 value). Fluorescence decays were fitted using an exponential function, shown in equation (2-2):⁵⁶

$$I(t) = \sum_i \alpha_i e^{-\frac{t}{\tau_i}} \quad (2-2)$$

where $I(t)$ is the intensity at a given time, α represents the pre-exponential factor and determines the weighting of each lifetime component, t is the time and τ represents the individual lifetime components.

In this thesis, only monoexponential and biexponential fittings were used. For biexponential fits, the mean-weighted fluorescence lifetime (τ_{MWT}) was also used (equation 2-3):

$$\tau_{MWT} = \frac{\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2}{\alpha_1 \tau_1 + \alpha_2 \tau_2} \quad (2-3)$$

2.2.4 Microscopy

Both confocal and multiphoton microscopy was performed using a Leica SP5-II inverted scanning confocal microscope (Leica Microsystems Ltd). A schematic of this microscope is shown in Figure 2-11.

Both the internal lasers of the microscope itself, which have wavelengths of 458, 476, 488, 514 and 633 nm for single-photon excitation, and a pulsed Ti:sapphire laser, (Coherent Chameleon Vision II) with a wavelength range of 680-1080 nm for two-photon excitation, were used as excitation sources for imaging. The laser radiation passes through an objective, focusing it onto the sample. The objectives used for imaging were of 10x magnification (numerical aperture [NA] = 0.3), 20x (NA = 0.7), 40x (NA = 0.85), water immersion 63x (NA = 1.2) with a correction collar (11506279, Leica Microsystems Ltd). The laser radiation is scanned over the sample, and then fluorescence from the sample is collected by the same objective and directed back into the microscope. The fluorescence emission then travels through an acousto-optical beam-splitter (AOBS), a TeO₂ crystal whose reflection and transmission properties are controlled by acoustic waves generated by a mechanical transducer, which replaces the dichroic mirror typically found in confocal microscopes and allows fluorescence to be transmitted and excitation radiation to be filtered off. The fluorescence emission then travels through the confocal pinhole, before entering a prism which disperses the radiation (note that the pinhole was set to its maximum opening for two-photon excitation, which does not require a pinhole). The dispersed radiation then hits the detection system, which consists of three photomultiplier tubes (PMTs), whose wavelength detection region can be controlled due to the dispersion of the emission radiation by the prism.

Imaging at different temperatures was achieved through the use of a temperature-controlled microscope stage (E200, Lauda, Lauda-Königshofen, Germany), with 0.2 °C precision.

The emission spectra of the cells stained with thiophene rotor described in Chapter 6 were also performed using the Leica SP5-II microscope. This was achieved by using a constant excitation source and taking confocal images stepwise at different emission wavelengths, recording the fluorescence intensity of the image at each step (e.g. detection width 10 nm recording each 10 nm between 500 and 700 nm). The fluorescence intensity was plotted against the emission wavelength to generate an emission spectrum using the Leica SP5 image processing software.

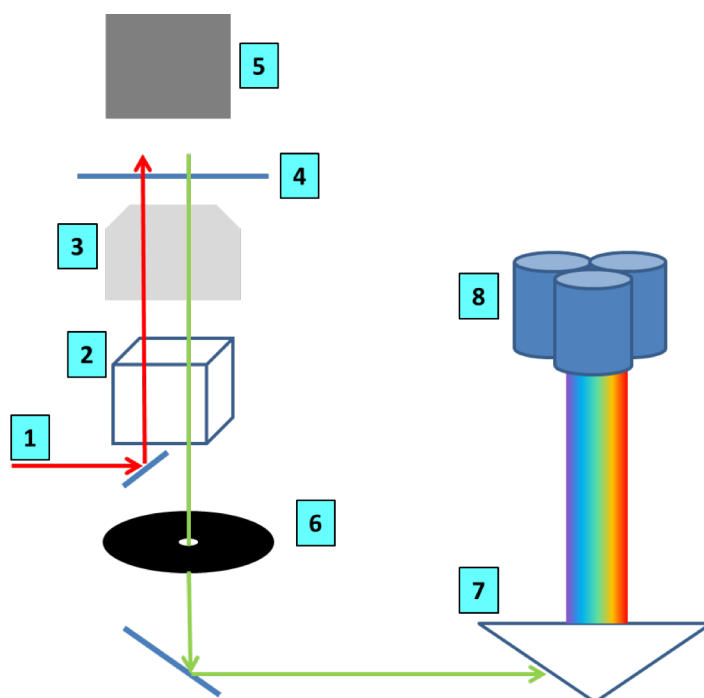


Figure 2-11 Schematic of the confocal microscope used in this thesis. The laser radiation (1) passes through the acousto-optic beam-splitter (AOBS, 2) and the microscope objective (3), irradiating the sample on the microscope stage (4). Transmitted light can be detected (5) and used to generate a transmission image. Fluorescence emission from the sample passes through the AOBS and the confocal pinhole (6) and enters the prism (7), which disperses the radiation. This is then collected by the photomultiplier tubes (PMTs).²⁸⁹

For the cell imaging experiments performed under shear stress described in Chapter 7, flow was generated using a peristaltic pump (MPII Mini-Peristaltic Pump, Harvard Apparatus, Holliston, Massachusetts, USA), connected to the flow chamber by a tube, which maintained a constant fluid velocity. HeLa cells were cultured in flow chambers designed for confocal imaging (μ -slide I 0.4 Luer ibiTreat: #1.5 polymer coverslip, tissue culture treated, sterilised, Ibidi, Munich, Germany), stained with the appropriate dye and media was flown over the top of the cells using the pump. The flow cell was placed on the microscope stage and lifetime images were obtained in the manner described above.

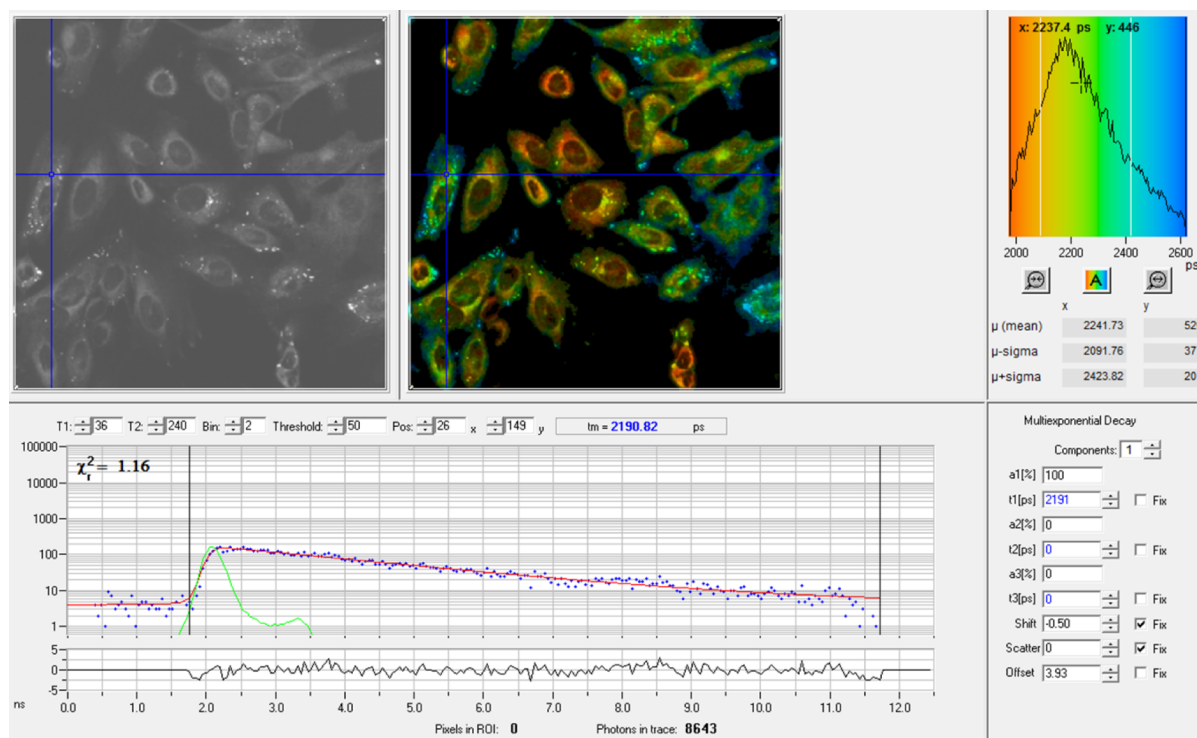


Figure 2-12 Screenshot of the SPCImage software used to process lifetime images (image shows HeLa cells stained with BODIPY-C10). Images were binned in order to give a photon count of above 100 counts in the peak channel in order to allow accurate monoexponential fitting, and a threshold was used to discount any pixels below a certain intensity to eliminate background signal (in this case the threshold was set to 50). The χ^2 for this decay is 1.16, indicating a good quality of fit. The red line indicates the monoexponential fitting, the blue points represent the photon count for that channel and the green curve represents the instrument response function (IRF). Underneath the plot showing the decay and fitting is a plot showing the residuals, which indicate the goodness of fit and help determine whether any deviation from the fitting is caused by systematic noise, or due to an inadequate fitting method. Shown in greyscale is the two-photon image taken over a set time (in this case 300 s), and the image shown in colour is the lifetime image, where the colour scale represents the fluorescence lifetime as determined through monoexponential fitting, with red representing the lower bound of the lifetime range (in this case 1950 ps) and blue representing the upper bound (in this case 2650 ps). For this image the mean lifetime, μ (mean) is 2251.73 ps.

2.2.5 Fluorescence lifetime imaging microscopy (FLIM)

FLIM was performed using the previously described Leica SP1 microscope, with a Ti:sapphire Coherent Chameleon Vission II 80 MHz femtosecond laser as an excitation source, over a wavelength range of 680-1080 nm, which enabled two-photon excitation, and a TCSPC card (SPC-830, Becker and Hickl GmbH). Images were recorded at a size of 256x256 pixels and 256 time bins for fluorescence decays. The second harmonic generation signal from urea crystals, which are efficient two-photon scatterers, were used to record the instrument response functions (IRFs).

With the exception of the lifetime images of the *E. coli* live cells and spheroplasts, lifetime images were analysed using SPCImage software (Becker and Hickl). An example of this software being used for fluorescence decay fitting is shown in Figure 2-12. If they did not have the 100 photon count in the peak channel required for monoexponential fitting, lifetime images were binned in order to

increase the signal at the expense of 2D resolution. For multiexponential decays, lifetime images were binned until they displayed a peak photon count of *ca.* 500 counts. Lower intensity pixels were discounted by setting a threshold to the image.

For the lifetime images of the *E. coli* live cells and spheroplasts, analysis was performed using the FLIMfit software developed at Imperial College London.¹⁵¹ To enable a photon count sufficient for biexponential decay fitting, regions of interest were manually drawn around individual cells and all the photons within this region were binned together giving a single decay with a high signal to-noise ratio, which was then fitted to a biexponential decay. See Figure 2-13 for an example screenshot illustrating this fitting process.

The phasor analysis described in Chapter 6 was performed by Aurimas Vyšniauskas using in-house MatLab software (MathWorks).

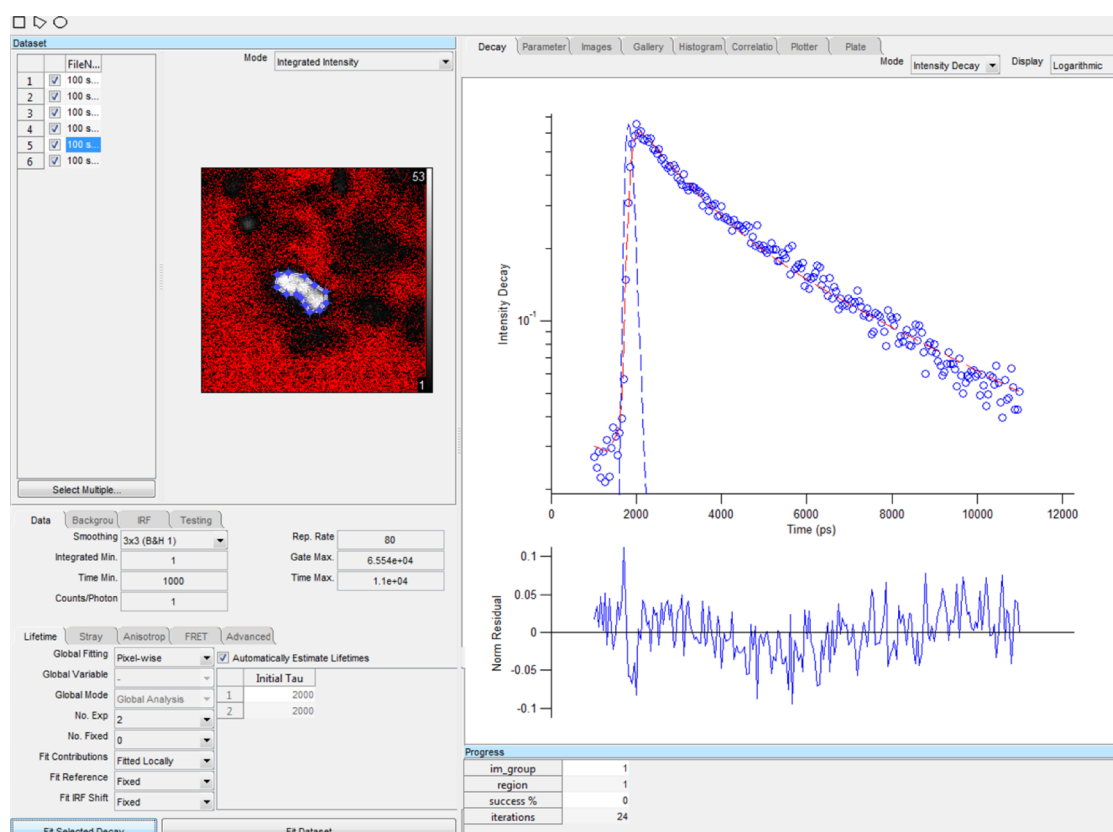


Figure 2-13 Screenshot of the FlimFit software used to analyse lifetime images of *E. coli*. Regions of interest were drawn around individual bacteria and all the pixels within this region were binned together to give one decay, which was then fitted using a biexponential fit. This was done in order to ensure that each bacterium gave a sufficiently high photon count to allow biexponential fitting to a sufficient degree of accuracy

BODIPY-based molecular rotors are known to exhibit aggregation induced quenching at high concentrations, resulting in a red shifted secondary emission peak between 630-670 nm with a long fluorescence lifetime, and a reduced fluorescence lifetime within the region of 500-550 nm as the excited state of the BODIPY-rotor is quenched. This leads to a biexponential decay and will cause incorrect calculations of the viscosity.⁴¹ In order to ensure that this was not occurring in the measurements described in this thesis, single fluorescence decays for the whole image were taken within wavelength ranges of 500-550 nm (encompassing the emission maximum of the non-aggregated BODIPY rotors) and 600-700 nm (encompassing the emission maximum of aggregated BODIPY). If the fluorescence decay was longer in the 600-700 nm range, this was taken as a sign of rotor aggregation and the experiment was repeated at a lower BODIPY rotor concentration. In general for lipid bilayers, a lipid:rotor molar ratio of 200:1 or greater was used to prevent dye aggregation.^{41,125}

2.2.6 Nuclear magnetic resonance (NMR) Spectroscopy

¹H and ¹³C NMR spectra were recorded using Bruker AV-400 (400 Hz) NMR Spectrometer, and spectra were analysed using MestReNova software (Mestrelab Research). The deuterated solvents that were used are specified in section 2.1.2 and were used as a lock.

2.2.7 Infrared (IR) spectroscopy

Infrared spectra were taken using a Spectrum 100 FT-IR Spectrometer (Perkin Elmer Inc.) and analysed using Perkin Elmer Spectrum 10 software.

2.2.8 Molecular dynamics simulations

The molecular dynamics simulations described in Chapters 3 and 6 were performed by Callum Dickson using the AMBER^{152,153} suite using lipid parameters described elsewhere.^{154,155} Briefly, diffusion of the dyes inside DOPC and DPPC bilayers was simulated at a range of temperatures in order to investigate the preferred orientation of the dye and the diffusion coefficient of the dye within the bilayer. To obtain the preferred orientation of the dye, the dye was placed in the water phase above the bilayer and the system was simulated for 100 ns at 303 K, during which time the dye entered the bilayer.

2.2.9 Fluorescence correlation spectroscopy (FCS)

FCS was performed by Niall Geoghegan from the University of Glasgow, using the Becker and Hickl DCS-120 confocal laser scanning system. The excitation source was a 473 nm 50 MHz pulsed diode laser, and a water immersion 40x (NA = 1.2) objective was used.

2.2.8 Wide angle X-ray scattering (WAXS)

The WAXS experiments described in Chapter 4 were performed with the assistance of Dr Nick Brooks. Experiments were performed using a custom built X-ray beamline designed for both small and wide angle X-ray scattering experiments, giving access to a d-spacing range of *ca.* 3-200 Å. Samples were held in a glass capillary (Capillary Tube Supplies, Ltd, Cornwall) and temperature was controlled using a peltier thermoelectric heater and monitored using a PT100 temperature sensor. Analysis was carried out using custom built software in the LabView package (National Instruments UK, Newbury) to find the centre and amplitude of the wide angle X-ray peak. Gel phase bilayers were identified by their characteristic wide angle peak at *ca.* 4.2 Å.

2.2.9 High pressure fluorometry

For the emission spectra recorded at high pressure discussed in Chapter 7, a home-built high pressure network was used to generate the high hydrostatic pressures (Figure 2-14). High-pressure apparatus was obtained from SITEC-Sieber Engineering AG (Zurich, Switzerland) and LEDs and their power sources were obtained from ThorLabs (Newton, New Jersey, USA). Emission spectra for BODIPY-C10 in LUVs were obtained using a 467 nm LED and an Ocean Optics Flame-S miniature spectrometer (Ocean Optics, Oxford), and spectra were recorded using home-built software on the LabView platform. Experiments were performed with the assistance of Leanne Baker (Undergraduate summer student, Department of Chemistry, Imperial College London)

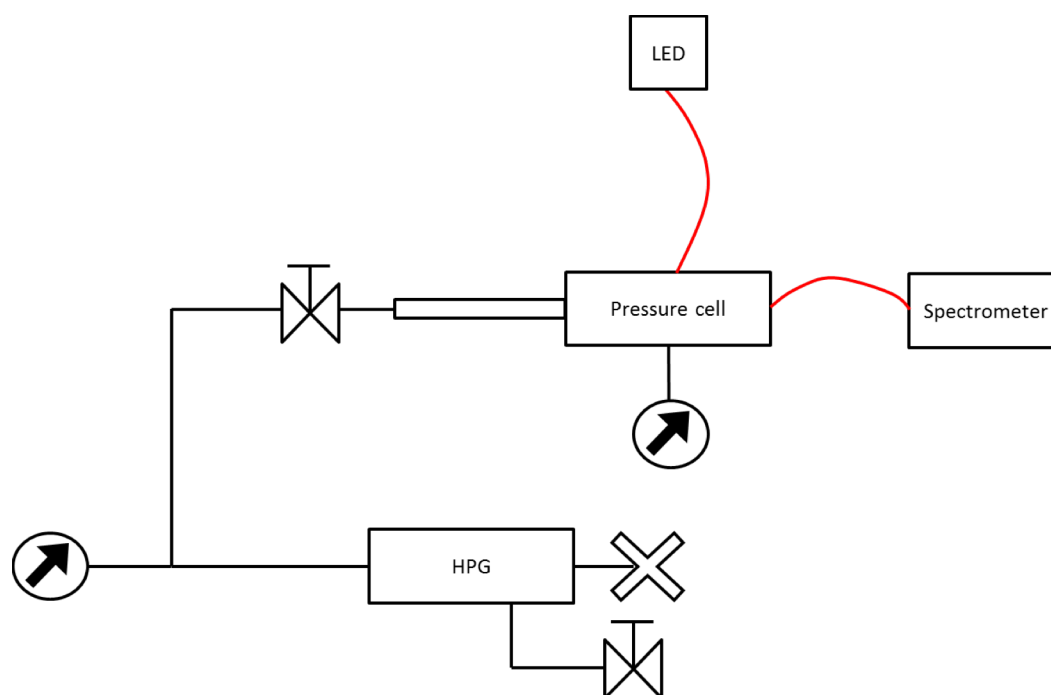


Figure 2-14 Schematic of the design of the cell used for obtaining emission spectra at high hydrostatic pressures

The pressure cell itself (Figure 2-14) consists of a high tensile strength steel body with a central cavity that contains the sample. The sample chamber is sealed on two sides by transparent sapphire windows, capable of withstanding pressures up to *ca.* 150 MPa, which are mounted on metal window holders. These are connected to fibre optic cables that lead to an LED that acts as an excitation source and a spectrometer that records the spectra. The sample chamber is also connected to a pressure transducer that converts pressure into voltage, allowing accurate measurement of the pressure within the sample chamber. Finally, the sample chamber is connected a water-filled pressure network, via a pressure separator that prevents mixing of the sample with the water from the network. Pressure is generated using a hand-operated pump, and water is fed into the system via a valve attached to this pump. This system provides an inexpensive method of recording spectra at high pressures, and its modular nature makes it highly flexible; different LEDs allow a variety of excitation wavelengths, and different spectrometers could also be potentially used for different applications. Additionally, this system also potentially allows the incorporation of other more complex systems, such as pressure jump apparatus.

3. Imaging Phase Separation in Model Membranes using BODIPY based molecular rotors

The work in this chapter is adapted from that published as Dent, M. R. et al. Imaging Phase Separation in Model Lipid Membranes Through the use of BODIPY Based Molecular Rotors. Phys. Chem. Chem. Phys. 17, 18393–18402 (2015).

All molecular dynamics simulations reported in this Chapter were performed by Callum Dickson and all FCS studies were performed by Niall Geoghegan

3.1 Introduction

The plasma membranes of live cells are highly complex and dynamic systems, and as such can be very difficult to study. Artificial lipid bilayers can provide a much simpler system for studying the physical properties of lipids and are therefore widely used within lipid research. Several types of artificial bilayer have been developed previously that display different characteristics for use in different situations, with examples including supported lipid bilayers (SLBs),¹⁵⁶ black lipid membranes¹⁵⁷ and lipid monolayers.¹⁵⁸ Large unilamellar vesicles (LUVs) are vesicles consisting of one continuous lipid bilayer at a size scale of between 30-400 nm, and provide a useful model membrane system, due to their ease of production in large quantities via the extrusion method.¹⁵⁹

Due to their size, which is on the same order as the size of cells,¹⁴³ allowing them to be imaged using optical microscopy, giant unilamellar vesicles (GUVs) are commonly used model lipid bilayers. GUVs can be easily made with a variety of compositions using the electroformation technique first pioneered in 1992 by Angelova *et al.*,¹⁶⁰ and, as such, make useful models of plasma membranes. Whilst phase-separation of lipids within the plasma membranes of live cells into coexisting liquid-ordered (L_o) and liquid-disordered (L_d) phases remains a contentious issue within the research community, it is well-characterised within GUVs (Figure 3-1).^{16,161} Phase-separation into coexisting L_o and L_d phases can be observed in GUVs consisting of ternary lipid mixtures, meaning that such mixtures can be used as simple models for the lipid raft hypothesis.¹⁶² GUVs can be built to a high degree of complexity, allowing reconstitution of membrane proteins thought to associate with L_o or “raft” phases within them,^{163,164} as well as membrane asymmetry¹⁶⁵, allowing accurate replication of certain properties of the plasma membrane.

Despite the fact that L_o - L_d phase separation can be readily observed within ternary lipid bilayers, and even within giant vesicles composed of cellular lipid extracts³², the fact remains that this phenomenon is not as easily observed *in vivo*. Yethiraj and Weisshaar postulated³⁴ that integral membrane proteins attached to the cytoskeleton prevent formation of the large lipid phases commonly observed in artificial bilayers. Their work highlights the fact that any conclusions drawn from studies within artificial membrane may not be applicable when describing the plasma membranes of living cells.

Fluorescence-based techniques have found widespread use in the study of the physical properties of lipids within a bilayer, owing to their capability of operating *in situ* with high speed and sensitivity, despite the potential drawback of requiring the use of a fluorescent probe, which may alter the bilayer's physical properties.¹⁶⁶ Another potential caveat to the use of fluorescence-based techniques to investigate lipid phase behaviour is the fact that the majority of lipid-soluble fluorescent probes and fluorescent lipid analogues will preferentially partition within the L_d phase, significantly reducing fluorescence signal from the L_o phase.^{166–168} This means that caution is necessary when choosing or designing fluorescent probes for use in phase-separated bilayers.

The phase characteristics of the lipids within a bilayer are known to affect the diffusion coefficients of both the lipids themselves and any membrane inclusions, with lipid diffusion coefficients within L_d phases being several times higher than in cholesterol-rich L_o phases.¹⁶⁹ Various fluorescence based techniques including fluorescence correlation spectroscopy (FCS),^{71,170} and fluorescence recovery after photobleaching (FRAP)¹⁷¹ have been used to extract diffusion coefficients from different lipid phases within model membranes.

Molecular rotors have also been used to investigate viscosity and phase behaviour within model

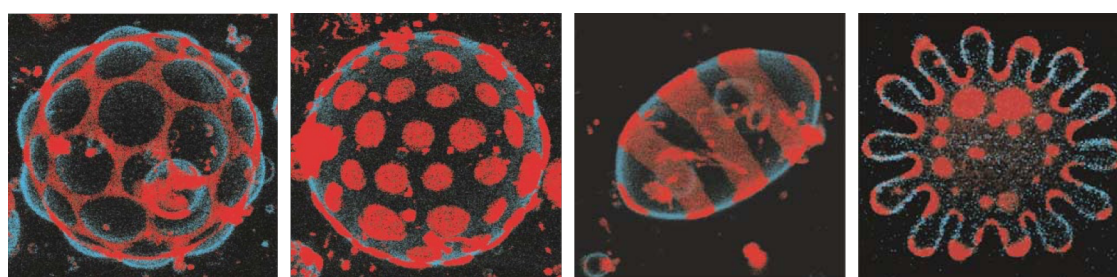


Figure 3-1 An example of the variety of different possible architectures and structures of phase-separated GUVs (adapted from¹⁶¹)

membrane systems. In 2002, Haidekker *et al*⁹⁵ synthesised membrane-soluble cyanine-based molecular rotors with the aim of targeting cellular plasma membranes, incorporating them into dimyristoyl-phosphatidylcholine (DMPC) vesicles and monitoring emission intensity with temperature. As temperature increases, bilayer viscosity decreases, meaning that emission intensity decreases with temperature. At the gel-to-liquid phase transition temperature of DMPC (23 °C), where the bilayer moves from a rigid and viscous gel phase to a fluid lamellar phase, they paradoxically observed an increase in emission intensity, which they attribute to the formation of local gel-phase domains, but may also suggest a change in bilayer orientation of the dye. They later¹⁷² incorporated another cyanine rotor into dilauroyl-phosphocholine (DLPC) GUVs and determined the effect of a number of compounds on membrane viscosity, including cholesterol, which was shown to increase bilayer viscosity. DLPC forms L_d phase bilayers at room temperature¹⁷³, meaning that the condensing effect of cholesterol could be expected to lead to an increase in bilayer viscosity.¹⁷⁴ By using a ratiometric system consisting of a cyanine rotor attached to non-rotor fluorophore,¹⁰² they later attempted to overcome the problem of local concentration affecting rotor quantum yield in heterogeneous environments, as discussed in Chapter 1. By calibrating their ratiometric rotor in solutions of known viscosity, they determined a bilayer viscosity of 61 cP in DLPC GUVs. By using cyanine-based molecular rotors to probe relative viscosity changes in lipid vesicles, Xu *et al*¹⁷⁵ showed that introduction of a local anaesthetic disrupts lipid chain packing in lipid bilayers, causing a reduction in bilayer viscosity, highlighting the potential for molecular rotors to be used as probes for drug analysis.

By imaging phase-separating GUVs using a BODIPY-based molecular rotor, BODIPY-C10 (Figure 3-2), Wu *et al*⁴¹ showed that the L_o phase within a model bilayer displayed a higher viscosity than an L_d phase, presumably owing to the increased molecular packing within the L_o phase. They did however note the poor partitioning of the BODIPY rotor into the L_o phases of the GUVs, which may cause issues when attempting to visualise similar “raft” phases within plasma membranes, owing to the reduced fluorescence signal. In addition to this, by using two-foci FCS, they determined the diffusion coefficient of the BODIPY rotor within a model L_d phase, finding a diffusion coefficient of $11.7 \mu\text{m}^2\text{s}^{-1}$, comparable to diffusion coefficients of non-rotor BODIPY-lipid analogues in the same system. They conclude that BODIPY rotors are membrane soluble, and that they show good potential for use in determining lipid viscosity and phase behaviour within lipid bilayers, potentially even within live cells.

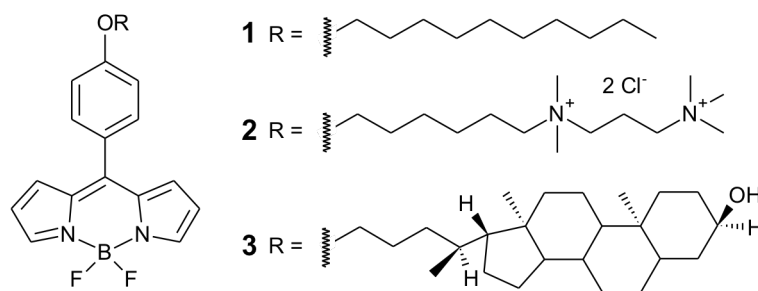


Figure 3-2 Structures of the three BODIPY rotors used in this chapter: (1) BODIPY-C10, (2) BODIPY- C++, (3) BODIPY-Chol

BODIPY-C10 and other BODIPY rotors with similar structures have previously been used to image live cells, rapidly internalising into the cell interior and staining organelles within the cytoplasm.¹²⁴ In 2009, Levitt *et al*¹³² used two membrane-soluble BODIPY rotors to image viscosity within SK-OV-3 cells. The rotors were both taken up into the cell interior, staining two organelles with distinct membrane viscosities of 160 ± 20 cP and $260 \pm$ cP, although the exact nature of the organelles was unknown. They went on to hypothesise that synthetic modifications to the BODIPY rotors may allow them to target certain regions of the cell.

Despite the fact that it was designed to be membrane-soluble, the rotor BODIPY-C10 was unable to image the plasma membranes of live cells, due to the rapid endocytosis mechanism taking it up into the cell interior. As a way of overcoming this, in 2014 López-Duarte *et al*¹³⁵ designed a BODIPY rotor BODIPY-C++ (Figure 3-2) with a double positive charge on the hydrocarbon tail attached to the phenyl group of the BODIPY core with the aim of reducing endocytosis. After incubation with SK-OV-3 cells at 4 °C in Mg^{2+} and Ca^{2+} free media with BODIPY-C++, selective plasma membrane staining was observed, allowing fluorescence lifetime imaging of the plasma membrane in order to investigate its viscosity. They reported a plasma membrane viscosity of *ca.* 270 cP, considerably lower than previously calculated for model L_o phases,⁴¹ with the membrane appearing to be homogeneous in terms of viscosity. As previously stated, many membrane soluble dyes, including BODIPY-C10, do not partition to any great extent into L_o phases of model bilayers, so it is hard to discern whether this observation is due to a lack of membrane phase separation within the SK-OV-3 plasma membranes or is due to the BODIPY-C++ rotor not effectively staining any L_o phases that do exist within the lipid bilayer.

In an attempt to address this potential shortcoming of the previously synthesised BODIPY rotors, the rotor BODIPY-Chol was synthesised by Ismael Lopez-Duarte in our group (Figure 3-2), where a cholesterol moiety was covalently attached to the BODIPY rotor core with the aim of targeting

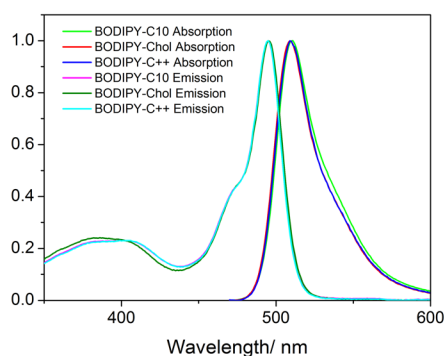


Figure 3-3 Overlaid absorption and emission spectra of the three BODIPY rotors

cholesterol rich regions in cells and model bilayers. Cholesterol is known to interact in a similar way to cholesterol inside lipid bilayers, albeit with weaker interactions.^{176,177} Because L_o phases are rich in cholesterol, cholesterol or cholesterol containing fluorophores should partition preferably into the L_o phase,¹⁷⁸ provided that their structures do not disrupt the ordered packing within the L_o phase. A variety of cholesterol derivatives of non-rotor BODIPY dyes have previously been synthesised,^{179–181} with some having even been reported to target L_o phases within model lipid bilayers.^{178,182} In 2013, Solanko *et al*¹⁷⁸ synthesised two non-rotor BODIPY-cholesterol derivatives, showing that they preferentially targeted the L_o phase of phase-separating GUVs. They were able to stain the plasma membranes of cultured cells using these dyes, although they did not observe any hindered diffusion upon studying these plasma membranes using FCS, suggesting that the probes were not diffusing between different lipid phases within the membrane. If the rotor BODIPY-Chol is also capable of partitioning into the L_o phase of model bilayers, it could potentially be used to probe phase behaviour within the plasma membranes of live cells, detecting the differences in viscosities between L_o and L_d phases.

In this chapter we investigate the use of BODIPY-based molecular rotors BODIPY-C10, BODIPY-C++ and the previously unpublished BODIPY-Chol as probes to determine viscosity within model lipid bilayers. By first calibrating the rotors within solutions of known viscosity and then calculating membrane viscosity within different lipid phases of model bilayers, we use the rotors to discern between coexisting L_o and L_d phases within GUVs. In addition to this, we use molecular dynamics simulations and fluorescence correlation spectroscopy (FCS) to investigate the diffusional properties of BODIPY rotors within lipid bilayers, in order to relate this to the bilayer viscosities calculated from the fluorescence lifetimes of the rotor probes.

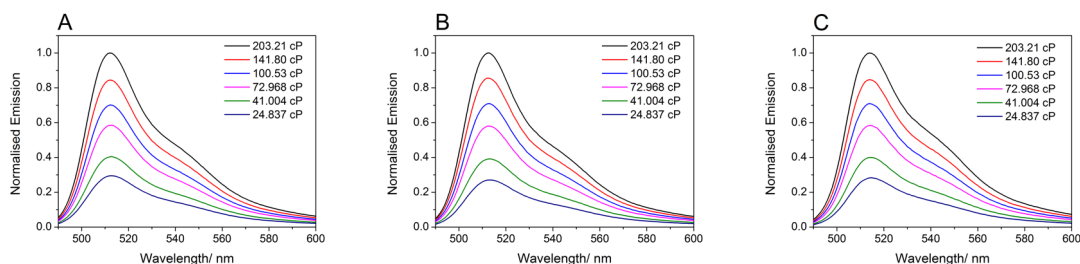


Figure 3-4 Overlaid emission spectra of (A) BODIPY-C10, (B) BODIPY-C++, (C) BODIPY-Chol in 30/70 methanol/glycerol solutions at a range of temperatures

3.2.1 Photophysical characterisation of the three BODIPY rotors

Overlaid absorption and emission spectra of the three rotors are shown in Figure 3-3. The spectra are almost completely identical, suggesting that the electronic structures of the fluorescent components of the three rotors are very similar, and that the different groups covalently attached to the BODIPY rotors do not significantly affect the fluorescence properties. Given the fact that none of the substituents of BODIPY-C10, BODIPY-C++ or BODIPY-Chol will affect the conjugation of the π -structure of the BODIPY cores, this is as expected.

In order to assess the viscosity sensitivity of the three rotors, they were dissolved in methanol/glycerol solutions of known viscosity, and fluorescence lifetime and quantum yield were determined. Figure 3-4 shows overlaid emission spectra of the three rotors, BODIPY-C10, BODIPY-C++ and BODIPY-Chol, in viscous solutions of 70% glycerol/30% methanol at a range of temperatures, corresponding to a range of viscosities. The emission intensity of the three rotors increases with increasing solution viscosity, suggesting molecular rotor-like behaviour.

Figure 3-5 shows fluorescence decay traces of the previously unpublished fluorescent dye BODIPY-

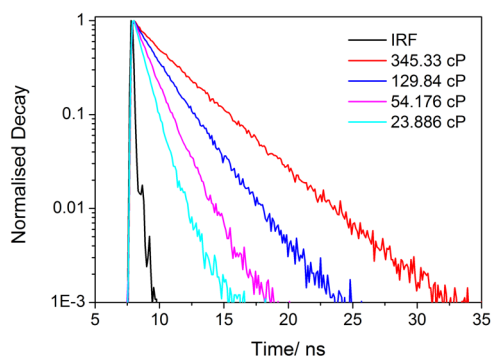


Figure 3-5 Fluorescence decays of BODIPY-Chol in methanol/glycerol solutions of differing viscosity

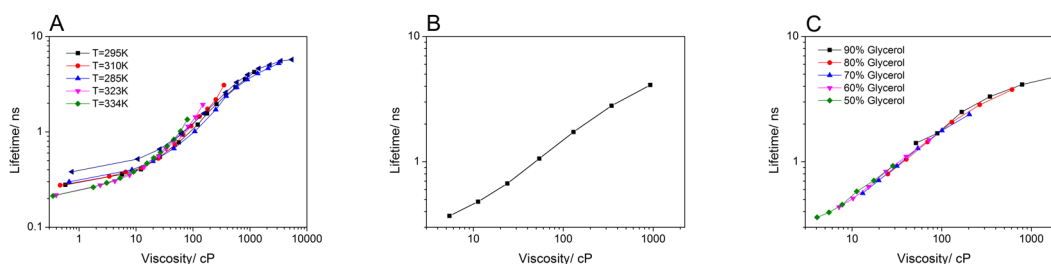


Figure 3-6 Calibration plots for (A) BODIPY-C10,⁴¹ (B) BODIPY-C++,¹³⁵ and (C) BODIPY-Chol. Lifetimes were determined in methanol/glycerol solutions of known viscosity at a range of temperatures, and the linear fit on a log-log scale between 10-1000 cP was used to generate a calibration for the BODIPY rotors

Chol in a range of glycerol/methanol solutions of differing viscosity at 25 °C, where fluorescence lifetime can also be seen to be increasing with increasing solution viscosity. Taken together with the decay traces of the other two well-characterised BODIPY rotors, this further confirms that these BODIPY dyes are acting as molecular rotors. The fluorescence decays of all three BODIPY derivatives conform to a monoexponential decay with a good χ^2 . By fitting fluorescence decays monoexponentially, the fluorescence lifetimes of the three BODIPY molecular rotors were plotted against solution viscosity in order to generate a “calibration” plot, allowing us to use rotor lifetime to determine an unknown viscosity. Such calibrations have been performed previously for BODIPY-C10⁴¹ and BODIPY-C++,¹³⁵ and are shown as Förster-Hoffmann plots in Figure 3-6 along with the plot for BODIPY-Chol, which has not been previously established. The calibration plots show a linear dependence of lifetime against viscosity on a double logarithmic scale between 10-1000 cP, in agreement with the Förster-Hoffmann equation within this biologically relevant viscosity range,^{124,132,135,183} although the rotors do deviate from this linearity above 1000 cP and below 10 cP. Additionally, the fact that the lifetime values do not deviate significantly from the linear fit, even for results obtained at different temperatures, suggests that the photophysical properties of the rotors are not significantly affected by temperature. The equations linking lifetime with viscosity for the three rotors are as follows:

BODIPY-C10¹²⁵

$$\log \tau = 0.4569 \log \eta - 0.75614 \quad (3-1)$$

BODIPY-C++¹³⁵

$$\ln \tau = 0.5000 \ln \eta - 1.9422 \quad (3-2)$$

BODIPY-Chol (this work)¹³⁸

$$\ln \tau = 0.5178 \ln \eta - 1.8385 \quad (3-3)$$

By comparison with a standard of fluorescein in 0.1 mol dm⁻³ aqueous NaOH solution, the change in fluorescence quantum yield of BODIPY-Chol was determined with solution viscosity (Figure 3-7). As predicted by the Förster-Hoffmann approach, this plot is also linear on a log-log scale. Using the quantum yields and fluorescence lifetime values for BODIPY-Chol in methanol/glycerol solutions, it was possible to determine the change in radiative decay constant k_r , and non-radiative decay constant, k_{nr} , with viscosity, using equations 3-4 and 3-5, shown below:⁵⁶

$$\Phi_f = \frac{k_r}{k_{nr} + k_r} \quad (3-4)$$

$$\tau_f = \frac{1}{k_{nr} + k_r} \quad (3-5)$$

Where ϕ_f is fluorescence quantum yield and τ_f is fluorescence lifetime. A plot showing change in k_r and k_{nr} with viscosity is shown in Figure 3-7. As expected for a molecular rotor, k_r is unaffected by viscosity, whereas k_{nr} decreases sharply with increasing solution viscosity. This is consistent with the idea that the viscosity sensitivity of molecular rotors is controlled by the non-radiative decay pathway.

Several previously studied molecular rotors⁸⁷ have been shown to display highly polarity sensitive emission, something that could potentially limit their usefulness as probes of heterogeneous biological environments, where the polarity of the surrounding environment may be unknown. Additionally, the polarity within a lipid bilayer is likely to be very different from that experienced by the molecular rotors in the relatively polar methanol/glycerol calibration system. At low viscosities the lifetime of BODIPY rotors is influenced by solvent polarity, which is illustrated by the large

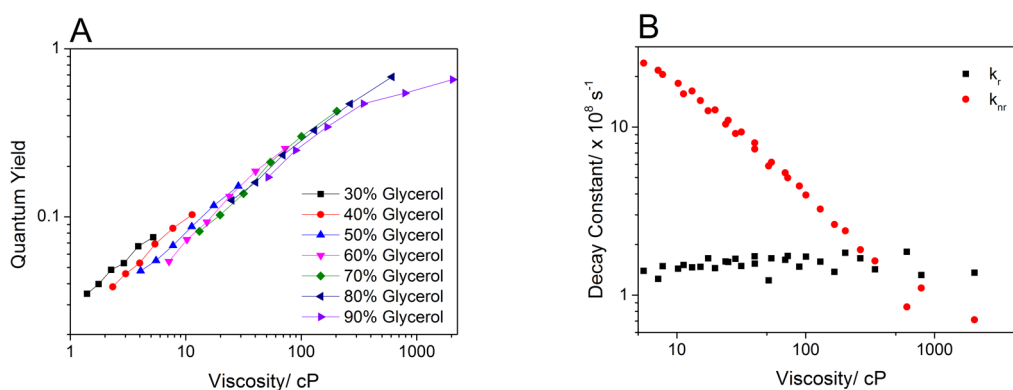


Figure 3-7 (A) change in quantum yield and (B) change in k_r and k_{nr} of BODIPY-Chol with viscosity

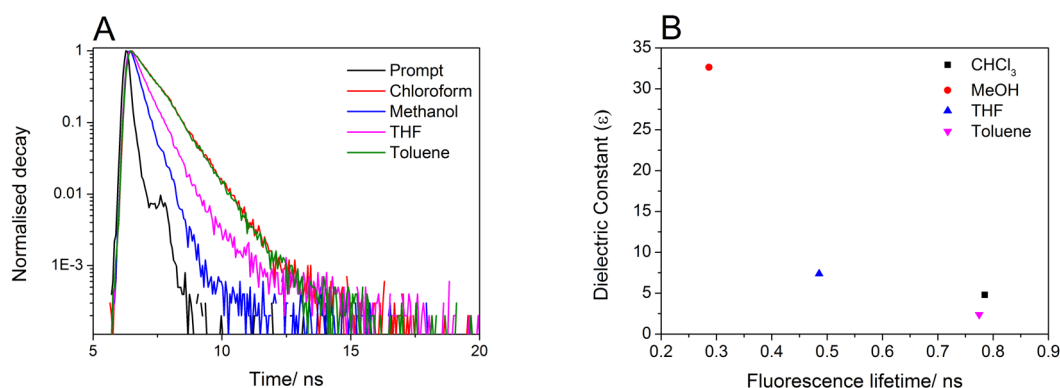


Figure 3-8 (A) Fluorescence decays of BODIPY-C10 in nonviscous solvents of varying polarity, (B) plot of fluorescence lifetime of BODIPY-C10 against solution dielectric constant at 25 °C (ϵ)^{184,185}

differences in lifetime observed for BODIPY-C10 dissolved in nonviscous solvents of differing dielectric constant^{184,185} (i.e. polarity) shown in Figure 3-8. Despite all having similar viscosities of below 1 cP,¹⁸⁶ the fluorescence lifetime of BODIPY-C10 varies by up to a factor of 4 between the different solvents (the fluorescence lifetime value of BODIPY-C10 in THF and toluene, *ca.* 0.8 ns, would be associated with a viscosity of *ca.* 25 cP in the methanol/glycerol calibration). The plot shown in Figure 3-8 (B) suggests a loose inverse correlation between solution polarity and fluorescence lifetime, with low polarity solutions such as toluene showing a higher BODIPY-C10 lifetime than polar methanol.

Despite these observations in solutions of low viscosity, we hypothesised, similarly to Haidekker *et al*⁸⁷, that any effect of solvent polarity on fluorescence lifetime would decrease with increasing viscosity. In order to test this, we compared the calibration plot of BODIPY-C10, obtained in the polar methanol/glycerol system, with fluorescence lifetimes of BODIPY-C10 in castor oil, a viscous and

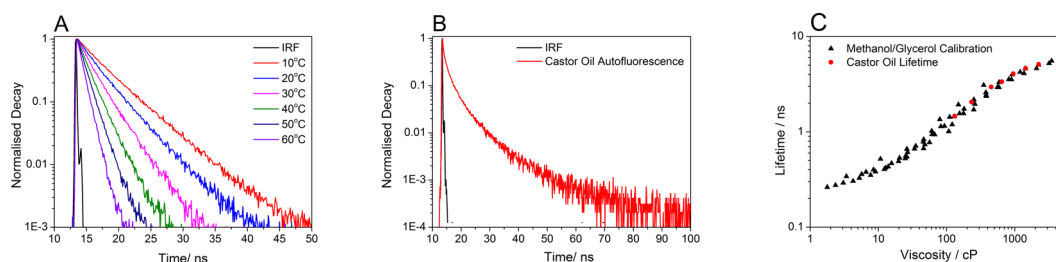


Figure 3-9 The effect of polarity on BODIPY rotor lifetime. (A) Fluorescence decays of BODIPY-C10 in castor oil, a nonpolar viscous oil, at different temperatures, (B) fluorescence decay of pure castor oil, showing castor oil autofluorescence, (C) comparison of the methanol/glycerol calibration of BODIPY-C10 with the fluorescence lifetimes of BODIPY-C10 in castor oil

highly nonpolar solvent, at different temperatures (rheometry measurements of the castor oil were performed by Craig Young). Based on the results shown in Figure 3-8, if polarity continues to affect BODIPY rotor lifetime at high viscosities then the lifetimes in castor oil may be higher than the lifetimes observed in methanol/glycerol mixtures at the same viscosities. This would render the methanol/glycerol calibration system unsuitable for viscosity measurements within lipid bilayers. The results are shown in Figure 3-9, with the lifetimes of BODIPY-C10 in castor oil showing excellent overlap with the methanol/glycerol plot. This suggests that, at high viscosities, polarity has little effect on BODIPY fluorescence lifetime, and, as such, BODIPY rotors are suitable for probing viscosities in environments of varying polarity, provided the viscosity is above a certain threshold. At this point it should be noted that castor oil is intrinsically fluorescent, resulting in biexponential decays for solutions of BODIPY-C10 in castor oil. The autofluorescence from the castor oil contributed a small percentage of the total signal (*ca.* 20% at room temperature, Figure 3-9) with a lifetime unaffected by temperature, and so was attributed one component of a biexponential fit, with the other attributed to the monoexponential, viscosity-sensitive fluorescence decay of BODIPY-C10.

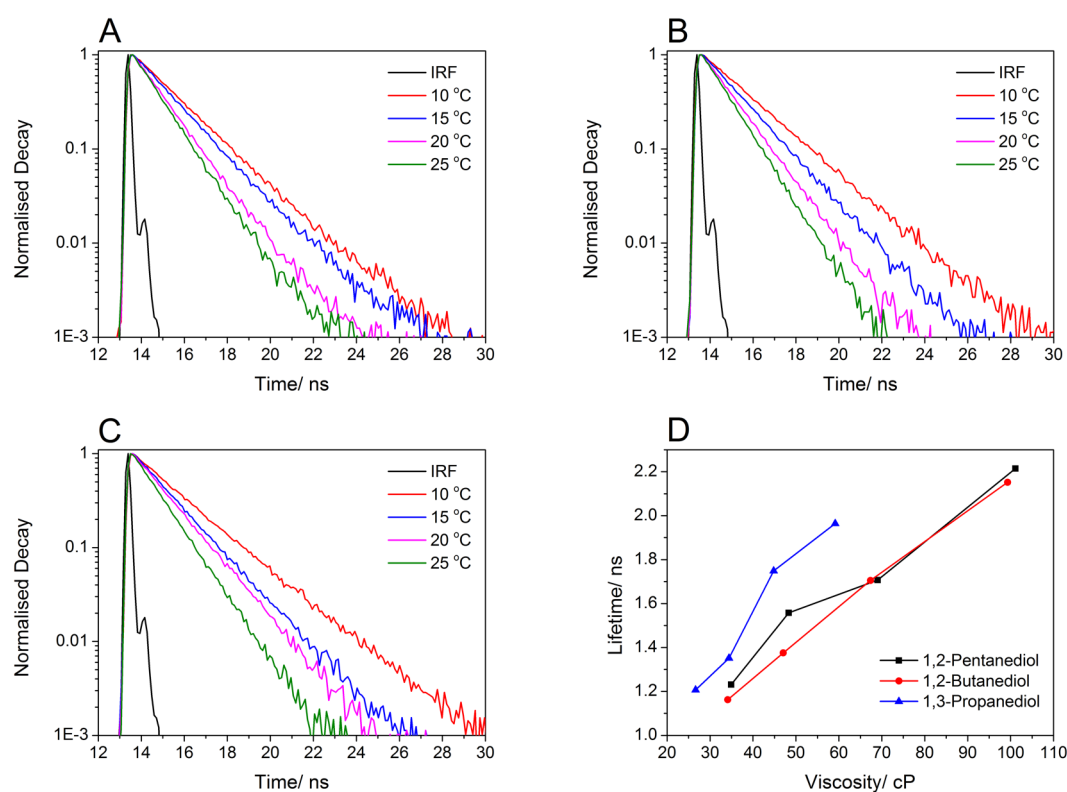


Figure 3-10 Lifetimes of BODIPY-C10 in viscous diols between 10-100 cP. Fluorescence decays of BODIPY-C10 in (A) 1,3-propanediol, (B) 1,2-butanediol and (C) 1,2-pentanediol at different temperatures; (D) change in fluorescence lifetime of BODIPY-C10 with viscosity within the viscous diols.

To further investigate the impact of polarity on BODIPY rotor lifetime at low viscosities, and to potentially set a threshold for the minimum viscosity where solution polarity has a negligible impact on BODIPY-rotor fluorescence lifetime, BODIPY-C10 was dissolved in three different viscous diols. BODIPY-C10 was dissolved in 1,2-pentanediol, 1,2-butanediol and 1,3-propanediol, viscous diols with differing polarities, and fluorescence lifetime was determined at a range of temperatures, giving a viscosity range of *ca.* 20-100 cP (rheometry measurements performed again by Craig Young). Figure 3-10 shows a plot of diol viscosity against BODIPY-C10 lifetime. At low viscosities, there is poor overlap between the BODIPY lifetimes within the three diols, which is likely as a result of the polarity differences between them. However, as viscosity increases, the overlap becomes better, confirming our hypothesis that polarity has a diminishing effect on BODIPY rotor lifetime at increasing viscosity, with polarity difference becoming negligible at viscosities of over 100 cP.

3.2.2 Viscosity studies in large unilamellar vesicles

In order to test the three BODIPY rotors as probes of membrane viscosity, they were incorporated into large unilamellar vesicles (LUVs) of 200 nm diameter produced via gas extrusion¹⁵⁹ made up of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine

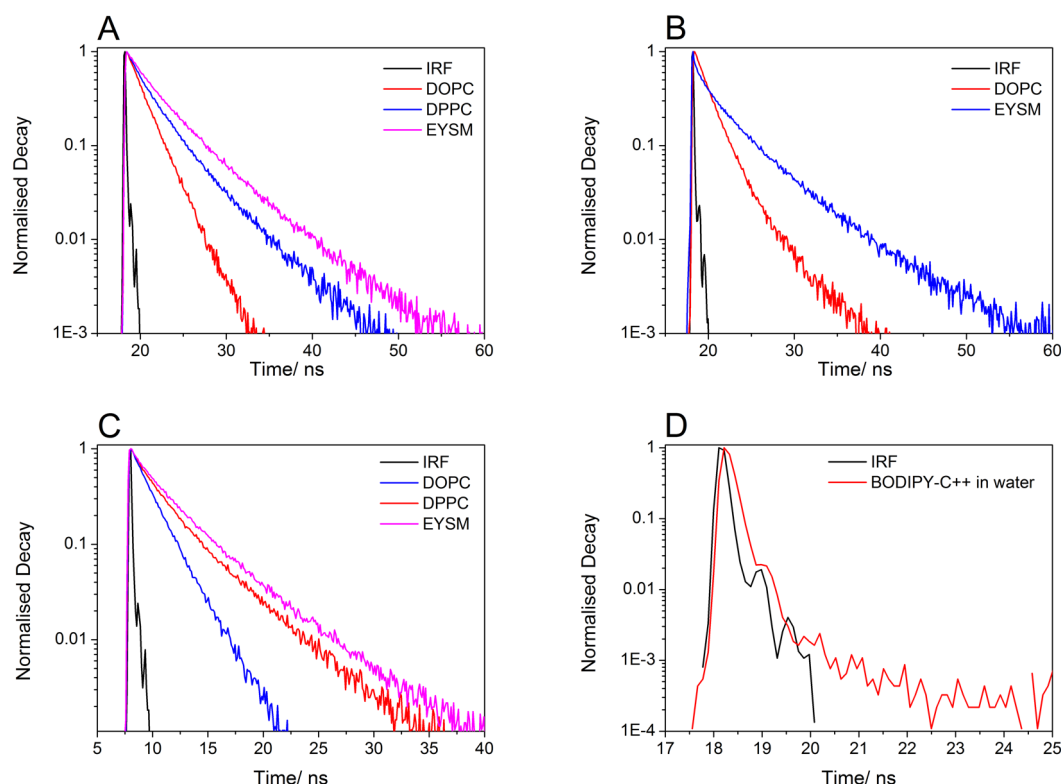


Figure 3-11 Decays of (A) BODIPY-C10, (B) BODIPY-C++, (C) BODIPY-Chol in LUVs of liquid and gel phase lipids, (D) decay of BODIPY-C++ in water

(DPPC) or egg yolk sphingomyelin (EYSM). DOPC is an unsaturated lipid and as such forms fluid lamellar, or liquid, phase bilayers at room temperature, and DPPC and EYSM are saturated lipids, forming gel phase bilayers at room temperature. Fluorescence lifetime was then determined using time-correlated single photon counting (TCSPC), with the dye:lipid molar ratio being always kept below 1:250 to prevent the BODIPY rotors from displaying aggregation-induced quenching, which has previously been shown to cause biexponential decay characteristic and reduced fluorescence lifetime, leading to artificially low calculated viscosities.⁴¹

At room temperature, BODIPY-C10 and BODIPY-Chol gave monoexponential decay traces in DOPC and biexponential decays in the saturated lipids (Figure 3-11). BODIPY-C++ gave biexponential decay traces in DOPC, with one component with a very short lifetime on the order of 500 ps and one component with a lifetime very similar to those of BODIPY-C10 and BODIPY-Chol in DOPC. BODIPY-C++ is water soluble, and since the short lifetime component matched the lifetime of BODIPY-C++ in the aqueous phase, it was attributed to the small percentage of the rotor partitioned in the water

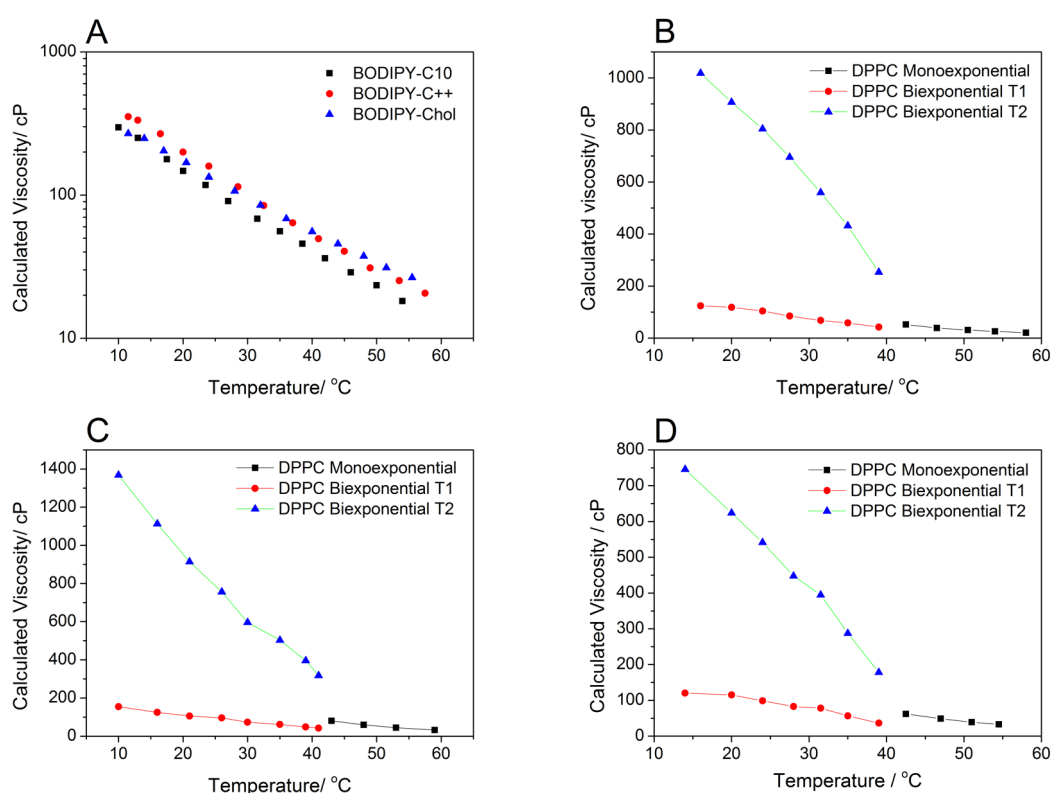


Figure 3-12 Calculated viscosities in LUVs. (A) Viscosities of the three rotors in DOPC LUVs at a range of temperatures; calculated viscosities for (B) BODIPY-C10, (C) BODIPY-C++ and (D) BODIPY-Chol in DPPC LUVs at a range of temperatures, calculated from both components of the biexponential decays below the gel-to-liquid transition temperature and from the monoexponential decays above it

phase (15% of the decay, Figure 3-11 (D)). The long lifetime component was treated as the viscosity-dependent lifetime of BODIPY-C++ in the DOPC bilayer.

By using the methanol/glycerol calibration plots, viscosity values were calculated from the fluorescence lifetimes of the three rotors in LUVs. Figure 3-12 shows the viscosities calculated from the lifetimes of the three BODIPY rotors within DOPC LUVs at a range of temperatures. As temperature increases, the fluorescence lifetimes of the BODIPY rotors decrease, indicating a reduction in the bilayer viscosity. The three rotors gave very similar viscosity values, and this suggests that the viscosity sensitive parts of the rotors reside within the same part of the bilayer. The similarity between the calculated viscosity values also suggests that BODIPY rotors are reliable probes for determining membrane viscosity in disordered liquid membranes, within the biologically relevant viscosity range of *ca.* 20-500 cP.^{124,132,135}

The effect of changing temperature on the fluorescence lifetime derived viscosities of the three rotors in DPPC LUVs is shown in Figure 3-12, with the viscosities calculated from each of the three rotors again agreeing well with each other. Above 41 °C, the gel transition temperature of DPPC, the

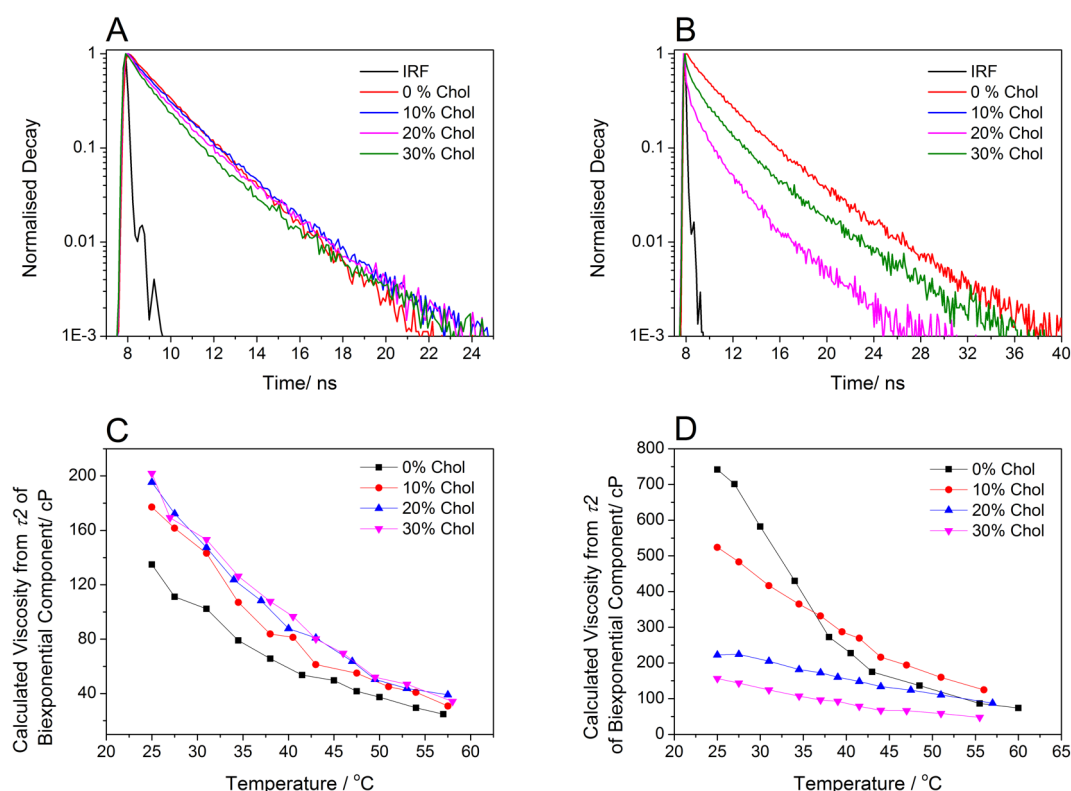


Figure 3-13 Effect of varying concentration of cholesterol on the fluorescence decays of BODIPY-Chol at 25 °C in (A) DOPC and (B) EYSM; (C) calculated viscosities of BODIPY-Chol in DOPC LUVs with varying concentrations of cholesterol, (D) calculated viscosities of BODIPY-Chol in EYSM LUVs with varying concentrations of cholesterol

rotors exhibit monoexponential fluorescence decay behaviour, and the viscosity values are similar to DOPC at these temperatures. However, below this temperature the decay traces become biexponential, with a similar phenomenon being observed in EYSM. The biexponential decays of BODIPY rotors in gel phase bilayers have previously^{41,187} been attributed to the rotors taking on two conformations within the bilayer, causing them to show two different “viscosities”, as the two environments hinder the intramolecular rotation of the non-radiative decay processes differently. The orientation of the BODIPY probes within the bilayer will be discussed further in section 3.2.3. It has previously⁴¹ been shown that the presence of the two components is concentration independent, and is as such not due to aggregation of the rotors. Each lifetime component of the biexponential decay was converted into a viscosity value, with the plot of viscosity against temperature showing a sharp change in gradient at 41 °C, reflecting the gel transition temperature. The significantly higher viscosity shown by the τ_2 component suggests that gel phases display a higher viscosity than liquid phases, something that can be rationalised by the increased molecular packing within the gel phase. These results suggest that molecular rotors can indeed be used to distinguish between different lipid phases, and can also be used to detect phase transitions within lipid bilayers.

Finally, the effect of cholesterol on the viscosity of lipid bilayers was investigated. In both DOPC/Cholesterol and EYSM/Cholesterol LUVs, BODIPY-Chol showed biexponential fluorescence decays, and the change in the viscosity calculated from the τ_2 components of the decays with temperature is shown in Figure 3-13. The addition of cholesterol to the bilayer caused a significant decrease in the viscosity of EYSM LUVs, but an increase in the viscosity of DOPC LUVs. Increasing the amount of cholesterol from 10% up to 30% had little effect on the viscosity of DOPC LUVs, but further decreased the viscosity within the EYSM LUVs. It is well established that cholesterol increases the ordering of unsaturated lipids, and decreases ordering of saturated lipids,¹⁸⁸ and one of the main roles of cholesterol within the plasma membrane is to mediate lipid ordering,¹⁸⁹ so it is perhaps not surprising that the addition of cholesterol brings the viscosities of EYSM and DOPC closer to each other. For saturated lipids such as EYSM, the addition of cholesterol disrupts the tight packing of the gel phase lipid molecules, forming a liquid-ordered phase,¹⁹⁰ which can be seen in Figure 3-13, where the addition of cholesterol prevents the sharp change in viscosity associated with the gel transition temperature of EYSM. Cholesterol is also known to condense unsaturated lipids such as DOPC, increasing the viscosity and lipid ordering,¹⁷⁴ which is consistent with the results of our experiments.

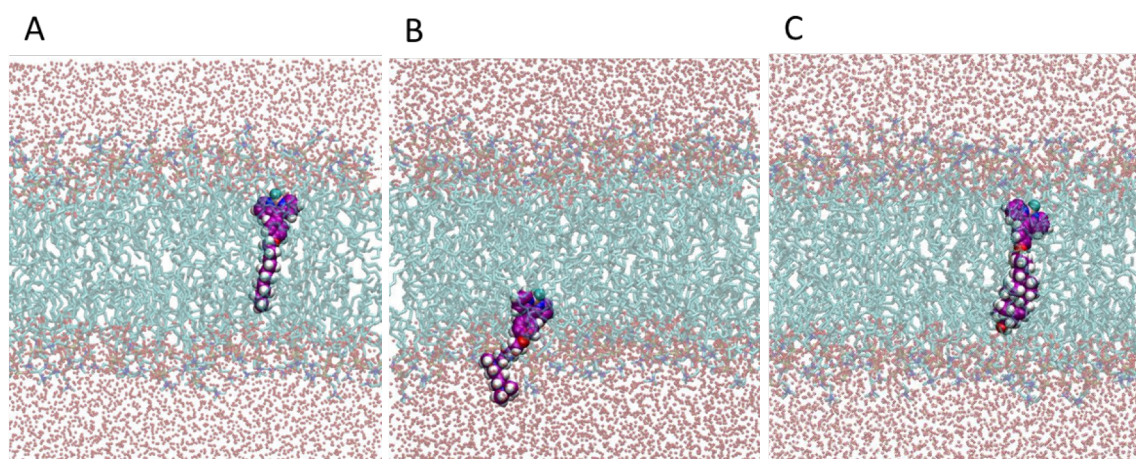


Figure 3-14 Molecular dynamics simulations showing the preferred orientations of the BODIPY rotors in the bilayer. (A) BODIPY-C10, (B) BODIPY-C++, (C) BODIPY-Chol in DOPC

3.2.3 The position of the BODIPY rotors within the bilayer

In order to be able to reliably use a molecular rotor as a probe of membrane viscosity it is essential to know its position within the bilayer. Due to various intermolecular interactions, the lateral pressure varies greatly between the head and tail groups of the lipids within the bilayer (see Chapter 1),⁵³ meaning that resistance to intramolecular rotation of a molecular rotor will be different in the tail region of the lipid bilayer compared with the headgroup region. Therefore, different molecular rotors that take on different orientations may give different viscosity values for the same bilayer.¹⁸⁷ Due to the similarities in the calculated viscosities for the three rotors in both DOPC and DPPC bilayers, we hypothesised that they all take on similar orientations within the bilayer.

In order to determine the localisation of the BODIPY probes, the molecular dynamics software AMBER¹⁵² was used by Dr. Callum Dickson to simulate the behaviour of the BODIPY rotors within DOPC bilayers, with the final orientations of the dyes shown in Figure 3-14.

The simulations showed that the “head” groups of the BODIPY rotors are similar distances from the core of the bilayer for each of the rotors in DOPC. Figure 3-15 shows the calculated electron density profiles of three rotors in DOPC, showing the positions of the groups within the rotors relative to the bilayer as a whole. Despite the tail groups taking on different orientations, with the charged tail of BODIPY-C++ extending into the aqueous phase, the BODIPY headgroups all have a similar bilayer position, *ca.* 10 Å from the bilayer cores. The fact that the three rotors report such similar viscosities in DOPC bilayers suggests that it is the headgroup of the BODIPY rotors that is responsible for their

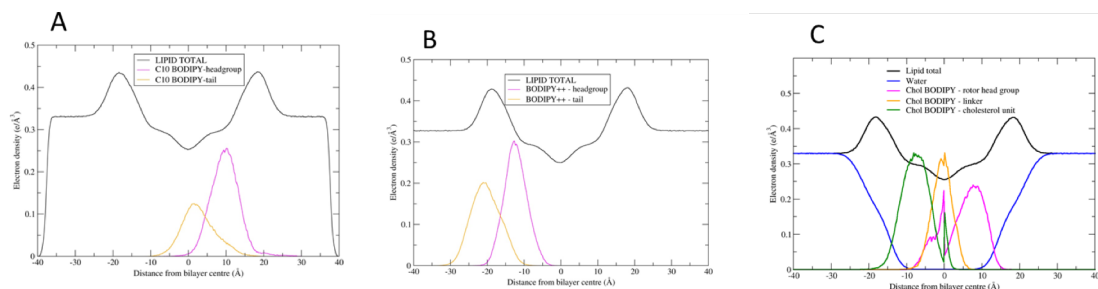


Figure 3-15 Electron-density plots of the three rotors in DOPC bilayer as determined from AMBER MD simulations. (A) - BODIPY-C10, (B) - BODIPY-C++, (C) - BODIPY-Chol

viscosity sensitivity. The fact that the dyes take on one preferred orientation is consistent with the monoexponential fluorescence decays observed for the rotors in DOPC bilayers.

For the DPPC simulation (Figure 3-16), two preferred orientations for BODIPY-C10 were observed, one orientation where the whole molecule resides within the bilayer core in a similar position to BODIPY-C10 within DOPC bilayers, and another where the rotor aligns alongside the bilayer lipids, with the viscosity sensitive headgroup positioning itself within the lipid headgroup region. These two distinct preferred orientations explain the two different components of the biexponential fluorescence decay behaviour of the BODIPY rotors within gel phase systems, in agreement with the fluorescence anisotropy study performed by Olšinova *et al*¹⁸⁷ on BODIPY rotors in gel phase bilayers, which suggested that the rotors adopt two orientations: one perpendicular and one parallel to the membrane normal.

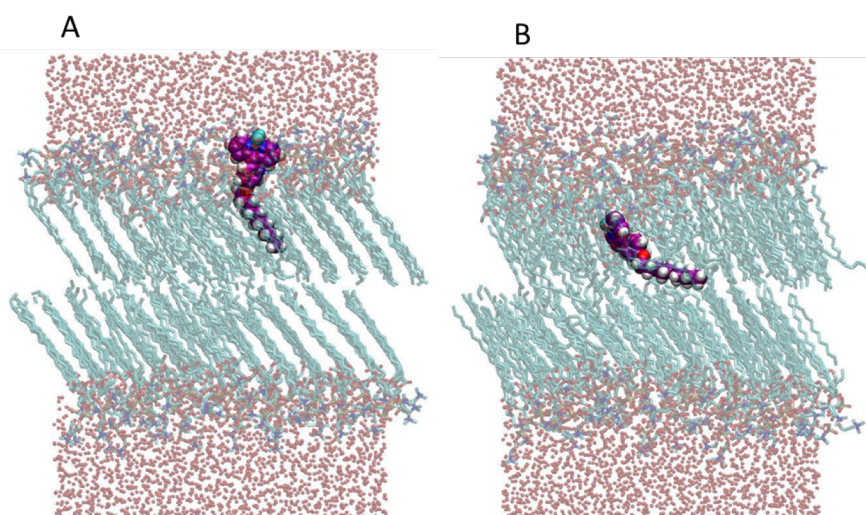


Figure 3-16 Molecular dynamics showing the two preferential orientations of BODIPY-C10 within a gel-phase DPPC bilayer, (A) within the lipid headgroup region and (B) within the lipid tail region

Overall, the molecular dynamics simulations suggest that the three BODIPY rotors behave in a similar manner within lipid bilayers, independent of the nature of the R group attached to the BODIPY phenyl ring. Additionally, the simulations show that the BODIPY rotors intercalate within the lipid bilayer, rather than creating a cavity between the two leaflets of the bilayer, suggesting that the lipid viscosity measurements from the BODIPY rotors are reliable measures of lipid ordering. This data taken together with the high lipid:dye ratio gives us confidence that the bilayers under study are not disrupted by the presence of the rotor.

3.2.4 Estimating diffusion coefficients within the bilayer

A potential drawback in the use of methanol/glycerol solutions of known viscosity for the calibration of BODIPY rotors is that a homogeneous bulk solution with free diffusion in three dimensions may not be a good representation of lipid bilayers, which are quasi-two-dimensional systems with a high degree of heterogeneity. It should also be noted that “viscosity” is a macroscopic property, and is somewhat poorly defined for a heterogeneous microscale system such as a lipid bilayer, where the term “microviscosity” can be interpreted as a measure of the molecular crowding within the immediate vicinity. In a lipid bilayer, the intramolecular rotation that leads to the nonradiative decay pathway of a molecular rotor will subsequently be affected by the lipid packing within its surrounding environment, meaning that the viscosity value calculated by the molecular rotor will represent a measure of the lipid packing within the bilayer.¹⁸⁷ This also means that higher membrane viscosities will correspond to lower diffusion coefficients of species within the membrane, as previously established by Saffman and Delbrück.¹⁹¹

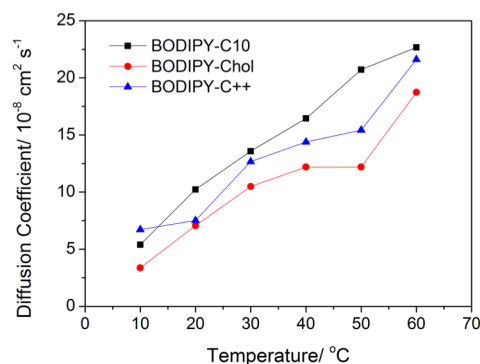


Figure 3-17 Plot showing change in simulated diffusion coefficient with temperature for the three BODIPY rotors in DOPC bilayers

Table 3-1 Parameters used in the Saffman-Delbrück calculations (*BODIPY-rotor radius from molecular dynamics simulations*)

Parameter	Value	Reference
η_m	<i>From lifetime</i>	n/a
η_f	0.8903 cP	¹⁹³
a (<i>BODIPY-C10</i>)	6.20 Å	n/a
a (<i>BODIPY-C++</i>)	6.97 Å	n/a
a (<i>BODIPY-Chol</i>)	8.28 Å	n/a
DOPC bilayer thickness	37 Å	¹⁵⁵

In order to investigate whether the fluorescence lifetime of BODIPY rotors gives an accurate estimate of membrane viscosity, we compared diffusion coefficients calculated for the three BODIPY rotors within DOPC bilayers obtained from three complementary methods: fluorescence correlation spectroscopy¹⁹² (FCS, performed by Dr Niall Geoghegan) of DOPC giant unilamellar vesicles (GUVs), molecular dynamics simulations (performed by Dr Callum Dickson, see Figure 3-17 for change in simulated diffusion coefficient with temperature for the three rotors in a DOPC bilayer) and by calculating diffusion coefficients from the bilayer viscosities, determined using the three BODIPY rotors. To calculate the diffusion coefficients from the viscosity values, the Saffman-Delbrück equation was used with the parameters shown below in Table 3-1.^{155,193}

The Saffman-Debrück equation is shown in Equation 3-6

$$D_{sd} = \frac{k_B T}{4\pi\eta_m h} \left[\ln \left(\frac{2L_{sd}}{a} \right) - \gamma \right] \quad (3-6)$$

Where D_{sd} is the Saffman-Delbrück diffusion coefficient, a is the radius of the membrane inclusion, h is the bilayer thickness, η_m is the membrane viscosity, γ is the Euler-Mascheroni constant ($\gamma \approx 0.577$), and L_{sd} is the Saffman-Delbrück length, given by equation 3-7:

$$L_{sd} = \frac{h\eta_m}{2\eta_f} \quad (3-7)$$

where η_f is the viscosity of the surrounding fluid.

The diffusion coefficients calculated using the three methods for the three rotors at a range of temperatures in DOPC bilayers are shown in Table 3-2. The diffusion coefficient values are similar for each of the methods used, overlapping particularly well at 313 K. There is particularly good

Table 3-2 Diffusion coefficients calculated for the three rotors in DOPC bilayers using molecular dynamics simulations, FCS and the Saffman-Delbrück equation to calculate diffusion coefficients from the viscosity values calculated from the fluorescence lifetimes of the BODIPY rotors

Temperature / K	Diffusion Coefficient/ $\mu\text{m}^2\text{s}^{-1}$								
	BODIPY-C10			BODIPY-C++			BODIPY-Chol		
	Simulated	FCS	Lifetime	Simulated	FCS	Lifetime	Simulated	FCS	Lifetime
293	10.22	10.52	3.78	7.52	8.75	2.91	7.06	7.25	3.23
313	16.45	18.06	11.11	14.39	12.32	8.73	12.20	14.18	8.21
333	22.67	22.65	32.94	21.61	19.91	27.77	18.74	21.18	20.52

agreement between FCS and the molecular dynamics simulations, typically within $1 \mu\text{m}^2 \text{s}^{-1}$ of each other, suggesting that the simulations are an accurate reflection of real membrane systems under these conditions. Whilst the diffusion coefficients calculated using the Saffman-Delbrück equation are less accurate, they are still generally within a factor of two of the FCS and molecular dynamics simulations, which, given the inherent inaccuracies of the Saffman-Delbrück equation, shows good agreements with the other two methods. This suggests that the fluorescence lifetime of the BODIPY probes provide an accurate measure of the membrane viscosity, and that the methanol/glycerol mixtures are indeed a suitable calibration system. It should be noted, however, that there may be a systematic error within the results described in Table 3-2, with all lifetime-derived estimates of diffusion coefficients being underestimates at 292 K, and both BODIPY-C10 and BODIPY-C++ lifetimes giving overestimates at 333 K relative to the FCS and simulated results. This could be as a result of the value of $37.5 \text{ \AA}$ taken for the bilayer thickness, which may change at reduced temperature, or indeed for the estimates of molecular radius for the BODIPY probes, which were derived from simulations run at 313 K. Future work could involve the use of a more mathematically rigorous means of linking diffusion coefficient with viscosity, which would go beyond the limitations of the Saffman-Delbrück approach.

3.2.5 Viscosity studies in giant unilamellar vesicles

Giant unilamellar vesicles (GUVs) provide a useful model system to mimic the plasma membranes of cells, as they can be easily made with a variety of compositions and are on the same order of size as cells, allowing imaging using optical microscopy. In order to evaluate the practicality of using the three rotors as probes of phase separation within lipid bilayers, we prepared phase-separating ternary GUVs made up mixtures of DOPC:EYSM:Chol using electroformation. Example confocal

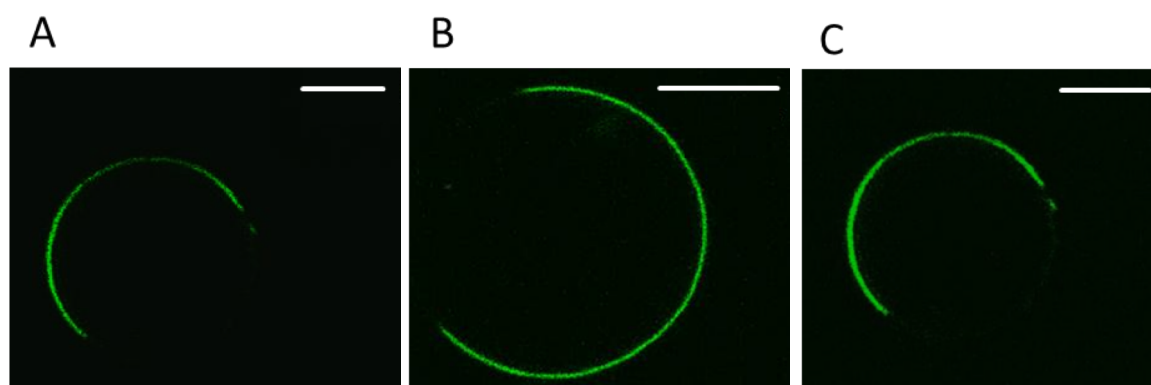


Figure 3-18 Confocal images of (A) BODIPY-C10, (B) BODIPY-C++ and (C) BODIPY-Chol in phase separating GUVs consisting of DOPC/EYSM/Cholesterol. In each of the images, the poor partitioning of the BODIPY rotor into the L_o phase can be observed

images of ternary GUVs showing L_o - L_d phase separation stained with the three rotors are shown in Figure 3-18, with all three probes showing a strong preference for the L_d phase.

Whilst BODIPY-C10 has previously been shown to partition poorly into the L_o phase,⁴¹ it is perhaps surprising that BODIPY-Chol also shows a poor partitioning into the L_o phase, given the fact that cholesterol is known to associate with ordered phases in lipid bilayers,^{176,177} and that other BODIPY-cholesterol probes have previously been shown to partition well into the L_o phase of model bilayers.¹⁹⁴ This could be explained by the presence of the phenyl ring on the BODIPY rotor probes, which are not typically present in the L_o targeting BODIPY dyes. The phenyl ring is bulky and not coplanar with the BODIPY headgroup in the ground-state,¹²⁰ and as such may limit the ability of the BODIPY rotors to partition into the rigid lipid environment of the L_o phase.

Despite the large difference in relative BODIPY concentration between the two phases causing a significant reduction in fluorescence signal from the L_o phase, it was possible to obtain fluorescence lifetime images of the BODIPY rotors in both L_o and L_d phases. Since fluorescence lifetime is concentration independent,¹⁰¹ reliable viscosity values for both L_o and L_d phases could be extracted using fluorescence lifetime imaging microscopy (FLIM). The preferential partitioning of the rotors into the L_d phase may however limit the usefulness of BODIPY based rotors in the study of lipid ordering within complex and live biological membrane systems, where any ordered domains are likely to be small and short lived.⁷⁵

The phase diagram for a ternary mixture of DOPC:EYSM:Chol is shown in Figure 3-19 (adapted from work by Bezlyepkina *et al*¹⁸), and highlights the L_o - L_d phase coexistence region, as well as the tie lines across this region. At the critical point the compositions of the L_o and L_d phases are identical, and near this point their compositions should be similar meaning the L_o phase is more disordered in character, and will likely have a similar viscosity to the L_d phase, which should allow the BODIPY probes to partition to a greater extent into the L_o phase. The lipid composition of the plasma membrane is believed to be near the critical point, with plasma membrane extracts being known to phase-separate at low temperatures,^{19,31} meaning that if the BODIPY rotors can easily discern between phases nearer to the critical point they may be applicable in the search for phase-separation within cellular plasma membranes and membrane extracts.

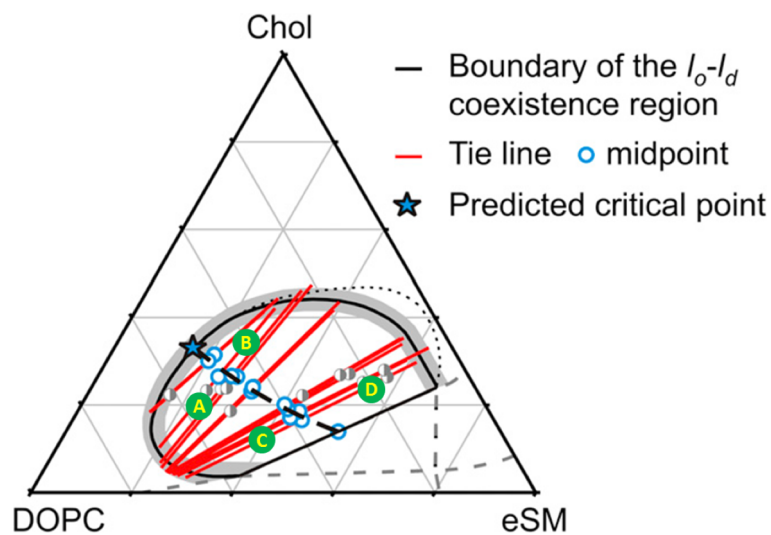


Figure 3-19 Phase diagram of DOPC/Chol/EYSM (labelled eSM), as adapted from¹⁸). Compositions of GUVs A, B, C, D (Figure 3-20) are shown on the phase diagram

Using BODIPY-C10 for imaging, GUVs composed of four different ratios of DOPC:EYSM:Cholesterol (A – 56:24:20, B – 40:25:35, C – 48:40:12 and D – 20:55:25, see Figure 3-19 for compositions displayed on the phase diagram for DOPC:EYSM:Cholesterol) were produced along two different tie lines in the phase diagram. As they were located on the same tie line, lipid mixtures A and B were expected to have L_o phases of the same compositions and L_d phases of the same compositions but with differing ratios of L_o phases to L_d phases. This was also expected of mixtures C and D. FLIM was used to determine the fluorescence lifetimes, and therefore the viscosities, of the four different GUV compositions of room temperature (see Figure 3-20 for sample lifetime images), with the mean L_o and L_d phase viscosities of 10 GUV images being shown in Table 3-3.

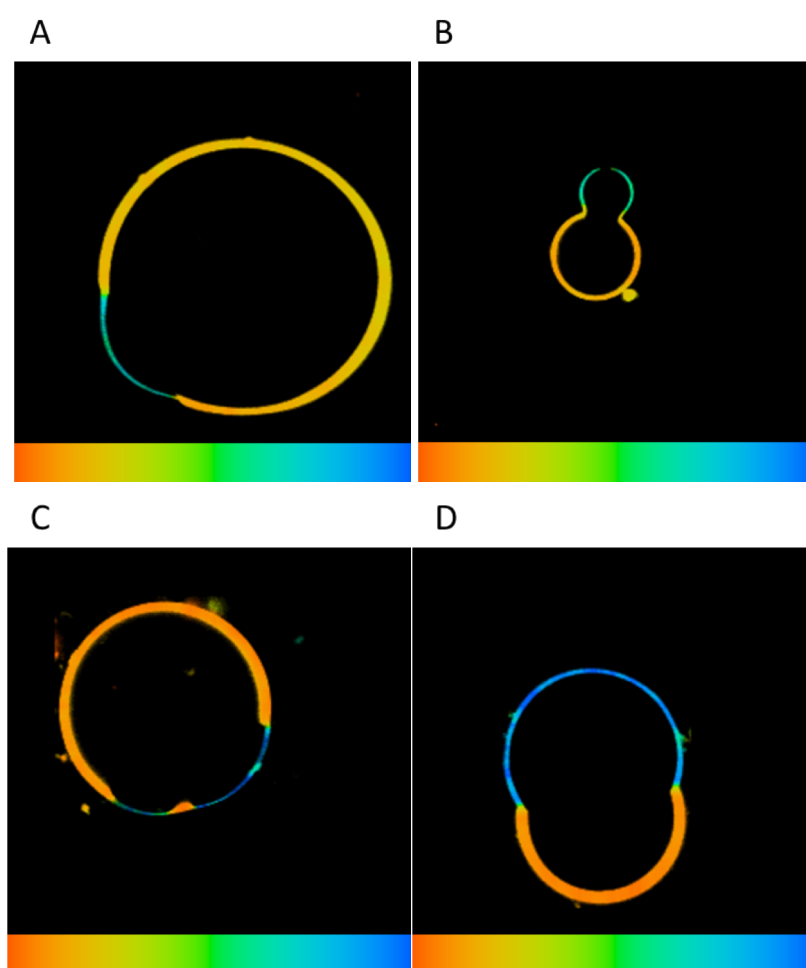


Figure 3-20 Representative fluorescence lifetime images of GUV from lipid mixtures A, B, C and D, imaged with BODIPY-C10. The lipid compositions of GUVs A and B lie on the same tie line within the DOPC:EYSM:Chol phase diagram (see Figure 3-19), hence the compositions and therefore viscosities of the L_o (blue / green) and L_d (orange) phases in GUVs A and B are very similar, which is reflected in the fluorescence lifetimes. The same is true for GUVs C and D, however they lie on a different tie line to GUVs A and B and this results in a significant difference in the L_o phase lifetime between these two pairs of vesicles. The fluorescence lifetime range shown by the colour bar = 1500 – 3200 ps, and represents a viscosity range of 94.6 – 454.3 cP. Note that the bulging of the different phases is a result of the difference in membrane thickness and molecular motion between the L_o and L_d phases, which leads to a line tension between these membrane regions and an energetic drive to minimise the length of the line interface.²⁹⁰

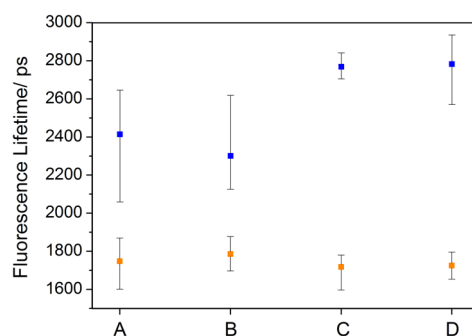


Figure 3-21 Plot showing mean fluorescence lifetimes of L_o (shown in blue) and L_d (shown in orange) phases for GUV mixtures A, B, C and D. Error bars represent maximum and minimum lifetime values from the 6 GUVs imaged

As can be seen from the representative lifetime images in Figure 3-20, which are plotted in the same colour scale (the mean viscosity values shown in Table 3-3 and Figure 3-21), the lifetimes of the L_o phases in A are very similar (within 10%) to those in B, and the L_d phases of A and B are also very similar. A similar observation is made for the lifetimes of GUVs C and D, which lie on a different tie line of the phase diagram. This suggests that the compositions of the L_o phases along the same tie line are identical, hence the similar viscosities, with the same being true for the L_d phases. On the phase diagram shown in Figure 3-19, the L_d compositions of A, B, C and D are close together, hence the similar viscosities, however the L_o composition of A and B is significantly different to that for C and D, which is highlighted by their different viscosities. The smaller percentage of DOPC in the L_o phases of C and D explains their more ordered nature and higher viscosity when compared with A and B. Mixtures A and B are relatively close to the critical point in composition, yet BODIPY-C10 still detects a significant difference in lifetime between the two phases, suggesting that BODIPY rotors can still be used to distinguish between different lipid phases close to the critical point. Histograms showing the lifetime distributions for the lifetime images shown in Figure 3-20, are shown in Figure

Table 3-3 - Calculated viscosities of L_o and L_d phases for GUV compositions A, B, C and D at room temperature using BODIPY-C10*

Composition	Mean L _d Viscosity/ cP	Mean L _o viscosity/ cP
A	128.9	251.1
B	134.7	227.3
C	124.4	333.2
D	125.4	336.7

*Calculated as an average of several GUV lifetime images – see Figure 3-21 for the lifetimes and error bars used to calculate these values

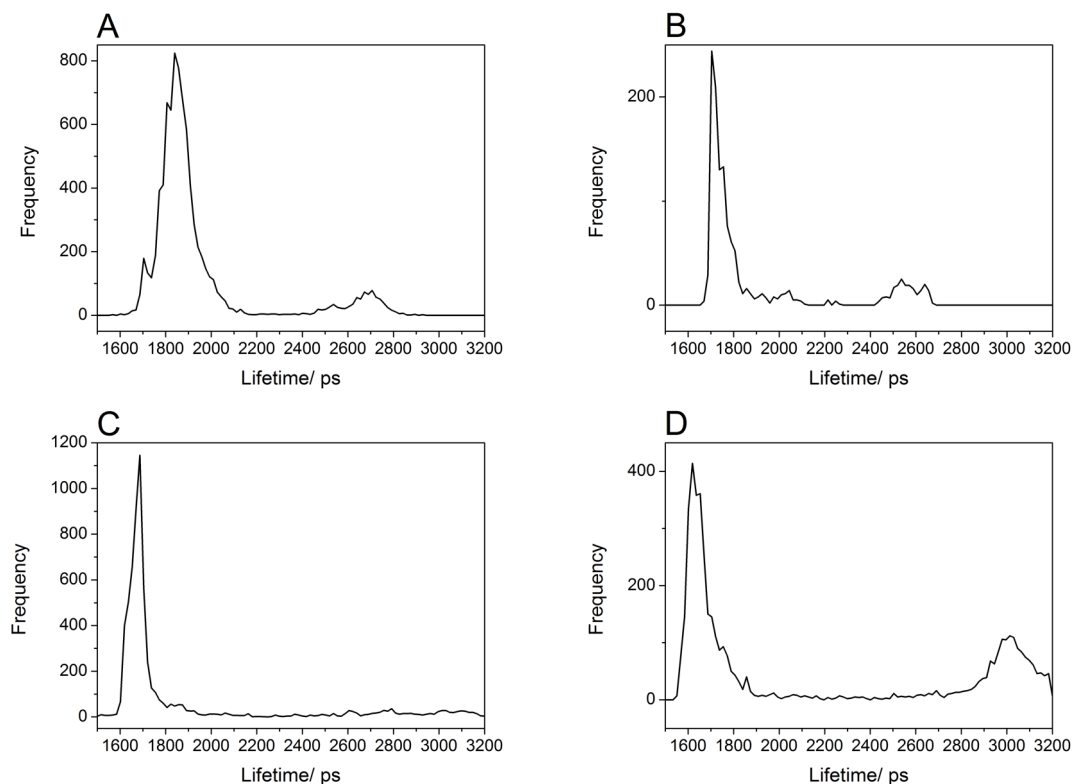


Figure 3-22 Histograms showing the lifetime distribution for the GUVs shown in Figure 3-20

3-22, with the small frequencies for the L_o phases reflecting the preferential partition of BODIPY-C10 into the L_d phase, which causes a significantly higher fluorescence signal from the L_d phase.

3.3 Conclusions

In this chapter, the three BODIPY-based molecular rotors were used to image viscosity in model lipid bilayers, in order to assess the suitability of BODIPY-based rotors for imaging of membrane systems and to investigate the effects of lipid phase behaviour on membrane viscosity.

The fluorescence lifetimes of the three rotors were first determined in methanol/glycerol mixtures of known viscosity in order to “calibrate” the rotors using a Förster-Hoffmann plot. In order to investigate whether solution polarity affected the fluorescence lifetimes of the rotors at high viscosities, the fluorescence lifetimes of BODIPY-C10 in viscous, nonpolar castor oil were compared with the methanol/glycerol calibration. Both systems gave very similar lifetimes for BODIPY-C10 at a range of viscosities, implying that BODIPY lifetime is not strongly affected by polarity at high

viscosities, with further experiments between 10-100 cP showing that polarity has a diminishing effect on lifetime as viscosity increases.

The three rotors were then incorporated into LUVs, in order to investigate viscosity within lipid bilayers. In DOPC, which forms fluid-lamellar (liquid) phases in the temperature range investigated, the three rotors reported very similar viscosities at a range of temperatures, reporting a viscosity range of *ca.* 30-450 cP within the temperature range of 9-55 °C. We hypothesised that this overlap in reported viscosities was due to the BODIPY rotors occupying a similar position within the bilayer, something we later confirmed using molecular dynamics simulations. We then went on to determine membrane viscosity within LUVs made up of DPPC and EYSM, lipids which form gel phase bilayers at room temperature and display a gel-to-liquid phase transition at *ca.* 40 °C, finding a large change in membrane viscosity at this temperature. These experiments showed that gel phase bilayers show a significantly higher viscosity than liquid-phase ones and that molecular rotors can be used to detect phase changes within lipid systems. To investigate the effect of cholesterol on membrane viscosity, we incorporated BODIPY rotors into DOPC and EYSM LUVs with varying concentrations of cholesterol, and determined fluorescence lifetime of the BODIPY probe at a range of temperatures. We found that addition of cholesterol causes a decrease in the viscosity of EYSM LUVs, as the cholesterol disrupts the molecular packing of the lipid molecules within the bilayer, forming an L_o phase. Conversely, addition of cholesterol to a DOPC bilayer causes a small-scale increase in viscosity, owing to cholesterol's lipid-condensing properties.

In order to test the accuracy of the viscosity calculated from BODIPY rotor lifetime, the viscosity values of the three rotors within DOPC LUVs at three different temperatures were used to calculate corresponding diffusion coefficients for the rotors, using the Saffman-Delbrück equation. These diffusion coefficients were then compared with diffusion coefficients for the BODIPY rotors in DOPC bilayers obtained through FCS experiments and molecular dynamics simulations, which were performed by Niall Geoghegan and Callum Dickson, respectively. The diffusion coefficients obtained from the three different methods agreed well with each other, despite the inherent inaccuracies of the Saffman-Delbrück equation, suggesting that BODIPY-rotor lifetime is an accurate method of determining membrane viscosity.

We then incorporated the three rotors into GUVs displaying L_o - L_d phase separation, finding that none of the three rotors partition well into the L_o phase, not even the cholesterol-conjugated BODIPY-Chol rotor. Despite this, we went on to use BODIPY-C10 to image four phase-separating GUV

compositions along two different tie-lines in the phase diagram of DOPC/EYSM/Cholesterol. By performing fluorescence lifetime imaging of these GUVs, we were able to show a clear difference in fluorescence lifetime between L_o and L_d phases, confirming earlier work⁴¹ that suggested that L_o phases display a higher viscosity than L_d phases. Additionally, we showed that the GUVs with compositions along the same tie-line showed very similar viscosities for their L_d phases, and also for their L_o phases.

Overall, we demonstrated that BODIPY-based molecular rotors can be used as reliable probes of viscosity within lipid bilayers, showing the differences in viscosity between different lipid phases both in LUVs and phase-separating GUVs. Whilst in principle this could provide a useful tool in investigating the presence of ordered microdomains in cellular plasma membranes, the strong partitioning of the three rotors into the L_d phase, as well as the tendency of the rotors to internalise within cultured cells may potentially limit their usefulness. The following chapters will discuss imaging of bacterial cells using BODIPY rotors, and the synthesis and characterisation of a fluorescent BODIPY probe, designed to act as a molecular rotor and to target the plasma membranes of cells, whilst also partitioning equally between phases.

4. Measuring Viscosity in the Plasma Membranes of Live *Escherichia coli* Bacteria Using BODIPY-based Molecular Rotors

The work in this chapter is adapted from that published as Mika, J. T. et al. Measuring the viscosity of the Escherichia coli plasma membrane using molecular rotors. Biophys. J, 111, 1528-1540 (2016)

All E. coli bacteria imaged in this chapter were cultured by Dr Jacek Mika. Confocal images in Figure 4-8, Figure 4-9 and Figure 4-10 were obtained by Dr Alex Thompson.

4.1 Introduction

4.1.1 Overview

In the previous chapter, we discussed the use of BODIPY-based molecular rotors to investigate viscosity within model lipid bilayers, determining that increased lipid ordering correlates with increased membrane viscosity and that the membrane viscosity as calculated from the fluorescence lifetimes of molecular rotors can be related to the diffusion coefficient of the rotor within the membrane. In this chapter we bring this idea forward, using BODIPY-based molecular rotors to investigate viscosity and lipid ordering within the plasma membranes of *Escherichia coli* (*E. coli*) bacteria at different temperatures.

4.1.2 *Escherichia coli*

A potential biological application of molecular rotors is the investigation of viscoelastic properties within prokaryotic cells. In addition to the more obvious differences, such as the large difference in size and lack of a nucleus in prokaryotic cells,¹⁹⁵ they also differ from eukaryotic cells on the molecular level, showing a much higher degree of macromolecular crowding, leading to a reduced diffusion coefficient of molecules within the cell interior.¹⁹⁶ A previous study¹⁸³ using BODIPY molecular rotors to investigate membrane viscosity within live cells and spores of the gram-positive bacterium *Bacillus subtilis* also revealed a much higher viscosity than previously observed within the plasma membranes of eukaryotic cells,¹³⁵ suggesting that bacterial membranes may be more ordered or gel-like in character. A thorough understanding of the membrane viscosity is therefore

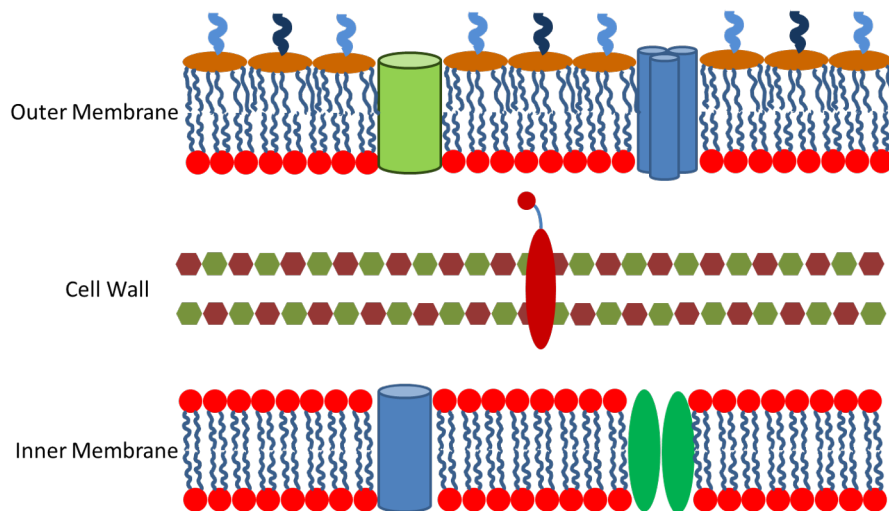


Figure 4-1 General structure of the gram-negative bacterial cell envelope (adapted from²⁹¹). Cylinders and ellipses represent membrane proteins, and the larger lipids on the outer leaflet of the outer membrane represent lipopolysaccharides

necessary in order to fully understand the makeup and behaviour of prokaryotic cells, with such applications as the treatment of disease and the prevention of food spoilage.¹⁸³

Escherichia coli, or *E. coli*, is a very well-studied bacterium, and is therefore often used as a model for understanding the physical properties of bacterial and prokaryotic cells. It is a gram-negative bacterium, measuring around 1 μm long by 0.35 μm thick, that is highly abundant and easy to culture,¹⁹⁷ and, as such, has found widespread usage as a model organism for a number of different applications, in fields as diverse as the development of pharmaceuticals,¹⁹⁸ evolutionary biology¹⁹⁹ and even the understanding of the role of DNA in encoding proteins.²⁰⁰ As a gram-negative bacterium, it has a fairly complex cell envelope structure which consists of the inner membrane, the peptidoglycan cell wall and an outer membrane (see Figure 4-1 for a schematic of this structure from the inside to the outside of the cell). The plasma membrane (also known as the inner membrane) plays a similar role to plasma membranes in eukaryotic cells, containing a wide array of membrane proteins which carry out different cellular functions, and acting as a boundary between the cell interior and the external environment.²⁰¹ The gram-negative bacterial cell wall is typically quite thin compared with the cell walls of other cells, and is approximately 20-30 nm thick. It occupies the space between the two membranes known as the “periplasmic space”, and can be thought of as an exoskeleton which helps to maintain the bacterial cellular structure.²⁰¹ Finally, the outer membrane is essentially a lipid bilayer rich in proteins, with about half the mass of the outer membrane consisting of proteins.¹⁹⁵ This outer membrane resembles the plasma membrane, although it is highly asymmetric in nature, with the inner leaflet of the lipid bilayer primarily consisting of phospholipids and the outer leaflet containing macromolecules known as lipopolysaccharides.¹⁹⁵

This outer membrane represents an additional permeability barrier to the cell interior, meaning that many powerful antibiotics are of no therapeutic use for the treatment of gram-negative bacterial infections.²⁰¹

Much work has previously gone into understanding the composition and phase behaviour of the *E. coli* plasma membrane, as well as the pathways to lipid biosynthesis within *E. coli* bacteria.²⁰² The lipid composition of the *E. coli* plasma membrane is believed to consist mainly of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) lipids,²⁰³ and *E. coli* bacteria are believed to tightly control the exact makeup of their plasma membranes in order to preserve a liquid-crystalline phase at a range of growth temperatures.²⁰⁴ Additionally, bacteria in general do not have significant amounts of cholesterol in their plasma membranes, unlike eukaryotic cells, and generally do not biosynthesise sterols,²⁰⁵ which may have an interesting effect on the phase behaviour of their membrane lipids.

Organisms such as *E. coli* that exist as individual cells, rather than as part of a multicellular body, are prone to experiencing wide shifts in temperature. These large scale changes in temperature are likely to have a major impact on the membrane viscosity and phase behaviour, leading to a profound impact on membrane structure and function. Transition from the fluid to the gel phase is thought to reduce the activity of many membrane associated enzymes, slow the rate of lateral protein diffusion within the bilayer, and also increase the permeability of the bilayer to cations and water, due to the packing defects that may form at the boundaries between gel and fluid phase lipids.²⁰⁶ Bacteria such as *E. coli* are therefore thought to adapt the composition of their plasma membrane to reduce the effect of temperature on their ability to function, with a number of biosynthetic pathways that regulate the lipid composition of the plasma membrane.²⁰⁷ A similar phenomenon is believed to take place within the plasma membranes of microorganisms exposed to extreme conditions, such as barophilic²⁰⁸ or thermophilic bacteria.²⁰⁹

4.1.3 The homeoviscous adaptation model

In a landmark paper in 1974,²¹⁰ Sinensky proposed the “homeoviscous adaptation” model, which suggests that *E. coli* bacteria regulate the relative proportions of saturated and unsaturated lipids within their plasma membranes in order to maintain a constant membrane viscosity at different growth temperatures, so as to regulate the activity of membrane-bound enzymes for optimal functioning. By growing *E. coli* cultures at a range of different temperatures, and then extracting the plasma membrane lipids and performing electron spin resonance (ESR) to determine the membrane

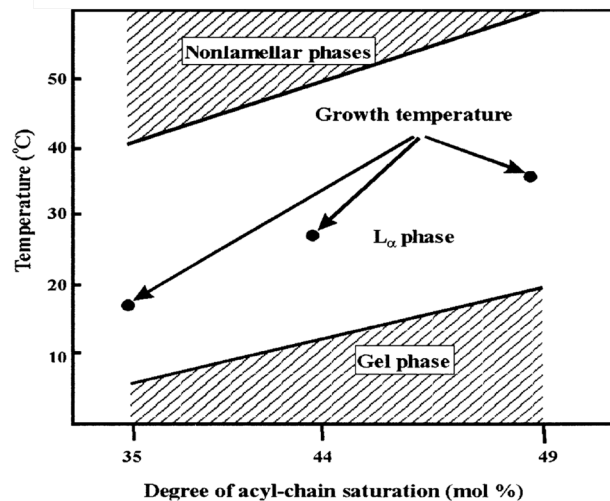


Figure 4-2 The homeoviscous adaptation model. *E. coli* bacteria are believed to regulate the lipid composition of their plasma membranes depending on their growth temperature, in order to maintain a "window" between the gel phase and a nonlamellar lipid phase (adapted from²¹²)

viscosity, Sinensky determined that the membrane viscosity of the extracts was constant if measured at the grown temperature, at ca. 190 cP. Additionally, he determined that the growth temperature was consistently ca. 15 °C above the gel-to-liquid phase transition temperature of the lipid extracts. This work was later supported by Nakayama *et al*,²¹¹ who extended the investigation to the effect of growth temperature on the phase transition temperature of the outer membrane, finding that, whilst the phase transition temperature of the outer membrane lipids is also sensitive to growth temperature, it is affected less than the plasma membrane, something they attributed to the presence of lipopolysaccharides in the outer membrane. A direct measurement of the plasma membrane viscosity within live *E. coli* cells has, to the best of our knowledge, not yet been performed, and whether homeoviscous adaptation actually maintains a constant viscosity in the membrane remains to be seen.³⁹

E. coli bacteria are currently believed to regulate their membrane lipid composition to keep the bilayer in a liquid crystalline (L_α) state in a "window" between the gel phase and a reversed non-lamellar phase (Figure 4-2).²¹² They do this by regulating the structure of the acyl chains of the plasma membrane lipids, with the relative fraction of saturated lipids within the *E. coli* membrane increasing with growth temperature, thereby increasing the gel-to-liquid phase transition temperature.²¹³ Maintaining the position of the plasma membrane within this "window" may be important, in order to ensure the thermodynamic distance of the membrane to the nonlamellar inverted hexagonal (H_{II}) phase,²⁰⁶ which has been hypothesised to act as an intermediate in membrane fusion.²¹⁴

The homeoviscous adaptation model is not without controversy, however. It is based on the Fluid-Mosaic model,⁸ which assumes that there is no long range order or lateral structuring within the plasma membrane. In 1999 Jin *et al*²¹⁵ hypothesised that *E. coli* bacteria regulate their membrane composition to ensure that the critical point of the constituent lipid mixture is close to their growth temperature, in order to allow small scale local phase separation so as to modulate reaction rates and molecular interactions. Other work²¹⁶ has suggested that the plasma membranes of *E. coli* bacteria are able to undergo phase separation in a manner similar to that described in the lipid raft hypothesis, although this observation was made from a detergent extraction experiment, which, as discussed in Chapter 1, has been shown to be an unreliable method of showing phase separation in the membranes of live cells.²⁷ Fluorescence-based techniques have also been used to investigate the possibility of coexisting lipid phases within the plasma membrane of *E. coli*, with a 2000 study²¹⁷ showing uneven distribution of a fluorescent-tagged lipid in the plasma membranes, which was interpreted as evidence of membrane heterogeneity and possible phase separation. In 1999, Fishov and Woldringh²¹⁸ observed uneven distribution of a lipophilic dye in the plasma membrane upon division of *E. coli* bacteria, which they interpreted as the formation of membrane domains by DNA-membrane interactions. By using a Langmuir trough to generate a stress-strain profile of monolayers comprised of *E. coli* polar lipid extracts, López-Montero *et al*²¹⁹ found that the monolayers displayed a nonlinear regime beyond a critical strain, which they interpreted as evidence of phase coexistence, with increasing strain favouring the formation of more ordered lipid phases. They suggest that this may point towards the existence of coexisting lipid phases within live *E. coli* plasma membranes, although they do acknowledge that lipid monolayers represent a very simplified model and their observations may not apply in the plasma membranes of live cells. How the question of membrane phase behaviour fits in with the homeoviscous adaptation model remains to be seen, and may become an absorbing area of research over the coming years.

4.1.4 The viscosity of the *E. coli* plasma membrane

There have been several previous attempts to determine bilayer viscosity within *E. coli* plasma membrane extracts. In his paper proposing the homeoviscous adaptation model, Sinensky²¹⁰ used the methanol/chloroform lipid extraction procedure described by Bligh and Dyer²²⁰ to obtain *E. coli* membrane lipids, which were then separated into polar and nonpolar fractions. The polar fractions were then formed into bilayers before undergoing electron spin resonance (ESR), finding the average viscosity as 190 cP. Spooner *et al*²²¹ also used ESR to investigate the viscosity of a mixture of polar *E. coli* lipid extracts and DOPC, finding an increased membrane viscosity of 300 cP, despite the addition

of the unsaturated and nonviscous¹³⁸ DOPC. Additionally, fluorescence-based techniques, such as the use of dipyrrenylpropane,²²² which forms excimers to varying degrees depending on the viscosity of the surrounding environment, and fluorescence polarisation studies²²³ have been used to assess relative changes in the membrane viscosity of *E. coli* lipid extracts, without giving a quantitative estimate of the viscosity value. By assuming that membrane viscosity is directly related to the gel-to-liquid phase transition temperature of the lipids (i.e. the lower the transition temperature, the less viscous the membrane), Casadei *et al* used differential scanning calorimetry of *E. coli* plasma membrane extracts to investigate the effects of growth temperature on membrane viscosity.²²⁴ They found that the gel transition temperature, and therefore the membrane viscosity, increased with growth temperature, supporting the homeoviscous adaptation hypothesis, although their assessment of viscosity was again qualitative rather than quantitative.

It is worth bearing in mind that the lipid extraction process itself, which involves a chloroform/methanol extraction of the whole organism, rather than just the plasma membrane, may not be an accurate representation of the true nature of the *E. coli* plasma membrane, which is a complex and dynamic mixture of lipids and proteins, supported by the peptidoglycan cell wall and so these viscosity measurements may not reflect the viscosity within the native membrane.

Despite the large amount of previous research done on the phase behaviour of *E. coli* plasma membranes and the homeoviscous adaptation model, there have, to the best of our knowledge, never been any direct measurements of the viscosity of native *E. coli* plasma membranes, with viscosity values instead being calculated from diffusion coefficients. In 2016, Oswald *et al*²²⁵ investigated the diffusion coefficients of membrane proteins within the *E. coli* plasma membrane, using the Saffman-Delbrück model¹⁹¹ to calculate a plasma membrane viscosity in the range of 1000-1200 cP, a value much higher than previously calculated in *E. coli* membrane extracts, possibly reflecting the increased molecular crowding of proteins and other species within the live membrane compared with membranes formed from lipid extracts. In 2014, Nenninger *et al*⁶⁶ used FRAP to investigate the diffusion of a BODIPY probe very similar in structure to our probe BODIPY-C10 within the plasma membrane of live *E. coli*, grown at 37 °C, at different temperatures. They showed a large difference in the diffusion coefficients at around 28 °C, which they interpreted as evidence of a phase transition. Comparisons between diffusion coefficients and membrane viscosities in *E. coli* plasma membranes and membranes of *E. coli* extracts will be discussed in further detail in the Results and Discussion section of this Chapter.

In this chapter, we stain the plasma membranes of live *E. coli* cells with BODIPY-C10, and use FLIM to quantitatively investigate the plasma membrane viscosity at different temperatures. Additionally,

we use BODIPY-C10 to investigate membrane viscosity within *E. coli* lipid extracts, for comparison with the plasma membranes of live cells. We find that the membrane viscosity of live plasma membranes is much higher than that of membrane extracts suggesting a high degree of lipid ordering within the plasma membranes of live *E. coli* bacteria.

4.2 Results and Discussion

4.2.1 Studies in *E. coli* plasma membrane extracts

Many of the studies in the literature focusing on the viscosity of the *E. coli* plasma membrane, including the report by Sinensky that first proposed the homeoviscous adaptation model,²¹⁰ have relied on the use of *E. coli* lipid extracts. We therefore began our investigation by using the molecular rotor BODIPY-C10 to investigate the viscous properties of artificial bilayers made from total lipid extracts from *E. coli* bacteria cultured at 37 °C, obtained from Avanti Polar Lipids (Alabaster, Alabama, USA), in order to compare our results with those in the literature. We incorporated BODIPY-C10 into LUVs made up of the lipid extracts and performed both time-resolved and steady-state emission measurements.

Figure 4-3 (A) shows overlaid emission spectra of the LUVs at a range of temperatures. As expected, BODIPY-C10 displays a maximum emission wavelength of 515 nm and the emission intensity decreases with increasing temperature, reflecting the decrease in membrane viscosity. Figure 4-3 (B) shows the change in emission intensity of BODIPY-C10 at its maximum emission intensity of 515 nm with temperature. We expected to observe a sharp change in the gradient at the gel transition temperature, similar to what we observe from BODIPY-C10 in DPPC LUVs at 41 °C (Figure 4-4). Despite the predicted lipid phase transition 15 °C below the growth temperature,²¹⁰ there is no

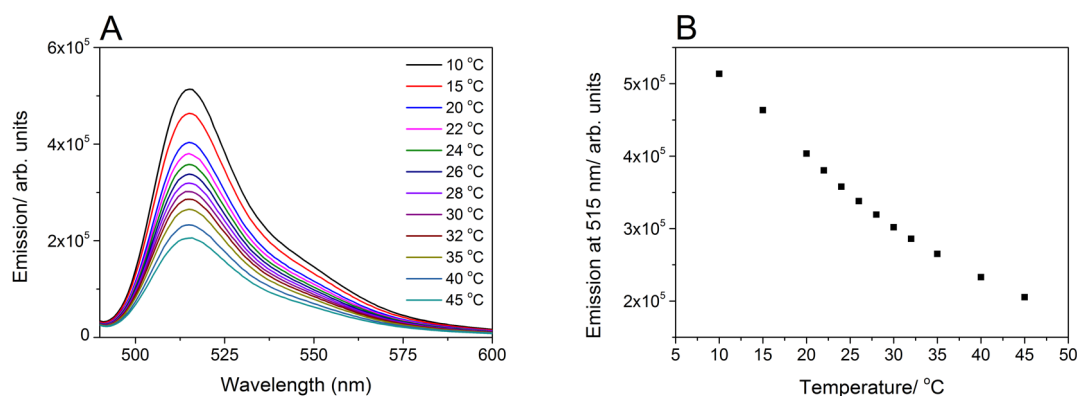


Figure 4-3 (A) overlaid emission spectra of BODIPY-C10 in total *E. coli* lipid extract LUVs at a range of temperatures; (B) emission intensity at 515 nm of BODIPY-C10 in the lipid extract LUVs

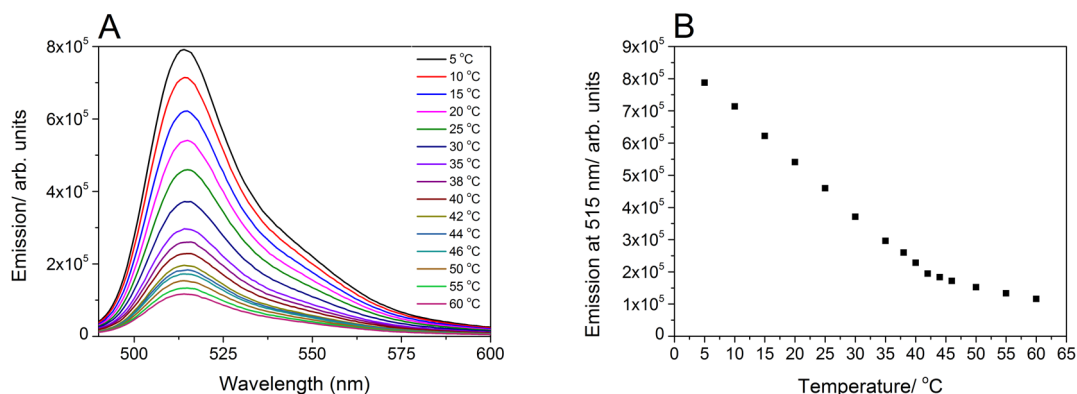


Figure 4-4 A) overlaid emission spectra of BODIPY-C10 in DPPC LUVs at a range of temperatures; **(B)** emission intensity at 515 nm of BODIPY-C10 in the DPPC LUVs

sharp change in the gradient of the curve, as might be expected for the viscosity change associated with a phase transition. Instead there is a gradual change in gradient between *ca.* 30 and 25 °C. DPPC is a single lipid whereas *E. coli* lipid extracts are a complex mixture of lipids, meaning that the phase transition may give a more subtle change in viscosity and may occur over a range of temperatures as a gradual process, meaning that will not give as clear a difference in viscosity as the phase transition in DPPC.

We then went on to perform TCSPC of the lipid extract LUVs at a range of different temperatures, and example fluorescence decays are shown in Figure 4-5. The fluorescence decays were biexponential within the temperature range that we studied, potentially reflecting different preferred orientations of BODIPY-C10 within the bilayer, owing to either the complex nature of the lipid mixture or the bilayer phase behaviour, similar to what has been previously observed for BODIPY-C10 in gel phase bilayers.¹³⁸ In order to analyse these decays, a biexponential fitting model was used with χ^2 values below 1.5 in all cases, suggesting a good fit. In addition to the individual lifetime components of the biexponential decays, τ_1 and τ_2 , an intensity-weighted mean lifetime was calculated using equation 4-1, shown below:

$$\tau_{\text{mean-weighted}} = \frac{\tau_1^2 \alpha_1 + \tau_2^2 \alpha_2}{\tau_1 \alpha_1 + \tau_2 \alpha_2} \quad (4-1)$$

where α_1 and α_2 are the amplitudes of the two different lifetime components. The intensity-weighted mean lifetime represents an average of the two lifetime components, and would therefore give an average membrane viscosity from the two different BODIPY-C10 lifetime components.⁵⁶ This may not be suitable if BODIPY-C10 takes on two discrete orientations in a homogeneous bilayer, leading to two discrete “viscosities”, as an average lifetime value would not represent either BODIPY-C10 orientation and would therefore be meaningless. Additionally, if a biexponential decay was caused by coexisting lipid phases then a mean-weighted lifetime would not represent the viscosity of any part of the bilayer, so would also be unsuitable. However, if the biexponential nature of the decay represents areas of different viscosity within the LUVs, potentially due a continuous distribution of BODIPY-C10 orientations, then it may make sense to use the mean-weighted lifetime to calculate the viscosity, as it would represent an average viscosity for the bilayer.

Plots showing the change in τ_1 and τ_2 and mean-weighted lifetime with temperature are shown in Figure 4-6 (A-C). Using the lifetime-viscosity calibration data for BODIPY-C10 to convert BODIPY-C10 lifetime into viscosity (equation 3-1 in Chapter 3), plots of bilayer viscosity against temperatures were generated, and are shown in Figure 4-6 (D-F). Much like the fluorescence emission intensity, each lifetime component of BODIPY-C10 decreased with increasing temperature, reflecting the reduction in bilayer viscosity. Additionally, there appears to be a change in gradient in each of the graphs between 25 °C and 30 °C, which may correspond to a change in lipid phase, although this change is not as sharp as the one observed in DPPC LUVs (see Chapter 3) and the decays remain biexponential regardless of temperature.

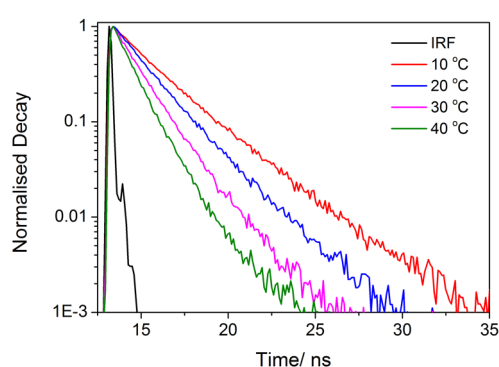


Figure 4-5 Example fluorescence decays of BODIPY-C10 in LUVs comprising of *E. coli* lipid extracts at a range of temperatures

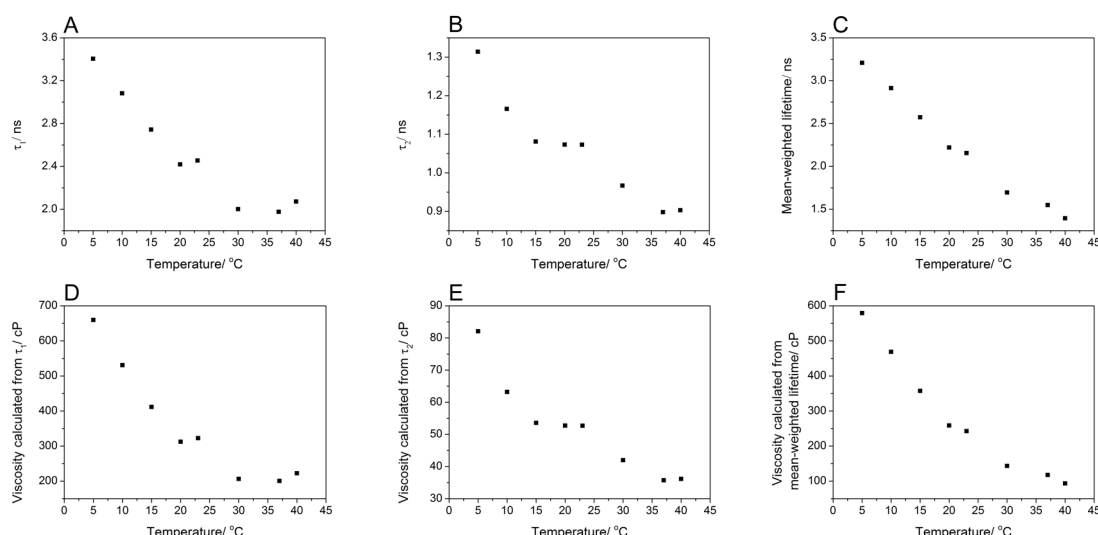


Figure 4-6 (A-C) Plots showing the change in different components of the biexponential decay of BODIPY-C10 in LUVs comprised of *E. coli* lipids with temperature: (A) τ_1 , (B) τ_2 and (C) mean-weighted lifetime; (D-F) Plots showing the change in bilayer viscosity of the *E. coli* extract LUVs, as calculated from the different lifetime components of the biexponential BODIPY-C10 decays at a range of temperatures: (D) viscosity calculated from τ_1 , (E) viscosity calculated from τ_2 and (F) viscosity calculated from mean-weighted lifetime. The BODIPY-C10 methanol/glycerol calibration described in Chapter 3 (equation 3-1) was used to calculate viscosities.

The viscosity calculated from the τ_2 component of the decay was in the range of 85-35 cP between 5 °C and 55 °C, much lower than other viscosities calculated for lipid bilayers, and below even DOPC (see Chapter 3), a simple unsaturated lipid which would be expected to have a low viscosity relative to other more complex lipid structures. The τ_2 component was therefore attributed to a second orientation of the BODIPY-C10 dye, similar to its behaviour in gel phase bilayers, and the viscosity calculated from the τ_1 component was taken as representative of the viscosity of the tail region of the bilayer. At 37 °C, the growth temperature of the bacteria that the lipid extracts were obtained from, the viscosity from the τ_1 component was calculated as 210 cP. This corresponds well with the value of 180 cP calculated by Sinensky for *E. coli* bacteria grown and measured at 37 °C. As temperature decreases, membrane viscosity increases, with it reaching 320 cP at 23 °C and 660 cP at 5 °C. These high viscosity values are close to the viscosities associated with model L_o phases at these temperatures (see viscosities for DPPC and EYSM LUVs Chapter 3), and may suggest that the bilayer is in a more ordered lipid phase.

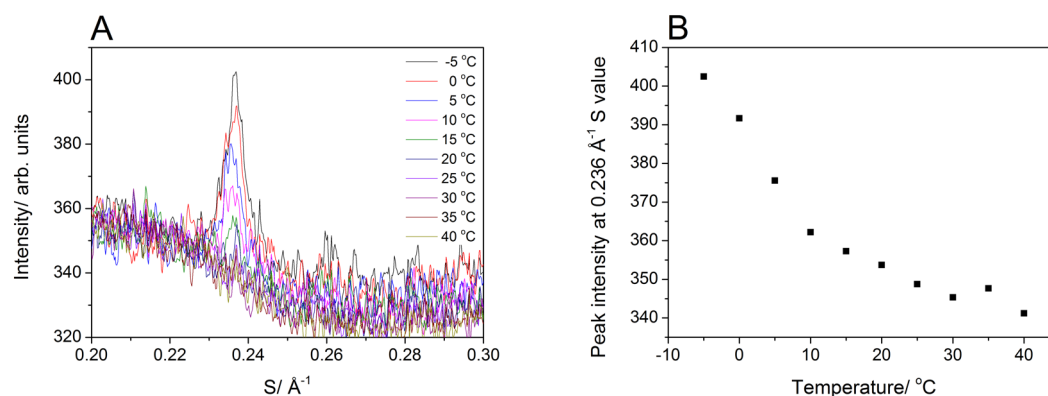


Figure 4-7 (A) Wide-angle X-ray diffraction patterns from *E. coli* total lipid extracts at a range of temperatures, highlighting the characteristic gel phase peak at *ca.* 0.24 Å⁻¹, (B) height of this peak with temperature

Since we observed no clear evidence of a phase transition from the fluorescence-based studies of the extracts, wide-angle X-ray scattering (WAXS) techniques were then used in order to further investigate the phase behaviour of the *E. coli* lipid extracts. Figure 4-7 (A) shows WAXS diffraction patterns from the *E. coli* lipid extracts at a range of temperatures. At *ca.* 0.24 Å⁻¹, a peak characteristic of the gel phase¹³⁶ can be observed that decreases in intensity with increasing temperature, appearing to disappear at 25 °C, suggesting a phase transition into a fluid phase. The plot in Figure 4-7 (B) that displays the change in the intensity of this peak, shows a rapid reduction in the peak intensity up to 10 °C, followed by a more gradual decline up to 25-30 °C. This supports the data from the lifetime measurements, which suggest a phase transition within this temperature region, and agrees with the hypothesis put forward by Sinensky²¹⁰ which states that the gel transition temperature of *E. coli* lipid extracts is *ca.* 15 °C below the growth temperature (37 °C for lipid extracts obtained from Avanti Polar Lipids).

4.2.2 Studies in the plasma membranes of live *E. coli* bacteria

The experiments performed using the *E. coli* lipid extracts to probe the phase behaviour agreed with the literature studies. Because molecular rotors allow us to directly study the undisturbed plasma membranes of live *E. coli* bacteria, we subsequently went on to use BODIPY-C10 to investigate viscosity within the plasma membranes of live *E. coli* bacteria.

A BODIPY-based probe very similar in structure to BODIPY-C10 has previously⁶⁶ been shown to stain the plasma membrane of live *E. coli* bacteria, and so we hypothesised that BODIPY-C10 would stain *E. coli* plasma membranes in a similar manner. In order to investigate the partitioning of BODIPY-C10

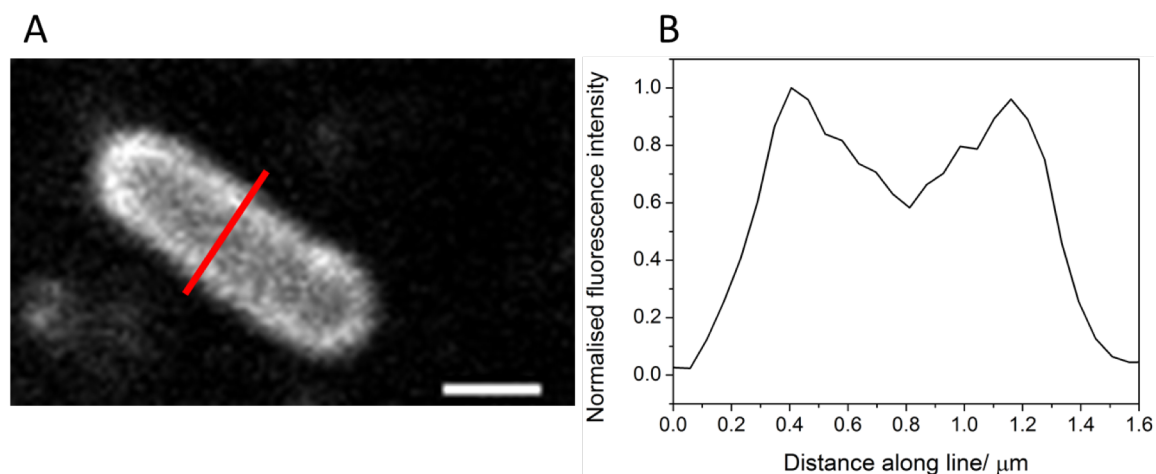


Figure 4-8 (A) Confocal image of an *E. coli* bacterium stained with BODIPY-C10 (scale bar = 1 μm , image taken by Dr Alex Thompson), **(B)** pixel intensity across the red line in (A), showing the fluorescence intensity across the bacterium

within live cells, *E. coli* bacteria cultured by Dr Jacek Mika (Katholieke Universiteit Leuven) stained with BODIPY-C10 were imaged using confocal microscopy (Figure 4-8, microscopy performed by Dr Alex Thompson, Department of Chemistry, Imperial College London). This revealed a ring-like structure bordering the rod-like shape of each bacterial cell, with a plot of pixel intensity across the bacterium shown in Figure 4-8 (B) showing two fluorescence maxima, corresponding to the position of the cell membrane, suggesting that BODIPY-C10 may indeed be staining the plasma membrane.

Because *E. coli* bacteria have two membranes in the cell envelope, the inner (plasma) membrane and an outer membrane, with the cell wall in between, we then sought to investigate which of the two membranes was being stained. In order to this, following a similar methodology to the one described by Nenninger *et al*,⁶⁶ Drs Jacek Mika and Alex Thompson performed an osmotic upshift experiment. When *E. coli* cells are suddenly placed in a medium of significantly increased osmolality compared with their growth medium, water leaves their cytoplasm, causing a contraction of the cell interior.²²⁶ The inner (plasma) membrane of the bacterium follows this contraction, losing its rod-like shape and eventually causing visible gaps between the inner membrane and the cell wall to appear, which are termed visual plasmolysis spaces (VPS).^{227,228} However, the outer membrane, which is attached to the more rigid cell wall, retains its rod-like shape.²²⁹ By investigating the effect of this osmotic shock on the fluorescence profile of the bacterium, the preferred localisation of BODIPY-C10 can therefore be identified.

To facilitate observation, *E. coli* cells were treated with cephalixin, an antibiotic that causes bacterial cells to elongate,²³⁰ before being subjected to osmotic shock using medium containing 15% sucrose. In these cells, the membrane stain no longer appears rod-like, instead appearing perturbed with observable VPS (Figure 4-9). This suggests that BODIPY-C10 is staining the plasma membrane of the *E. coli* cells, rather than the outer membrane.

This conclusion was supported by a further experiment, where spheroplasts were generated from live *E. coli* cells stained with BODIPY-C10 by digestion with lysozyme.¹⁴⁵ This removes the *E. coli* outer membrane, yielding spheroplasts, which only possess the plasma membrane of the original *E. coli* bacteria. As is shown in Figure 4-10, membrane staining is still present, with the pixel intensity plot shown in Figure 4-10 (B) showing the maximum fluorescence intensity at the edges of the cell. This further implies that BODIPY-C10 is staining the plasma membranes of the *E. coli* bacteria.

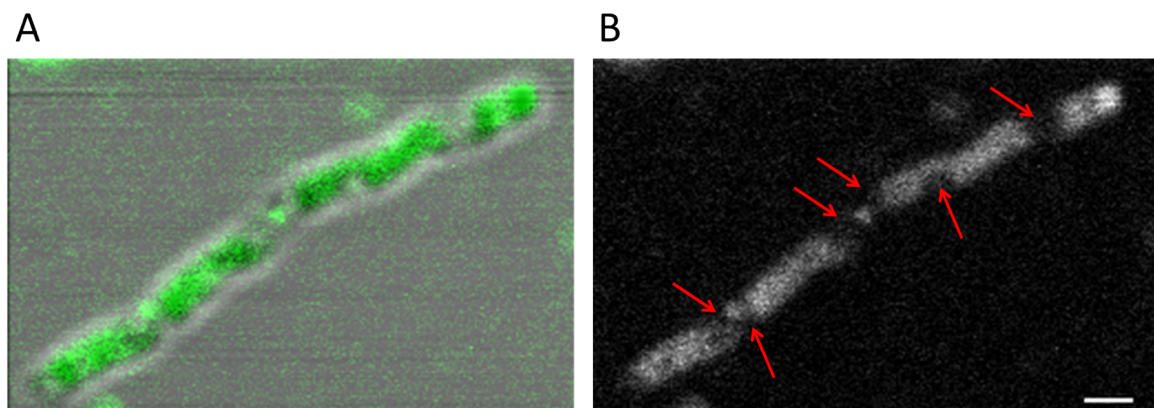


Figure 4-9 (A) Overlaid confocal and bright field image of *E. coli* bacteria stained with BODIPY-C10, elongated with cephalixin and then subjected to osmotic shock with media containing 15% sucrose. This image highlights the contraction of the cell interior. **(B)** Confocal image of the same bacteria, with the areas of VPS highlighted by red arrows (scale bar = 1 μm , osmotic shock experiment performed by Dr Jacek Mika and microscopy performed by Dr Alex Thompson).

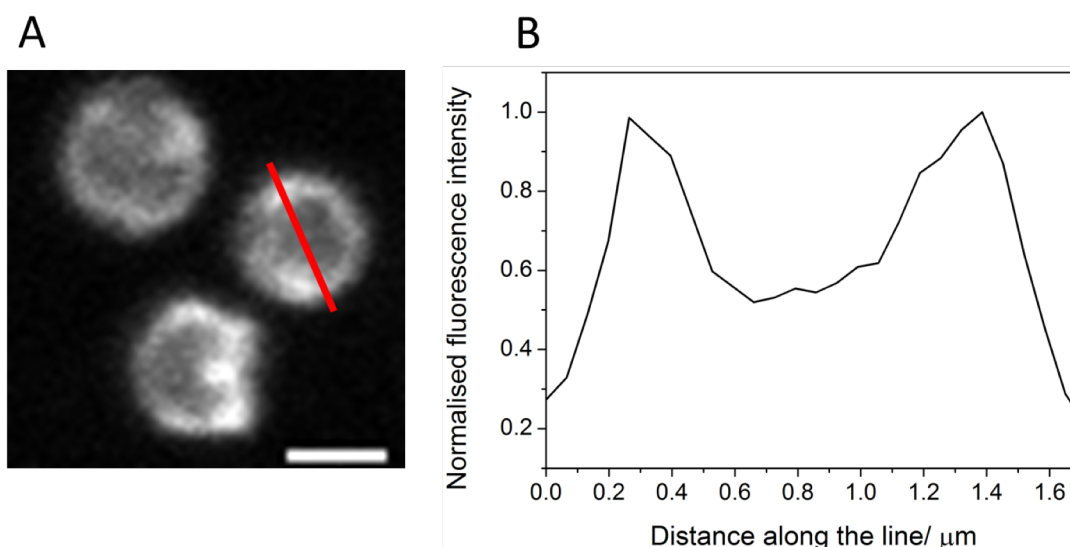


Figure 4-10 (A) confocal image of spheroplasted *E. coli* cells (scale bar = 1 μm , image taken by Dr Alex Thompson), **(B)** pixel intensity across the red line in (A), showing the fluorescence intensity across the bacterium

Following on from this evidence that BODIPY-C10 primarily stains the plasma membrane of *E. coli* bacteria, we went on to perform FLIM of BODIPY-C10 stained *E. coli* bacteria at different temperatures in order to investigate the viscous properties of the plasma membrane. As in the studies using lipid extracts, the time-resolved fluorescence decays of BODIPY-C10 were again biexponential in nature (see Figure 4-11 for an example fluorescence decay and biexponential fit for a whole cell). Figure 4-12 shows example lifetime images of both live *E. coli* bacteria and spheroplasts, grown and imaged at 37 $^{\circ}\text{C}$, with the colour scale showing the variation in τ_1 . Much like our experiments in *E. coli* lipid extracts, we chose to interpret the τ_1 value as representative of the membrane viscosity. This was because the shorter τ_2 lifetime component was too low to represent the viscosity of the lipid tail region of the membrane leaflet and was thus more likely to represent a second localisation of BODIPY-C10 within the lipid headgroup region of this bilayer. This interpretation was previously supported by the molecular dynamics simulations described in Chapter 3.

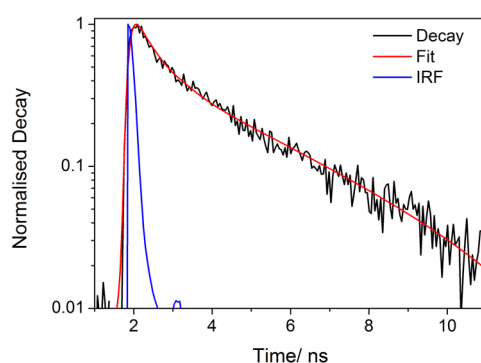


Figure 4-11 Example fluorescence decay of BODIPY-C10 in live *E. coli* bacteria

Our FLIM measurements of the live cells typically gave a low photon count per pixel of the lifetime image, below the *ca.* 500 counts required for biexponential fitting. Lengthy imaging times led to photobleaching of the BODIPY-C10 dye and blurring of the lifetime images, so an alternative method was developed to ensure a photon count sufficient for biexponential analysis. Regions of interest (ROIs) were drawn manually around individual cells, and all photons within the ROI were binned into a single decay. This was then fitted with a biexponential curve to establish an average fluorescence lifetime for the whole cell. Whilst this reduced the resolution of the FLIM technique and prevented us from establishing any information about subcellular heterogeneities, it allowed us to obtain a high signal-to-noise ratio and a high enough photon count to enable reliable biexponential fitting and extraction of quantitative viscosity values.

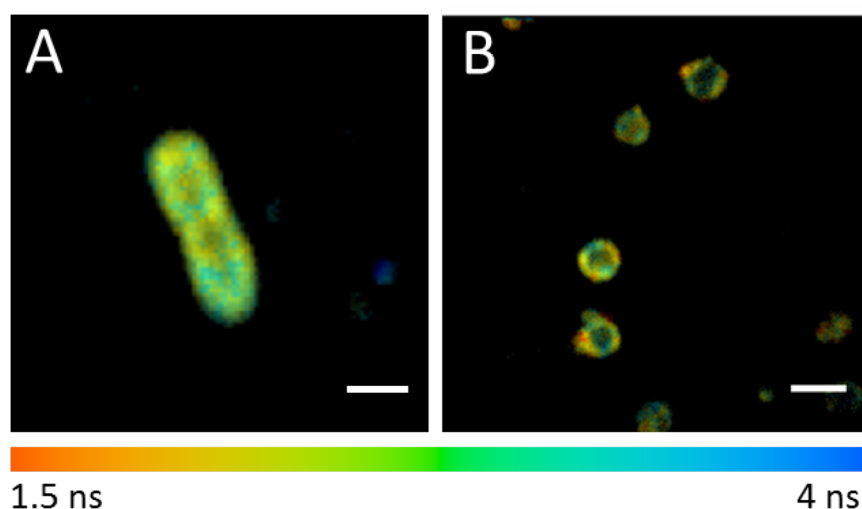


Figure 4-12 Sample lifetime images of (A) a live *E. coli* bacterium and (B) spheroplasts, both from bacteria cultured at 37 °C and imaged at 37 °C

We then set out to use FLIM of BODIPY-C10 in the plasma membranes of *E. coli* bacteria to investigate the homeoviscous adaptation model. We cultured BODIPY-C10 stained *E. coli* bacteria at 37 °C and performed FLIM at a range of different temperatures, and compared this with FLIM of *E. coli* bacteria grown at a range of different temperatures and imaged at the growth temperature. The *E. coli* bacteria grown at 37 °C showed an increase in τ_1 , τ_2 and the mean-weighted lifetime with decreasing imaging temperature (Figure 4-13), suggesting an increase in membrane viscosity. This was expected, as the *E. coli* bacteria would be unlikely to respond to the change in temperature in the time frame between culturing and imaging, so homeoviscous adaptation would not have compensated for the changing temperature by adjusting the membrane viscosity. Additionally, the τ_1 values shown in Figure 4-13 remained fairly constant above 25 °C, but increased significantly below that temperature. This may be evidence of a plasma membrane phase transition at this temperature, consistent with our observations for *E. coli* lipid extracts.

The *E. coli* bacteria grown and imaged at the same temperature also showed an increase in τ_1 , τ_2 and mean-weighted lifetime at reduced temperatures, indicating an increase in viscosity (Figure 4-14). This implies that no homeoviscous adaptation is being observed. However, the errors in the plot are very large, and a plot showing the individual lifetimes of all the cells imaged in this experiment (Figure 4-15) suggests that the majority of the points have a fairly consistent lifetime, and that the increasing trend in mean τ_1 with decreasing temperature may instead be due to individual outliers.

We therefore repeated this experiment in order to investigate whether a different *E. coli* culture would show less inter-cell heterogeneity and enable a conclusion to be drawn about the homeoviscous adaptation model. Figure 4-16 (A-C) shows the different lifetime components of

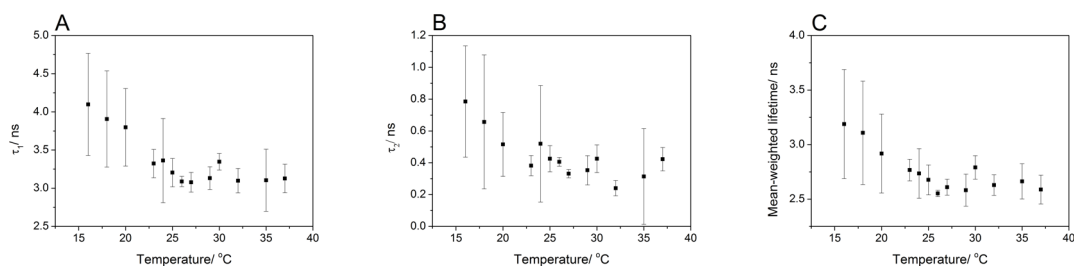


Figure 4-13 Initial experiments on *E. coli* bacteria grown at 37 °C and imaged at a range of temperatures. Plots show the variation in different lifetime components of the biexponential fits for BODIPY-C10 in the plasma membranes of live *E. coli* cells, with (A) showing τ_1 , (B) showing τ_2 and (C) the mean-weighted fluorescence lifetime. In all cases, 10-35 cells were imaged for each point, except 26 °C, where 4 cells were imaged, and the points represent the mean lifetime component. Error bars represent standard deviations.

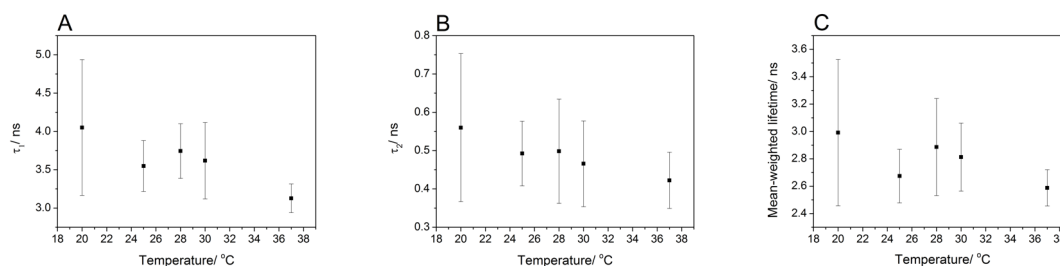


Figure 4-14 Initial experiments on *E. coli* bacteria imaged at their growth temperature. Plots show the variation in different lifetime components of the biexponential fits for BODIPY-C10 in the plasma membranes of live *E. coli* cells, with (A) showing τ_1 , (B) showing τ_2 and (C) the mean-weighted fluorescence lifetime. In all cases, 20-35 cells were imaged for each point, and the points represent the mean lifetime component. Error bars represent standard deviations.

BODIPY-C10 in *E. coli* plasma membranes grown and imaged at the same temperature, at a range of different temperatures.

Unlike the initial results shown in Figure 4-14, the fluorescence lifetimes remained fairly constant, without any observable trend in the data to correlate with increasing or decreasing temperature. This data may therefore be suggesting that the *E. coli* bacteria are maintaining a constant plasma membrane viscosity regardless of their growth temperature, supporting the homeoviscous adaptation model. Further experiments repeating the lifetime imaging of *E. coli* bacteria grown at 37 °C and imaged at varying temperatures, shown in Figure 4-16 (D-F) suggests a small increase in fluorescence lifetime at reduced temperature. Additionally, the τ_1 values observed here, in the range of 4-4.5 ns, are much higher than the ones observed in the initial experiments. To investigate this discrepancy, we repeated the experiment where the *E. coli* was grown at 37 °C and imaged at a range of different temperatures (Figure 4-17). Much like the studies shown in Figure 4-16, the τ_1 values were in the region of 4-4.5 ns, and there was little correlation between imaging temperature and fluorescence lifetime, due to the degree of inter-cell variation. Due to the comparatively low lifetime values of the initial experiments compared with our subsequent experiments, only the lifetime values from the final two experiments were used in subsequent data analysis.

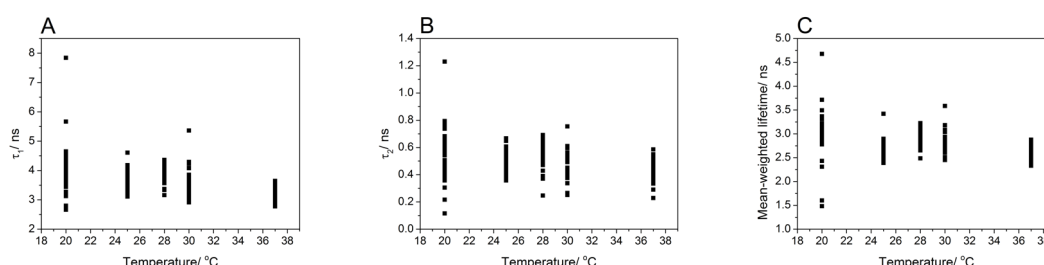


Figure 4-15 Plots showing the same data as Figure 4-14, but showing calculated lifetimes for each individual cell that was imaged

Due to the large inter-cell variability (for example, the spread in the data observable in Figure 4-15), the results were inconclusive as to whether the *E. coli* were exhibiting homeoviscous adaptation or whether there were any changes in lipid phase within the plasma membranes of live cells. We therefore focused our analysis solely on *E. coli* bacteria grown at 37 °C and imaged at 37 °C and 23 °C (i.e. room temperature), and used the lifetime data from BODIPY-C10 to infer information about the membrane viscosity, allowing us to investigate membrane structure and ordering. By combining the data from our final two experiments, we were able to analyse data from a much larger number of cells, allowing us to overcome the variation between individual cells to draw conclusions on the viscosity of the plasma membranes.

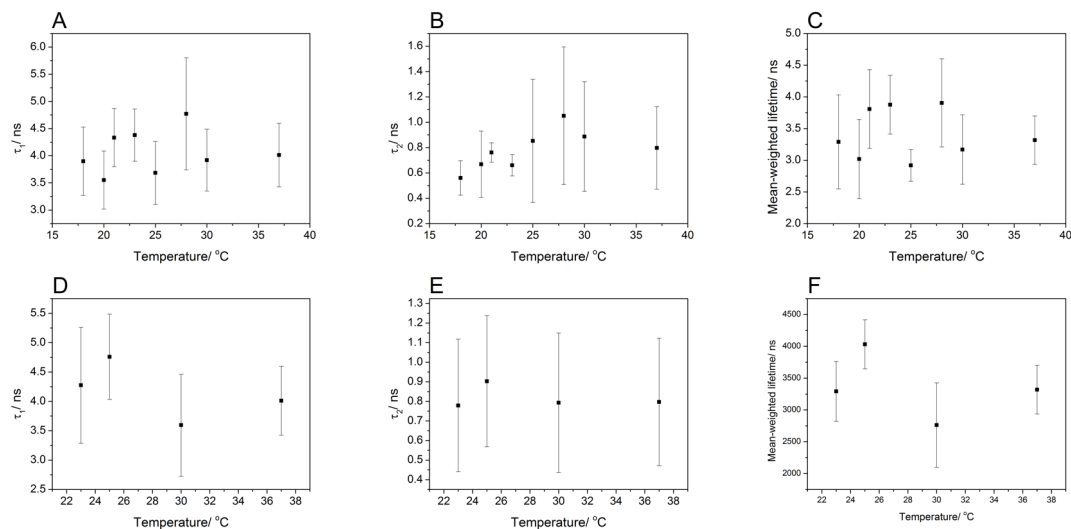


Figure 4-16 Repeat of the experiments shown in Figure 4-13 and Figure 4-14 investigating the fluorescence lifetime of BODIPY-C10 in *E. coli* bacteria. (A-C) show plots of BODIPY-C10 lifetime components in *E. coli* bacteria imaged at their growth temperature, at a range of temperatures, where (A) represents τ_1 , (B) represents τ_2 and (C) represents the mean-weighted lifetimes. (D-F) show plots of BODIPY-C10 lifetime component in *E. coli* bacteria grown at 37 °C and imaged at a range of temperatures, where (D) represents τ_1 , (E) represents τ_2 and (F) represents the mean-weighted lifetimes.

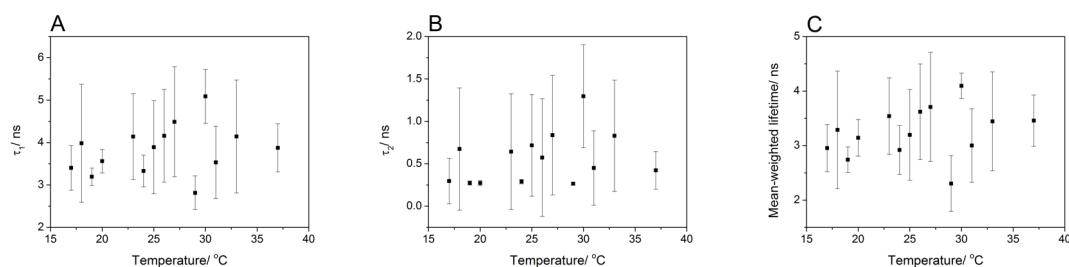


Figure 4-17 Second repeat of the experiments shown in Figure 4-13, where *E. coli* bacteria grown at 37 °C were imaged at a range of temperatures

Using the lifetime-viscosity calibration for BODIPY-C10 (equation 3-1, Chapter 3), we calculated the plasma membrane viscosities for *E. coli* bacteria at 23 °C and 37 °C. We also performed FLIM and calculated membrane viscosities for spheroplasts at 23 °C and 37 °C. Because live *E. coli* bacteria have two membrane systems, and the outer membrane is removed when spheroplasts are formed, leaving only the plasma membrane, a close overlap of spheroplasts membrane viscosity and live cell

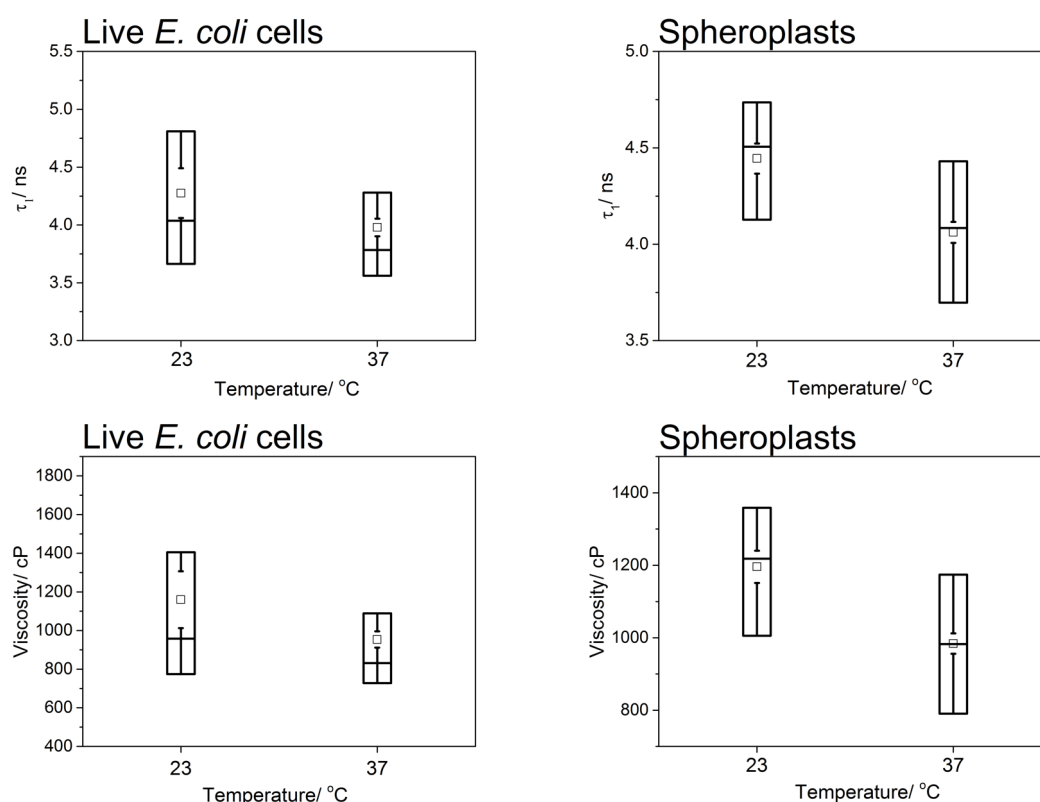


Figure 4-18 Box plots showing the τ_1 components of the fluorescence decays of BODIPY-C10 and the viscosities calculated from these in the plasma membranes of live *E. coli* cells and spheroplasts, at 23 °C and 37 °C, the bacterial growth temperature. Boxes represent the interquartile range, whiskers (error bars) represent one standard error from the mean, horizontal lines show the median values and open squares represent the mean values. Number of measurements (from the two independent measurements shown in Figure 4-16 and Figure 4-17): cells at 23 °C – 21 cells, cells at 37 °C – 57 cells, spheroplasts at 23 °C – 28 spheroplasts, spheroplasts at 37 °C – 58 spheroplasts

plasma membrane viscosity would help support out conclusion the BODIPY-C10 localises primarily within the *E. coli* inner membrane, i.e. the plasma membrane. Figure 4-18 shows box plots displaying the mean and median τ_1 values for a set of live *E. coli* and spheroplast membranes in addition to the interquartile ranges and standard deviations, at 23 °C and 37 °C, as well as the calculated viscosities for each of these values.

The mean τ_1 value for live cells at 37 °C was 3.98 ± 0.08 ns, corresponding to 950 ± 42.4 cP. For spheroplasts at 37 °C, the mean τ_1 was 4.06 ± 0.06 ns, corresponding to a mean viscosity of 980 ± 27.6 cP. For cells measured at 23 °C, the mean τ_1 was determined to be 4.28 ± 0.22 ns, corresponding to a mean viscosity of 1160 ± 146 cP, and for spheroplasts at 23 °C the mean τ_1 was 4.45 ± 0.08 ns, corresponding to a mean viscosity of 1200 ± 45.4 cP. Information regarding the medians, interquartile ranges and standard errors (standard deviation/square root of sample size) of the datasets can be seen in Table 4-1.

Box plots showing statistical information about the other lifetime components, and the viscosities

Table 4-1 Statistics of the lifetime data of BODIPY-C10 in the membranes of live *E. coli* cells and spheroplasts

Cells

	τ_1 / ns		Viscosity/ cP	
	23 °C	37 °C	23 °C	37 °C
Mean	4.28	3.98	1160	950
Standard Error	0.22	0.08	146	42.4
Median	4.00	3.78	960	830
1st Quartile	3.67	3.56	770	730
3rd Quartile	4.81	4.28	1410	1090
IQR	1.15	0.72	630	360

Spheroplasts

	τ_1 / ns		Viscosity/ cP	
	23 °C	37 °C	23 °C	37 °C
Mean	4.45	4.06	1200	980
Standard Error	0.08	0.06	45.4	27.6
Median	4.51	4.08	1220	980
1st Quartile	4.13	3.7	1010	790
3rd Quartile	4.74	4.43	1360	1170
IQR	0.61	0.73	350	380

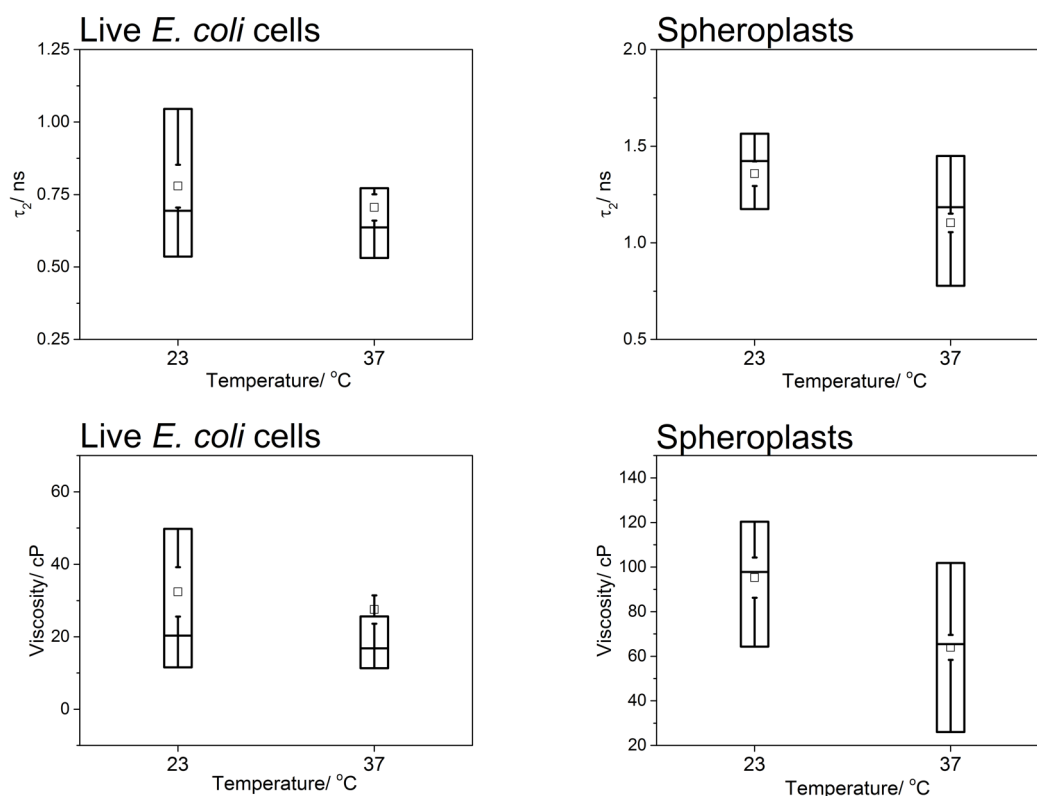


Figure 4-19 Box plots showing the τ_2 (A and B) components of the fluorescence decays of BODIPY-C10 and the viscosities (C and D) calculated from these in the plasma membranes of live *E. coli* cells and spheroplasts, at 23 °C and 37 °C, the bacterial growth temperature. Boxes represent the interquartile range, whiskers (error bars) represent one standard error from the mean, horizontal lines show the median values and open squares represent the mean values

calculated from these components, of BODIPY-C10 in live cells and spheroplasts can be seen in Figure 4-19 and Figure 4-20. As discussed earlier, the τ_1 lifetime component was determined to be representative of membrane viscosity.

As can be seen from Table 4-1 and Figure 4-18, the viscosities measured for the spheroplasts closely match those measured in cells. It is likely that the outer membrane has a different viscosity value than the inner membrane, and so, because spheroplast do not possess an outer membrane, we interpreted this as further evidence that BODIPY-C10 localises in the inner membrane of *E. coli* cells. Additionally, the membrane viscosity for both live *E. coli* cells and spheroplasts is higher at 23 °C, compared with 37 °C. This increase in viscosity with decreasing temperature would be expected for a lipid bilayer,¹³⁸ and the fact that the increase is consistent between spheroplasts and live *E. coli* cells suggests that no membrane adaptation is taking place in the live cells. This viscosity increase could be interpreted as evidence that a phase transition into a more ordered, viscous lipid phase has taken place, although the small difference in viscosity compared with the large viscosity difference

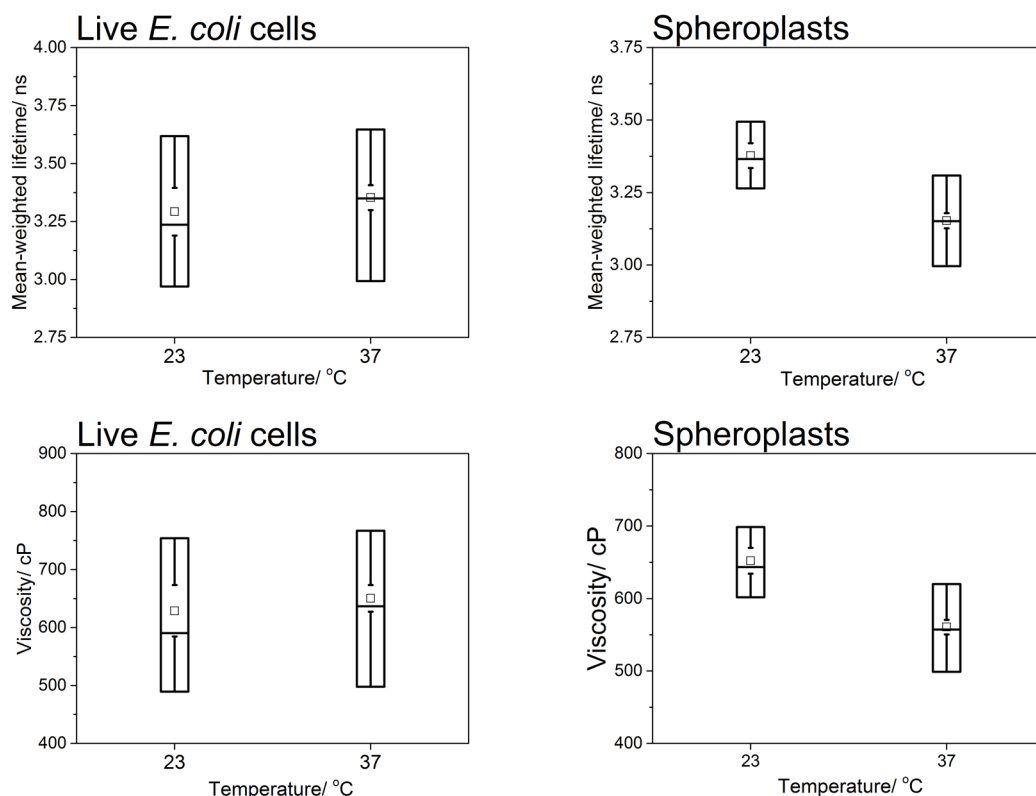


Figure 4-20 Box plots showing the mean-weighted lifetime (A and B), calculated from both components of the fluorescence decays of BODIPY-C10 and the viscosities (C and D) calculated from these in the plasma membranes of live *E. coli* cells and spheroplasts, at 23 °C and 37 °C, the bacterial growth temperature. Boxes represent the interquartile range, whiskers (error bars) represent one standard error from the mean, horizontal lines show the median values and open squares represent the mean values

observed in simple phase transitions such as DPPC (see Chapter 3) and the large spread in the data makes it difficult to assert this to a high degree of confidence.

Also observable from the box plots is the high spread in fluorescence lifetimes, and therefore viscosities, of BODIPY-C10 within the cells, with a greater spread in τ_1 at 23 °C compared with 37 °C. This spread in data suggests a high degree of inter-cell heterogeneity and may help to disguise any overall increase in viscosity and lipid ordering. *E. coli* have been previously reported to display a high degree of variation between isogenic cells in the same culture; an example of this is the variation in diffusion coefficients of bacterial cytosolic proteins, which may vary by up to an order of magnitude between seemingly identical cells within the same culture.^{196,227,231} The fact that spread is greater at 23 °C suggests that some cells may respond more readily to a shift in temperature than others. Also noteworthy is the fact that the larger spread in data in cells at 23 °C compared with 37 °C is not observed in spheroplasts, suggesting that spheroplasts membranes may respond to a change in temperature in a more homogenous manner when compared with intact *E. coli* cells.

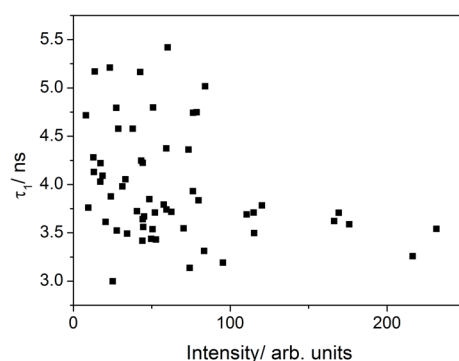


Figure 4-21 Plot showing intensity against T1 for live *E. coli* cells grown and imaged at 37 °C

Importantly, there was no correlation between cell fluorescence intensity and fluorescence lifetime (Figure 4-21), showing that the amount of BODIPY-C10 taken up by the cells did not have an effect on the calculated membrane viscosity. This suggests that it was not our chosen fitting model that caused the spread in the results, and also that BODIPY-C10 aggregation was not occurring, leading to aggregation-induced quenching and reduced BODIPY-C10 lifetime, as this would cause a correlation between cell fluorescence intensity and lifetime.

Table 4-2 shows the calculated viscosities of the live cell plasma membranes, spheroplast membranes and LUVs composed of *E. coli* lipid extracts in comparison with other lipid bilayer systems at 37 °C and 23 °C. The calculated viscosities within the live cells and spheroplasts are significantly higher than the viscosities calculated in *E. coli* lipid extracts, which may highlight the differences between the different systems. LUVs comprised of *E. coli* lipid extracts are much simpler systems than *E. coli* plasma membranes, containing little to no proteins, and may also contain lipids from the *E. coli* outer membrane. *E. coli* plasma membranes have a high protein concentration,²³² and the actin filaments that make up the prokaryotic cytoskeleton are also thought to play a role in membrane organisation of the plasma membrane.²³³ This added complexity and increased molecular crowding caused by the presence of membrane proteins may be the cause of the perceived increase in membrane viscosity. Previous studies have suggested that diffusion coefficients of membrane proteins may be up to 2 orders of magnitude higher in model membranes than in cells,^{232,234} and so our observation may be reflecting this. This highlights the differences between model membrane systems and native plasma membranes, and emphasises the caution that should be used when using artificial bilayers to investigate the physical properties of native membranes, such as viscosity.

Table 4-2 BODIPY rotor lifetimes and the corresponding viscosities for a variety of lipid bilayer systems at 23 °C and 37 °C. Note that for biexponential BODIPY rotor decays, such as EYSM and bacterial membranes, the lifetime component thought to correspond to the bilayer viscosity was used as the fluorescence lifetime. Note that for SK-OV cells, BODIPY-C++ (see Chapter 3 for further discussion) was used to determine membrane viscosity, but in all other cases BODIPY-C10 was used

23 °C

Lipid System	BOCIPY rotor lifetime/ ns	Calculated Viscosity/ cP	Reference
DOPC	1.67	140	138
EYSM	5.27	1720	138
EYSM/20% Chol	4.85	1430	138
SK-OV plasma membrane	2.2	250	135
Bacillus spores inner membrane	4.0	960	183
Bacillus vegetative cell membrane	2.7	400	183
<i>E. coli</i> plasma membrane	4.28	1160	<i>This study</i>
<i>E. coli</i> spheroplast membrane	4.44	1200	<i>This study</i>
<i>E. coli</i> lipid extracts	2.46	320	<i>This study</i>

37 °C

Lipid System	BOCIPY rotor lifetime/ ns	Calculated Viscosity/ cP	Reference
DOPC	1.11	60	138
EYSM	3.33	630	138
EYSM/20% Chol	3.26	600	138
<i>E. coli</i> plasma membrane	3.98	950	<i>This study</i>
<i>E. coli</i> spheroplast membrane	4.06	980	<i>This study</i>
<i>E. coli</i> lipid extracts	1.98	200	<i>This study</i>

Table 4-2 also highlights the high viscosity of the *E. coli* plasma membrane compared with eukaryotic plasma membranes (SK-OV-3 ovarian cancer cells)¹³⁵ and the plasma membrane of vegetative Bacillus cells. Bacillus is a genus of Gram-positive bacteria²³⁵, and the high viscosity of *E. coli* bacteria may represent a higher degree of lipid ordering compared with these two systems, although it does overlap well with the inner membrane of Bacillus spores. The viscosities of the *E. coli* plasma membrane are also close in value to model L_o phase artificial bilayers (EYSM/20% cholesterol), which suggests that *E. coli* plasma membranes display a high degree of lipid ordering. This increased viscosity compared with eukaryotic membranes may be explained in part by the high abundance of

phosphatidylethanolamine (PE) lipid within the *E. coli* membrane; PE lipids are believed to make up about 70% of the lipid content of the *E. coli* membrane,^{236,237} and have been previously shown to reduce diffusion coefficients of lipids within model systems,^{238,239} which has been attributed to increased membrane viscosity. This is believed to be caused by increased molecular packing²⁴⁰ and additional hydrogen bonds^{238–240} in the PE headgroup compared with the PC headgroup commonly found in eukaryotic membranes.

4.2.3 Diffusion Coefficient Calculations

In order to allow comparison with the *E. coli* plasma membrane diffusion coefficients reported in the literature, we then converted our calculated viscosity values into diffusion coefficients using the Saffman-Delbrück equation (equation 4-2).¹⁹¹

$$D_{sd} = \frac{k_B T}{4\pi\eta_m h} \left[\ln \left(\frac{2L_{sd}}{a} \right) - \gamma \right] \quad (4-2)$$

Where D_{sd} is the Saffman-Delbrück diffusion coefficient, a is the radius of the membrane inclusion, h is the bilayer thickness, η_m is the membrane viscosity, γ is the Euler-Mascheroni constant ($\gamma \approx 0.577$), and L_{sd} is the Saffman-Delbrück length, given by equation 4.3:

$$L_{sd} = \frac{h\eta_m}{2\eta_f} \quad (4-3)$$

Where η_f is the viscosity of the surrounding fluid. For Saffman-Delbrück calculations for *E. coli* membranes, the membrane thickness was taken as 3.75 nm, as reported by Mitra *et al.*²⁴¹ The other parameters used in the calculations are outlined in Chapter 3.

A comparison between the calculated diffusion coefficients within *E. coli* plasma membranes, spheroplast membranes and LUVs made up of *E. coli* lipid extracts and diffusion coefficients calculated through FRAP and single molecule tracking (SMT) for *E. coli* plasma membranes is shown in Table 4-3. Our calculated values for BODIPY-C10 in *E. coli* plasma membranes are 0.63 and 0.78 $\mu\text{m}^2\text{s}^{-1}$ at 23 °C and 37 °C, respectively. These values are comparable to the diffusion coefficients obtained by Nenninger *et al.*⁶⁶, who used the chemically similar BODIPY FL-C₁₂ probe and FRAP to obtain diffusion coefficients of 0.6 and 1.2 $\mu\text{m}^2\text{s}^{-1}$ at 23 °C and 37 °C, respectively, although somewhat less comparable to the results of Oswald *et al.*,²²⁵ who used SMT of BODIPY FL-C₁₂ within *E. coli* plasma membranes to obtain a diffusion coefficient of 1.5 $\mu\text{m}^2\text{s}^{-1}$. Oswald *et al* did, however use a Saffman-Delbrück approach to determine the viscosity of the *E. coli* membrane, finding a value of 1200 cP, which overlaps well with our calculated viscosity.

Table 4-3 Comparison of the diffusion coefficients calculated using the Saffman-Delbrück equation in *E. coli* live cell plasma membranes, spheroplasts and LUVs composed of lipid extracts with diffusion coefficients calculated in live *E. coli* plasma membranes using the structurally similar BODIPY FL-C₁₂ probe. * denotes that single molecule tracking techniques were used and ** denotes that FRAP was used.

23 °C

Lipid System	Probe	Diffusion Coefficient/ $\mu\text{m}^2\text{s}^{-1}$	Reference
<i>E. coli</i> plasma membrane	BODIPY-C10	0.63	<i>This study</i>
<i>E. coli</i> spheroplast membrane	BODIPY-C10	0.61	<i>This study</i>
<i>E. coli</i> lipid extracts	BODIPY-C10	2.1	<i>This study</i>
<i>E. coli</i> plasma membrane	BODIPY FL-C ₁₂ *	1.5	²²⁵
<i>E. coli</i> plasma membrane	BODIPY FL-C ₁₂ **	0.6	⁶⁶

37 °C

Lipid System	Probe	Diffusion Coefficient/ $\mu\text{m}^2\text{s}^{-1}$	Reference
<i>E. coli</i> plasma membrane	BODIPY-C10	0.78	<i>This study</i>
<i>E. coli</i> spheroplast membrane	BODIPY-C10	0.76	<i>This study</i>
<i>E. coli</i> lipid extracts	BODIPY-C10	3	<i>This study</i>
<i>E. coli</i> plasma membrane	BODIPY FL-C ₁₂ **	1.2	⁶⁶

We must, however, bear in mind that the Saffman-Delbrück approach has a number of limitations associated with it (discussed in further detail in Chapter 1 and Chapter 3), namely that it assumes that a membrane is a homogeneous liquid with no lateral structuring,¹⁹¹ rather than a heterogeneous bilayer whose lateral pressure profile changes with depth into the bilayer,⁵³ and that it assumes that the membrane inclusion is cylindrical in shape and spans the whole bilayer,¹⁹¹ which is untrue for BODIPY-C10, which localises within the tail region of the bilayer.¹³⁸ Additionally, there are reports that the Saffman-Delbrück approach fails for larger membrane inclusions (although this may be less relevant for BODIPY-C10, a small molecule).^{40,55,242} Despite this, the Saffman-Delbrück approach is still a widespread method for linking membrane viscosity with the diffusion coefficient, and allows us to compare our calculated viscosities with diffusion coefficients from the literature, in order to investigate the accuracy of our results. Overall, the similarities between our results and the diffusion coefficients in the literature suggest that our viscosity calculations are indeed accurate.

4.3 Conclusions

In this chapter, the BODIPY-based molecular rotor BODIPY-C10 was used to investigate the viscoelastic properties of the plasma membranes of live *E. coli* bacteria and artificial bilayers comprised of lipids extracted from *E. coli* bacteria. Our study, to the best of our knowledge, represents the first direct measurement of viscosity within the plasma membranes of live *E. coli* bacteria, and aimed to investigate the lipid phase behaviour and potentially shed further light on the homeoviscous adaptation model first postulated by Sinensky in 1974,²¹⁰ which stated that *E. coli* bacteria regulate the lipid composition of their plasma membranes based on their growth temperature, in order to maintain a constant membrane viscosity with a gel-to-fluid phase transition occurring *ca.* 15 °C below their growth temperature.

BODIPY-C10 was incorporated into LUVs composed of total lipid extracts obtained from *E. coli* bacteria grown at 37 °C, and emission spectroscopy and TCSPC were performed at a range of temperatures, in order to investigate the presence of transition in lipid phase and to determine the viscosity of the bilayer of the lipid extracts. Much like the simple single component bilayers discussed in Chapter 3, both emission intensity and the fluorescence decay time decreased with increasing temperature, suggesting a decrease in viscosity. A plot of maximum emission intensity against temperature did not suggest a clear phase transition similar to the one observed in DPPC at 41 °C, but a plot of the fluorescence decay components against temperature did indicate a transition at *ca.* 25 °C. This was further confirmed by WAXS, supporting Sinensky's original hypothesis. At room temperature (23 °C), the viscosity of these extracts was determined to be 322.6 cP, consistent with the viscosities calculated for model L_o phases in Chapter 3. This was found to reduce to 200. cP at 37 °C, and may indicate a phase transition to a more fluid-like state.

BODIPY-C10 was subsequently introduced to cultured live *E. coli* cells, which were investigated by confocal microscopy and FLIM. Experiments based around the osmotic shock of the live cells and also around the investigation of spheroplasts, *E. coli* bacteria stripped of their outer membrane and cell wall, suggested that BODIPY-C10 localises primarily in the inner, or plasma, membrane of the live *E. coli* bacteria. This allowed further experimentation to determine the plasma membrane viscosity of live *E. coli* bacteria grown and imaged at a range of different temperatures.

In order to investigate the homeoviscous adaptation model first postulated by Sinensky, we performed FLIM of *E. coli* bacteria grown and imaged at the same temperature and compared the lifetimes with *E. coli* bacteria grown at 37 °C and imaged at different temperatures. The initial experiments however, showed little evidence of homeoviscous adaptation, with each of the fluorescence lifetime components increasing at lower temperatures for both sets of *E. coli* bacteria

imaged. If homeoviscous adaptation was being observed, we would have expected the bacteria grown at 37 °C and imaged at reduced temperature to show an increase in lifetime but not the bacteria grown at reduced temperatures. Further experimentation failed to obtain conclusive proof of homeoviscous adaptation, which may have been caused by the wide spread in lifetimes between individual cells.

Due to this large inter-cell heterogeneity, we instead determined the BODIPY-C10 lifetime components of *E. coli* bacteria grown at 37 °C and 23 °C, and used the lifetime/viscosity calibration for BODIPY-C10 derived from methanol/glycerol solutions at known viscosity to calculate plasma membrane viscosities for the live bacterial cells and also for spheroplasts at the same temperatures. In live *E. coli* cells at 37 °C and 23 °C, we determined the plasma membrane viscosity to be 950 cP and 1160 cP, respectively. These viscosities overlapped well with viscosities calculated in spheroplasts, but were significantly higher than the viscosity values calculated in *E. coli* lipid extracts, suggesting a large difference in the structure of the bilayer between the native *E. coli* plasma membrane and LUVs comprised of membrane extracts. This was as expected, given the complex and highly crowded nature of the *E. coli* plasma membrane which contains large quantities of proteins and is supported by the peptidoglycan cell wall, compared with an artificial bilayer comprised purely of membrane extracts. The viscosities calculated in the plasma membranes of the live *E. coli* cells are also much higher than those observed in eukaryotic cells, and suggest that bacterial membranes may display a significantly higher degree of lipid ordering within their plasma membranes.

Finally, we used the Saffman-Delbrück approach to calculate diffusion coefficients for BODIPY-C10 in the plasma membrane using our calculated viscosities. The calculated diffusion coefficients agreed well with diffusion coefficients previously calculated in the literature, suggesting that our method of determining *E. coli* plasma membrane viscosity was indeed accurate.

Overall, this work displays the ability of molecular rotors to determine membrane viscosity in a diverse array of biological settings. Despite the fact that BODIPY-based rotors do not partition into L_o phases in phase-separating bilayers, they are still useful probes of lipid ordering, although they may struggle in the search for phase coexistence within complex biological membranes. For this task, new molecular rotors that partition equally between different lipid phases may need to be developed.

5. Synthesis and Characterisation of a Planar *meso*-unsaturated BODIPY Rotor

5.1 Introduction

5.1.1 Overview

In the previous chapter we explored the use of BODIPY-based molecular rotors for imaging viscosity within the plasma membranes of *E. coli* bacteria, finding that these membranes display a higher degree of lipid ordering than had previously been observed within eukaryotic cells. The BODIPY rotors are, however, limited by their unequal partitioning between L_o and L_d phases in model membranes, potentially limiting their utility as tools to investigate the phase behaviour of lipids within the plasma membrane. Here we investigate a *meso*-unsaturated planar BODIPY dye reported in the literature to behave as a molecular rotor. We first synthesise the planar BODIPY dye, before investigating the effect of viscosity on its fluorescence lifetime and intensity in methanol/glycerol solutions. Despite initially promising results, the dye was found to be insensitive to viscosity within lipid bilayers. Because of this, we determine that the dye does not act as a molecular rotor, contrary to what was reported in the literature. Moreover, the dye did not partition into L_o phases in model lipid bilayers, despite its planar structure. These results meant that we did not further explore the use of planar BODIPY structures as molecular rotors for investigating phase behaviour within lipid bilayers.

5.1.2 Phase partitioning of BODIPY dyes

Whilst BODIPY molecular rotors such as the ones discussed in Chapter 3 have been shown to be useful for assessing viscosities in a number of biological environments,³⁸ there are still a number of limitations to their use within biological membranes. As discussed in Chapter 3 and elsewhere,^{41,138} BODIPY rotors do not partition well into the L_o phases of phase-separating lipid bilayers, potentially limiting their utility in the search for ordered microdomains within the plasma membranes of live cells, where any ordered domains are likely to be on a submicron scale and exist on a microsecond timescale.³⁵ Additionally, with the exception of BODIPY-C++,¹³⁵ the previously reported BODIPY rotors are known to rapidly internalise into the interior of cultured cells.^{124,132} A group of BODIPY rotors that targeted the plasma membranes of live cells could give more room for synthetic modification, allowing the development of more targeted BODIPY rotors.

Whilst, to the best of our knowledge, no BODIPY rotor that partitions equally between L_o and L_d phases has been developed to date, there are examples of non-rotor BODIPY fluorophores that do partition into L_o phases. Li *et al*¹⁸⁰ synthesised cholesterol derivatives of BODIPY dyes that were shown to partition into the L_o phase, something that was further explored by Ariola *et al*,¹⁹⁴ who used the L_o phase-targeting BODIPY-Cholesterol dye to investigate membrane viscosity in phase-separating GUVs. Additionally, ganglioside-linked BODIPY dyes have been associated with ordered phases in lipid monolayers²⁴³ and bilayers,²⁴⁴ owing to the separation between the bulky BODIPY fluorophore and the tightly-packed lipid headgroups by a sugar spacer.¹⁶⁷

In general, bulky fluorophores, even those covalently attached to saturated lipid chains, will not partition into the L_o phase due to the disruption of the tightly-packed lipids that they would cause. Conversely, a bulky group would be more easily accommodated into a disordered lipid structure. Dyes that do partition into the L_o phase are often planar, allowing them to intercalate between the ordered chains of the L_o phase lipids.¹⁶⁷ We therefore hypothesised that if the bulky *meso*-aryl group were removed, a BODIPY rotor may be able to better partition into the L_o phase. The BODIPY group itself is planar in the ground state, with the aryl groups taking on a dihedral angle of 90° ,²⁴⁵ which may be a factor in their exclusion from the L_o phase. However, very few BODIPY-based molecular rotors without the *meso*-aryl substitution have been reported to date.

5.1.3 A planar BODIPY rotor

In 2014, Zhu *et al*¹⁴¹ reported a BODIPY dye without the *meso*-aryl substitution that was still capable of acting as a molecular rotor, the “planar BODIPY” dye that we have termed “PlanB” (structure shown in Figure 5-1). By performing density functional theory (DFT) calculations, they showed that the *meso*-aldehyde group of PlanB can rotate into the plane of the BODIPY ring, causing the BODIPY framework to distort and lose its planarity. They went on to plot the relative energy gaps between the ground state and first excited state as a function of the dihedral angle between the aldehyde group and the BODIPY framework, showing that it was at its lowest when the aldehyde group was in the plane of the BODIPY group. They hypothesised that this leads to a non-radiative decay pathway for PlanB, meaning that the non-radiative decay constant, k_{nr} was directly controlled by the rate of rotation of the aldehyde group. This therefore meant that in viscous media, where the barrier to rotation would be increased due to friction imparted by the solvent, the fluorescence emission of the dye would increase, and that PlanB would act as a molecular rotor. They went on to show that PlanB, despite having a very low quantum yield in a range of non-viscous solvents, displayed a marked increase in emission intensity in glycerol, a very viscous solvent. They then characterised the

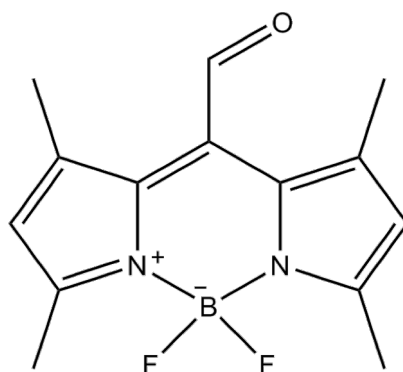


Figure 5-1 Structure of the aldehyde-substituted BODIPY dye, PlanB

increase in emission intensity in water/glycerol mixtures, plotting $\log$ (emission intensity) against $\log$ (viscosity), showing a linear correlation in agreement with the Förster-Hoffmann description of the viscosity sensitivity of molecular rotors.⁹⁷ They also observed an increase in the length of the fluorescence decay of PlanB with viscosity and determined the change in lifetime of PlanB with viscosity in water/glycerol mixtures. Despite not reporting whether the decays were monoexponential or not, and giving little information on how the lifetimes were derived from the fitting, they showed a linear plot of $\log$ (lifetime) against $\log$ (viscosity), also in agreement with the Förster-Hoffmann relationship. By relating fluorescence lifetime and fluorescence quantum yield of PlanB, they determined the change in the radiative and non-radiative rate constants, k_r and k_{nr} , respectively, with viscosity, finding a decrease in k_{nr} and an increase in k_r with increasing viscosity. This contrasts the general theory of molecular rotors, i.e. that the viscosity of the surrounding solution only affects the non-radiative decay pathway,^{38,82} and suggests that the emission of PlanB may be affected by more than just viscosity, despite the claims to the contrary made in the publication by Zhu *et al.*¹⁴¹ Despite these inconsistencies in the literature data for PlanB, the planar structure of this fluorophore and the dependence of its emission on the solution viscosity warranted further investigation. These *meso*-unsaturated BODIPY dyes may make an attractive alternative to *meso*-aryl substituted BODIPY rotors, as the lack of a bulky aromatic group and more planar structure may allow these dyes to intercalate better into the rigid structure of ordered lipid phases, potentially allowing these dyes to partition more strongly into the L_o phase of phase-separating lipid bilayers.

The potential of BODIPY dyes with a non-aryl unsaturated group at the *meso*-position to act as molecular rotors was further described by Lincoln *et al.*,¹³⁹ who used computational and spectroscopic techniques to investigate the mechanism for the non-radiative decay pathways of a series of *meso*-unsaturated BODIPY dyes, including PlanB. They disagreed with the mechanism for the non-radiative decay pathway suggested by Zhu *et al.*,¹⁴¹ instead suggesting that rotation of the

unsaturated *meso*-substituent into the plane of the BODIPY group only causes puckering of one of the pyrrole groups rather than the whole molecule. They showed that the rotation of this substituent can overcome the steric repulsion of the methyl groups at the C1 and C7 position, leading to a thermal deactivation pathway of the excited state which outcompetes the fluorescence pathway, causing a very low quantum yield. In this sense, they agreed with Zhu *et al* in that the rate of rotation of the *meso*-substituent could in principle be affected by the rigidity of the surrounding environment, outlining the potential of these compounds to act as molecular rotors. If these *meso*-unsaturated compounds are indeed capable of effectively acting as molecular rotors, they may be able to overcome some of the problems associated with the aryl-substituted BODIPY rotors discussed in Chapter 3, such as their inability to partition into L_o phases of phase-separating lipid bilayers and their tendency to rapidly internalise into the interiors of cultured cells, rather than staining the plasma membrane.^{124,132}

The synthesis of these *meso*-unsaturated “planar” BODIPY dyes, including PlanB, was first reported in 2010 by Krumova *et al*,²⁴⁵ who also noted their tendency to display very low quantum yield values in non-viscous media (although they did not explore the effect of viscosity on the emission of these dyes, and Zhu *et al*¹⁴¹ were the first to experimentally investigate their use as molecular rotors). They did, however, note the potential of aldehyde-substituted dyes such as PlanB to undergo reaction with nucleophiles such as alcohols or amines to yield highly emissive products, something that could affect the utility of these dyes in a biological setting, where nucleophiles such as water are abundant.

This Chapter describes the synthesis and characterisation of the aldehyde-substituted BODIPY dye PlanB. We first investigate whether PlanB is capable of acting as a molecular rotor in bulk solution and lipid bilayers, finding that its emissive properties are much more complex than initially suggested by Zhu *et al*, before investigating whether it could partition into the L_o phases of phase-separating lipid bilayers. We determine that, whilst it may indeed act as a molecular rotor, its small dynamic range in fluorescence response to changing solution viscosity, in addition to the potential side reactions yielding emissive properties, as well as its poor partitioning into L_o phases, make PlanB unsuitable as a molecular rotor for use in lipid bilayers, and discourage the further synthetic exploration of these *meso*-unsaturated BODIPY dyes.

5.2 Results and discussion

5.2.1 Synthesis of PlanB

The planar BODIPY dye PlanB was synthesised in an analogous fashion to Zhu *et al.*¹⁴¹ Commercially available acetoxyacetyl chloride **1** was condensed with 2,4-dimethylpyrrole followed by an *in situ* complexation with $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in the presence of a base,²⁴⁶ in this case Et_3N , to generate BODIPY **2**. The primary hydroxyl group of **2** was deprotected by hydrolysis with NaOH to generate BODIPY **3**, which was then oxidised in the presence of pyridinium chlorochromate to give PlanB (Figure 5-2, overall yield 0.5%, further information and NMR data described in Chapter 2). We then investigated the photophysics and viscosity sensitivity of PlanB in order to determine whether or not it acted as a molecular rotor.

5.2.2 Photophysical characterisation of the planar BODIPY dye

Normalised absorption and emission spectra of PlanB in 30/70 glycerol/methanol are shown in Figure 5-3 (A), with a maximum absorption wavelength of 512 nm and a maximum emission wavelength of 537 nm. For comparison, normalised absorption and emission spectra of the three BODIPY rotors discussed in Chapter 3 are shown in Figure 5-3 (B). The shapes of the absorption

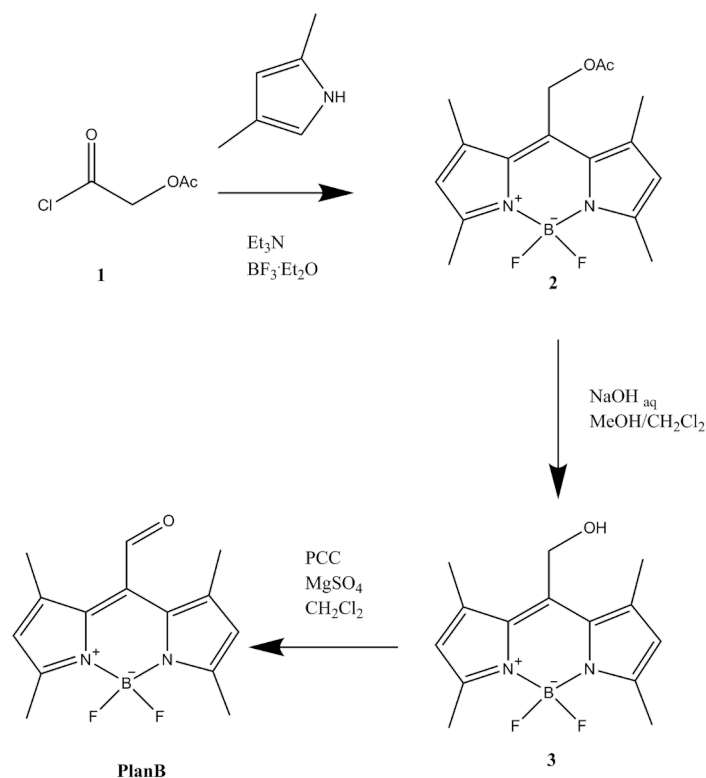


Figure 5-2 Reaction scheme showing the synthesis of PlanB

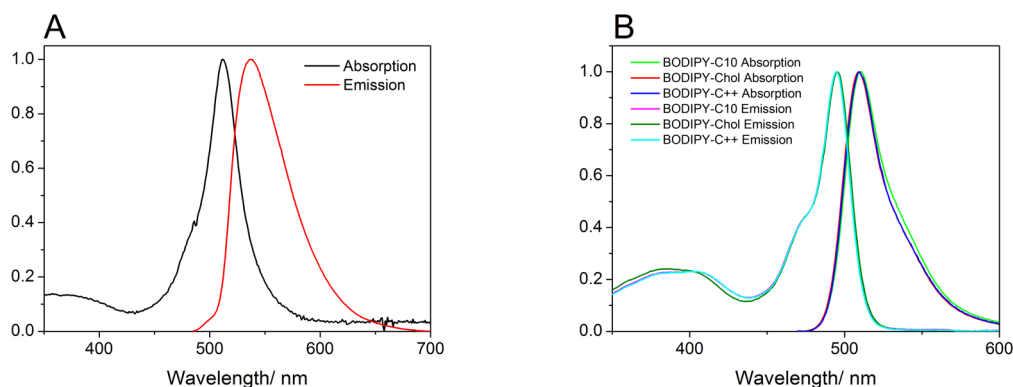


Figure 5-3 A) Normalised absorption and emission spectra of PlanB in a 30/70 glycerol/methanol solution, B) normalised absorption and emission spectra of the three BODIPY rotors discussed in Chapter 3 for comparison

profiles are similar for both PlanB and the three BODIPY rotors, and the Stokes shift is also similar. This may suggest a similar absorption and emission mechanism between the four dyes within methanol glycerol mixtures, although it does not imply that PlanB will display viscosity sensitive emission like the other rotors.

To investigate whether PlanB displayed viscosity sensitive emission, it was dissolved in a 90/10 glycerol/methanol mixture and emission spectra were taken at a range of temperatures, corresponding to a range of viscosities (Figure 5-4). Emission intensity was found to increase with increasing viscosity, consistent with the hypothesis that PlanB acts as a molecular rotor and in agreement with the photophysical studies performed by Zhu *et al.*¹⁴¹ In their study, they also plotted $\log(\text{emission intensity at } 542 \text{ nm})$ against $\log(\text{viscosity})$, finding a linear correlation with a gradient of 0.62. In Figure 5-4 we plot this same graph for PlanB in 90/10 glycerol/methanol, finding a good linear correlation ($R^2 = 0.97$) and a gradient of 0.29. This gradient value is lower than that quoted by Zhu *et al.*, and may reflect the fact that their calibration experiments were done in water/glycerol mixtures, or the fact that in our experiments the variable viscosity was achieved by varying temperature. The potential effects of solvent polarity and temperature on PlanB emission will be discussed later on.

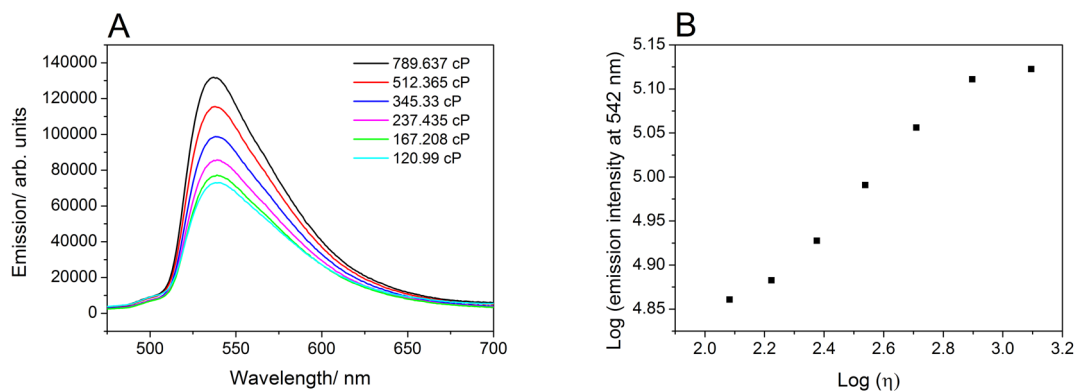


Figure 5-4 (A) emission spectra of PlanB in 90/10 glycerol/methanol at a range of temperatures

We next determined the quantum yield of PlanB in 70/30, 80/20 and 90/20 glycerol/methanol mixtures at a range of temperatures, using an excitation wavelength of 480 nm (similar to Zhu *et al*), and comparing absorption and emission data with fluorescein in 0.1 mol dm^{-3} aqueous NaOH (quantum yield = 0.95)⁵⁶ as a reference. The results are shown in Figure 5-5, on both linear and logarithmic scales. For every glycerol/methanol solution, the quantum yield increases with increasing viscosity, and this increase is linear on the double logarithmic scale shown in Figure 5-5 (B), in agreement with the Förster-Hoffmann equation.

There is little to no overlap in the plot however, between the quantum yields of PlanB in the different glycerol/methanol mixtures, which is further highlighted by the double logarithmic scale. Whilst the reduced calculated quantum yield in 90/10 glycerol/methanol may be a result of the poor solubility of PlanB in this mixture, there is still little overlap between the other mixtures. This suggests that the emission of PlanB may be affected by other factors in addition to solution viscosity.

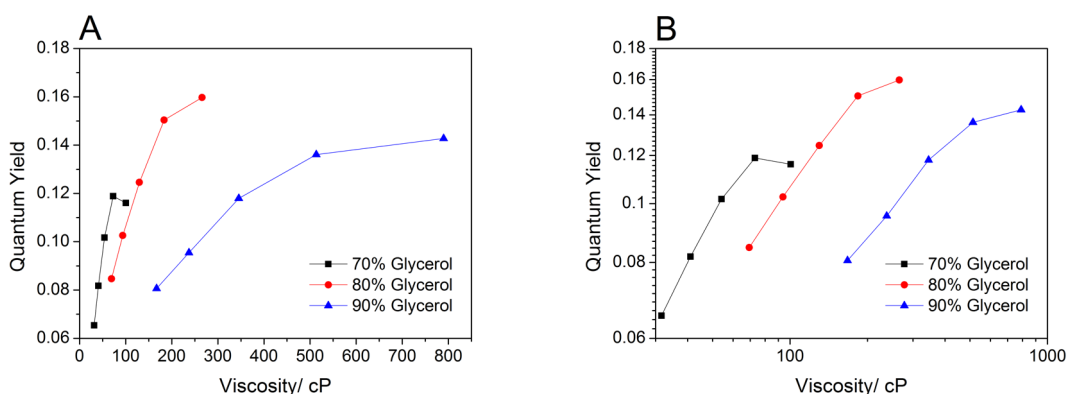


Figure 5-5 Calculated quantum yields for PlanB in 70/30, 80/20 and 90/10 glycerol/methanol at 15, 20, 25, 30 and 35 °C, at (A) linear and (B) double logarithmic scales

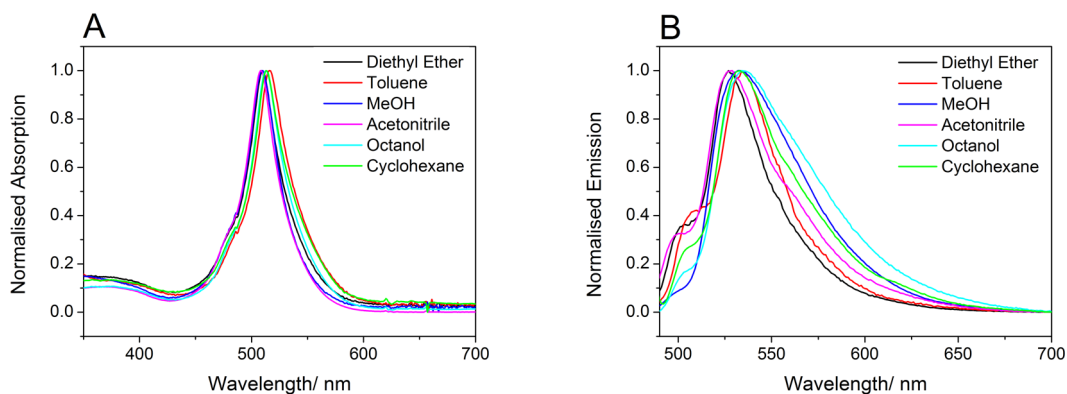


Figure 5-6 Normalised (A) absorption and (B) emission spectra of PlanB in a range of different solvents

Temperature, in addition to viscosity, is known to potentially affect the fluorescence behaviour of certain molecular rotors¹⁰⁹ by affecting the balance in the relative populations of excited states, and has previously been shown to influence the fluorescence behaviour in other BODIPY dyes designed as molecular rotors,¹²⁸ and so may be leading to the poor overlap in quantum yields shown in Figure 5-5. If temperature was affecting the emission of PlanB beyond affecting the viscosities of the glycerol/methanol solutions, it could make viscosity and temperature effects on the emission impossible to decouple, reducing the utility of PlanB as a viscosity-sensitive rotor. Additionally, several previously studied molecular rotors show polarity dependent emission, especially in twisted intramolecular charge transfer (TICT) rotors⁸⁷, and one of the main advantages of the BODIPY rotors discussed in Chapter 3 is their relative insensitivity to polarity at high viscosities. If PlanB is sensitive to either polarity or temperature, this may impair its ability to sense viscosity within biological systems, where the polarity or temperature may not be known and may not be controllable. Moreover, the quantum yields shown in Figure 5-5 are significantly lower than those calculated for the BODIPY rotors discussed in Chapter 3, and may also hinder the use of PlanB in FLIM, where photon counts of 100-1000 are required per pixel for accurate decay fitting.

In their initial proposal of PlanB as a molecular rotor,¹⁴¹ Zhu *et al* showed the maximum emission

Table 5-1 Maximum absorption and emission wavelengths of PlanB in varying solvents of differing dielectric constant (ϵ)

Solvent	ϵ	$\lambda_{\max}$ (Abs)/ nm	$\lambda_{\max}$ (Em)/ nm
Diethyl Ether	4.335	510	527
Toluene	2.379	516	534
Methanol	32.63	509	532
Acetonitrile	37.50	508	529
Octanol	10.30	513	534
Cyclohexane	2.023	513	533

wavelength changing by 20 nm across different solvents. This implies that solution polarity may have an effect on its emission. To further investigate the effects of polarity on the emissive properties of PlanB, it was dissolved in several solvents of varying polarity and absorption and emission spectra were taken (Figure 5-6), and the maximum absorption and emission wavelengths are shown alongside the solution dielectric constants, which provide a measure of the solution's polarity (Table 5-1 shows max absorption and emission wavelengths).¹⁸⁴ Whilst both the maximum absorption and emission wavelengths do change slightly with solution polarity, they do not change by a large amount, comparable with other BODIPY rotors such as those discussed in Chapter 3, which suggests that PlanB does not show significant solvatochromism.

Previous work by Krumova and Cosa²⁴⁵ suggested that BODIPY dyes with an aldehyde substitution at the *meso*-position may react with methanol, leading to the formation of a hemiacetal (Figure 5-7), which causes a large increase in the quantum yield of the dye. If this was the case with PlanB, then any perceived viscosity sensitivity of the dye could in reality be explained by this reaction. To investigate whether this process was in fact leading to the increase in quantum yield between different glycerol/methanol solutions, we took fluorescence spectra for PlanB dissolved in methanol every 15 minutes for 90 minutes to see if there was any observable increase in emission intensity. The overlaid emission spectra are shown in Figure 5-8 (A). There is a large increase in the emission intensity over the 90 minutes, with the increase in intensity reaching a plateau after 60 minutes. Additionally, the normalised emission spectra shown in Figure 5-8 (B) show a shift in the maximum emission wavelength, which could be explained by the formation of another emissive species, such as the hemiacetal product of the reaction with methanol.

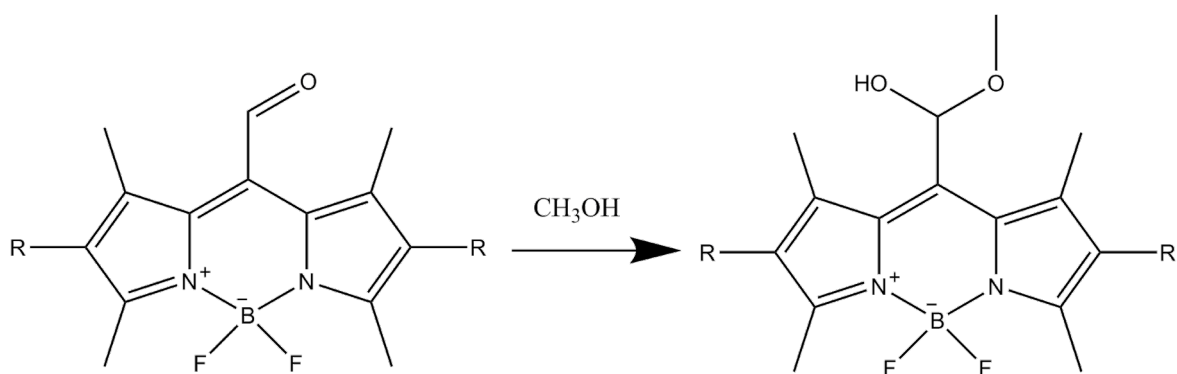


Figure 5-7 Potential reaction between *meso*-substituted aldehyde BODIPY dyes and methanol to form a hemiacetal, as described in ref²⁴⁵

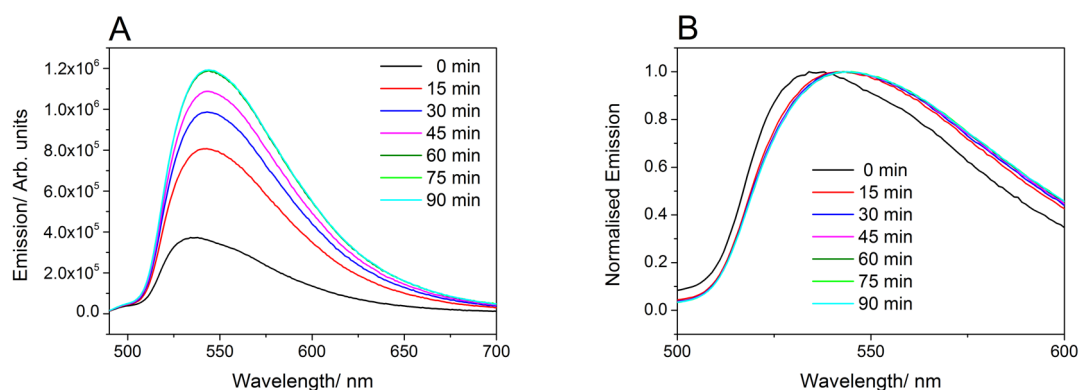


Figure 5-8 (A) Overlaid emission spectra of PlanB in methanol at 20 °C taken every 15 minutes for a period of 90 minutes, (B) normalised emission spectra of the emission spectra shown in (A), highlighting the shift in maximum emission wavelength

This supports the hypothesis that PlanB is in fact reacting with the methanol to generate a much more emissive hemiacetal^{139,245}, and may explain the poor overlap between the calculated quantum yields shown in Figure 5-5. This may also suggest that PlanB is not actually acting as a molecular rotor, and the experiments performed by Zhu *et al*¹⁴¹ in water/glycerol that they interpreted as confirmation of the rotor properties of PlanB were being influenced by this reaction and were thus flawed. Note that water has a higher polarity than methanol and may therefore act as a stronger nucleophile in the reaction with PlanB. This highlights an important facet of any molecular rotor, namely that it must be relatively chemically inert in order to not undergo any reaction with its surrounding environment causing a change in its fluorescence properties, leading to a mistaken perceived increase in viscosity. These experiments suggest that PlanB may be unsuitable as a molecular rotor for probing biological systems, where a lot of water is present, which could act as a nucleophile to react with the aldehyde group of PlanB.

In their work investigating the mechanism of the radiative and non-radiative decay pathways of *meso*-substituted BODIPY dyes that included PlanB, Lincoln *et al*¹³⁹ showed that excited state rotation of a conjugated *meso*-substituent into the plane of the BODIPY ring leads to a non-radiative deactivation of the excited state, essentially the same mechanism that is thought to lead to non-radiative decay in other BODIPY rotors with a phenyl substitution at the *meso*-position.¹²⁰ The fact that the C1 and C7 positions on PlanB bear methyl groups, which would prevent a BODIPY dye with an aryl substituent at the *meso*-position from acting as a molecular rotor due to the increased steric hindrance preventing rotation, does not prevent the smaller aldehyde group of PlanB from rotating into the plane of the BODIPY group. Lincoln *et al* hypothesised that this could allow molecules such as PlanB to act as molecular rotors, if the kinetic barrier to rotation was engineered correctly.¹³⁹

Because of this, we set out to investigate the effects of viscosity on the fluorescence lifetime of PlanB, to investigate whether it behaved as a molecular rotor, despite the discouraging results shown in Figure 5-8. If PlanB indeed reacts with methanol to form a fluorescent product, there should be two lifetime components representing both PlanB and the hemiacetal derivative. If either component were sensitive to viscosity, then this system may still be usable as a molecular rotor under certain circumstances. PlanB was dissolved in 60/40, 70/30, 80/20 and 90/10 glycerol/methanol, left to equilibrate for 1 hour, and the fluorescence lifetime was determined at a range of temperatures. Fluorescence decays of PlanB in each solution at 10 °C are shown in Figure 5-9. The fluorescence decays are biexponential in character, perhaps owing to the existence of two fluorescent species: PlanB and the hemiacetal product of its reaction with methanol. The fluorescence decays were longer in more viscous solutions, although the increase in length between 53.595 cP and 1246.1 cP is much less significant than the corresponding increase for other BODIPY rotors (see Figure 5-9 (B) for an example), suggesting that PlanB shows much less sensitivity to viscosity in its fluorescence lifetime than other previously characterised, BODIPY rotors. This may cause problems in using the fluorescence lifetime of PlanB to determine viscosity, as a small error in lifetime could lead to a very large error in viscosity.

Due to the biexponential nature of the fluorescence decays, we calculated both components of the decays, τ_1 and τ_2 , and also calculate the mean-weighted fluorescence lifetime, using equation 5-1:

$$\tau_{\text{mean-weighted}} = \frac{\tau_1^2 \alpha_1 + \tau_2^2 \alpha_2}{\tau_1 \alpha_1 + \tau_2 \alpha_2} \quad (5-1)$$

We then plotted these different lifetimes against the viscosity of the surrounding solution, in order to generate calibration plots of the different lifetimes against viscosity (Figure 5-10). Unlike the

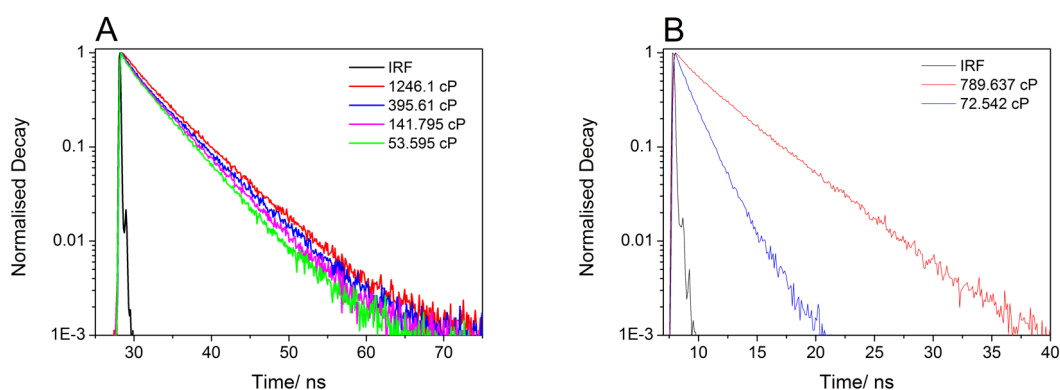


Figure 5-9 (A) Fluorescence decays of PlanB in 90, 80, 70 and 60% glycerol solutions at 10 °C, (B) fluorescence decays of BODIPY-Chol (see Chapter 3) in glycerol/methanol solutions for comparison

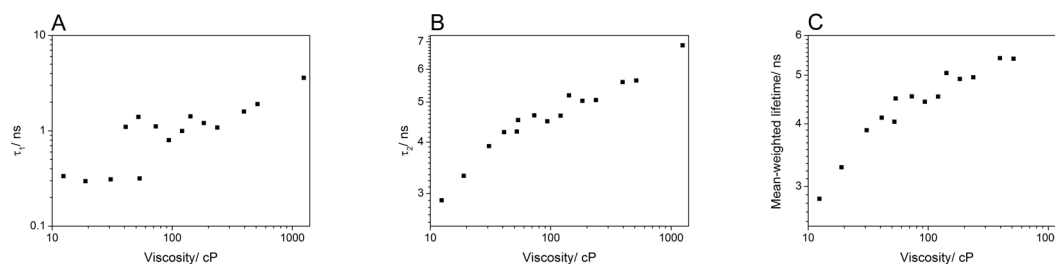


Figure 5-10 Change in (A) τ_1 , (B) τ_2 and (C) mean-weighted lifetime of PlanB in glycerol/methanol solutions of changing viscosity

results for quantum yield, the points on the graphs overlap well, even though the lifetimes were calculated at a range of temperature. This indicates that the fluorescence lifetime of PlanB is not significantly influenced by temperature. Additionally, each lifetime component appear to increase linearly with viscosity on a double logarithmic scale between 100-1000 cP, in agreement with the Förster-Hoffmann relationship.^{38,97} This suggests that PlanB may indeed be acting as a molecular rotor, although it would be difficult to decouple the potential reaction with methanol from this to give a reliable calibration, and there is a chance that the changes in calculated lifetimes may be merely reflecting different relative concentrations of PlanB and its fluorescent hemiacetal derivative.

5.2.3 Is the planar BODIPY sensitive to viscosity in LUVs?

To investigate whether PlanB could be used to investigate changes in viscosity within lipid bilayers, we incorporated it into LUVs and recorded emission spectra and time-resolved decays using TCSPC. Figure 5-11 (A) shows overlaid emission spectra of PlanB in DOPC LUVs at a range of temperatures. The emission intensity of PlanB decreases with temperature, suggesting that it may be sensing the change in membrane viscosity associated with the change in temperature. The fluorescence decays for PlanB in DOPC LUVs at a range of temperatures in Figure 5-11 (B) are also shorter at higher temperatures. Much like the decays shown in Figure 5-9, however, this is a very modest decrease and makes accurate quantification difficult. This is shown by the calculated τ_1 , τ_2 and mean-weighted lifetime values shown in Table 8, which show a fairly poor fitting and do not decrease with temperature, despite the observable shortening of the decays in Figure 5-11.

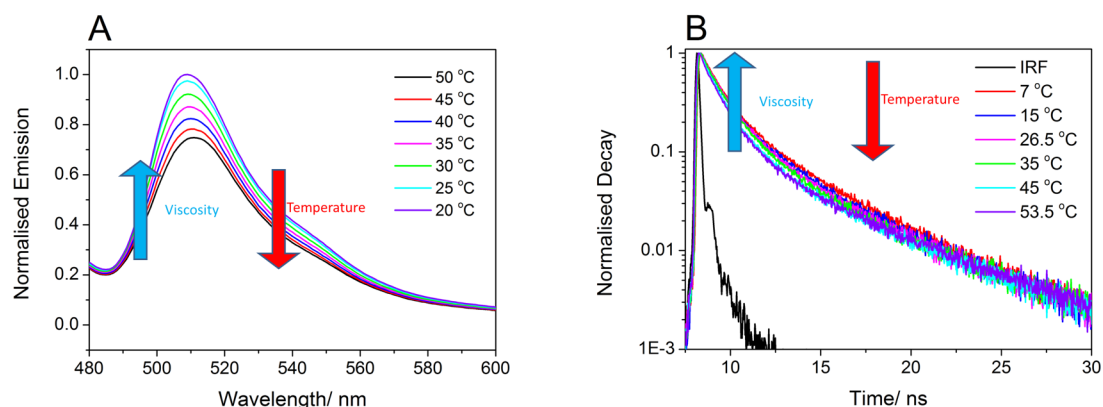


Figure 5-11 (A) Overlaid emission spectra and (B) fluorescence decays of PlanB in DOPC LUVs at a range of temperatures

It is also noteworthy that decays shown in Figure 5-11 (B) are of a significantly different shape to the decays used for the calibration (Figure 5-9). This could potentially reflect the dye taking on multiple orientations, as discussed for BODIPY rotors in gel phase bilayers in Chapter 3. It may also reflect other phenomena such as excited state aggregation, despite the 1:250 dye to lipid ratio used for formation of LUVs, or a reduction in the rate of formation of the hemiacetal derivative. This difference in the shapes of the decays between glycerol/methanol mixtures and LUVs may also mean that the lifetime-viscosity calibrations may not be applicable for lipid systems. Overall this data in LUVs suggests that PlanB may not be suitable as a molecular rotor for probing changes in viscosity in lipid bilayers.

Due to the limitations of PlanB that became apparent through these experiments in solutions and model lipid bilayers, we decided to investigate whether a different BODIPY dye with a similar structure could overcome these issues and act as a molecular rotor. The BODIPY dye “BODIPY-alkene” was kindly donated to us by the group of Gonzalo Cosa from McGill University in Canada (structure shown in Figure 5-12, synthesis described elsewhere¹³⁹). This dye has a similar structure to PlanB, and as such may be able to act as a molecular rotor, and may be less reactive than PlanB,

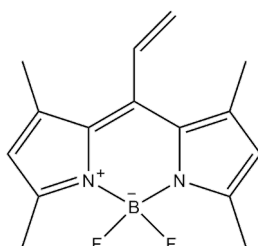


Figure 5-12 Structure of BODIPY-alkene, donated to us by the group of Gonzalo Cosa

making it more suitable for biological environments. Unpublished data from the Cosa group suggested that BODIPY-alkene displays a marked increase in quantum yield between solution phase and incorporation into a glass, suggesting that it may display an increased sensitivity to viscosity compared with PlanB.

In order to investigate whether BODIPY-alkene could act as a molecular rotor capable of investigating membrane viscosity without the drawbacks associated with PlanB, we incorporated it into DOPC LUVs and performed TCSPC at a range of temperatures, to see whether BODIPY-alkene could detect the accompanying change in membrane viscosity.

Fluorescence decays of BODIPY-alkene in DOPC LUVs at different temperatures are shown in Figure 5-13, with no observable change in the fluorescence decay with temperature. This suggests that planar BODIPY dyes like PlanB and BODIPY-alkene are much less sensitive to viscosity than the more established aryl-substituted BODIPY rotors such as those discussed in Chapter 3. The alkene and aldehyde groups of the planar BODIPY rotors are much smaller in size than the bulky aryl substituents of the BODIPY rotors discussed in Chapter 3, and may therefore have a lower kinetic barrier to rotation, even with the methyl groups on the C1 and C7 positions of the BODIPY group. This may therefore explain the reduced sensitivity to viscosity exhibited by both PlanB and BODIPY-alkene.

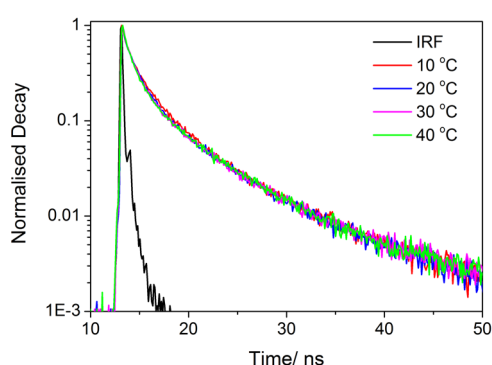


Figure 5-13 Fluorescence decays of BODIPY-alkene in DOPC LUVs at a range of temperatures

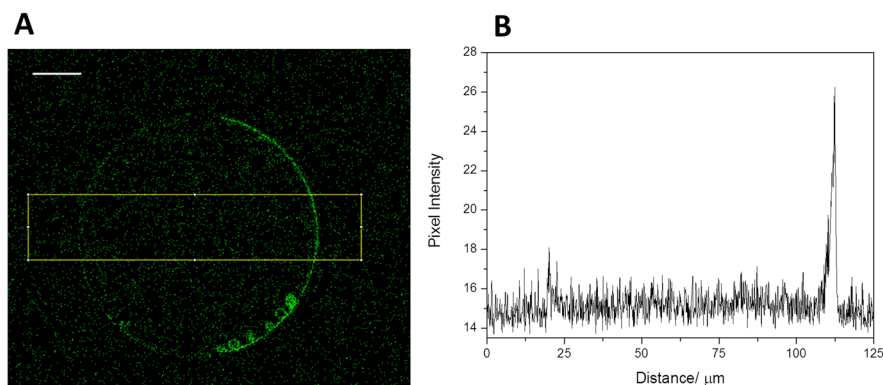


Figure 5-14 (A) Confocal microscopy image of a 1:1:1 DOPC:DPPC:cholesterol phase-separating GUV stained with PlanB, scale bar = 20 μm , **(B)** plot showing the difference in pixel intensity through the box shown in **(A)**, highlighting the difference in fluorescence signal between the L_o and L_d phases

5.2.4 Studies in GUVs

Despite the obvious limitations to the use of PlanB as a molecular rotor as explained in the previous sections, we decided to investigate whether or not it targeted the L_o phase in phase-separating GUVs. If it could successfully target L_o phases, then there could potentially be scope for synthetic modifications of PlanB that could result in a molecular rotor that could be used for probing plasma membrane phase separation.

To investigate this, we produced phase-separating GUVs of 1:1:1 ratio of DOPC:DPPC:Cholesterol, with a 1:250 dye:lipid ratio to prevent any dye aggregation, and imaged them using confocal microscopy (Figure 5-14). This image shows a fluorescence signal from the L_d phase of the GUV, suggesting that PlanB has been successfully incorporated into the GUV. However, there is very little fluorescence signal from the L_o phase, with the pixel density plot shown in Figure 5-14 (B) showing fluorescence signal from the L_o phase that is barely distinguishable from the background noise. This suggests that, despite the lack of a *meso*-phenyl group, PlanB does not partition into the L_o phase, much like the BODIPY rotors discussed in Chapter 3. This, combined with the relative insensitivity to viscosity suggested by the lifetime studies in both LUVs and bulk solution and low quantum yield values, as well as the potential effect of polarity and reaction with methanol on the fluorescence behaviour, suggests that PlanB is not a suitable molecular rotor for bilayer studies.

5.3 Conclusions

In this chapter, we report the synthesis and characterisation of a BODIPY dye with an aldehyde group on the *meso*-position, that we termed “PlanB”, that has previously been reported to act as a molecular rotor. We began by investigating its photophysical properties in bulk solution and the effect of solution viscosity on its quantum yield and fluorescence lifetime, before investigating its fluorescence properties within model lipid bilayers.

PlanB was initially dissolved in a 90/10 glycerol/methanol solution and emission spectra were taken at a range of temperatures. Promisingly, the emission intensity decreased with increasing temperature, something that could be attributed to PlanB sensing the change in viscosity associated with an increase in temperature. When we investigated the effect of viscosity on the quantum yield of PlanB in glycerol/methanol solutions at different temperatures, however, there was very little agreement in calculated quantum yield between the glycerol/methanol solutions, although for each solution there was an observed increase in quantum yield with solution viscosity. Additionally, the quantum yield values were significantly lower than those of other BODIPY rotors at similar viscosity, which could present problems for using PlanB in conjunction with FLIM in lipid bilayers, where photon counts of 100-1000 photons per pixel are required to accurately fit the fluorescence decays.

In order to determine what was responsible for this poor agreement in quantum yield between solutions, we first investigated the effect of polarity on the absorption and emission of PlanB. Whilst the maximum absorption and emission wavelengths did change between the different solutions, there was no clear correlation between solution polarity and the maximum absorption or emission wavelengths, and PlanB did not show a significant degree of solvatochromism.

We subsequently investigated a hypothesis initially postulated by Krumova and Cosa²⁴⁵ that the aldehyde group on the *meso*-position of PlanB can react with nucleophiles such as methanol to give a highly fluorescent product. By dissolving PlanB in methanol and taking emission spectra every 15 minutes, we observed a significant increase in the emission intensity with time, suggesting that this reaction is indeed taking place. Whilst this may not mean that PlanB is definitely not acting as a molecular rotor, it certainly suggests that glycerol/methanol mixtures (or indeed glycerol/water mixtures as used in the original research article for PlanB¹⁴¹) are not a suitable calibration system for investigating the effect of viscosity on the emission of PlanB. Furthermore, if PlanB reacts with nucleophiles such as methanol and water, then it may be unsuitable for use in imaging biological systems, where water is abundant.

In order to further investigate whether or not PlanB can act as a molecular rotor it was again dissolved in glycerol/methanol solutions, and the change in fluorescence lifetime was determined with viscosity. The decays were biexponential and, although they did change with solution viscosity, they did not change nearly as much with viscosity as the BODIPY rotors investigated in Chapter 3, suggesting that they are much less sensitive to changes in solution viscosity. Additionally, the reaction between PlanB and methanol to give the fluorescent hemiacetal product raises questions as to the accuracy of the lifetime calibration, and adds complexity to the fluorescence decay profiles.

Finally, despite the drawbacks of PlanB as a viscosity sensor that arose during the solution-phase experiments, we went on to investigate whether it could be used to sense changes in lipid bilayers, and whether molecular rotors based around this synthetic design could partition into the L_o phase to investigate phase behaviour within biological membranes. We initially incorporated PlanB into DOPC LUVs and obtained emission spectra at a range of temperatures, observing that the emission intensity of PlanB decreased at higher temperatures, perhaps suggesting that it was sensing the decrease in viscosity that accompanies the increase in temperature. Based on this observation, we then performed TCSPC to determine the effect of membrane viscosity on the fluorescence lifetime of PlanB. Much like the fluorescence decays observed for the glycerol/methanol mixtures, the decays were biexponential and did not change significantly with temperature, but did show a reduction with increasing temperature, potentially indicating sensitivity to viscosity. Due to the very small change in the decays and poor quality of the fitting, however, there was no reduction in the calculated lifetimes with decreasing viscosity, indicating that PlanB cannot be used to detect changes in bilayer viscosity.

We then went on to use a planar BODIPY dye with a similar structure to PlanB, termed “BODIPY-alkene”. We incorporated this dye into DOPC LUVs and performed TCSPC at varying temperature, but did not observe a change in the fluorescence decay. This highlights the viscosity insensitivity of planar BODIPY dyes such as PlanB and BODIPY-alkene and illustrates the challenges facing the design and synthesis of this type of BODIPY if a higher sensitivity to viscosity is to be achieved.

Despite the apparent inadequacies of PlanB as a molecular rotor, we went on to incorporate it into phase-separating GUVs in order to see whether it could partition into the L_o phase and potentially justify further synthetic work to engineer a planar BODIPY molecular rotor. Unfortunately, much like the BODIPY rotors discussed in Chapter 3, PlanB did not partition into the L_o phase of the phase-separating GUV.

Overall, PlanB was determined to have several serious drawbacks that prevent its use as a probe of phase separation in lipid bilayers, discouraging further investigation into the use of BODIPY rotors without the aryl substituent on the *meso*-position of the BODIPY group. It was found to have a low quantum yield and also to show little sensitivity to viscosity compared with previously studied molecular rotors. The chemical instability of the dye was also an issue, with PlanB reacting with methanol to give a highly fluorescent hemiacetal product,²⁴⁵ which may have given the illusion of a heightened sensitivity to viscosity. Additionally, the fluorescence decays of PlanB were biexponential, potentially owing to the fluorescent by-products formed from reactions of PlanB, adding complexity to the fluorescence decay character. Finally, PlanB does not partition into the L_o phase of phase-separating GUVs, despite the lack of an aryl substituent that was hypothesised to be the reason why the BODIPY rotors discussed in Chapter 3 did not partition into the L_o phase. For these reasons, no further work was done on these planar BODIPY dyes, and we moved on to looking at other potential molecular rotors for probing phase separation in biological membranes, which will be discussed in the next Chapter.

6. Imaging plasma membrane phase behaviour in live cells using a thiophene-based molecular rotor

The work in this chapter is adapted from that published as Dent, M. R. et al. Imaging plasma membrane phase behaviour in live cells through the use of a thiophene based molecular rotor Chem. Commun., 52, 13269-13272 (2016)

All molecular dynamics simulations were performed by Callum Dickson and phasor plots were drawn by Aurimas Vyšniauskas

6.1 Introduction

6.1.1 Overview

Whilst molecular rotors have great potential as probes of viscosity and lipid organisation within biological membranes, there are still several drawbacks to their use within the plasma membranes of live cells, as well as within complex model membrane systems. Other than BODIPY-C++,¹³⁵ all molecular rotors reported to date are known to internalise rapidly into the cell interior,^{106,124,132} making the selective staining of the plasma membrane very difficult and adding complexity to the process of image analysis.

Additionally, as discussed in Chapter 3, BODIPY-based molecular rotors, including BODIPY-C++, do not partition into liquid ordered phases in lipid bilayers, which may limit their use as probes of phase separation within plasma membranes where any coexisting lipid phases are believed to exist on a very small length scale, below the diffraction limit of optical microscopy.³⁵ This is a common problem for membrane-soluble dyes,¹⁶⁶ as the highly packed lipid molecules within the L_o phase often exclude the exogenous fluorescent molecules. This issue is something that must be bypassed before molecular rotors can be used as probes of phase separation within plasma membranes.

In this chapter, we introduce a thiophene-based molecular rotor that not only selectively stains the plasma membranes of live cells, but also displays an equal partitioning between L_o and L_d phases. We characterise this thiophene rotor in lipid bilayers, initially in LUVs before performing FLIM of phase-

separating GUVs stained with the rotor. We go on to image the plasma membranes of a range of live cells, finding evidence of suboptical phase separation.

6.1.2 Detecting phases within the plasma membrane using fluorescence and phasor analysis

Whilst progress has been limited with molecular rotors, in recent years there have been several developments in the design and use of other fluorescent dyes that can distinguish between L_o and L_d phases, with the aim of shedding light on the existence of ordered “raft” phases within the plasma membranes of live cells.

Perhaps the simplest answer to the problem of visualising L_o phases is the use of fluorophores that partition preferably into the L_o phase to investigate the localisation and size of L_o phases within the plasma membrane. Several probes that preferentially partition into the L_o phase have been reported,¹⁶⁶ which generally have certain structural characteristics to encourage favourable interactions with highly ordered lipid environments, such as being highly planar or being covalently linked to saturated alkyl groups¹⁶⁷. This idea has been previously implemented to study phase separation within plasma membrane extract vesicles, and the preferential partitioning of different membrane proteins.³² Whilst this technique has found fairly widespread usage in model membrane systems,¹⁶⁶ it has found somewhat limited application within live cell membranes, as partitioning between L_o and L_d depends strongly on the lipid compositions of the two phases, leading to imprecise partitioning within plasma membranes, which are complex lipid mixtures typically near the critical point for phase separation.^{19,31} This highlights the fact that phase separation within the plasma membrane, if it does actually occur, is unlikely to strongly resemble the L_o - L_d phase separation observable within model membrane systems, such as those discussed in Chapter 3

Probes that partition between both L_o and L_d phases and display different emissive properties in each phase may therefore be more useful in distinguishing between different liquid phases within the plasma membrane. Molecular rotors fall under this category, with both fluorescence lifetime and quantum yield increasing within the more viscous L_o phases.^{41,138} Solvatochromic dyes have also found widespread application in the imaging of coexisting lipid phase in both model membranes systems and live cell plasma membranes. Solvatochromic dyes display changes in their emissive properties depending on the polarity of their surrounding environments. Because different lipid phases show clear differences between both the distributions of water molecules across the bilayer and the dynamics of these water molecules,²⁴⁷ with the increased lipid packing of L_o phases leading to lower levels of hydration and therefore polarity,¹⁶⁶ solvatochromic dyes may become useful tools for probing the existence of different lipid phases. By synthetically modifying their chemical structures, a range of solvatochromic dyes that display different localisations within lipid bilayers

have been synthesised, with properties such as equal staining of different lipid phases, and even selectively staining one leaflet of the bilayer over another.¹⁶⁶

One of the most commonly used solvatochromic probes of membrane ordering is Laurdan (6-acyl-2-dimethylaminonaphthalene, see Figure 6-1 for structure), which partitions evenly between L_o and L_d phases in model bilayers.¹⁶² When incorporated into model bilayers, it has been reported to show a 50 nm red-shift in emission intensity between gel and fluid phases,²⁴⁸ which has been attributed to solvent relaxation. This red-shifted emission is caused by a reduction in the mobility of the water molecules present at the level of the phospholipid glycerol backbones.²⁴⁹ Laurdan has been used to detect coexisting lipid phases in model membranes and membranes reconstituted from plasma membrane extracts, allowing quantification of the relative amounts of L_o and L_d phases.^{162,248,250,251}

Despite the fact that Laurdan is rapidly internalised into the cell interior when introduced to live cells, complicating data analysis and introducing uncertainty into the conclusions of membrane imaging, it has been previously used to investigate ordering within live plasma membranes. In 2003, Gaus *et al*²⁵² labelled different live cell lines with Laurdan and used the shift in its emission wavelength to imply information about the lipid ordering within the plasma membrane. From this study, they determined that there is a significant degree of lipid ordering within the plasma membrane, with ordered regions associating with specific cellular structures, such as filopodia. However, in this work, the Laurdan probe internalised to a large extent into the cell interior, which may impact the accuracy of the results. In 2012, Owen *et al*²⁵³ used Laurdan and another solvatochromic dye, di-4-ANEPPDHQ, in conjunction with FLIM to investigate lipid ordering within the plasma membranes of live HeLa cells.

Laurdan is known to change its fluorescence decay properties between L_o and L_d phases, and through the use of phasor analysis,²⁵⁴ they were able to quantify the relative ratio of L_o to L_d phases, determining that 76% of the plasma membrane was in the L_o phase, with the phases being below the diffraction limit of optical microscopy. Phasor analysis is a model-free assessment of the composition

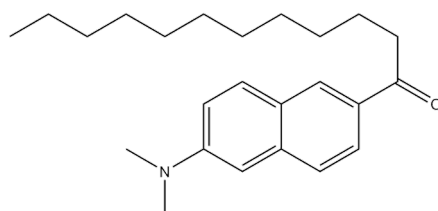


Figure 6-1 Structure of Laurdan

of fluorescence decays²⁵³ that has a number of advantages associated with its use that will be discussed further on. This result contrasts with work by Gaus *et al*²⁵² discussed in this paragraph, which suggested that only 10-15% of the cell membrane consists of ordered regions. Recent research by Niko *et al*²⁵⁵ using a membrane-targeting pyrene-based solvatochromic dye has further implied that the plasma membranes of live cells display high levels of lipid ordering, with the authors even suggesting that the plasma membrane more resembles a model L_o phase than an L_d phase.

Phasor analysis has several advantages over conventional methods for analysing fluorescence lifetime images. Firstly, as it is a model-free assessment of the composition of fluorescence decays,²⁵³ it does not require fitting to a number of exponential decay components. This allows it to avoid the problem of deciding the number of exponentials to fit a decay to, which can lead to uncertainties in the fitting process.²⁵⁴ The method of phasor analysis performs a Fourier transform of the fluorescence decays from each pixel of the fluorescence lifetime image and plots the real (g) against the imaginary (s) component of the Fourier transform. The components are calculated using the equations (6-1) and (6-2), shown below:

$$g(\omega) = \frac{\int_0^{\infty} I(t) \cos(\omega t) dt}{\int_0^{\infty} I(t) dt} \quad (6-1)$$

$$s(\omega) = \frac{\int_0^{\infty} I(t) \sin(\omega t) dt}{\int_0^{\infty} I(t) dt} \quad (6-2)$$

Where I(t) is the fluorescence decay, ω is the angular frequency derived from the frequency of the pulsed laser source ($2\pi \times 80$ MHz) and t is time. If the fluorescence decay is monoexponential, the point produced by a phasor transform lies on the semicircle centred at g = 0.5, s = 0, the so-called “universal circle”.¹¹⁴ In the case of a multiexponential decay, the point lies within a semicircle. The phasor transform of a FLIM image produces a “cloud” of points where the points correspond to the individual decays in the FLIM image, although for clarity, the centre of each cloud can be shown.

Phasor analysis has also been applied to the fluorescence decays of molecular rotors within biological environments, where the fluorescence decays may be too complex for accurate multiexponential analysis, such as determining the mechanism of protein aggregation¹¹⁴ or probing specific interactions between small molecules and DNA.²⁵⁶ Additionally, phasor analysis can be used to determine whether or not a fluorescence decay is monoexponential or multiexponential by whether it lies on the universal circle or not, which can also be used to glean information about the surrounding environment of the molecular rotor.¹⁸⁷ Phasor analysis does not, however, give a value such as fluorescence lifetime, and is therefore more suitable for qualitative comparison and

detecting changes within an individual system, rather than being used to determine absolute viscosity values.

6.1.3 Thiophene-based dyes for probing membranes

Recently,¹⁴⁰ Prof Harry Anderson's group at Oxford University synthesised a range of solvatochromic thiophene-based push-pull dyes that were shown to selectively stain the plasma membranes of live cells. These dyes were originally designed as probes for use in second harmonic generation (SHG) and two-photon excited fluorescence (TPEF) application due to their high molecular first hyperpolarisability, β . The dyes were shown to display low dark toxicity and rapid uptake into the plasma membrane of cells, allowing easy imaging of the cell membranes using two-photon excitation.

In addition to this, the membrane-targeting thiophene dyes were shown to have an increased fluorescence quantum yield and lifetime within the plasma membranes of the live cells compared with the dyes in free solution. In toluene, a highly nonpolar solvent (dielectric constant, $\epsilon = 2.4$),²⁵⁷ the lifetime was measured to be 0.66 ns and the quantum yield was 0.026, which, despite being much higher than the values in water ($\epsilon = 78.4$),²⁵⁷ were still much lower than those observed in the plasma membrane. This means that the difference in lifetimes observed between the fluid solution and membranes could not be explained by a difference in polarity alone, and we therefore hypothesised that this was due to the thiophene probes acting as molecular rotors, with the higher viscosity within the plasma membrane leading to the increased fluorescence signal observed. Given the planar structure of the thiophene dye, we also hypothesised that it may be able to partition more equally between L_o and L_d phases within the bilayer than the BODIPY based molecular rotors described in the previous chapters. A molecular rotor that targets the plasma membrane and partitions equally between different liquid phases could help to shed further light on the lateral organisation of lipids within the plasma membrane and the lipid raft hypothesis.

In this chapter we investigate the fluorescence properties of thiophene-based dye "thiophene-rotor" (Figure 6-2) within model lipid bilayers and the plasma membranes of live cells. We first

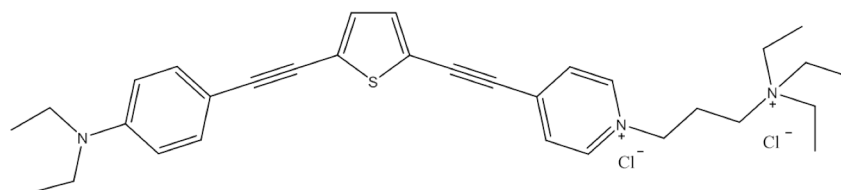


Figure 6-2 Structure of thiophene-rotor

demonstrate its viscosity sensitivity in model lipid bilayers, before investigating the effect of cholesterol on the fluorescence properties of thiophene-rotor. We move on to show that thiophene-rotor partitions equally between L_o and L_d phases within model bilayers and is therefore an ideal candidate for investigating lipid organisation within the plasma membranes of live cells. Finally we image plasma membranes of live cells stained with thiophene rotor, finding evidence of suboptical phase separation within the plasma membrane, in agreement with contemporary ideas about the existence of microdomains within the plasma membrane.

6.2 Results and discussion

6.2.1 Characterisation of the thiophene rotor in LUVs

In order to investigate our hypothesis that thiophene-rotor behaves as a molecular rotor, we needed to investigate its fluorescence properties in a system with a range of different viscosities. Castor oil, which has a large dynamic range of viscosities at variable temperatures and was used in Chapter 3 to address the viscosity sensitivity of the BODIPY rotors, was unsuitable as thiophene-rotor is insoluble in highly nonpolar viscous solvents. Additionally, the polarity sensitivity¹⁴⁰ of thiophene-rotor means that the methanol/glycerol calibration system is also unsuitable for investigating its viscosity sensitivity due to the large spectral shift of thiophene-rotor across solvents with changing polarity.¹⁴⁰ Therefore, in order to assess whether the fluorescence properties of thiophene-rotor responded to changes in viscosity, it was incorporated into LUVs and emission intensity and fluorescence lifetime were investigated with temperature. From our experiments using BODIPY rotors in lipid bilayers (see Chapter 3 and reference¹³⁸), we know that the BODIPY rotors detect viscosity within the tail region of the lipid bilayer, and that DOPC LUVs have a viscosity range of ca. 20-400 cP within the temperature range of 5-55 °C, and so, as thiophene-rotor is a dye designed to incorporate into lipid bilayers, we decided to use DOPC LUVs of known viscosity as a calibration system for thiophene-rotor.

Overlaid emission spectra of thiophene-rotor in DOPC LUVs at a range of temperatures are shown in Figure 6-3 (A), showing a maximum emission wavelength of 596 nm at 25 °C. Similar to what was observed for BODIPY rotors in DOPC LUVs in Chapter 3, as temperature increased, emission intensity decreased, corresponding to the reduction in bilayer viscosity. In DPPC LUVs, a max emission wavelength of thiophene-rotor at 25 °C of 569 nm was observed, and, much like DOPC, the emission intensity of thiophene-rotor decreased with increasing temperature (Figure 6-3 (B)). The change in relative emission intensity for thiophene-rotor in DOPC and DPPC at 596 nm and 569 nm, respectively, is shown in Figure 6-3 (C). Thiophene-rotor in DOPC shows emission intensity decreasing as a smooth curve with increasing temperature, and thiophene-rotor in DPPC shows a change in gradient at *ca.* 41 °C, corresponding with the gel-to-liquid phase transition temperature, after which the curves for thiophene-rotor in DOPC and DPPC overlap very well. The differences between DOPC and DPPC, as well as the change in gradient upon transition from the gel phase to the liquid phase suggest that it is the viscosity change that the thiophene-rotor is responding to, rather than simply the change in temperature, and that the thiophene-rotor is acting as a molecular rotor.

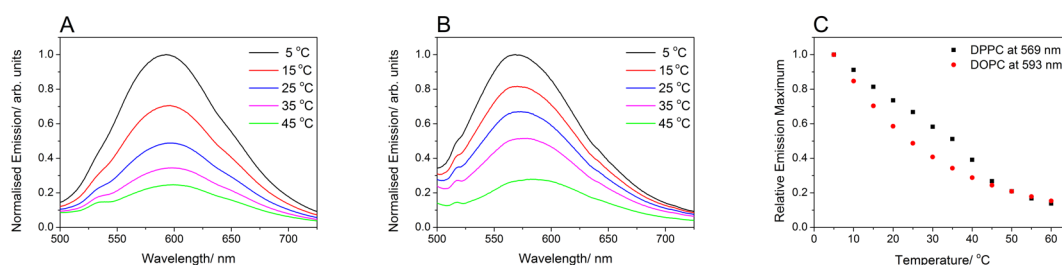


Figure 6-3 Overlaid emission spectra of thiophene rotor in (A) DOPC and (B) DPPC LUVs at a range of temperature, (C) Change in relative emission intensity at the emission max wavelength of thiophene in DOPC and DPPC LUVs at a range of temperatures

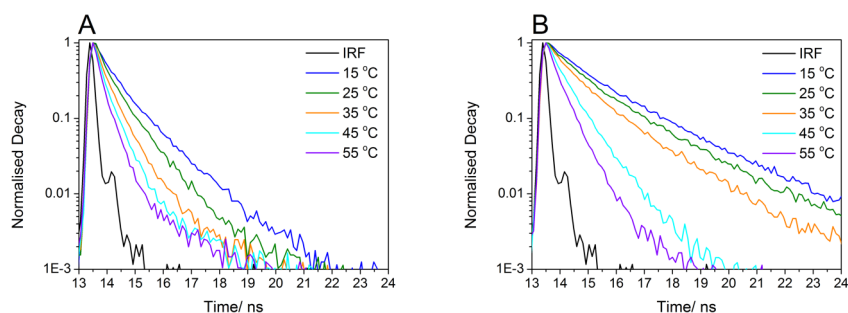


Figure 6-4 Fluorescence decays of thiophene-rotor in (A) DOPC and (B) DPPC LUVs

The viscosity-sensitivity of thiophene-rotor was further investigated by performing TCSPC on LUVs stained with thiophene-rotor in order to investigate changes in fluorescence lifetime. As can be seen from the fluorescence decays of thiophene-rotor in DOPC and DPPC LUVs (Figure 6-4), an increase in temperature causes a shortening of the fluorescence decay times. Between 35 and 45 °C for thiophene-rotor in DPPC, there is a large difference between the lengths of the decays, again corresponding to the gel-to-liquid phase transition temperature.

Several previously reported molecular rotors have fluorescence lifetimes in the picosecond range, meaning that complex and expensive instrumentation is required to analyse the fluorescence decays, potentially prohibiting the use of FLIM and TCSPC as a means of determining viscosity.¹⁰² To investigate the feasibility of lifetime analysis of thiophene-rotor, we calculated fluorescence lifetime values of thiophene-rotor in DOPC and DPPC LUVs. The fluorescence decays shown in Figure 6-4 highlight the fact that thiophene-dye in both DOPC and DPPC bilayers exhibits biexponential fluorescence decay behaviour. The mean-weighted fluorescence lifetime was therefore calculated from both lifetime components (τ_i) and their amplitudes (α_i) using equation (6-3):

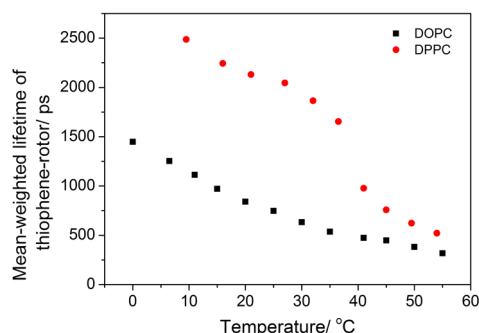


Figure 6-5 Change in mean-weighted lifetime of thiophene-rotor in DOPC and DPPC LUVs with temperature

$$\tau_{mean-weighted} = \frac{\tau_1^2 \alpha_1 + \tau_2^2 \alpha_2}{\tau_1 \alpha_1 + \tau_2 \alpha_2} \quad (6-3)$$

The mean-weighted fluorescence lifetimes of thiophene-rotor in both DOPC and DPPC LUVs were then plotted against temperature (Figure 6-5). Whilst the trend is smooth for thiophene-rotor in DOPC, the sharp change in gradient for DPPC at *ca.* 40 °C reflects the gel-to-fluid gel transition temperature.

We next compared the mean-weighted lifetimes of thiophene-rotor recorded in DOPC at variable temperature with the fluorescence lifetimes of BODIPY-C++ (see chapter 3 and reference¹³⁵) at identical conditions, so as to calibrate the viscosity sensitivity of thiophene-rotor within the bilayer. The molecular dynamics software AMBER¹⁵³ was used to investigate the bilayer positioning of thiophene-rotor and compare it with the bilayer positioning of BODIPY-C++ (simulations performed by Callum Dickson), in order to investigate whether this was a valid comparison. The simulations showed that both thiophene-rotor and BODIPY-C++ occupy a similar position within a DOPC bilayer (see Figure 6-6 for comparative electron density plots and still images showing the final orientations of the dyes in DOPC bilayers), suggesting that both dyes will sense a similar ‘viscosity’ based on their bilayer positioning.

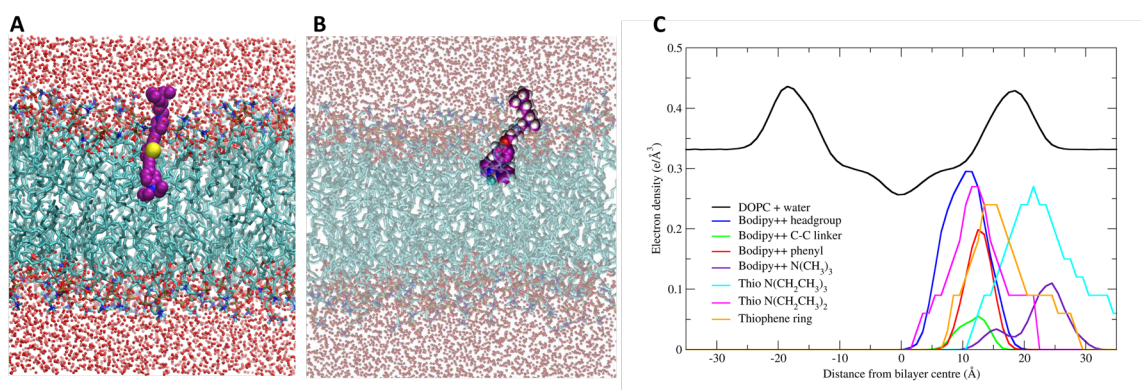


Figure 6-6 Molecular dynamics simulations performed by Callum Dickson comparing the bilayer positioning of thiophene-rotor and BODIPY-C++ in DOPC bilayers. (A) Thiophene-rotor in DOPC and (B) BODIPY-C++ in DOPC at 25 °C, (C) electron density plot comparing the exact orientation of thiophene-rotor with BODIPY-C++ in DOPC

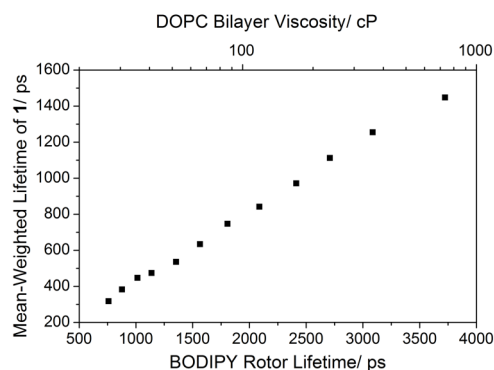


Figure 6-7 Viscosity calibration of thiophene-rotor in DOPC LUVs from comparison with BODIPY-C++ in DOPC

This means that the fluorescence lifetime of BODIPY-C++ can be related to that of thiophene-rotor so a calibration plot of DOPC bilayer viscosity (obtained from C++-BODIPY lifetime in DOPC) against thiophene-rotor lifetime can be generated (Figure 6-7). The calibration plot shown in Figure 6-7 displays a linear correlation between thiophene-rotor lifetime and DOPC bilayer viscosity on a single logarithmic scale, in agreement with the Förster-Hoffmann equation,⁹⁷ further confirming the hypothesis that thiophene-rotor is behaving as a molecular rotor and that mean-weighted fluorescence lifetime can be related to the bilayer viscosity.

Molecular dynamics simulations were also used to investigate effect of temperature on the bilayer positioning of thiophene-rotor. Figure 6-8 shows the final average positions of the thiophene-rotor after four 100 ns simulations at 20 °C within pure DOPC and DPPC bilayers, showing a similar orientation.

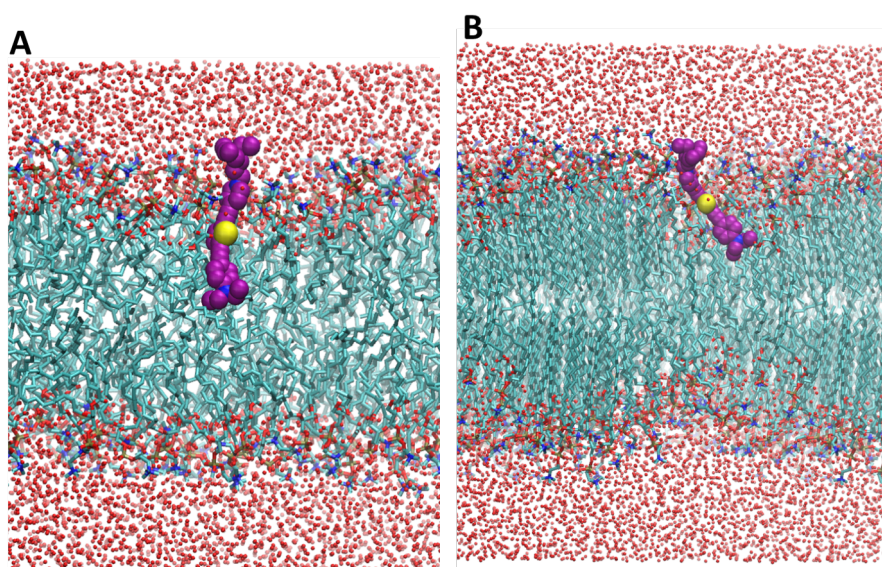


Figure 6-8 Molecular dynamics simulations (performed by Callum Dickson) showing the preferred orientations of thiophene-rotor in (A) DOPC and (B) DPPC

Electron density plots showing the average positioning of thiophene-rotor in DOPC and DPPC bilayers at 5, 23 and 60 °C relative to the bilayer and the water phase are shown in Figure 6-9. In both cases, as temperature increases, the thiophene-rotor is increasingly internalised into the bilayer, facing out into the water phase less and less. This means that the thiophene-rotor moves into a position with a higher electron density, representing the charged headgroups of the lipids. This effect can be observed experimentally by a shift in the maximum emission wavelength with temperature for thiophene-rotor in both DOPC and DPPC (Figure 6-10). Thiophene-rotor has previously¹⁴⁰ been shown to display solvatochromic emission, with maximum emission wavelength increasing with solvent polarity. In both systems, the maximum emission wavelength increases with temperature, suggesting that the environment is becoming increasingly polar with increasing temperature, reflecting the increased surrounding electron density shown in the simulations.

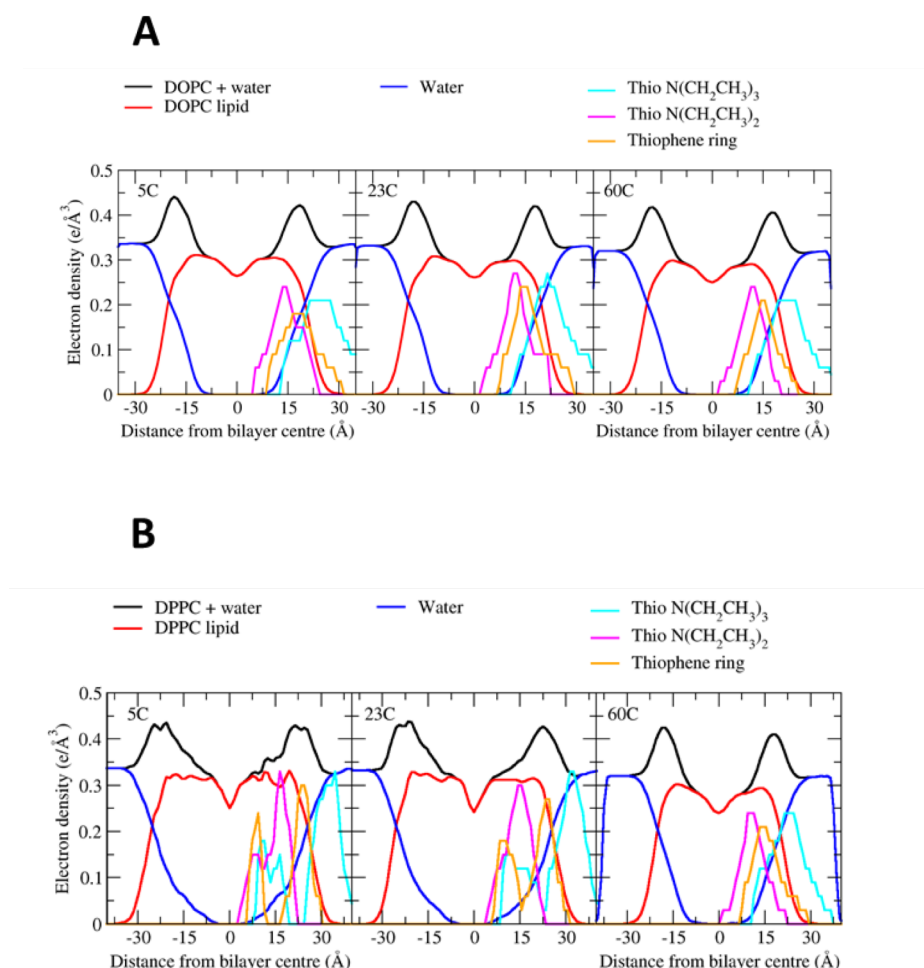


Figure 6-9 Electron density plots from molecular dynamics simulations performed by Callum Dickson showing the average position of thiophene-rotor within the bilayer at 5, 25 and 60 °C in (A) DOPC and (B) DPPC

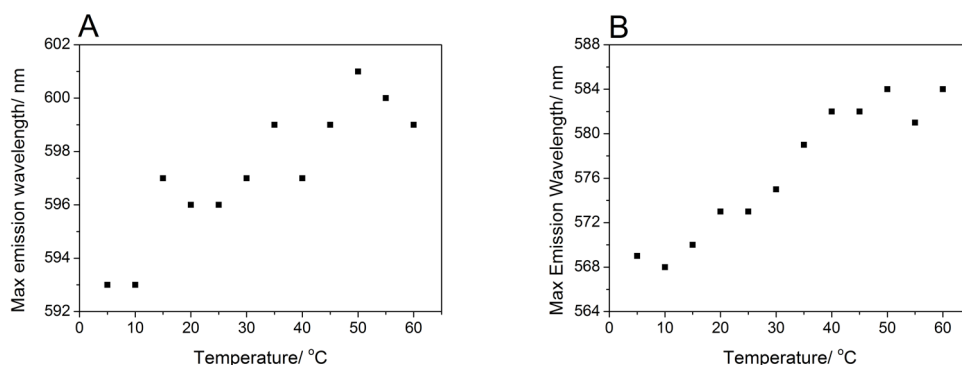


Figure 6-10 Max emission wavelength of thiophene-rotor in (A) DOPC and (B) DPPC LUVs with temperature

Table 6-1 Mean-weighted lifetime of thiophene-rotor in DOPC and DPPC LUVs with varying cholesterol content

% Cholesterol	DPPC	DOPC
	Mean-weighted Lifetime/ ps	Mean-weighted Lifetime/ ps
0	2190	970
5	2900	
10	2930	990
20	3380	1300
30	3350	1410
40	3180	
50	2910	

6.2.2 The effect of cholesterol on the lifetime of the thiophene rotor

The fluorescence decays of thiophene-rotor in DOPC and DPPC LUVs with varying concentrations of cholesterol are shown in Figure 6-11. In both systems, the addition of cholesterol causes a lengthening of the fluorescence decay and an increase in the mean-weighted lifetime (Figure 6-11, Table 6-1). Cholesterol is known to condense unsaturated lipids,¹⁸⁸ leading to an increase in viscosity¹³⁸, meaning that this observation was as expected for the DOPC bilayers. In saturated lipid bilayers, however, cholesterol disrupts the tight packing of the gel-phase lipid molecules, forming a liquid-ordered phase.¹⁸⁸ This would normally be expected to be accompanied by a large decrease in the membrane viscosity,¹³⁸ meaning that the observed increase in thiophene-rotor lifetime was unexpected.

In DPPC/Cholesterol mixtures, the lifetime of thiophene-rotor increases with increasing cholesterol content up to 20% cholesterol, before decreasing with additional cholesterol. Figure 6-12 shows the low cholesterol region of the phase diagram for DOPC/DPPC/Cholesterol mixtures, in order to highlight the phase behaviour of binary DPPC/Cholesterol mixtures. Up to 5 mol% cholesterol, the bilayer would remain in the gel (S_o) phase, meaning a similar viscosity value would be expected. Up

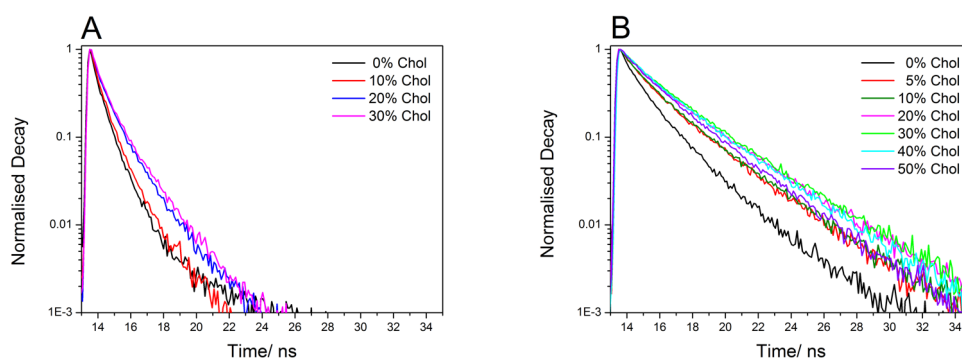


Figure 6-11 Fluorescence decays of thiophene-rotor in (A) DOPC and (B) DPPC LUVs with varying cholesterol content

to *ca.* 25 mol% cholesterol, S_o - L_o phase coexistence is expected, and at higher concentrations of cholesterol, only the pure L_o phase is present. This may explain the decrease in thiophene-rotor lifetime beyond 30% cholesterol, as the increasing cholesterol content reduces the gel-phase character, thereby reducing membrane viscosity. Up to this point, the increase in lifetime with increasing cholesterol content is perhaps puzzling.

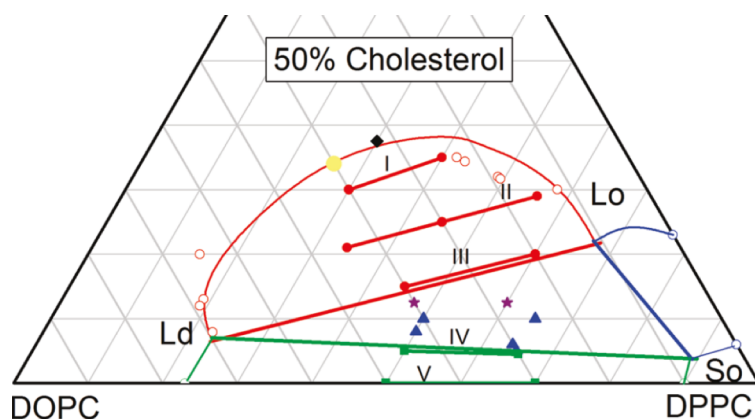


Figure 6-12 Phase diagram for DOPC/DPPC/Cholesterol ternary bilayers (adapted from ref²⁹²)

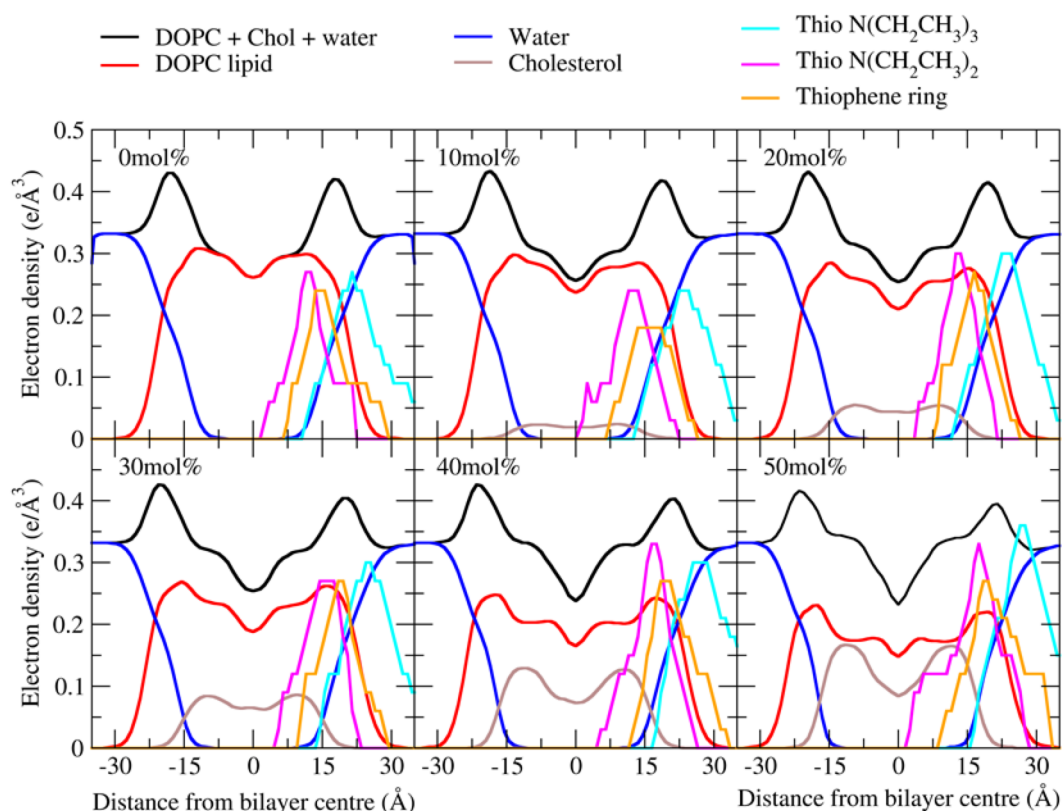


Figure 6-13 electron density plots from molecular dynamics simulations performed by Callum Dickson showing the simulated position of thiophene-rotor in DOPC bilayers at a range of cholesterol concentrations

We hypothesised that this change in thiophene-rotor lifetime was caused by a change in its bilayer positioning with increasing cholesterol content. Changes in bilayer positioning affecting the fluorescence lifetime of molecular rotors have been previously explored by Olšinova *et al*¹⁸⁷ and in Chapter 3 of this thesis, and it is reasonable to suggest that this may be affecting the lifetime of thiophene-rotor in this case. In order to investigate this hypothesis, the molecular dynamics software AMBER¹⁵³ was used by Callum Dickson to simulate the behaviour of thiophene-rotor within the bilayer and the effects of cholesterol on its bilayer positioning.

Figure 6-13 shows electron density profiles of four thiophene-rotor molecules within a DOPC bilayer up to 50 mol% cholesterol. The orientation of the dye molecules does not appear to change greatly with increasing cholesterol content, although they do appear to be pushed slightly upward and out of the membrane with the addition of cholesterol.

In addition to electron density plots, in order to quantify the bilayer orientation of thiophene-rotor, a molecular order parameter ($\langle P_2 \rangle$) was calculated for thiophene-rotor using the angle θ between the long axis of inertia of the molecule and the membrane normal, using equation 6-4, shown below:

$$P_2 = \frac{1}{2} \langle 3 \cos^2 \theta - 1 \rangle \quad (6-4)$$

$\langle P_2 \rangle$ can take a value between 1, indicating orientation with the membrane normal (i.e. aligned with lipid tails) and -0.5, which indicated alignment with the membrane plane. A value of 0 indicates random orientation. The average $\langle P_2 \rangle$ order parameter of thiophene-rotor molecules in a DOPC bilayer with increasing cholesterol content is shown in Figure 6-14, where the order parameters have been grouped as greater than $\langle P_2 \rangle = 0.25$, meaning that the angle between the thiophene-rotor molecule and the membrane normal is above 45° , and less than $\langle P_2 \rangle = 0.25$, meaning that the angle between the thiophene-rotor molecule and the membrane normal is below 45° . In the plot, the size of each symbol represents the number of thiophene-rotor molecules (out of a total of four) that are in each group. It can be seen from Figure 6-14 that, above 30 mol% cholesterol, there is an approximately equal split between thiophene-rotor molecules slightly aligned with the membrane normal and random alignment. Below 30 mol% cholesterol, there is no clear trend in thiophene-rotor orientations.

Overall, these results suggest that there is no large-scale change in bilayer orientation of thiophene-rotor in DOPC with increasing cholesterol content, with a small scale change in position in bilayer position, and no observable change in the angle of orientation relative to the bilayer. This correlates with the 45% increase in mean-weighted lifetime observed from 0% to 30 % cholesterol content, and may represent both the increase in viscosity with addition of cholesterol, and the small scale change

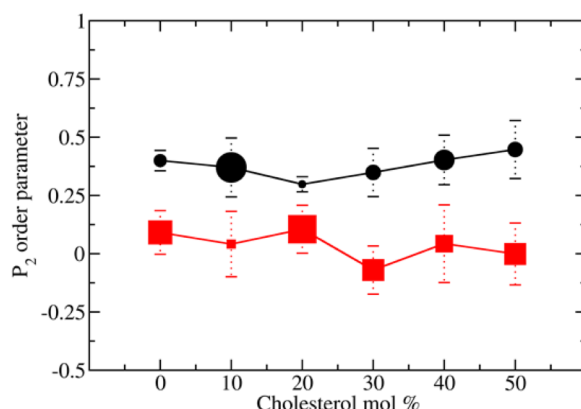


Figure 6-14 Change in $\langle P_2 \rangle$ order parameter of thiophene-rotor in a simulated DOPC bilayer with increasing cholesterol content (from molecular dynamics simulations performed by Callum Dickson)

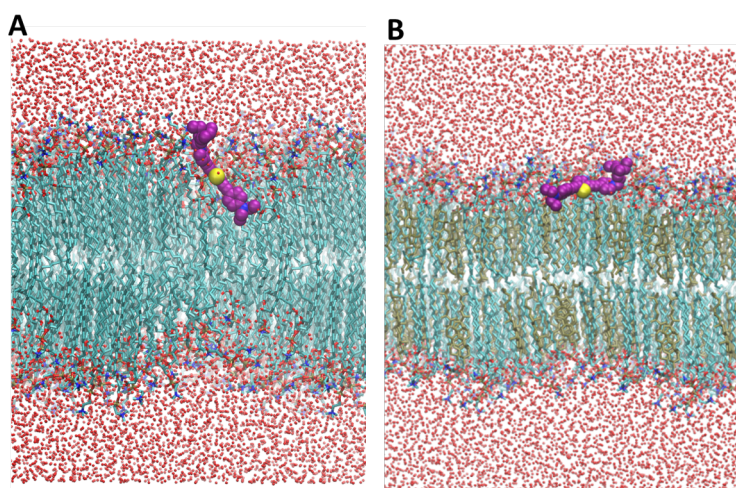


Figure 6-15 Average bilayer positions of thiophene-rotor in a simulated DPPC bilayer with (A) 0 mol% and (B) 50 mol% cholesterol (simulations performed by Callum Dickson)

in bilayer position, although it is hard to decouple these from each other (the corresponding increase in BODIPY-Chol lifetime from 0% to 30% cholesterol is 23%, as determined in Chapter 3).

Figure 6-15 shows the final positions of thiophene-rotor in the molecular dynamics simulations for DPPC with 0 and 50 mol% cholesterol. There is a clear difference in the orientations, with the thiophene-rotor aligning with the membrane normal at 50 mol% cholesterol and aligning with the lipid tails at 0 mol% cholesterol, similar to its orientation in pure DOPC (see Figure 6-8). This represents a large change in the orientation, which could explain the unexpected increase of thiophene-rotor fluorescence lifetime in DPPC LUVs with increasing cholesterol.

To further investigate this, simulated electron density profiles for thiophene-rotor in DPPC bilayers with increasing cholesterol content were obtained (Figure 6-16). Figure 6-16 shows that the change in orientation of thiophene-rotor in DPPC changes with cholesterol content. From 0 to 30 mol% cholesterol, the thiophene-rotor molecules appear to be increasingly pushed from the bilayer core by the cholesterol, facing out increasingly into the water phase. Above 30 mol% molar fraction of cholesterol the dye transitions from orientation of aligned along the membrane normal to aligned along the membrane plane. Consequently, at 40 and 50 mol% there is a mixture of dye alignments – the signal remains for dye molecules aligned along the membrane normal, yet a new signal appears for dye aligned along the plane. From this, it can be inferred that thiophene-rotor has two preferred orientations at high cholesterol content, compared with a single preferred orientation at low/zero cholesterol content. This observation, where the thiophene-rotor is increasingly pushed from the bilayer core up to 30 mol% cholesterol, before beginning to align with the membrane normal may

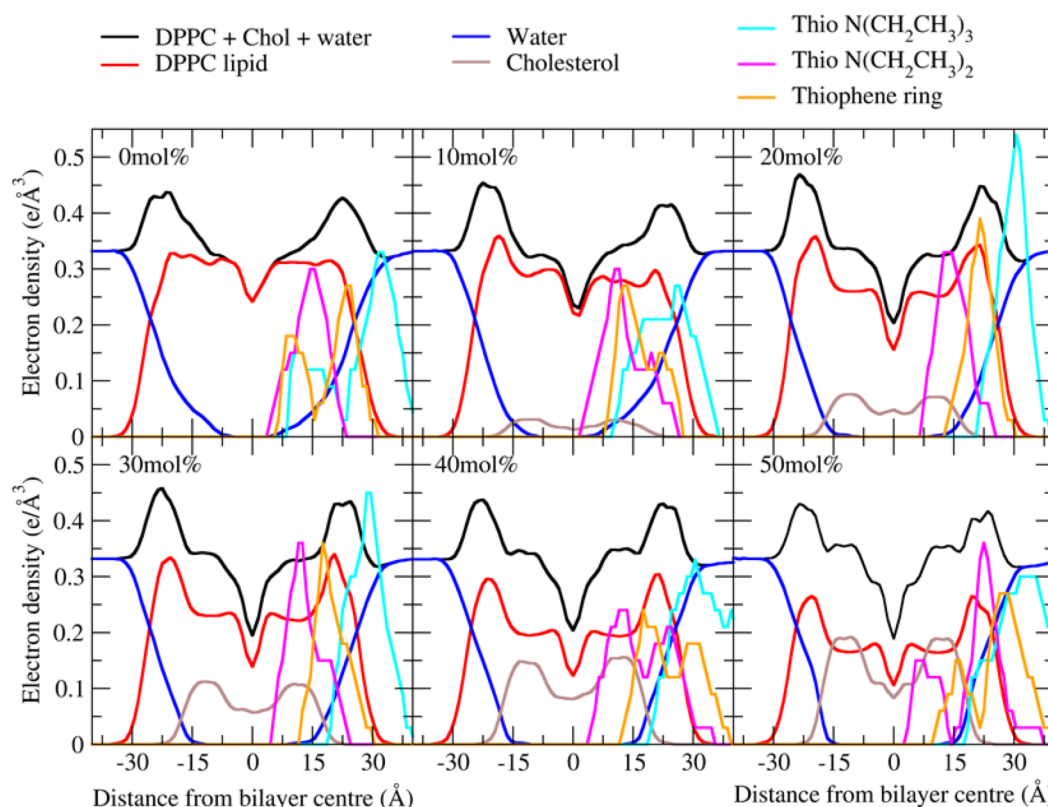


Figure 6-16 electron density plots from molecular dynamics simulations performed by Callum Dickson showing the simulated position of thiophene-rotor in DPPC bilayers at a range of cholesterol concentrations

explain the increase in thiophene-rotor fluorescence lifetime up to 30 mol% cholesterol, followed by the gradual decrease in lifetime at higher cholesterol content.

The average $\langle P2 \rangle$ order parameter of thiophene-dye in DPPC with increasing mol% cholesterol further supports this conclusion (Figure 6-17). At 0 and 10 mol% cholesterol the majority of thiophene-rotor molecules are aligned along the membrane normal, with a small number aligned along the membrane plane. At 20mol%, all thiophene-rotor molecules are aligned along the membrane normal (with low fluctuation, note the smaller error bars). Starting at 30 mol% the number of thiophene-rotor molecules aligned with the membrane normal steadily decreases, at the same time the number of thiophene-rotor molecules transitioning to alignment with the membrane plane concurrently increases. Overall, we see a shift in the orientation of the thiophene-rotor molecules predominantly aligned along membrane normal to the molecules predominantly adopting random/aligned along membrane plane orientation starting at 30 mol% cholesterol.

Overall, this data from the molecular dynamics simulations helps to explain the trend in fluorescence lifetime of thiophene-rotor in LUVs with cholesterol. The shift in thiophene-rotor orientation with increasing cholesterol concentration suggests that the pure DOPC “viscosity calibration” may be

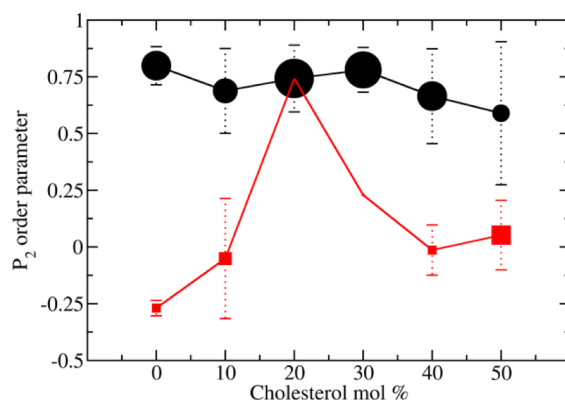


Figure 6-17 Change in $\langle P_2 \rangle$ order parameter of thiophene-rotor in a simulated DPPC bilayer with increasing cholesterol content (from molecular dynamics simulations performed by Callum Dickson)

unsuitable for thiophene dye for cholesterol-containing bilayers, such as phase-separating GUVs and the plasma membranes of live cells, which are known to be rich in cholesterol.²⁵⁸ Despite this, there was still a large difference in thiophene-rotor fluorescence lifetime between L_d phase LUVs (DOPC with cholesterol) and L_o phase LUVs (DPPC with cholesterol). To investigate whether thiophene-rotor could be used to differentiate between coexisting L_o and L_d phases within artificial bilayers, we next incorporated it into phase-separating GUVs.

6.2.3 Studies in giant unilamellar vesicles

As explained earlier in Chapters 3 and 5, the poor partitioning of the BODIPY-based molecular rotors between L_o and L_d phases within model bilayers is a major limitation to their use in probing phase separation within the plasma membranes of live cells. To investigate whether thiophene-rotor partitioned into both L_o and L_d phases, and whether its lifetime was significantly different between the different phases, it was incorporated into phase-separating GUVs (DOPC/DPPC/Cholesterol, Figure 6-18, and DOPC/EYSM/Cholesterol at 1:1:1 molar ratios, Figure 6-19) and FLIM of the GUVs was performed.

Confocal microscope images suggest equal partitioning between L_o and L_d phases of the DOPC/DPPC/Cholesterol vesicles, and the pixel-by-pixel fluorescence intensity shown in Figure 6-18 (B) from the L_o phases is comparable to that of the L_d phases. This suggests that thiophene-dye is able to partition approximately equally between L_o and L_d phases, setting itself apart from other molecular rotors reported to date.¹⁶⁶

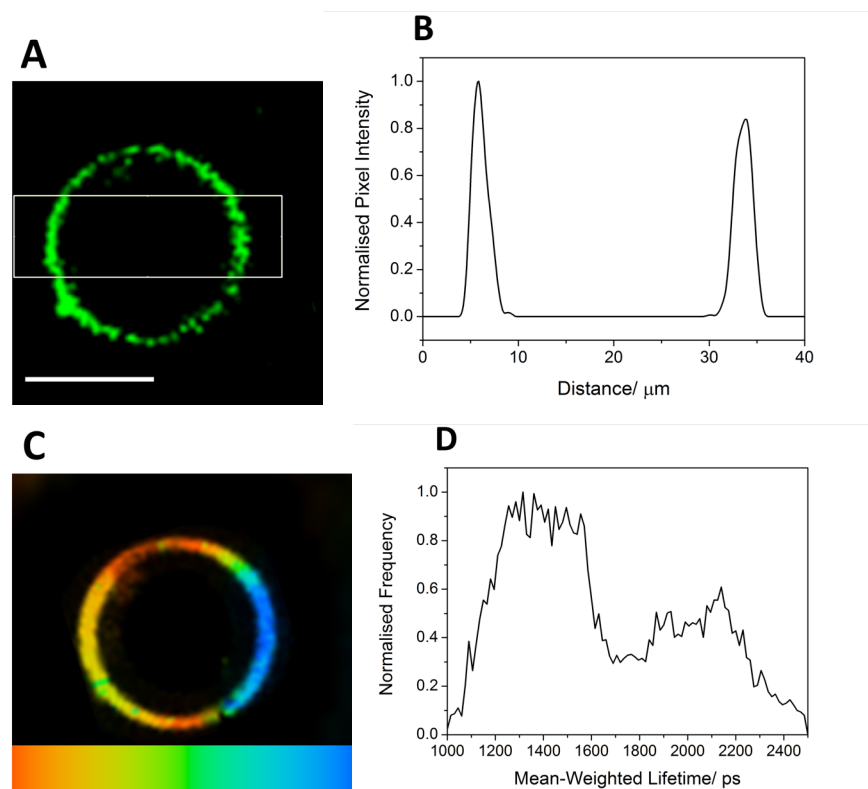


Figure 6-18 (A) Confocal image of a 1:1:1 DOPC/DPPC/cholesterol GUV displaying L_o L_d phase separation (scale bar = 20 μm), (B) plot showing pixel intensity in the box shown over the GUV in (A), (C) fluorescence lifetime image showing the distribution in mean-weighted fluorescence lifetimes over the same GUV (colour scale = 1000-2500 ps), (D) summed histogram of 10 individual GUV lifetime images

Fluorescence lifetime imaging of the GUVs of both compositions was performed, and representative lifetime images as well as histograms showing the lifetime distributions of 10 GUVs are shown in Figure 6-18 and Figure 6-19. The L_o phases show a significantly higher fluorescence lifetime than the L_d phases, suggesting a higher viscosity, in agreement with the work described in chapter 3 and elsewhere.⁴¹ The equal L_o - L_d phase partitioning in addition to the clear difference in thiophene-rotor fluorescence lifetime between the two different phases, suggests that thiophene-rotor may make an ideal candidate for probing phase behaviour within the plasma membranes of live cell lines, overcoming the problems associated with many other molecular rotors.¹⁶⁶

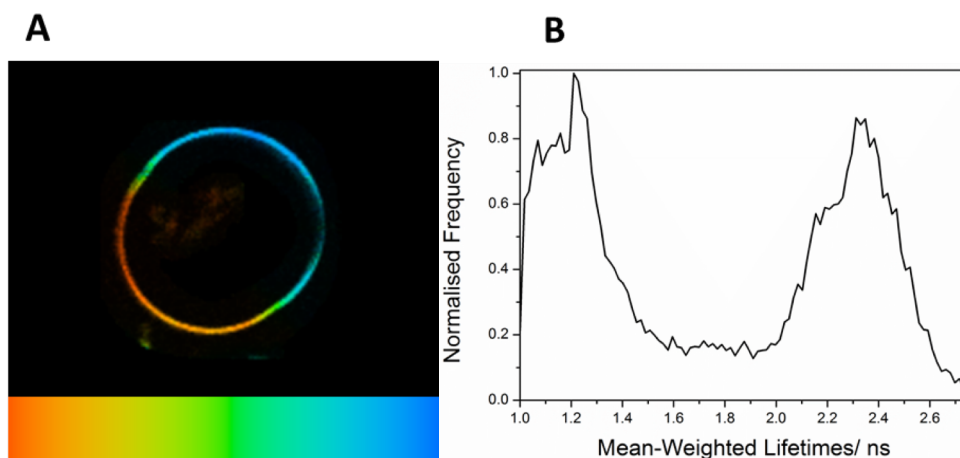


Figure 6-19 (A) Representative fluorescence lifetime image of a phase separating GUV (1:1:1 DOPC/EYSM/Cholesterol) and **(B)** summed histogram of 10 separate GUV images

6.2.3 Studies in live cells

To investigate whether thiophene-rotor could indeed be used to investigate phase behaviour within the plasma membranes of live cells, four cell lines: BE, HCT 116, NIH 3T3 and neuroblastoma, were cultured and incubated with thiophene-rotor. Thiophene-rotor rapidly stained the plasma membranes of the cell lines, allowing confocal and lifetime imaging of the cells. The emission spectrum of thiophene-rotor within the plasma membrane of the neuroblastoma cell line is shown in Figure 6-20. The emission maximum is at *ca.* 605 nm, which is higher than that observed in either DOPC or DPPC LUVs and suggests that the plasma membranes of the neuroblastoma cells are more hydrated than the model bilayers.

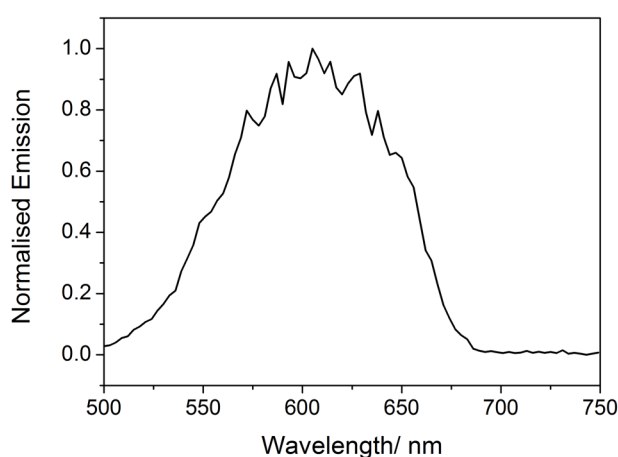


Figure 6-20 Emission spectrum of thiophene-rotor in the plasma membranes of neuroblastoma cells

Figure 6-21 shows representative lifetime images of thiophene-rotor in each cell line. Each pixel of lifetime image was fitted to a biexponential decay, and the mean-weighted lifetime of thiophene-rotor was calculated. 10 lifetime images of confluent, healthy cells were obtained, with summed and normalised histograms for the 10 images being shown in Figure 6-22 (the mean lifetimes for BE, HCT 116, NIH 3T3 and neuroblastoma cell membranes were 1725, 1602, 1786 and 1758 ps, respectively). These lifetime values are all in the region of 1.7 ns, which is essentially halfway between the mean-weighted lifetimes for L_o and L_d phases observed in phase-separating GUVs (Figure 6-18 and Figure 6-19), and could be interpreted as evidence of suboptical L_o - L_d phase separation within each pixel of the lifetime image of the cell line plasma membranes. Individual lifetime images appear to be homogeneous, which suggests that any L_o - L_d phase separation, if present, is either below the optical resolution of the microscope, or below the temporal resolution of the FLIM technique, which used a

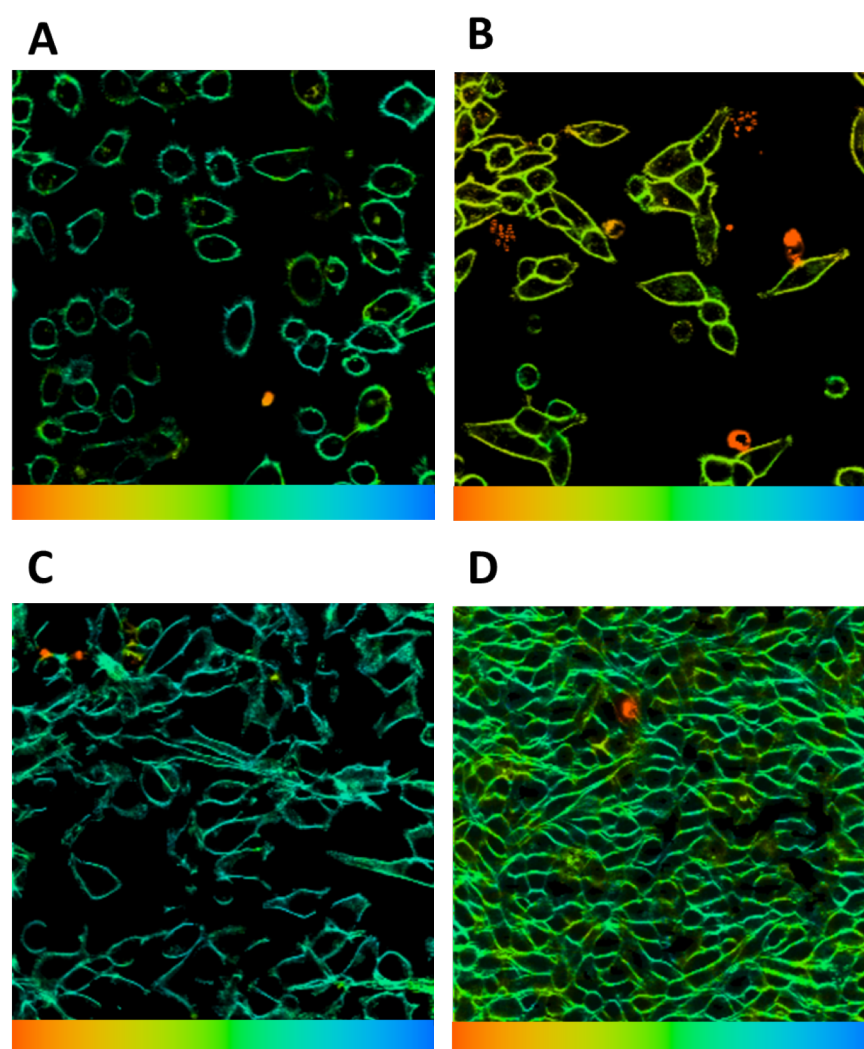


Figure 6-21 Sample lifetime images of thiophene-rotor in (A) BE cells, (B) HCT 116 cells, (C) NIH 3T3 and (D) neuroblastoma cells, with the colour range representing 1250-2150 ps

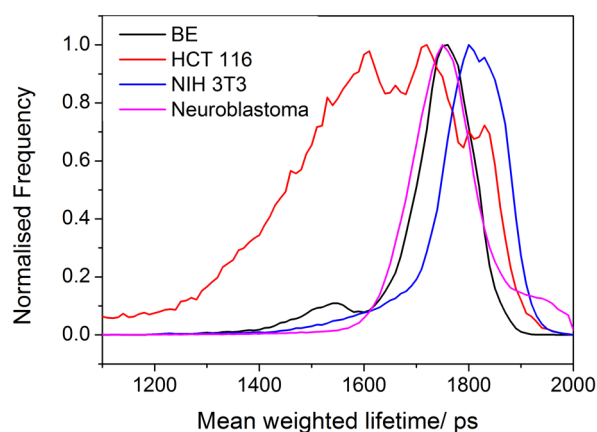


Figure 6-22 Histograms showing the lifetime distributions of the thiophene-rotor within the plasma membranes of the live cell lines

collection time of 300 seconds for each image.

Given possible parameter uncertainties in fitting the biexponential decays of thiophene-rotor, and in order to further investigate the possibility of coexisting L_o and L_d phases within the plasma membranes of the cell lines, we used phasor analysis²⁵⁴ to compare fluorescence decays of thiophene-rotor within model L_o phases (DPPC/Cholesterol LUVs), model L_d phases (DOPC/Cholesterol LUVs), and the plasma membranes of the live cell lines (Figure 6-23). The decays recorded for **1** in live cell plasma membranes fell on the straight line between the pure L_o phases, formed from DPPC/Cholesterol mixtures, and the pure L_d phases, formed from DOPC/Cholesterol mixtures. Progression along a straight line in phasor space suggests the existence of two viscosity

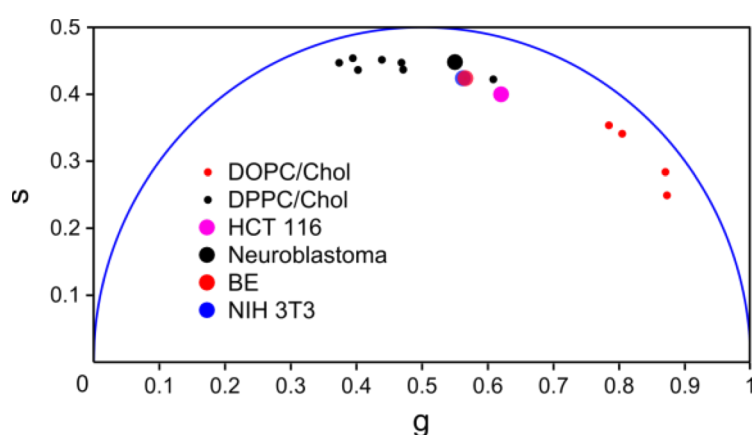


Figure 6-23 Phasor transforms of the fluorescence decays of thiophene-rotor in model membranes and the membranes of the live cell lines. The model membrane decays come from the LUVs composed of DOPC/Cholesterol (model L_d phases) and DPPC/Cholesterol (model L_o phases) shown earlier in Figure 6-11

components in the decay, which may be attributed to the existence of two different lipid phases that display different viscosities. This result therefore suggests a coexisting mixture of L_o and L_d in plasma membranes of live cells. This is consistent with the recent study performed by Owen *et al.*,²⁵³ who used phasor analysis of Laurdan in the plasma membranes of live cells to suggest the coexistence of L_o and L_d domains within the plasma membrane. Whilst the exact size and nature of these domains remains unclear, and a quantitative assessment of the viscosities of the different phases is inaccessible using phasor analysis, this could still represent important evidence suggesting that the lipids within the plasma membrane do indeed phase-separate into coexisting L_o and L_d domains, although any phase separation within plasma membranes is likely to be less clear cut than large scale L_o - L_d phase separation in model membranes, and the fact that large scale phase separation does not occur within the plasma membrane suggests that this process may be thermodynamically disfavoured and thus may be highly transient. The implications for the functions of any ordered lipid microdomains within the plasma membrane are still unclear.

6.3 Conclusions

In this chapter, the thiophene-based dye “thiophene-rotor” was postulated as a molecular rotor that could target the plasma membranes of live cells, and could therefore be used to investigate lipid phase-behaviour within plasma membranes. We characterised the photophysical behaviour of thiophene-rotor within model lipid bilayers, before using it to perform lifetime imaging on the plasma membranes of four live cell lines.

Thiophene-rotor was first incorporated into DOPC and DPPC LUVs, and the steady state emission spectroscopy and time resolved spectroscopy via TCSPC was performed at a range of temperatures, in order to investigate the effect of membrane viscosity on emission intensity and fluorescence lifetime of thiophene-rotor. Both emission intensity and lifetime were found to increase with increasing bilayer viscosity, suggesting that thiophene-dye is capable of acting as a molecular rotor. By comparing the fluorescence lifetime of thiophene-rotor in DOPC with that of BODIPY-C++, which was shown using MD simulations to take on a similar orientation within DOPC bilayers, a “calibration plot” of bilayer viscosity against thiophene-rotor lifetime was generated. By measuring the change in fluorescence lifetime with temperature for thiophene-rotor in DPPC LUVs, a large change in lifetime was observed at the gel-to-liquid transition temperature of DPPC, similar to what has previously been observed for other molecular rotors in DPPC, and suggesting that thiophene-rotor is viscosity-sensitive, rather than temperature-sensitive.

The effect of cholesterol on the fluorescence lifetime of thiophene-rotor within the bilayer was then investigated. In both DOPC and DPPC, which are unsaturated and saturated lipids, respectively, cholesterol was found to cause an increase in the fluorescence lifetime of thiophene-dye. This was unexpected, as cholesterol would be expected to decrease the viscosity of a saturated lipid bilayer. In order to further investigate this, MD simulations were performed in order to probe the effect of cholesterol on thiophene-rotor positioning within the bilayer. The simulations revealed that cholesterol causes the thiophene-rotor to change position, which explained the change in fluorescence lifetime. This means that the DOPC calibration is unsuitable for thiophene-rotor in cholesterol-containing bilayers, and that an absolute viscosity value cannot as of yet be obtained for cholesterol-containing bilayers such as cellular plasma membranes. Despite this, the thiophene-rotor displayed significant difference in fluorescence lifetime between cholesterol-containing L_o and L_d phases in model bilayers, potentially allowing it to still be used to discern between the two phases.

To investigate whether thiophene-rotor could be used to discern between coexisting L_o and L_d phases in model bilayers, as well as its partitioning between the two phases, it was incorporated into phase-separating GUVs and imaged through confocal microscopy and FLIM. Contrary to the majority of previously reported molecular rotors, thiophene-rotor was found to partition equally between L_o and L_d phases. Additionally, FLIM revealed a large difference in thiophene-rotor lifetime between the two phases, suggesting that it could be used to probe phase behaviour within the plasma membranes of live cells, where any coexisting phases are likely to be very small and potentially difficult to detect.

Thiophene-rotor was then incorporated into four live cell lines, targeting the plasma membranes of each of the cell lines. FLIM was performed, allowing us to measure the fluorescence lifetime of thiophene-rotor within the plasma membranes. The lifetime of thiophene-rotor within the plasma membranes of the four cell lines was found to be close to halfway between its fluorescence lifetime within model L_o and model L_d phases. This could suggest suboptical phase separation into coexisting L_o and L_d phases within each pixel of the lifetime image. To further investigate this, phasor analysis was performed of the fluorescence decays of thiophene-rotor in plasma membranes as well as in LUVs of model L_o and L_d phases. The resulting phasor plot showed the fluorescence decays within the cells to be on the straight line between the model L_o and L_d phases, again suggesting that there may be a degree of L_o - L_d phase separation within the plasma membranes of the live cells.

7. Conclusions

Diffusion-limited processes are ubiquitous in cell biology, and often control the rate of mass transport of various reagents within the cell. One of the main parameters controlling the rate of diffusion is the viscosity of the system, and a thorough understanding of the viscoelastic properties of a biological system is therefore necessary in order to fully understand how it functions.³⁸ This is particularly important within biological membranes, where membrane viscosity directly affects the diffusive properties and therefore activities of the proteins within the membrane.³⁹ The structure and function of membrane proteins is a major area of research, and membrane proteins are the targets of over 50% of modern medicinal drugs.² An intimate understanding of the physical makeup of these biological membranes is therefore necessary for us to fully understand the functioning of these proteins.

Despite the apparent importance of viscosity within cells and biological membranes, there are still comparatively few ways of actually measuring viscosity on the microscale. The use of molecular rotors, viscosity sensitive organic fluorophores, in conjugation with fluorescence lifetime imaging (FLIM) provides a novel approach to the measurement of microviscosity, allowing the generation of a map of the different viscosities across a heterogeneous biological sample. In this thesis, we used molecular rotors based around boron dipyrin (BODIPY) and thiophene moieties to investigate membrane viscosity across a range of different systems.

First, we used three BODIPY-based molecular rotors to investigate viscosity and phase behaviour within model lipid bilayers. The plasma membrane is widely believed to transiently phase-separate into coexisting ordered (L_o) and disordered (L_d) liquid phases, the so-called “lipid raft hypothesis”,²⁰ although these phases are generally believed to be below the diffraction limit of optical radiation in size,³⁵ making visualising them a highly challenging endeavour. We therefore investigated whether BODIPY-based molecular rotors were able to discern between L_d and L_o phases and whether they could therefore be used to shed further light on the lipid raft hypothesis.

We began this study by first characterising the three BODIPY rotors, termed BODIPY-C10, BODIPY-C++ and BODIPY-Chol, in methanol/glycerol solutions of known viscosity in order to generate calibration plots of fluorescence lifetime against viscosity, thereby allowing us to predict the viscosity of an unknown sample from the fluorescence lifetime of the molecular rotor. By determining fluorescence lifetime at a range of different temperatures and viscosities, we showed that the emissive properties of the BODIPY rotors are insensitive to temperature within the

temperature range that we investigated. Following on from this, we studied the polarity sensitivity of the rotors by comparing the fluorescence lifetime of BODIPY-C10 in castor oil, a viscous and highly nonpolar solvent, with the methanol/glycerol calibration, finding that solution polarity does not significantly affect the fluorescence lifetime of BODIPY-C10 at viscosities within the biologically relevant range of 100-1000 cP. This relative insensitivity to polarity and temperature is important for the investigation of heterogeneous biological environments, where both of these parameters may not be easily controlled.

We then went on to investigate the properties of the three BODIPY rotors within simple lipid systems in large unilamellar vesicles (LUVs). By calculating the viscosity of DOPC LUVs at a range of temperatures, we showed that the three rotors gave similar viscosity values, suggesting the three rotors occupy a similar position within the bilayer, something we later backed up using molecular dynamics simulations. By repeating this experiment using DPPC as the lipid, which forms a gel phase bilayer at room temperature with a gel-to-liquid phase transition temperature at 41 °C, we showed that liquid and gel phases have significantly different viscosities. We therefore hypothesised that BODIPY rotors may be able to sense different coexisting lipid phases based on their viscosities.

In order to investigate whether our calibration in bulk three-dimensional solution still applied in lipid bilayers, which are quasi two-dimensional highly heterogeneous systems, we compared diffusion coefficients in DOPC bilayers calculated from the viscosity given by the BODIPY rotor fluorescence lifetime¹⁹¹ with diffusion coefficients for the BODIPY rotors in DOPC bilayers obtained from molecular dynamics simulations and fluorescence correlation spectroscopy. The three methods predicted similar diffusion coefficients, evidence that the viscosity calculated by the BODIPY rotors within the bilayer is an accurate estimate of membrane viscosity.

Next, we incorporated our BODIPY rotors into phase-separating giant unilamellar vesicles (GUVs), in order to investigate whether BODIPY rotors could reliably discern between coexisting liquid phases within a bilayer. Whilst the BODIPY rotors did show that the L_o phase has a higher viscosity than the L_d phase, each of the three rotors displayed very poor partitioning into the L_o phase, similar to the majority of membrane-soluble dyes.¹⁶⁷ This presented a problem because, although BODIPY rotors can provide an accurate estimate of viscosity within a lipid bilayer, if they are unable to partition to a significant extent into the L_o phase it could hamper the search for lipid rafts, which may be below 200 nm in size and highly transient.

Following on from our initial studies in model lipid membranes, we set out to use our BODIPY rotors to image biological systems. With the exception of BODIPY-C++,¹³⁵ BODIPY rotors generally rapidly

internalise into the cell interior of eukaryotic cells,^{124,132} preventing imaging of the plasma membrane. BODIPY rotors have, however, previously been successfully used to image membrane viscosity within bacteria,¹⁸³ so we therefore set out to investigate viscosity and lipid ordering within the plasma membranes of live *E. coli* bacteria. *E. coli* bacteria are thought to regulate the lipid content of their plasma membranes depending on their growth temperature in order to maintain a constant viscosity, with a lipid phase transition occurring at *ca.* 15 °C below their growth temperature,²¹⁰ making this a fertile area for exploration using molecular rotors.

We began this investigation by using BODIPY-C10 to investigate the viscosity of LUVs comprising of total lipid extracts from *E. coli* bacteria grown at 37 °C. By observing the change in fluorescence lifetime of BODIPY-C10 within these LUVs with temperature, we observed a subtle change in viscosity at *ca.* 25 °C, which we interpreted as evidence of a phase transition. This conclusion was supported by wide-angle X-ray diffraction experiments on the total lipid extracts, which showed the characteristic gel phase peak disappearing around this temperature.

Based on these initial experiments, we then introduced BODIPY-C10 into cultured *E. coli* bacteria, performing confocal microscopy experiments to determine the localisation of the probe within the cell. Initial experiment revealed that BODIPY-C10 localised in a region at the edge of the cell, which we hypothesised was the plasma membrane. As gram-negative bacteria, *E. coli* cells have two membranes in the cell envelope: an inner (plasma) membrane and an outer membrane, separated by the peptidoglycan cell wall, and so we performed further experiments to investigate which one of the two membranes was being stained. Through osmotic shock experiments and then by using lysozyme to digest the outer membrane and cell wall to give spheroplasts, we showed that it was indeed the inner, or plasma, membrane that was being stained by BODIPY-C10. This allowed us to progress with our investigation into the viscoelastic properties of the *E. coli* plasma membrane.

By performing FLIM of the live *E. coli* bacteria stained with BODIPY-C10, as well as the spheroplasts, we were able to calculate viscosities for the *E. coli* plasma membranes. Our initial experiments, comparing the fluorescence lifetimes of BODIPY-C10 in live *E. coli* grown at 37 °C and imaged at a range of temperatures with *E. coli* grown and imaged at the same temperature, were inconclusive, potentially due to the large spread in data between different individual cells, meaning that we were unable to derive detailed information about the temperature adaptation of the *E. coli* plasma membrane. We instead chose to focus on *E. coli* bacteria grown at 37 °C and imaged at both 37 °C and 23 °C, in order to obtain information about the viscosity and lipid ordering within the plasma membrane.

Using the methanol/glycerol calibration system, we then converted the lifetimes of BODIPY-C10 within the *E. coli* plasma membrane into viscosities. The calculated viscosities for spheroplasts and live cells agreed well with each other at both temperatures, providing further evidence that BODIPY-C10 was localised primarily within the inner membranes of the cells. In both spheroplasts and cells, the mean viscosity for the cells increased with the reduction in temperature, consistent with the observations in simple lipid bilayers in Chapter 3. The viscosity values observed in live cells and spheroplasts were significantly higher than those in the LUVs comprised of *E. coli* lipid extracts, possibly owing to the high protein concentration in live cell membranes and the effects of the cytoskeleton on lipid ordering. This result highlights the potential pitfalls associated with using extracts and model lipid bilayers to investigate live cell membranes, which are highly complex and may be very different from the model system. The high viscosity of the *E. coli* plasma membrane is on the same scale as the viscosities calculated in model L_o phases, and may reflect an increased lipid ordering in the membrane compared with the plasma membranes of eukaryotic cells, which have a much lower viscosity.¹³⁵

By using the Saffman-Delbrück equation,¹⁹¹ we were able to then calculate diffusion coefficients for BODIPY-C10 in the *E. coli* plasma membrane based on the calculated membrane viscosity, allowing us to compare our results with results from the literature based on a similar fluorescent probe.⁶⁶ Our calculated diffusion coefficients agreed well with those from the literature, despite the inherent inaccuracies of the Saffman-Delbrück approach, suggesting that our estimate of the *E. coli* plasma membrane viscosity was accurate.

Whilst our work in *E. coli* bacteria clearly outlines the utility of BODIPY-based molecular rotors in investigating the viscoelastic properties of lipid bilayers within a variety of different biological systems, there are still potential issues with the use of BODIPY rotors to investigate structure and ordering within biological membranes. The tendency of BODIPY rotors to internalise into the interior of eukaryotic cells coupled with the fact that they do not partition well into the L_o phase of phase-separating lipid bilayers means that they are not ideal probes of phase separation within the plasma membrane. We therefore began to search for alternatives to the BODIPY rotors that our work had been previously based on.

Many fluorescent probes that are known to target the L_o phase of phase-separating bilayer are planar in structure,¹⁶⁷ allowing them to intercalate into the rigid tail region of the bilayer. We therefore hypothesised that a more planar BODIPY rotor not possessing a phenyl substituent on the *meso*-position may be able to target the L_o phase, much like the cholesterol-conjugated BODIPY dyes previously developed in the literature.¹⁹⁴ Zhu *et al*¹⁴¹ had recently claimed to have developed a

planar BODIPY dye capable of acting as a molecular rotor that targeted the plasma membranes of live cells, and so we set out to synthesise and characterise this dye, in order to investigate whether it could be a molecular rotor capable of targeting the L_o phase.

After first synthesising this planar BODIPY dye, which we termed “PlanB”, we investigated its photophysical properties in methanol/glycerol solutions of varying viscosity and temperature. As reported by Zhu *et al*,¹⁴¹ the fluorescence quantum yield and lifetime were found to increase with increasing solution viscosity, although there was very poor overlap between different methanol/glycerol solutions on the plot of quantum yield against viscosity. We initially hypothesised that PlanB may be polarity sensitive, but absorption and emission spectra of PlanB in solvents of varying polarity revealed only a small change in emission and absorption maxima, suggesting that polarity would be unlikely to have such a profound effect on the emission of PlanB.

It has been previously speculated that BODIPY dyes such as PlanB may undergo reactions with nucleophiles such as methanol, yielding a highly emissive hemiacetal product.²⁴⁵ We therefore investigated whether this reaction may have been taking place in the methanol/glycerol solutions, leading to a perceived sensitivity to viscosity. By dissolving PlanB in methanol and taking emission spectra over a 90 minute period, we showed a marked increase in the emission intensity, suggesting that this reaction was indeed taking place. The potential for this side reaction therefore makes PlanB highly unsuitable for use as a molecular rotor in biological environments, where nucleophiles such as water are highly abundant.

Despite this initial discouraging result, we went on to investigate whether PlanB could at least behave as a molecular rotor within a lipid bilayer. Previous computational studies^{139,141} had indicated that BODIPY dyes such as PlanB may be able to display viscosity sensitivity, and so we hypothesised that structurally similar but less reactive probes may still have some utility as probes of lipid bilayer sensitivity, especially if they could target the L_o phase of a phase-separating bilayer. Lifetime experiments in DOPC LUVs, however, showed a very small reduction in the length of the fluorescence decay of PlanB at increased temperature, meaning that there was no detectable reduction in fluorescence lifetime, suggesting that PlanB cannot be successfully used as a molecular rotor within this viscosity range. Experiments in L_o - L_d phase-separated GUVs also showed that PlanB does not partition into the L_o phase, much like the other BODIPY rotors discussed in earlier chapters.

Due to these discouraging results, we decided that the synthesis of a BODIPY rotor that could overcome these serious shortcomings would be a very challenging endeavour, and thus turned our attention away from alternative BODIPY rotors. We instead chose to focus on a recently-synthesised

thiophene-based dye that was known to target the plasma membranes of live cells.¹⁴⁰ These thiophene-based dyes had been found to show an increased fluorescence quantum yield and lifetime in the plasma membranes of live cells compared with free solution, and we therefore hypothesised that they may be capable of acting as molecular rotors. Given their planar structure, we also hypothesised that they may be capable of partitioning more equally than BODIPY probes between the L_o and L_d phases of phase-separating lipid bilayers. We therefore began work on a thiophene-based dye that we termed “thiophene rotor”.

Due to the highly polarity-sensitive nature of its fluorescence emission and the fact that it is insoluble in viscous nonpolar solvents such as castor oil, we decided to use DOPC LUVs as a calibration system for thiophene rotor. Emission spectra taken at a range of temperatures showed a decrease in emission intensity with increasing temperature, consistent with the behaviour expected of a molecular rotor. We then compared the fluorescence lifetimes of thiophene rotor in DOPC LUVs at a range of temperatures with those of BODIPY-C++, in order to generate a calibration plot of bilayer viscosity against lifetime, which was linear on a double logarithmic scale, consistent with other molecular rotors. Molecular dynamics simulations revealed that thiophene rotor and BODIPY-C++ have similar orientations within DOPC bilayers, legitimising the comparison between thiophene rotor and BODIPY-C++ in DOPC LUVs in the calibration plot.

As further evidence that thiophene rotor acts as a molecular rotor, we incorporated it into DPPC LUVs and determined its fluorescence lifetime at a range of temperatures. At the gel transition temperature of DPPC, a large change in the fluorescence lifetime of thiophene rotor was observed, corresponding to the change in viscosity between a liquid and a gel phase bilayer, and suggesting that thiophene rotor is viscosity sensitive, rather than temperature sensitive.

We then went on to investigate the effect of cholesterol on the fluorescence lifetime of thiophene rotor. In both DOPC/Cholesterol and DPPC/Cholesterol LUVs, we observed an increased thiophene rotor lifetime. Whilst this was expected in DOPC, where cholesterol has a condensing effect, leading to an increased viscosity,¹⁸⁸ cholesterol disrupts the tight packing of lipids within the gel phase, leading to the formation of an L_o phase,¹⁸⁸ meaning that a reduction in viscosity should occur in DPPC/Cholesterol mixtures. Molecular dynamics simulations were used to rationalise this observation, revealing that the addition of cholesterol to the bilayer causes a shift in the position of thiophene rotor within the bilayer. Whilst this precludes the use of the DOPC calibration plot to generate an exact viscosity value in cholesterol containing bilayers, thiophene rotor still showed a large difference in fluorescence lifetime between DOPC/cholesterol (L_d phase) and DPPC/cholesterol

(L_o phase) LUVs, and so we hypothesised that it could discern between coexisting L_o and L_d phases in phase-separating model bilayers.

We therefore incorporated thiophene rotor into phase-separating DOPC/DPPE/cholesterol GUVs and investigated them using confocal microscopy and FLIM. Unlike the BODIPY based molecular rotors studied in earlier chapters, thiophene rotor was found to partition approximately equally between the two phases, showing a large difference in lifetime between them. This suggested that it may even be able to detect ordered phases within the plasma membranes of live cells, which are highly contentious and notoriously difficult to detect using conventional microscopy techniques.

To investigate this, we cultured four different cell lines with thiophene rotor, finding that it does indeed target the plasma membranes. By performing FLIM, we showed that the lifetime of thiophene rotor was close to halfway between its lifetime in model L_o phases and model L_d phases, which could be interpreted as evidence of the existence of suboptical phase separation within each pixel of the lifetime image. To further investigate this, phasor analysis was used, which is a model-free method of comparing fluorescence decays. A phasor plot of thiophene rotor in the plasma membranes of cells compared with model L_o and L_d phases showed the fluorescence decays from the cells to be on the straight line between the model L_o and L_d phases, further suggesting a degree of L_o-L_d phase separation within the plasma membranes of the cells. This observation would be supported by recent developments in the literature,^{35,75,253} which have suggested a degree of transient suboptical phase separation within the plasma membrane.

In conclusion, this thesis describes the use of molecular rotors to investigate viscosity and lipid ordering within both model and biological membranes. We show that molecular rotors can accurately determine microscale viscosity within lipid bilayers and can be used in a variety of biological systems, ranging from *E. coli* bacteria to eukaryotic cells. We demonstrate the shortcomings of conventional BODIPY-based molecular rotors to image phase separation in lipid bilayer, and attempt to overcome this by synthesising an alternative BODIPY rotor, which was beset by several limitations. Through the use of a thiophene-based molecular rotor, we find evidence of suboptical phase separation within the plasma membranes of live cells.

8. Proof of concept applications and preliminary studies

8.1 Overview

This thesis has explored the use of molecular rotors to investigate structure and ordering within biological membranes. We concluded that molecular rotors provide a novel and useful platform to investigate structuring within membranes, with several advantages over other contemporary methods. In this Chapter we describe preliminary studies for future investigations using molecular rotors.

First, the use of molecular rotors to explore the effects of hydrostatic pressure on lipid structuring is discussed. We outline the design of a new apparatus for performing fluorescence spectra at high pressures and then describe preliminary work using BODIPY-C10 in LUVs to investigate pressure-induced phase transitions. Next we discuss the use of membrane-targeting molecular rotors such as BODIPY-C++ and thiophene-rotor to investigate the effects of shear stress on the viscosity of the plasma membrane and outline some preliminary FLIM studies. Finally we discuss the use of molecular rotors to investigate changes in erythrocyte membrane viscosity upon invasion by malarial parasites

8.2.1 The use of molecular rotors to investigate the effects of pressure on viscosity within lipid bilayers

Throughout this thesis, temperature has been widely employed as a thermodynamic variable in order to influence the viscosity of lipid bilayers, and the effect of temperature on the phase behaviour of lipids has been well studied.²⁵⁹ The use of high hydrostatic pressure to alter the phase behaviour of lipids is less well-studied, perhaps owing to the experimental difficulties associated with these techniques.²⁶⁰ The use of hydrostatic pressure as a thermodynamic variable does, however, offer several key advantages over temperature as a variable for probing lipid phase behaviour. Unlike temperature, pressure will not usually disrupt intramolecular bonding (below *ca.* 2 GPa), although large pressure changes can cause some proteins to denature.²⁶¹ Pressure can also be applied and released from a sample very rapidly, as pressure waves travel at the speed of sound.²⁶⁰ The effect of an increase in pressure tends to be the opposite of that of an increase in temperature and the pressure dependence of a lipid phase transition temperature, T_t , can be determined using the Clapeyron equation (Equation 8-1):

$$\frac{dT_t}{dP} = \frac{\Delta V_m}{\Delta S_m} = \frac{T_t \Delta V_m}{\Delta H_m} \quad (8-1)$$

where ΔS_m , ΔH_m and ΔV_m are the molar transition entropy, enthalpy and volume changes, respectively. Note that this equation is only valid at the phase boundary where the free energy change (ΔG) for the transition is zero.²⁶⁰

Applying pressure to any system will promote processes that lead to a reduction in volume for the system, causing an increase in chain ordering and reduction in motion of the hydrophobic tail groups within a lipid bilayer. Because the lipid head group is less influenced by pressure than the tail region, an increase in pressure will typically also lead to an increase in bilayer thickness (Figure 8-1). Pressure can therefore be used to drive phase transitions within lipid systems and can even be used to induce phase separation into coexisting lipid domains.^{262,263} Due to the high speed in which

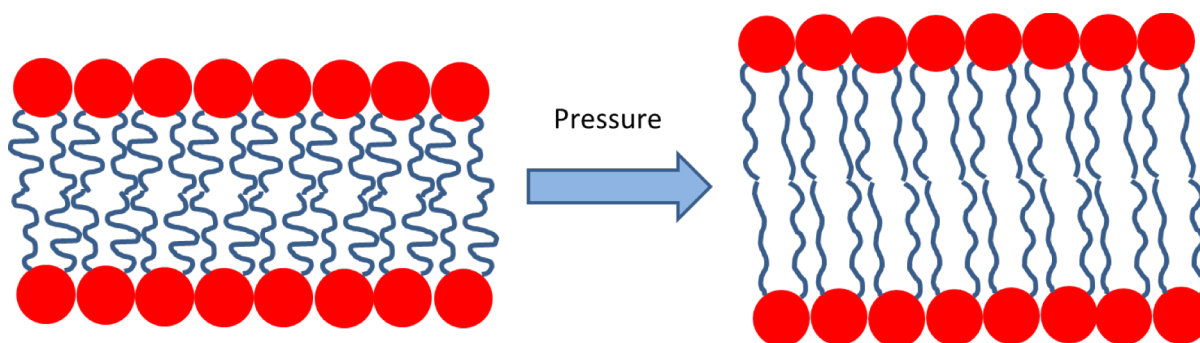


Figure 8-1 Schematic of the effect of an increase in pressure on a lipid bilayer²⁶⁰

pressure can be applied and released, the use of pressure jump techniques can allow the decoupling of the thermodynamic trigger variable from the structural or environmental change allowing the real time kinetics of these changes to be studied.²⁶⁰

In these preliminary studies we use molecular rotors to investigate the effects of hydrostatic pressure on viscosity within lipid bilayer systems, with future work aiming to quantify the effects of pressure on membrane viscosity and to also investigate the kinetics of domain formation with increased pressure. Recent research has focused on the use of fluorescence microscopy at high hydrostatic pressure to investigate the effects of pressure on bending rigidity²⁶⁴ and phase separation²⁶⁵ within model bilayers, and we propose using molecular rotors to expand this work and to link it to a quantitative assessment of the changes in viscosity associated with these processes.

As initial work to facilitate these experimental goals, we have developed a cell capable of recording emission spectra at hydrostatic pressure up to 150 MPa. A schematic of the system is shown in Figure 8-2 and is discussed in more detail in Chapter 2. The pressure cell itself consists of a high tensile strength steel body with a central cavity that contains the sample. The sample chamber is sealed on two sides by transparent sapphire windows capable of withstanding pressures up to *ca.*

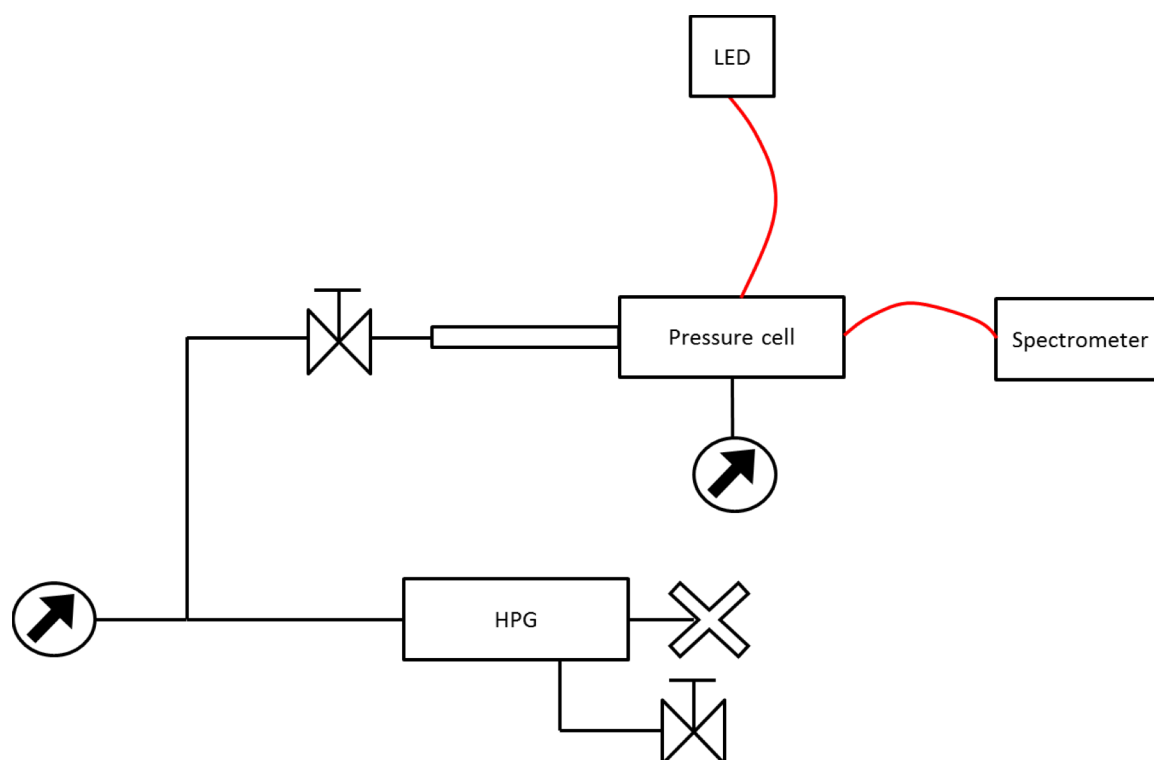


Figure 8-2 Schematic of the design of the cell designed for obtaining emission spectra at high hydrostatic pressures

150 MPa, which are then mounted on metal window holders. These are connected to fibre optic cables that lead to an LED that acts as an excitation source and a spectrometer that records the spectra. The sample chamber is also connected to a pressure transducer that converts pressure into voltage, allowing accurate measurement of the pressure within the sample chamber. Finally, the sample chamber is connected a water-filled pressure network via a pressure separator that prevents mixing of the sample with the water from the network. Pressure is generated using a hand-operated pump, and water is fed into the system via a valve attached to this pump. This system provides an inexpensive method of recording spectra at high pressures and its modular nature makes it highly flexible; different LEDs allow a variety of excitation wavelengths and different spectrometers could also be potentially used for different applications. Additionally, this system also potentially allows the incorporation of other more complex systems, such as pressure jump apparatus.

As initial experiments using this cell and with the assistance of Leanne Baker, we incorporated BODIPY-C10 into LUVs consisting of lipids with varying gel-to-liquid phase transition temperatures and obtained emission spectra at a range of hydrostatic pressures. Figure 8-3 shows emission spectra at a range of pressures for LUVs consisting of DOPC ($T_m = -16.5\text{ }^{\circ}\text{C}$)²⁶⁶, DMPC ($T_m = 24\text{ }^{\circ}\text{C}$)¹⁷³, SOPC ($T_m = 6.7\text{ }^{\circ}\text{C}$)²⁶⁷ and POPC ($T_m = -2\text{ }^{\circ}\text{C}$)¹⁷³ and BODIPY-C10. In all cases, increasing pressure leads

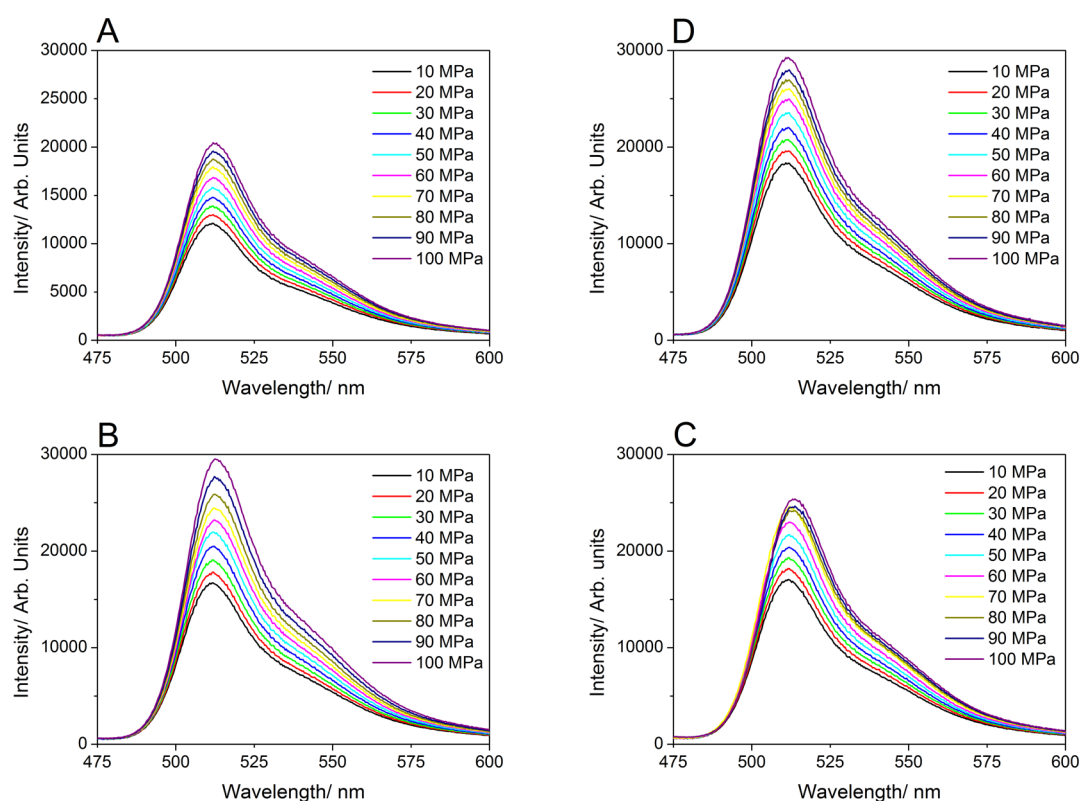


Figure 8-3 Overlaid fluorescence spectra of BODIPY-C10 in LUVs composed of (A) DOPC, (B) POPC, (C) SOPC and (D) DMPC at a range of pressures

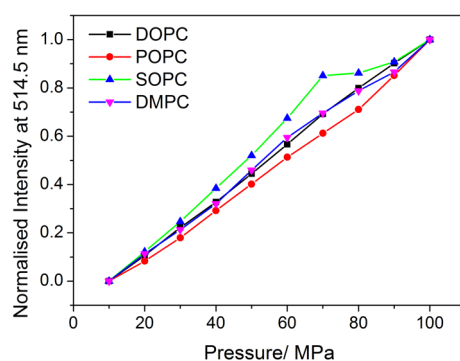


Figure 8-4 Maximum emission intensity of BODIPY-C10 in LUVs composed of different lipids at a range of pressures

to an increase in the emission intensity, suggesting that increasing pressure leads to an increased bilayer viscosity. A plot of the BODIPY-C10 maximum emission intensity at 514 nm with pressure (Figure 8-4) shows a steady increase in emission intensity with increasing pressures for all lipids except SOPC, which shows a sharp change in gradient at 70 MPa that may correspond to a transition into the gel phase. Of the lipids that form a liquid phase at room temperature, SOPC has the highest gel transition temperature, and so would be expected to show a transition into the gel phase at the lowest pressure. Interestingly, DMPC shows a similar gradient in the plot of maximum emission intensity against pressure despite being in the gel phase at room temperature, suggesting a similar relative change in viscosity with pressure for the gel phase as in the liquid phase.

Further work using this high pressure emission spectrometer could focus on determination of the gel transition pressures of a number of lipids, especially if the system can be strengthened to withstand even greater pressures. By adapting the cell to allow spectra to be taken at different temperatures, further work to investigate the effects of both temperature and pressure on the phase behaviour of different lipid systems may become possible. Furthermore, if this high pressure cell can be adapted for use with TCSPC, it will allow quantitative determination of the change in bilayer viscosity with pressure.

Finally, if high pressure equipment can be adapted for use with a confocal microscope setup, it could allow the use of FLIM in conjunction with molecular rotors to investigate viscosity and phase behaviour within a range of different membrane systems and biological samples. Of particular interest may be the membrane viscosity of barophilic bacteria, which are believed to adjust their membrane composition to maintain a constant membrane viscosity based on the pressure of their surrounding environment.²⁰⁸

8.2.2 The effects of shear stress on the plasma membrane viscosity of endothelial cells

Atherosclerosis is a condition where the artery walls thicken as a result of the formation of atherosclerotic plaques within the arteries,²⁶⁸ and is currently one of the leading causes of death in the developed world.²⁶⁹ The formation of atherosclerotic plaque in the vascular endothelium is believed to be related to changes in endothelial cell plasma membrane viscosity that occur as a response to the hydrodynamic shear stress imparted on the vascular wall by the flow of blood.²⁷⁰ A previous study⁴⁷ using the julolidine-based molecular rotor DCVJ showed that plasma membrane viscosity decreases with increased shear stress and vice versa. White and Frangos²⁷⁰ hypothesised that shear stress can destabilise the plasma membrane, leading to a decrease in plasma membrane viscosity which in turn causes the activation of various secondary signalling cascades.

Atherosclerotic plaque is believed to primarily form in areas of weaker hydrodynamic shear stress, where blood flow is slow and changes directions, whereas vessel regions with steady blood flow that are subjected to a higher and constant shear stress tend to remain disease free.²⁷¹ It therefore follows that shear stress plays an important role in the formation of atherosclerotic plaque, which may be related to the plasma membrane viscosity of the endothelial cells.²⁷²

A detailed understanding of the effects of shear stress on the viscosity of the plasma membranes of endothelial cells could therefore allow us to better understand the development of atherosclerosis, allowing early diagnosis and treatment of the condition. This study could build on the earlier work with DCVJ performed by Haidekker *et al*,⁴⁷ and could use lifetime imaging of molecular rotors to provide a quantitative picture of the effect of shear stress on the plasma membrane viscosity of endothelial cells. This could then be contrasted with the effects of shear stress on other cell lines, in order to investigate whether this effect of shear stress is unique to endothelial cells or is a physical effect that affects all plasma membranes.

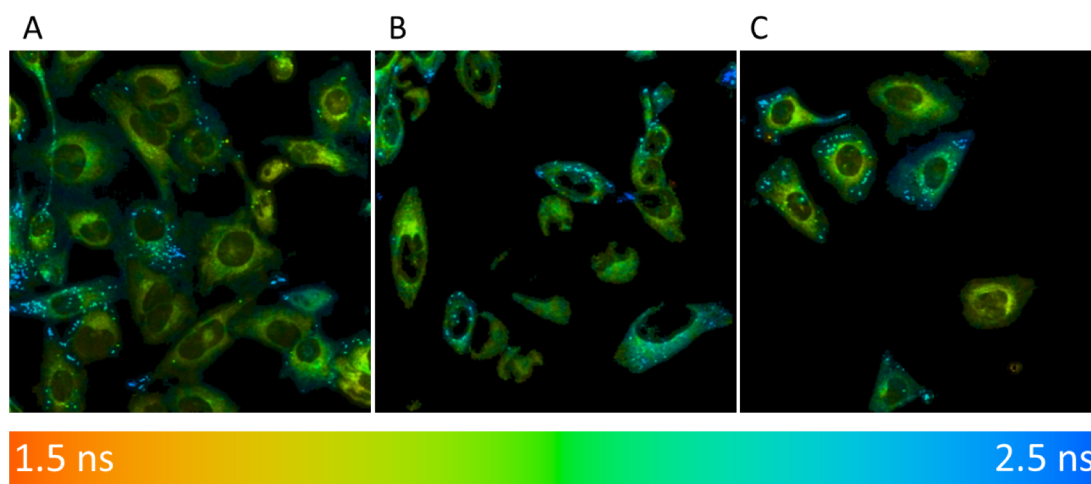


Figure 8-5 Representative lifetime images of HeLa cells stained with BODIPY-C10 under flow - (A) no flow, (B) 1 relative flow, (C) 2 relative flow, where 1 relative flow provides an arbitrary amount of shear stress, and 2 relative flow is double that. Future work will aim to quantify levels of shear stress in N m^{-2}

To this end, we performed initial experiments on live HeLa cells cultured in a flow chamber which allows media to be passed over the cells at differing levels of shear stress. Figure 8-5 shows example lifetime images of HeLa cells stained with BODIPY-C10 under varying amounts of flow (note that the shear stress was not accurately quantified in N m^{-2} and is thus referred to as “relative flow”). As previously reported,¹²⁴ BODIPY-C10 rapidly internalises, staining the cell interior, and the summed histograms of 5 lifetime images at different relative amounts of flow shown in Figure 8-6 suggest no effect of shear stress on the viscosity of the cell interior. This is perhaps not unexpected as the BODIPY-C10 probe does not stain the plasma membrane and it may be unlikely that shear stress would have a large impact on the viscosity of organelles within the cell interior.

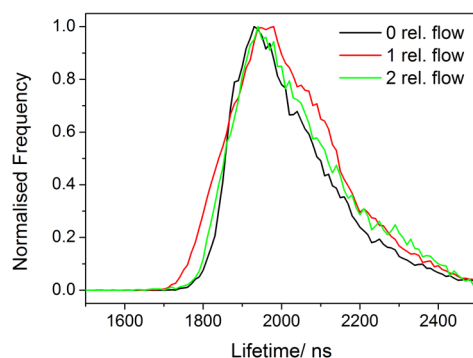


Figure 8-6 Summed normalised histograms showing the lifetime distributions for HeLa cells stained with BODIPY-C10 at differing rates of relative flow

Further experiments using BODIPY-C++, which is known to briefly stain the plasma membrane in cold media,¹³⁵ were less successful however. Figure 8-7 shows sample lifetime images of HeLa cells under flow, with a 10 μ M solution of BODIPY-C++ within the cell media in order to achieve membrane staining. The lifetime images show poor membrane staining however, which prevented accurate measurement of membrane viscosity under flow. Additionally, the large amount of cell media required for the flow cell experimental setup used a large amount of the BODIPY-C++ dye, which would be unsustainable for a long term experimental plan. BODIPY-C++ must stay in the medium to allow membrane staining, as incubation with BODIPY-C++ followed by flow with media with no BODIPY-C++ would lead to BODIPY-C++ leaching from the plasma membrane, due to its water solubility.

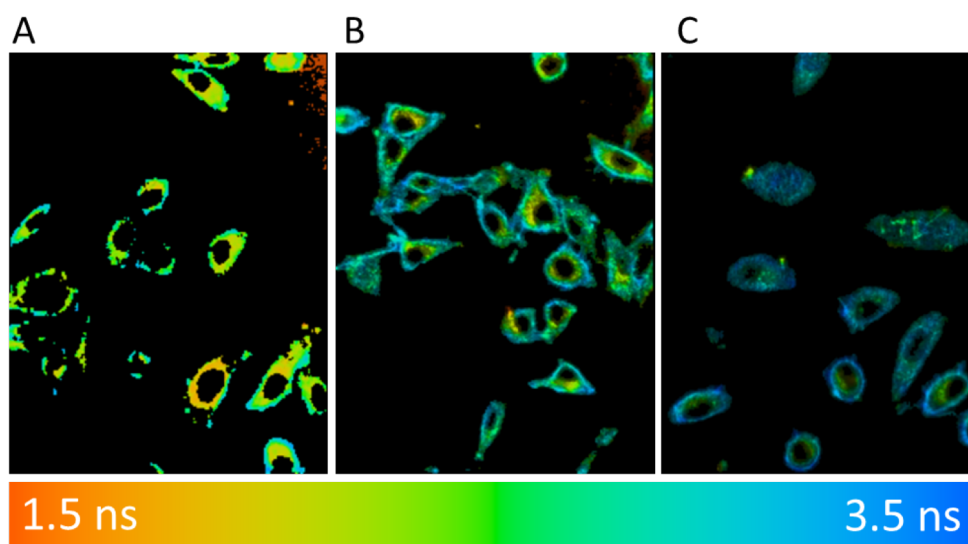


Figure 8-7 Representative lifetime images of HeLa cells stained with BODIPY-C++ under flow - (A) no flow, (B) 1 relative flow, (C) 2 relative flow

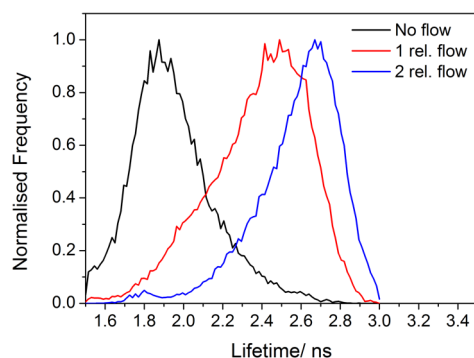


Figure 8-8 Summed histograms for 5 lifetime images of HeLa cells under flow stained with BODIPY-C++

There was, however, a noticeable increase in the fluorescence lifetime of BODIPY-C++ with increased flow (see Figure 8-8 for summed histograms of the HeLa cells under different levels of flow). This implies that the viscosity of the HeLa plasma membranes was increasing with flow, in contrast to the results presented by Haidekker *et al.*⁴⁷

A potential solution to the problems associated with this experimental setup could be the development of a flow cell that allowed a significantly lower amount of media to be used, and for the flow to be applied rapidly before the BODIPY-C++ dye could internalise into the cell interior, allowing FLIM of the plasma membrane under flow. A way of achieving this this could be a setup consisting of a microfluidic flow cell. A microfluidic chamber with flow potentially generated using automated syringe pumps would allow a significantly reduced amount of media. Additionally, previous research has demonstrated the potential of microfluidic devices as low-cost flow cells for use within biological applications.^{273,274}

If such a system were developed with an accurate method of measuring the exact flow within the cell, BODIPY-C++ could then be used to give an accurate idea of the exact change in viscosity of the plasma membranes of endothelial cells under varying levels of shear stress, with the aim of better understanding the formation of atherosclerotic plaques in model arteries.

8.2.3 The use of BODIPY-based molecular rotors to investigate structural changes within erythrocyte plasma membranes upon invasion by malarial parasites

Malaria is a mosquito-carried disease that is widespread in the Earth's tropical regions and is a major cause of mortality in the developing world.²⁷⁵ The disease itself is caused by parasitic protozoans from the *Plasmodium* genus, with the majority of deaths from malaria being caused by *Plasmodium falciparum*.²⁷⁶ Infection by the *Plasmodium* parasite begins with a bite from an infected female mosquito. After a brief silent infectious phase, merozoite forms of the parasite are passed into the bloodstream, allowing them to access circulating red blood cells (erythrocytes). The merozoites rapidly invade erythrocytes, and as they grow and replicate the intracellular parasites dramatically alter the structure of the cell, making it rigid and poorly deformable with a tendency to adhere to other cells.²⁷⁷ The new merozoites from the replication process then rupture the cell and begin to invade other erythrocytes, repeating this process. It is this process that gives rise to the symptoms of malaria.²⁷⁸

Much work has gone into understanding the mechanism of erythrocyte invasion by the merozoite form of the *Plasmodium* parasite. The parasite initially binds to the erythrocyte surface and reorients itself so that its apex directly contacts the erythrocyte plasma membrane, before forming a "tight" junction in the membrane, which is essentially a pore in the membrane that allows invasion of the host cell.²⁷⁹ This process is accompanied by a buckling of the erythrocyte structure, believed to be as a result of changes in the structure of the erythrocyte cytoskeleton as well as a reorganisation of the plasma membrane lipids.²⁷⁸ Once this tight junction has been formed, the parasite then forces its entry into the cell interior via an actomyosin motor.²⁷⁷

Whilst the parasite surface proteins that help to drive this process are well understood,²⁷⁷ the biophysical changes that occur in the erythrocyte plasma are not. Recent evidence²⁷⁹ has suggested that these biophysical changes may be driven by the parasite in order to make invasion more energetically favourable, a phenomenon that is well studied as a strategy for cell entry in viruses.^{280,281} Additionally, there is evidence suggesting that the binding of certain erythrocyte membrane proteins, such as glycophorin A^{282,283} and complement receptor 1,²⁸⁴ both of which are bound during merozoite invasion,²⁷⁷ can change membrane biophysical properties, such as membrane deformability.

We therefore propose an experiment where molecular rotors are incorporated into the plasma membranes of erythrocytes, in order to investigate the changes in membrane viscosity and lipid ordering upon merozoite invasion. This experiment could allow us to better understand the physical changes that occur within the erythrocyte plasma membrane upon invasion and to challenge the

current dogma that merozoite invasion is completely driven by the parasite rather than as balance between parasite and host cell contributions.²⁷⁹

As an initial experiment in collaboration with Marion Koch and Dr Katherine Wright, we stained donor erythrocytes with BODIPY-C10 and performed FLIM. Unlike other eukaryotic cells, BODIPY-C10 did not appear to be internalised and stained the plasma membranes of the cells, which is likely due to the simple structure and reduced cell machinery of erythrocytes compared with other cells. The fluorescence decays of BODIPY-C10 appeared monoexponential, and Figure 8-9 (A) shows a representative lifetime image of BODIPY-C10 stained erythrocytes. We initially varied the concentration of BODIPY-C10 within the media in order to investigate whether there was any concentration dependence on lifetime, which would show evidence of aggregation-induced quenching that may be expected at lipid:BODIPY ratios of less than 200:1,⁴¹ and the results are shown in Figure 8-9 (B). BODIPY-C10 concentrations of 1.0 and 0.5 μM showed very similar lifetime distributions, with a mean lifetime of *ca.* 1.9 ns suggesting that no aggregation was occurring.

We then introduced three proteins to erythrocytes stained with 1 μM BODIPY-C10: EBA, RH5 and RH2, which are known to be involved in the mechanism of parasite invasion,²⁷⁷ in order to investigate whether they had any effect on the viscosity of the erythrocyte plasma membrane. Figure 8-10 shows representative lifetime images of erythrocytes stained with BODIPY-C10 with the three malarial proteins. Summed histograms of 4 lifetime images, relative to erythrocytes with no malarial protein are shown in Figure 8-11.

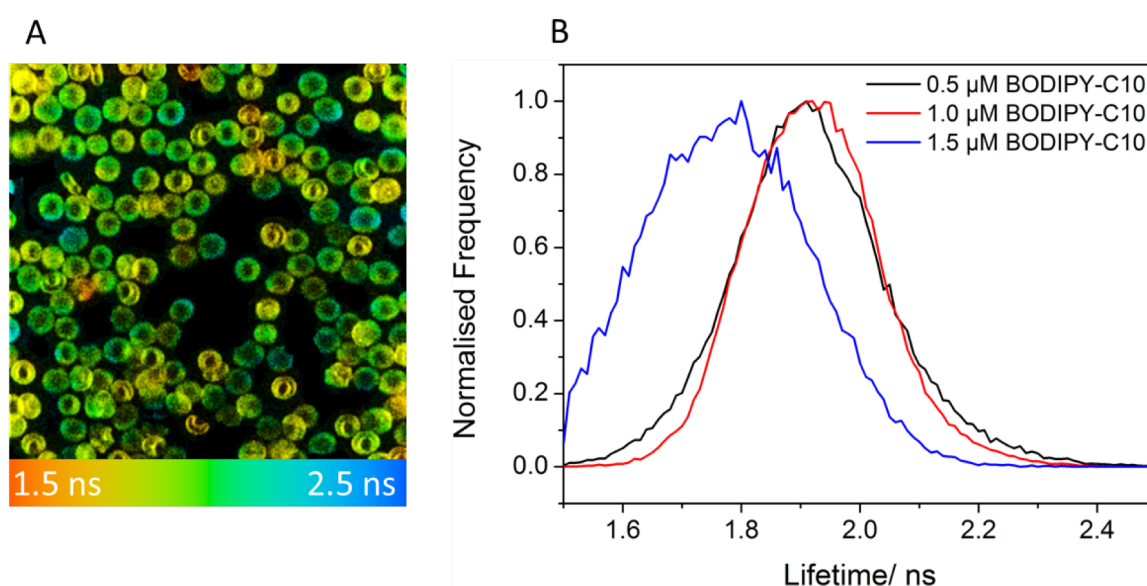


Figure 8-9 (A) Example lifetime image of erythrocytes stained with 1.0 μM BODIPY-C10, **(B)** summed and normalised histograms showing the lifetime distribution of 10 lifetime images of erythrocytes stained with varying BODIPY-C10 concentration

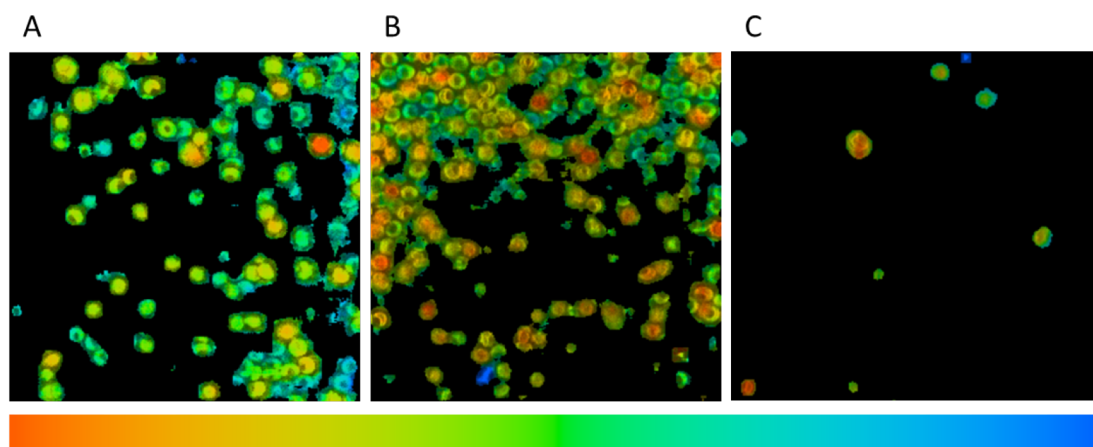


Figure 8-10 Sample lifetime images of erythrocytes stained with BODIPY-C10 and incubated with the malarial proteins (A) RH5, (B) RH2 and (C) EBA

In all cases, introduction of a protein caused a reduction in the quality of the fitting, with χ^2 values for monoexponential fitting in the range of 2-3. Additionally, the EBA protein, which binds to receptors in the erythrocyte plasma membrane,²⁷⁷ caused a significant clustering of the erythrocytes. Whilst there was an overall decrease in the calculated BODIPY-C10 lifetime for the EBA and RH2 proteins compared with erythrocytes with no malarial protein present, it is difficult to conclude whether this was caused by a reduction in the erythrocyte plasma membrane viscosity associated with the binding of the protein, or whether it was an artefact of the reduced fitting quality. We therefore investigated the cause of this reduction in fitting quality.

Red blood cells are strongly pigmented due to their haemoglobin content, and may therefore display a strong autofluorescence.²⁸⁵ Haemoglobin has an absorption profile²⁸⁶ that overlaps with that of BODIPY-C10 (maximum absorption wavelength = 495 nm, see Chapter 3), as well as a complex fluorescence decay profile.²⁸⁷ This autofluorescence may therefore contribute towards the

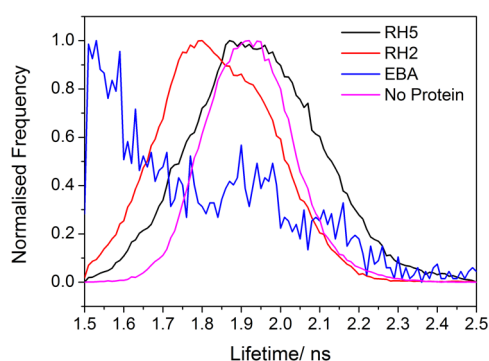


Figure 8-11 Summed histograms of erythrocytes stained with BODIPY-C10 with malarial parasites believed to be involved in parasite invasion. The data for EBA protein is a result of poor fitting, potentially due to erythrocyte autofluorescence

fluorescence signal of the erythrocytes stained with BODIPY-C10, affecting the calculated fluorescence lifetime. In order to investigate the magnitude of this autofluorescence, we performed FLIM of erythrocytes with no BODIPY-C10 (Figure 8-12). We found that autofluorescence gave a significant photon count in the lifetime image, meaning that it would provide a major contribution to the fluorescence decay of erythrocytes stained with BODIPY-C10, and may also cause a change in lifetime based on the concentration of BODIPY-C10 within the cells as autofluorescence will influence the fluorescence decay to a varying extent based on the BODIPY-C10 concentration.

Based on this, it therefore becomes very difficult to separate erythrocyte autofluorescence from the fluorescence signal from the BODIPY-C10 probe, preventing us from being able to confidently assert that any change in BODIPY-C10 lifetime upon introduction of malarial proteins is caused by changes in the viscosity of the erythrocyte plasma membrane.

There are a number of potential solutions to this problem of autofluorescence. Erythrocyte ghosts,²⁸⁸ which are cells that have been porated and resealed to remove as much intracellular structure as possible leaving only the plasma membrane, may be an answer, as much of the haemoglobin will have been removed from the cell. However, because the cell has been porated, the original membrane will likely not be intact, meaning that studies on the mechanism of merozoite invasion may not accurately represent the erythrocyte plasma membrane. Another alternative

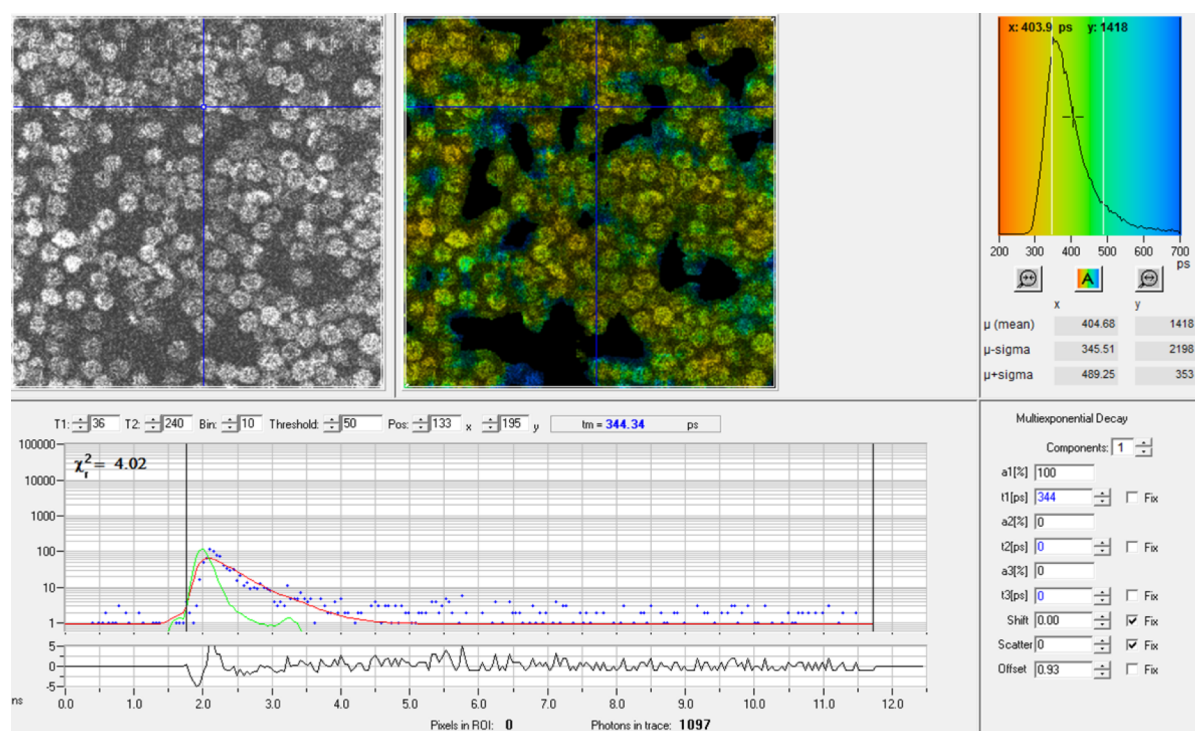


Figure 8-12 Screenshot of SPCImage FLIM processing software showing erythrocytes with no BODIPY-C10, in order to highlight autofluorescence

solution could involve the use of molecular rotors whose spectral emissions overlap less with the haemoglobin autofluorescence, allowing an easier decoupling of rotor fluorescence and autofluorescence. Whilst red-shifted BODIPY dyes based on the structure of BODIPY-rotors are believed to be sensitive to temperature rather than viscosity,¹²⁸ other molecular rotors with more red-shifted emission, such as the thiophene rotor discussed in Chapter 6 or the porphyrin dimers reported by Vyšniauskas *et al*¹⁰⁸, may prove to be useful.

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