

*Drug interactions with lipid membranes:
the effects of drug and lipid structure upon
rates of ester hydrolysis*

Duncan Robert Casey

Department of Chemistry, Imperial College, London

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All work is original and that of the author, except where noted. All outside works referred to are listed in Section 9, page 204.

1 Abstract

The transport of compounds around the body has been a topic of interest for many years, and the advent of non-invasive biological imaging in living tissue has made huge advances in the characterisation and localisation of cellular receptors for use in drug targeting.

However, there remains a significant paucity of knowledge regarding how the majority of drug molecules are transported about the body, when they often exhibit negligible aqueous solubility and the body expresses no trans-membrane pumps or chaperone proteins that recognise them and facilitate their movement. This leads to large attrition rates in drug discovery programmes, as compounds with high binding constants or inhibitive activity *in vitro* fail to perform *in vivo*, due to poor bioavailability or non-specific sequestration away from the tissue of interest.

In this study, the interactions between a number of drug and lipid molecules were investigated and the effects upon both the lipids' chemical and bulk membrane structures were analysed. This revealed some of the mechanistic causes of the previously observed hydrolytic activity a number of common drug compounds exhibit toward lipid membranes and identified parameters affecting the observed rates of reaction. The findings also suggest approaches by which this behaviour might be predicted, or even tuned to deliver optimum pharmacological characteristics.

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Finally, I'd like to thank my parents for not throwing me out when it became clear that I was delaying getting a real job for another four years, and the innumerable friends and family who have at various points humoured me when I got geekily excited by something, kept me sane or paid for my coffee/beer/rent when I needed it. They are too many to name but without them, this wouldn't have happened. Thanks, gang.

3 Introduction

(Adapted from A. Seddon, D. Casey *et al.*, Chem. Soc. Rev., 2009, 38, 2509–2519¹)

Understanding the mechanism at the molecular level by which drugs interact with cell membranes is of critical importance in pharmacological science. Many drugs, displaying a wide range of applications and with diverse structures, can cross or bind to lipid membranes and potentially modulate the physical properties of that membrane. However, the cell membrane is a carefully balanced environment and any changes inflicted upon its structure by a drug molecule must be considered in conjunction with the overall effect that this may have on the function and integrity of the membrane.

3.1 The Cell Membrane

3.1.1 Eukaryotic cell membranes

The plasma membrane of all cells is comprised of a double leaflet of lipids organised into a bilayer structure, and maintains the difference in electrolyte concentrations and electric field gradient between the extracellular environment and the cytosol within the cell. In eukaryotic cells, the interior of organelles such as the endoplasmic reticulum, the Golgi and the mitochondria are separated from the cytosol by a second membrane. This allows cells to maintain differences in pH or ionic strength inside organelles, especially in the case of mitochondria which rely on their internal membranes to isolate the harmful reactive oxygen intermediates formed during the synthesis of ATP.

3.1.2 Lipid composition of membranes

Biological membranes consist of a back-to-back arrangement of amphiphilic lipid molecules. The interior of the plasma membrane is comprised of hydrophobic fatty acid tails, with the hydrophilic lipid head-groups pointing outwards to the extracellular and cytosolic regimes. Although the hydrophobic interior acts as a barrier to the passage of polar molecules and ions in or out of the cell, a modulated flux of these species is required for the cell to function, meaning

regulated mechanisms by which molecules can cross the membrane are vital. The mechanisms by which molecules can enter or leave the cell will be discussed in detail below.

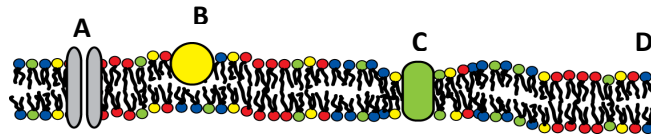


Figure 1: The fluid lipid bilayer structure is comprised of a variety of phospholipids, sphingolipids and sterols and contains channels and membrane-associated proteins. (A) membrane channel; (B) surface associated membrane protein; (C) transmembrane protein; (D) the lipid bilayer, with the hydrophilic lipid head-groups represented as coloured circles and the fatty acid tails as black lines¹.

The lipids that make up biological membranes are highly varied in terms of their head-group, chain length and degree of saturation. These membranes are approximately 5nm thick and are studded throughout with a range of trans-membrane and membrane-associated proteins, as shown above in Figure 1. The role of lipids *in vivo* extends beyond that of structural compartmentalisation as they are involved in cell signalling pathways and implicated in a number of disease pathologies. Furthermore, the composition of cellular membranes can be altered rapidly in response to environmental stimuli². A comprehensive overview of biological membranes and their structure has been written by van Meer *et al.* and references cited therein².

Recent research has shown that there is greater underlying structure to the lipid bilayer and that lateral ordering within the bilayer may play an important role in certain biological processes. The formation of microdomains, (or rafts), for example, is hypothesized to be involved in signalling complexes³. While the existence of rafts has been demonstrated in model membranes, their existence *in vivo* remains controversial. A full discourse of the intricacies of membrane structure is beyond the scope of this thesis and the interested reader is directed to recent reviews on this topic.

The structure of the cell membrane is maintained by enzyme activity both within the membrane itself and in the cytosol, synthesising or digesting lipid molecules in order to conserve its integrity. The plasma membrane of eukaryotic cells consists predominantly (~50%) of the glycerophospholipid phosphatidylcholine, with smaller fractions of phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidic acid. An example is presented below,

in Table 1. To add to the complexity, each lipid class contains fatty acid chains of varying length and degree of unsaturation⁴. These can contribute significantly to the curvature of each individual lipid: double bonds in lipid biosynthesis are almost exclusively *cis*, meaning that a single unsaturated bond can dramatically increase the volume occupied by the fatty chain⁵.

Phospholipid class	% of total lipid phosphorus
Acylphosphatidylglycerol	<0.1
Bis(monoacylglycero) Phosphate	0.32
Diphosphatidylglycerol	6.25
Phosphatidylglycerol	0.22
Phosphatidylethanolamine	26.3
Phosphatidylcholine	46
Phosphatidylinositol	9.88
Phosphatidylserine	3.19
Sphingomyelin	3.92
Lysophosphatidylcholine	0.86
Others	3.07

Table 1: The relative composition of the phospholipid membranes found in rat liver homogenates⁶.

By contrast, bacterial membranes such as that of *E. Coli* are 70% phosphatidylethanolamine with phosphatidylglycerol species constituting the remaining majority². Literature values for the lipid compositions of different tissue and organelle membranes within an organism are available but these are often unreliable and subject to considerable variation between measurements. The search for the ‘lipidome’ is particularly hampered by its dynamic nature in response to external stimuli².

Another class of structural lipids are the sphingolipids are another class of structural lipids, which contain a ceramide backbone. Sphingomyelin is the major sphingolipid component of mammalian cells, as well as the glycosphingolipids containing mono-, di-, or oligosaccharides based on glucosyl or galactosylceramide. Sterols comprise the major class of non-polar lipids in cell membranes. In mammals, the predominant sterol is cholesterol². These common head-groups are illustrated in Figure 2, and their phase behaviour is discussed in depth below. A comprehensive review on the different lipids found in both eukaryotic and prokaryotic membranes is given by Dowhan⁷, and for an in-depth discussion of their structures and nomenclature see Fahy *et al.*⁸.

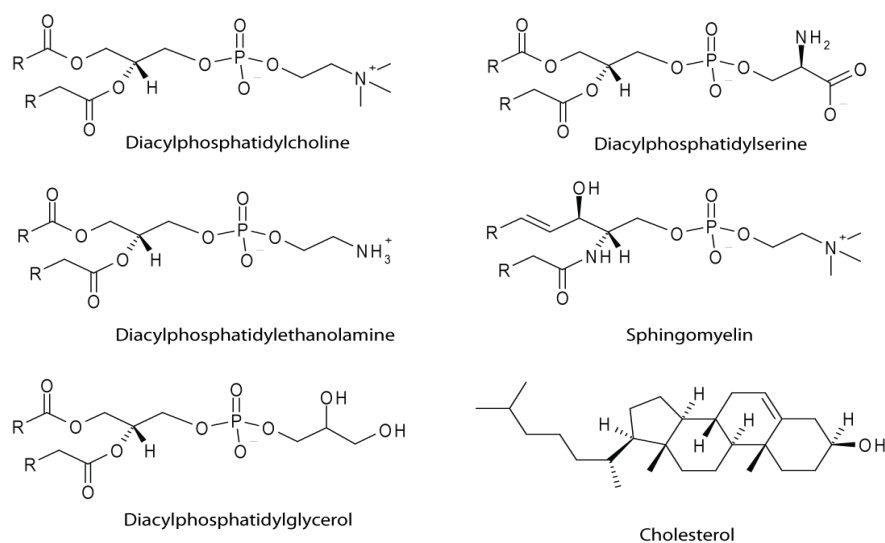


Figure 2: Head-group structures of commonly occurring membrane lipids and the structure of cholesterol. R indicates an alkyl chain.

3.1.3 Phase behaviour of lipids

Lipids can adopt a range of lyotropic phases in the presence of water, including the fluid lamellar, inverse hexagonal and inverse bicontinuous cubic phases⁹. In order to understand the formation of these phases, the total free energy of the system must be considered. Gruner *et al.*¹⁰ hypothesized that the total free energy of bilayer systems (g_{total}) is dominated by three contributions: the bending energy per unit area, g_c , the packing of the hydrocarbon chains, g_p , and g_{inter} which brings together hydration and electrostatic forces into a single free energy interaction term leading to:

Equation 1 ¹⁰

Under certain circumstances, the last term can be assumed to be, or experimentally arranged to be negligible, leaving the curvature elasticity and the packing of the hydrocarbon chains as the chief terms dictating the free energy of the system. The curvature elasticity is determined by reducing the lipid bilayer to an infinitely thin elastic surface. Deforming such a surface has an associated energy cost, which depends on changes in the mean curvature (Equation 2) and Gaussian curvature (Equation 3), where c_1 and c_2 are the principal curvatures of the surface under consideration and are illustrated in Figure 3.

Equation 2

Equation 3

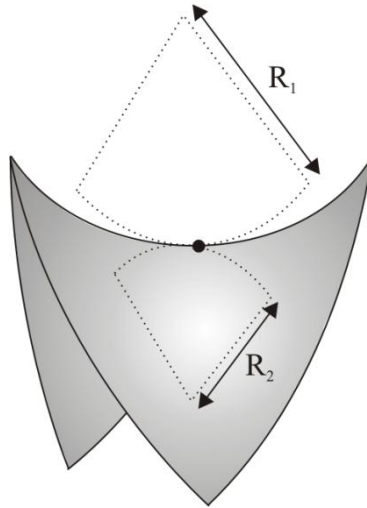


Figure 3: The radii of curvature of a surface, R_1 and R_2 . The principle curvatures of a surface, c_1 and c_2 , are given by the reciprocal of the radii of curvature of that surface, $1/R_1$ and $1/R_2$, respectively.

When combined with the bending modulus κ and Gaussian modulus κ_G which describe the energy cost per unit area of changing the mean and Gaussian curvature, this leads to the ‘Helfrich ansatz’ which describes the curvature elastic energy per unit area g_c for a membrane (Equation 4)¹⁰:

Equation 4

where H_0 is the mean curvature (or spontaneous mean curvature) of the surface when relaxed.

The spontaneous mean curvature depends on the distribution of lateral stresses acting on the membrane at varying depths (Figure 4a). The lateral pressure in the chain region which occurs due to thermally activated *trans-gauche* conformational changes is balanced by the interfacial tension at the polar / non-polar interface where the head-groups are attached to the chains. There will also be head-group interactions between lipids which may be repulsive or attractive (electrostatic, steric or hydrational). This is often represented by a lateral stress profile as shown in Figure 4b which is a plot of the lateral stress $\pi(z)$ against the distance z through the monolayer. The net lateral tension across this monolayer (the integral of the stress profile) must equal zero with the spontaneous curvature proportional to the first moment of the lateral stress across the monolayer¹¹.

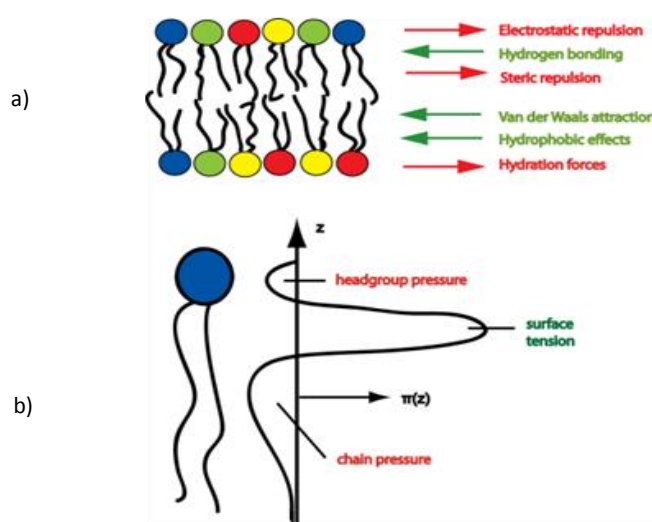


Figure 4: (a) The forces acting on the lipid bilayer at varying depths within the bilayer; (b) the lateral pressure profile.

The magnitude of each of these contributions is dictated by the steric and electronic structure of the head-group and fatty acid tail of each of its component lipids, leading to an average value of an otherwise vastly complex system. Head-group pressure is controlled by its cross-sectional area, any charges it contains and the hydration shells that these groups co-ordinate, as well as any interactions with neighbouring head-groups. Fatty acid interactions are dominated by hydrophobic effects and Van der Waals forces, meaning that fully-saturated, all-*trans* systems pack the most closely and thus occupy the smallest cross-sectional area. In order to simplify

these concepts, the lipids are split into three broad-brush categories, highlighted below in Figure 5.

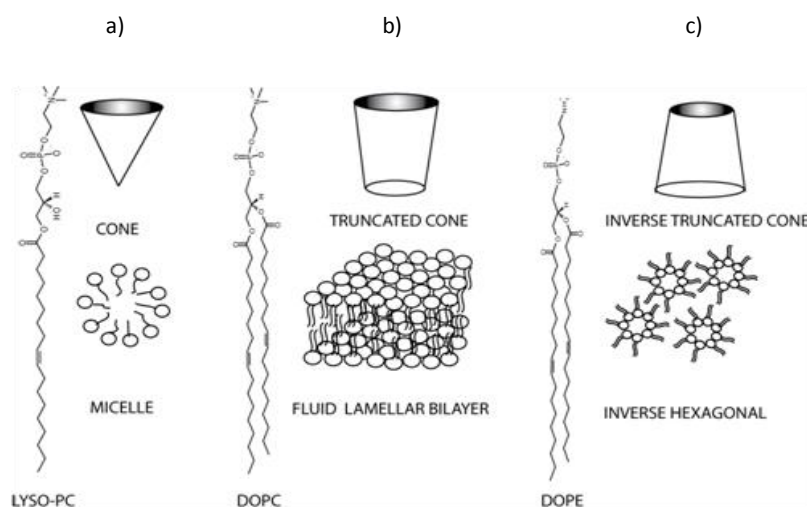


Figure 5: (a) Single chained lipids such as lyso-PCs will self-assemble into micelles; (b) DOPC, a commonly occurring bilayer lipid self assembles into a fluid lamellar phase; (c) lipids with a small head-group area and a propensity to hydrogen bond, for example DOPE, will tend to adopt curved structures such as the inverse hexagonal structure.

Commonly occurring lipids in biological membranes, such as 1,2-dioleoyl-*sn*-glycerophosphatidylcholine (DOPC) (Figure 5) have a small negative spontaneous curvature ($H_0 =$ between $-1/20$ and $-1/8.7\text{nm}^{-1}$), single chained lipids such as lyso-lipids have a positive spontaneous curvature (for example, palmitoyl lyso-PC, $H_0 = 1/6.8\text{nm}^{-1}$) (type I lipid; curvature away from the aqueous exterior) and double chained lipids with small head-group areas such as 1,2-dioleoyl-*sn*-glycerophosphatidylethanolamine (DOPE) have a large negative spontaneous curvature ($H_0 = -1/3\text{nm}^{-1}$), (type II lipids; curvature towards the aqueous exterior). Sensing and regulation of the curvature elastic energy has been shown to be a mechanism by which a variety of key biological processes operate: an increased curvature or curvature stress leads to the formation of defects within the otherwise relatively uniform membrane surface, leading to increased amphiphile binding. This process was first observed to regulate lipid biosynthesis¹², but has since been highlighted as a more generic mechanism for the sorting and activation of membrane proteins^{13,14}. This topic is explored in more detail in section 6 (page 147), but if it is considered that a number of amphiphilic drug compounds have previously been observed to induce serious changes in a membrane's curvature, rigidity and fluidity^{15,16}, it appears likely that the non-specific detergent effects of drugs within a membrane may play at least some role in their transport and biological activity.

H_0 is not expected to be zero, but when two identical monolayers are placed back to back in a bilayer, the bilayer spontaneous curvature (H_0^b) is zero so as to prevent the formation of energetically costly vacuums. If the spontaneous curvature of the monolayers is increased (physically achieved by changing the composition of the monolayer) there will come a point at which the curvature elastic energy becomes too great and a transition to an inverse phase occurs so as to allow the interface to bend, as in Figure 6. Such systems are more likely to be able to form the intermediate structures necessary for membrane fusion events to occur¹⁷ by lowering the energy required to create voids between the monolayers¹⁸.

This process is of major biological relevance as the same process must occur to allow the budding or fusion of vesicles in intra-cellular communication, for example at synapses in the nervous system. The resting state of such cell membranes lies in the fluid lamellar phase; when a budding event is initiated, regions of high curvature are created which closely mimic the proposed transition state between the $L_\alpha - H_{II}$ phase transition¹⁹, as shown below in Figure 6.

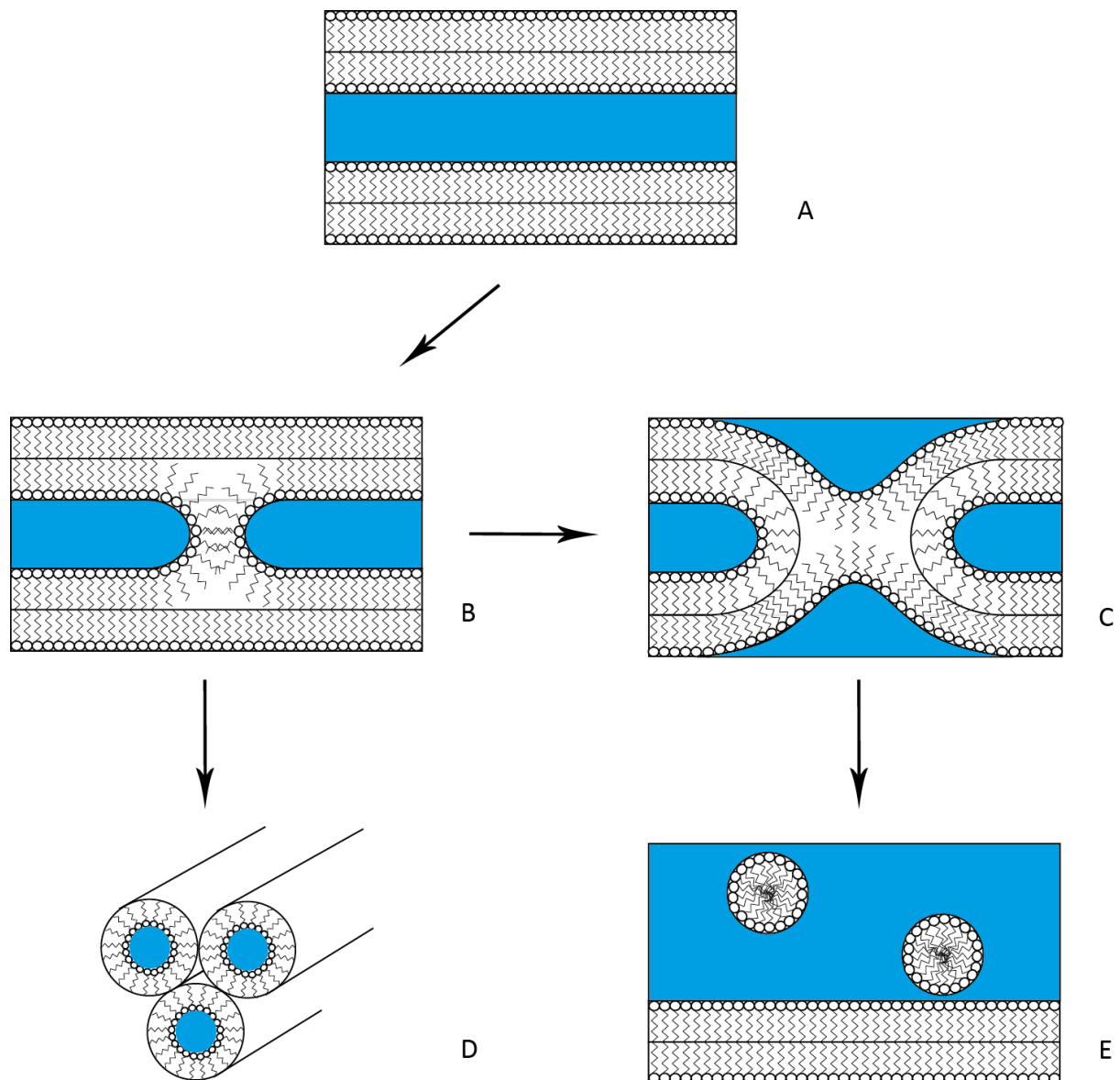


Figure 6: The stalk hypothesis model of phase transition between lamellar and more curved phases. As the lamellar sheets approach one another, either through dehydration or thermal undulation (a), contact points begin to form between the lammellae (b). These can link up to form inverse hexagonal cylinders (d), or can alternatively 'unzip' along the length of the sheet (c), leading to membrane fission and the creation of daughter vesicles, micelles and cubic phases (e). Adapted from Siegel¹⁹.

The void spaces which inhibit the formation of these non-bilayer structures are clearly visible in images b, c and d. The addition of strongly hydrophobic species such as hexane but also potentially a range of drug compounds can fill these energetically unfavourable regions, promoting the formation of highly curved phases and thus disrupting the morphology of the membrane. These curved phases behave very differently to lamellar sheets: their restricted geometry means that they effectively exclude water from all but a very limited volume, shown

schematically above in Figure 6d. The combination of narrow channel widths and static layers of water co-ordinated to the lipid head-groups leads to slow diffusion times throughout the enclosed water, combined with decreased lateral motion of the lipids themselves due to the increased steric stresses upon them. For a more detailed discussion of this and other mechanics of biological membranes see Seddon²⁰, Shearman *et al.*²¹ and Zimmerberg *et al.*²².

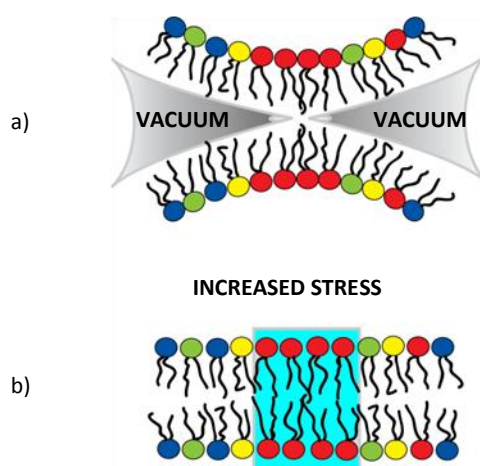


Figure 7: (a) The effect of monolayer curvature on curvature elastic stress in the bilayer. Monolayers containing type II lipids, such as those with PE head-groups, try to curve towards the water; (b) however they are constrained within a bilayer and are forced together, leading to an increase in stress within the membrane.

The lipids presented on the inner and outer leaflets of the bilayer differ greatly². This transverse asymmetry is believed to be controlled by a family of enzymes, called flippases, floppases (both ATP-dependent) and scramblases (calcium dependant), which enable the bidirectional translocation of lipids between the two leaflets. Flippases²³ aid the stereospecific movement of lipids from the outer to inner leaflet, displaying a high degree of head-group selectivity, with floppases²³ aiding translocation in the opposing direction to that of flippases (from the inner to outer) and scramblases²³ operating in both the ‘flip’ and ‘flop’ directions. Inevitably, the physical shape of the molecule and its trans-bilayer diffusion rate, which can vary dramatically between species, will also influence this asymmetry. *In vivo*, the loss of trans-bilayer asymmetry results in the exposure of phosphatidylserine to the outer leaflet, triggering the activation of a number of cell-lysis enzymes such as Phospholipase A₂ (PLA₂) and inducing apoptosis²⁴.

3.2 Moving Molecules across Membranes - Classical Models of Transport

In order to understand the interactions of drug molecules with the plasma membrane of cells, it is worth considering some basic biological concepts concerning the mechanisms by which molecules can cross cell membranes.

3.2.1 Passive transport

The mechanism by which small molecules may traverse a cell membrane spontaneously without any energy being expended by the cell is referred to as *passive transport*, (see Figure 8) with the simplest example being that of diffusion. Small, hydrophobic molecules will diffuse rapidly across membranes, (although a significant percentage may be retained), whereas small but uncharged polar molecules will take a greater period of time to cross. Ions face a very high energetic barrier to cross the hydrophobic core of the membrane due to their charge and hydration shells and so in practical terms, their rates of transit are generally low. In terms of drug molecules, non-ionized forms or neutral drugs like caffeine can readily cross the membrane by diffusion²⁵. The amount of ionized form of a drug versus the non-ionized form available will be dependent on the partition function ($\log P$, explained fully in section 3.2.3 below) and pK_a of the drug and the pH of the medium on either side of the membrane. If the drug has a fixed charge (for example, quaternary amines or some types of antibiotic) the drug is unlikely to enter the cell by diffusion alone. In the case of cationic amphiphilic drugs (CADs) (whose cationic groups are normally formed by secondary or tertiary amines) and other small amphiphiles, a grey area emerges²⁶. Recent molecular dynamics calculations suggest that for a strong base, $pK_a \sim 12$, the pK_a can shift by approximately 4.5 units upon entering a membrane²⁷, although the energetic cost associated with such a movement effectively prohibits such action. For slightly weaker, amphiphilic bases such as the CADs under discussion, the situation is less certain but it seems likely that the majority of such compounds would have difficulty traversing a membrane by diffusion alone. Should such a CAD enter a cell or cross the blood-brain barrier by simple diffusion, it is likely to become ionised almost immediately upon exposure to the intracellular environment: the pH of arterial blood plasma is around 7.4; cerebrospinal fluid is around 7.3 and cytosol is often around 7.2²⁸. As a result, any such compound is unlikely to be able to

diffuse out of a cell via the same mechanism. Furthermore, organelles such as lysosomes can have internal pH values below 5.5 and thus tend to attract basic drugs in high concentrations, leading to aggregation²⁹.

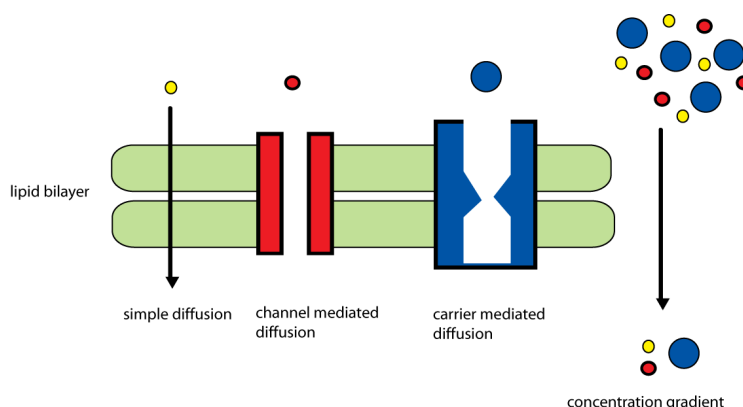


Figure 8: Passive transport can occur across membranes in a number of ways, the simplest of which is diffusion. Molecules can also cross the membrane without requiring the input of external energy by diffusion through a channel, the opening and closing (or ‘gating’) of which can be controlled by voltage, ligand binding or mechanical stress. Ion channels are often selective for a particular ionic species. Carrier proteins can also facilitate translocation across the membrane by a passive mechanism¹.

3.2.2 Measuring diffusion across membranes

The rate of diffusion of a drug across a membrane will be proportional to the concentration gradient, the lipid-water partition coefficient (the equilibrium ratio between the concentrations dissolved in water and an immiscible organic phase (typically 1-octanol), assuming the molecule is un-ionised) and the diffusion coefficient of the drug within that membrane. This can be summarised by Fick’s Law of Diffusion (Equation 5):

Equation 5

Where D is the diffusion coefficient of the drug in the membrane, P is the partition coefficient of the molecule between the membrane and the external medium, b is the thickness of the membrane and $X_1 - X_2$ is the concentration gradient across the membrane. Experimentally, however, if the lipid-water partition coefficient is too great then the drug is unlikely to leave the membrane and may merely be sequestered within it.

3.2.3 Binding and partitioning to membranes

The value of the partition coefficient or log P (see Equation 6), of a compound is often used as a crude measure of distribution of a compound *in vivo*, as Overton's rule suggests that lipophilic compounds will traverse a lipid bilayer faster than hydrophilic species and that this solubility was the major rate-determining factor in the species' permeability coefficient³⁰. Although this has been overtaken by more recent research into ion channels and membrane protein pumps³¹ like those described below, this remains the backbone of many predictive tools in drug-discovery.

Equation 6

The classical method of determining the octanol-water partition coefficient for a molecule involves dissolving the molecule of interest in a two-phase system containing octanol and water and determining the concentration in each fraction, most commonly by UV-Vis spectroscopy or alternatively by the addition of a small amount of a radiotracer. 1-Octanol is used as a mimic of the lipid environment; data can be extrapolated from the interaction of drug molecules with the octanol phase and applied to the partitioning of the drug within a lipid membrane. A more high-tech approach can be achieved using HPLC, correlating the retention time of the molecule with that of molecules with a known log P value³². While this method has the advantage of being high throughput, it assumes a degree of knowledge about that behaviour of a molecule which may not be available for *de novo* drugs.

Many drugs have some form of amphiphilic or detergent-like character, in particular the CADs, with critical micelle concentrations in the sub-millimolar range³³. This, combined with their often high octanol-water partition coefficients, means that they exert an effect upon any membrane in which they are resident should they reach a sufficient concentration, causing cell leakage, deformation and sometimes lysis due to their detergent-like solubilising effect on the membrane. For a comprehensive review of these effects and their physicochemical and biological implications, see Schreier¹⁶.

The distribution function, or log D, is a slightly more refined model³⁴, but one whose measurement requires significantly more effort. Log D is the ratio of the equilibrium

concentrations of all species, both unionized and ionized of a molecule dissolved in octanol to the same species dissolved in water at a given temperature, normally 25° C. It differs from Log P in that ionized species are considered as well as the neutral form of the molecule (see Equation 7).

Equation 7

In order to find a log D function, the partition function must be measured across a range of pH values and thus ionisation states. This is further complicated by the choice of buffer upon the system, and any Hofmeister effects^{35,36} that may arise as a result. Again, it gives no indication of any interactions the compound may have with proteins resident in the membrane, nor is 1-octanol a particularly good model for the often highly charged, amphiphilic lipids which make up such a barrier. Despite this, it is often one of the major physico-chemical properties which a pharmaceutical company will consider when debating the desired products of a high-throughput library.

At its most basic level, the Hofmeister effect^{35,36} describes the effect of an ion upon the structure of water around it. Chaotropes such as nitrate and iodide ions have very weak interactions with the surrounding water; kosmotropes such as sulphates and phosphates have very strong interactions, and tend to co-ordinate water around them, weakening the hydrogen bond network that water normally forms. This means that, at least in concentrated solutions, the volume of 'bulk' water (as opposed to surface-coordinated water used to solubilise or suspend the protein/colloid/membrane of interest) decreases and to compensate the solute is precipitated out, thus freeing up the low entropy surface coordinated water. This obviously has major implications for the observed partition/distribution function of a compound in a system as described above, as the relative concentrations measured in the two phases of the system are based on the assumption that all the solvent in the system is available to contribute to their solvation.

3.2.4 Non-specific binding

Labelled drug molecules, such as those used in positron emission tomography (PET) can sometimes be found to interact non-specifically with membrane structures. This leads to a high background signal as a result of this non-specific binding, which reduces the quality of PET data that can be collected from probing a specific receptor. Non-specific binding is thought to be correlated in part to a molecule's lipophilicity, or log P value; this is, however, an oversimplification. A general rule of thumb is that a molecule with a log P below 3 will be a good PET ligand. Within the brain, where a degree of lipophilicity is required to traverse the blood brain barrier, a log P value between 1.5 and 3 is preferred³⁷. Whilst the behaviour of many radiotracers correlates with these log P regimes, there are also many exceptions. As a result there is a need to understand the molecular basis of non-specific binding, in order to improve the design of PET ligands and further the fundamental understanding of how drugs interact with membranes. Log P, as discussed earlier, contributes significantly toward non-specific binding, but there are also active processes that can lead to the sequestration of drugs within membranes. This sequestration has two major effects. Firstly, a drug sequestered within a membrane will have reduced efficacy, being extremely unlikely to meet its target unless it is ubiquitous or the drug was administered topically. This requires more drug to be administered in order to achieve the desired therapeutic effect, thus increasing any side-effects and narrowing the therapeutic window of the compound. Secondly, and more insidiously, the drug concentration inside these membranes can become dangerously high (>50mM)³⁸, causing a range of potential effects varying from membrane disruption through to storage disorders such as phospholipidosis, which can cause a range of secondary pathologies³⁹. For example, amiodarone has been observed to cause clouding of the lens and cornea of the eye⁴⁰ and ketoconazole has been demonstrated to induce long-term hepatic effects through its primary metabolite, which is sequestered in the lipid domains it creates and is only very slowly metabolised⁴¹. It is clear that non-specific binding is a far more complex process than has been previously thought: whilst it can be an unwanted side product of drug-membrane interaction, it may be advantageous under certain circumstances to have a drug capable of non-specific binding, acting for example as a reservoir of drug bound within a membrane that can be released over a period of time.

3.2.5 Facilitated transport

In order for hydrophobic species such as ions, sugars and amino acids to be able to enter and leave the cell, transport proteins embedded in the membrane must be utilised: these are categorised as either carrier proteins or channel proteins. As the name implies, carrier proteins bind to the transported molecule and by means of conformational changes of the protein, carry the molecule across the membrane. Channel proteins form aqueous pores across the lipid bilayer that, when opened, allow the molecule to diffuse through the pore and thus cross the membrane. Often these channels will be highly selective, allowing only certain ions to pass through them. They are not continuously open, but instead open and close in response to stimuli such as voltage, mechanical stress or ligand binding. Each transport protein is specific for a particular class of molecule and often has specificity for a specific substrate within that class. Many transport proteins move molecules in the direction of the concentration gradient: as such they require no energy to do so. This is therefore a form of passive transport, known as *facilitated diffusion*. For a series of reviews describing each of the families of solute carrier proteins such as these, please see Hediger *et al.* and the accompanying special issue⁴².

To move an uncharged molecule across a membrane by facilitated diffusion simply requires a concentration gradient across the membrane. For a charged molecule to cross, the concentration gradient and the electrical potential difference across the membrane (the membrane potential) are combined to give a net driving force known as the electrochemical gradient.

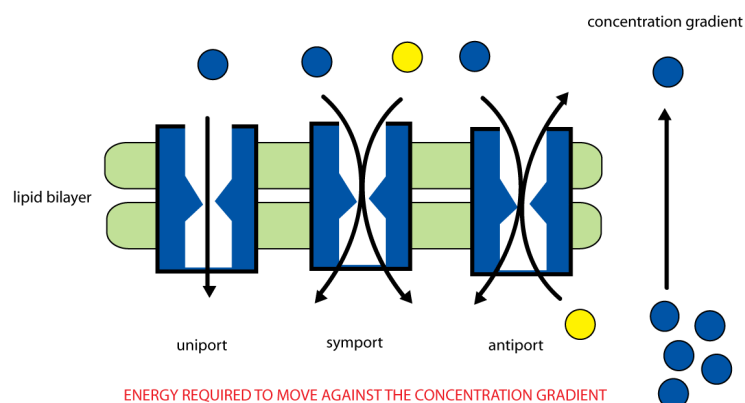


Figure 9: In active transport, molecules are transported against the concentration gradient using proteins as channels or transporters. In primary active transport, a substrate can be moved directly (uniport); in secondary active transport, a substrate is moved in conjunction with a secondary molecule in the same direction (symport) or in conjunction with a secondary molecule moving in the opposite direction (antiport).

3.2.6 Active transport

When a cell needs to move molecules across the membrane against a concentration gradient, the process will require an energy input in one of three ways. This energy can come from the hydrolysis of ATP, leading to what is known as *primary active transport*. Energy may also be derived from the coupling of the transport of a secondary species, usually via an ion gradient and is known as *secondary active transport*. Finally, the energy required can come from light; this is a highly specialised mechanism employed by some organisms and is mediated by light transduction proteins such as bacteriorhodopsin⁴³. Methods of active transport are illustrated in Figure 9.

3.2.6.1 Primary active transport

Primary active transport is driven by proteins which use chemical energy from ATP hydrolysis to translocate molecules across the membrane. Often these proteins are ion pumps, responsible for maintaining gradients of ions across the membrane⁴⁴. Creating ion gradients is also important for secondary active transport. Some carrier proteins, including the ATP-binding cassette (ABC) transporter super-family⁴⁵ are directly responsible for pumping a variety of molecules in and out of the cell.

Some drugs are actively taken up by cells by primary active transport, although this is the exception rather than the norm. For example, the tyrosine kinase inhibitor imatinib, used for the treatment of chronic myeloid leukaemia, is taken up by the human organic cation transporter protein hOCT1 as well as being expelled by P-glycoprotein meaning that differences in individual patients' expression levels can lead to the failure of drug therapy⁴⁶.

3.2.6.2 Secondary active transport

During secondary active transport, molecules are translocated across the membrane as the result of diffusion of another substance. The proteins responsible for this coupled transport are known as symporters or antiporters. A symporter moves a second solute in the same direction as the primary transport; an antiporter moves the second solute in the opposite direction. By using the energy stored in the electrochemical gradient of one molecule (or ion), symporters and antiporters can move a second molecule against the concentration gradient. Examples include the SVCT1 and 2 proteins, which transport vitamin C across the membrane via the simultaneous diffusion of Na⁺⁴⁷. The Na⁺ - Ca²⁺ antiporter drives the removal of calcium from the

cell against the concentration gradient by using the opposing movement of Na^+ . For a range of further examples, including structures, physiological distribution and pharmacokinetics, please refer to Terada and Inui⁴⁸.

3.2.6.3 Drug resistance

In eukaryotes, ABC transporters have attracted clinical attention as they are able to efflux hydrophobic drug molecules from the cell. In cancer cells, over-expression of P-glycoprotein (P-gp) and the multi-drug resistance protein (MDR) leads to the expulsion of chemotherapy drugs from the cell, conferring resistance to a wide range of therapeutic agents⁴⁶. Drug resistance of one type of malaria parasite, *Plasmodium falciparum*, is also controlled by an ABC transport protein, which causes the efflux of the anti-malarial agent, chloroquine⁴⁹. An excellent overview of the mechanisms and structure of bacterial ABC transporters is given by Moussatova *et al*⁴⁵.

Most secondary multidrug transporters work to efflux drugs using the movement of H^+ , known as the proton motive force, or gradient. Toxic compounds are expelled from the cell via a coupled exchange with protons. There are four classes of bacterial secondary multidrug transporters: the major facilitator super-family (MFS), the small multidrug resistance family (SMR), the resistance-nodulation cell-division family and the multidrug and toxic compound extrusion family (MATE). These families can transport a variety of substrates, including sugars, phosphate esters, small molecule dyes and a range of antibiotics and by extruding compounds from the interior of the cell, build up resistance of the cell to that compound. For comprehensive reviews on this subject see Putman *et al*.⁵⁰ and references cited therein.

3.3 *An alternative mechanism for drug translocation*

Neither passive nor active transport mechanisms, however, can adequately describe the translocation of bulky hydrophobic or amphiphilic molecules not recognised by the cell (for example CADs), across the membrane. In light of this failure, a third class of mechanism is required: one which considers direct chemical or physical interactions between the drug and lipid molecules. Simple examples include the non-specific effects that occur when amphiphilic compounds are sequestered or retained within a membrane as described above. This has been most widely studied in the case of anaesthetics, which have been observed to cause significant fluidisation and softening of membranes⁵¹. However, the doses at which these effects occur are significantly greater than those which are pharmacologically relevant, and later research has focussed upon the effects that the compounds have on the lateral pressure profiles of their surrounding lipids and proteins⁵².

Another candidate for this class was recently observed by Baciú *et al.*^{53,54}, in which the presence of a small molar fraction of CAD within a phosphatidylcholine membrane led to the rapid hydrolysis of the lipid molecules *via* their ester bonds. This hydrolysis was catalytic with respect to the drug, and generates a molecule of lyso-lipid, in this case mono-acyl phosphatidylcholine, and the concomitant fatty acid. The reaction is not believed to be regiospecific (although if it were, mono-acyl lipids have been shown to isomerise rapidly, favouring the *sn*-1 conformation⁵⁵). Furthermore, the reaction is not constrained by a highly specific active site as is the case with similar enzyme-mediated reactions. As a result, the lyso-lipid formed in the first reaction can itself form a substrate for hydrolysis, forming a second molecule of fatty acid and one of highly hydrophilic phosphatidylglycerol.

This process rapidly causes dramatic perturbations to the membrane topography, as degradation products begin to accumulate. Fluorescence microscopy studies have revealed that drug-bearing membrane fragments are ejected from the membrane within seconds of the compound's administration (see Figure 10, below), and it is therefore possible that this mechanism contributes toward its *in vivo* transport. These fragments are formed of lyso-lipid rich micelles and mixed lamellar bodies, which have been previously observed: Burack *et al.* have previously demonstrated that in gel-phase dipalmitoylphosphatidylcholine/monopalmitoylphosphatidylcholine/palmitic acid mixtures, where the lyso-lipid and fatty acid are added at a 1:1 ratio to mimic the effects of PLA₂ hydrolysis, a major phase change occurred

at ~8% lyso-lipid, as the multi-lamellar vesicles under observation collapsed to form small, disk-like micelles⁵⁶.

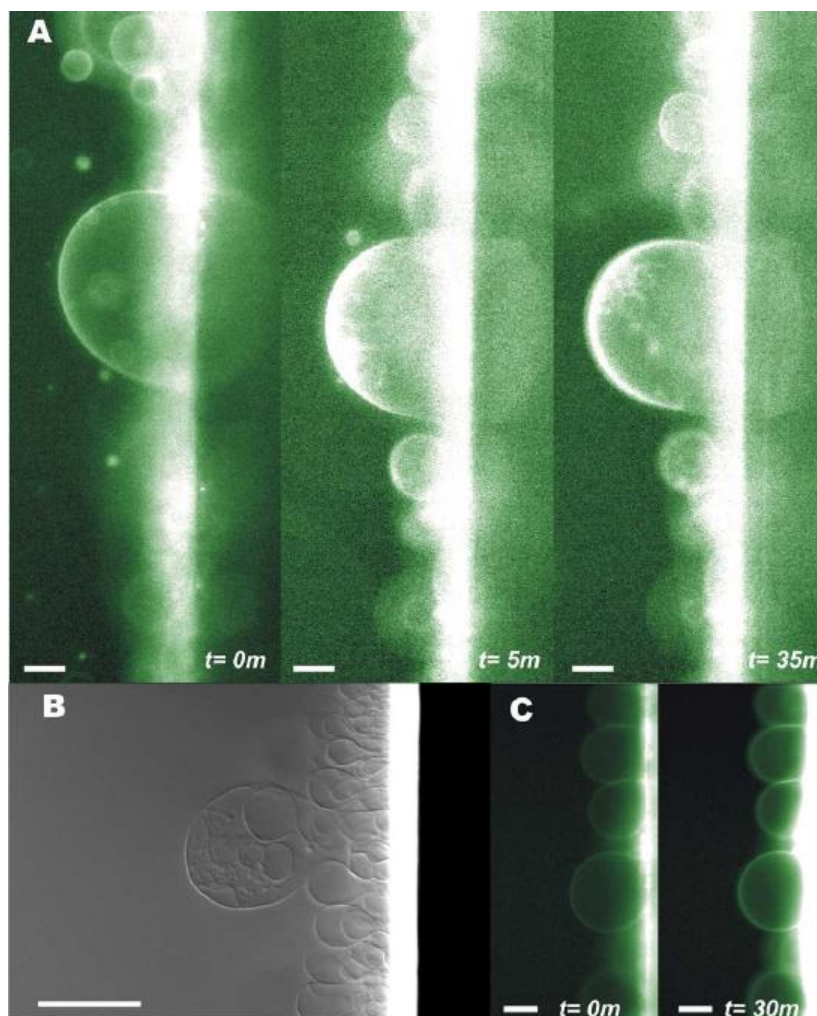


Figure 10: Fluorescence micrographs showing CAD-catalysed hydrolysis. (A) Fluorescence microscopy images showing the effects of labelled spiperone HCl on DOPC giant unilamellar vesicles (GUVs) over time; (B) TM image DOPC GUVs with haloperidol HCl; (C) The same experiment performed on ether-PC, showing no reaction. Scale bar = 20µm⁵³

The mechanism of formation of these bodies is still the topic of some debate. Lyso-PC has a relatively low critical micelle concentration of around 5µM, forming small micelles often containing as few as 140 monomers each⁵⁷ and has been observed to cause the formation of many such bodies upon its addition to egg-PC multilamellar vesicles⁵⁸. However, more recent studies contend that as the aqueous solubility of lyso-lipids is significantly greater than that of the di-acyl species, they rapidly leave the bilayer as monomers⁵⁹ and that the effects observed are caused by fatty acid and diacyl lipid interactions. In practice, both phenomena may contribute: if it is assumed, as a first approximation, that an exogenously applied drug remains

charged at all times and thus does not flip-flop between bilayers, it follows that all hydrolysis that it catalyses will also take place in the outer leaflet of the membrane. The flip-flop rate of a lyso-lipid has been shown by NMR to be negligible ($t_{1/2} > 12\text{hr}$)⁶⁰, whereas those of fatty acids have been shown to be extremely rapid⁶¹⁻⁶³. This implies that the fatty acids produced by hydrolysis will equilibrate across both leaflets of a membrane very quickly, leaving an effective 2:1 lyso-lipid: fatty acid ratio in the outer shell and local regions of high lyso-lipid concentrations, quickly producing regions of high curvature which eventually bud off into solution, (shown below in Figure 11). This hypothesis is somewhat supported by data from PLA₂ studies (described in more detail below), which indicate that after hydrolysis the membrane is significantly enriched in fatty acid relative to lyso-lipid⁶⁴.

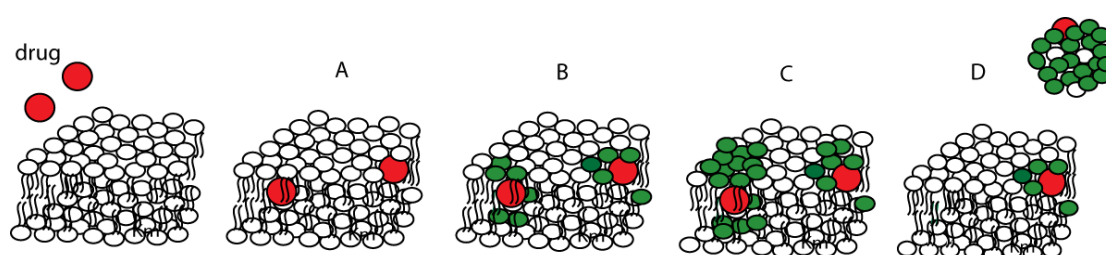


Figure 11: The proposed mechanism of drug transport: CAD molecules migrate to the membrane interface, driven by the hydrophobic effect, where they intercalate between lipid molecules (A). They rapidly hydrolyse a number of surrounding ester bonds (B), forming a high local concentration of tightly-curved lipid shown in green (C). This leads to changes in membrane morphology and eventually the formation of small micelles and vesicles, each containing a few molecules of the drug (D)¹.

3.3.1 Comparisons with enzyme-catalysed hydrolysis

This process has many similarities to the action of Phospholipase A₂ (PLA₂), an endogenous enzyme responsible for membrane maintenance and specifically in the pancreas for lipid metabolism. However, a number of important differences apply: for example, PLA₂ is highly selective in both its choice of substrate and its regiospecificity, only attacking the *sn*-2 ester⁶⁵; the mechanism described above appears, from our investigations, to apply to any acyl bond at a lipid interface. Furthermore, PLA₂ is activated and deactivated in response to specific stimuli, leading to ‘lag-burst’ kinetics^{66,67}. This does not appear to be the case for the CAD-catalysed reaction, and indeed there is no reason why it should as no three-dimensional ‘active site’ exists on the molecule.

There are some interesting parallels, however. Previous experiments have demonstrated that

the rate of CAD-catalysed reaction is affected by the lateral stress profile within the membrane: very different rates have been observed for membranes made of saturated *versus* unsaturated lipids⁵³. A similar effect is observed in the activation of PLA₂, whereby the full activation of the enzyme appears driven by the aggregation of degradation products produced in the low-rate 'lag' phase, either due to the increase in anionic fatty acid concentration⁶⁸ or due to the formation of membrane defects and/or lateral phase separation as lyso-lipid leaves the membrane^{56,69} and the system attempts to move towards the inverse hexagonal phase²¹.

3.3.2 Links to phospholipidosis

As described above, the hydrolysis of a proportion of the lipid or even the act of introducing a number of largely type I drugs⁷⁰ into a bilayer will attempt to increase the curvature of the membrane, leading to an increase in the curvature elastic stress within the bilayer. This stress has been shown to activate the biosynthesis of a number of lipid molecules and others by causing a conformational change within a number of membrane-bound proteins^{71,72}.

Furthermore, it has been shown that the enzymes which normally degrade any excess membrane fabric such as the PLA family are inhibited by the presence of a range of such drugs⁷³⁻⁷⁵. It is proposed that the effect of these two mechanisms is the build-up of excessive cellular lipid concentrations, commonly known as phospholipidosis: a lipid storage disorder commonly associated with the administration of a range of CADs. However, there are some suggestions that this 'disease' is, in fact, an evolutionary response to protect the cell from unwanted amphiphilic compounds by sequestering the CADs away from any sensitive proteins or organelles⁷⁶.

This sequestration can have a number of secondary effects which have been discussed previously in this chapter (see section 3.2.4), in terms of both non-specific binding and out-and-out toxicity. The apparent links outlined above have led to the hypothesis that a successful drug requiring systemic availability must strike a tight balance between showing sufficient hydrolytic activity to permit its release from its host membrane to travel around the body, whilst not causing such damage as to trigger a response from the enzymes responsible for maintaining homeostasis, potentially trapping and neutralising the compound.

3.4 Techniques for Studying Drug-Membrane Interactions

Although it is relatively easy to analyse the bulk effects of drug induced membrane disruption upon lipid polymorphism, it is considerably more difficult to quantitatively apply this to unilamellar vesicles which more closely mimic the cell membrane.

When characterizing the interactions of drugs with membranes, no single technique will be able to provide all the necessary information. Parameters that are of use in the determination of drug-membrane behaviour include drug location, orientation and conformation within a membrane, the phase behaviour and stability of that membrane on introduction of a drug molecule and the nature of any chemical modifications that the drug may inflict on the membrane or vice versa. All of the following techniques are described in detail in Wiese and Seydel⁷⁷ together with illustrative examples.

3.4.1 Classical approaches to drug membrane interactions

Standard laboratory spectroscopic techniques such as UV and FTIR spectroscopy can be employed with great effect in the study of drug membrane interactions. Often, polarized FTIR spectroscopy is used: by monitoring changes in the position, width and intensity of IR bands, it is possible to describe local changes in phospholipid organization and orientation on the introduction of a drug molecule. The temperature dependence of the wavenumber of the CH₂ symmetric stretching vibration within DPPC was characterized in this way in the presence of salicylic acid, leading to information on how drug interaction affected transition temperatures within the bilayer⁷⁸. The simple and relatively inexpensive technique of UV-Vis spectroscopy has proved to be an excellent way of estimating the degree of partitioning of a drug into a lipid membrane, in a manner analogous to standard log P measurements. For example, the partitioning of the anticancer drug derivative 4-hydroxytamoxifen into DPPC bilayers has been successfully determined in this way⁷⁹. Circular dichroism can also be used to study changes in drug location and conformation. Drugs with anthracycline rings, such as doxorubicin, daunorubicin and idarubicin exhibit a variety of π - π^* and n - π^* transitions which are sensitive to the nature of the environment within which they reside. The electrostatic interactions of positively charged lipid head-groups on the depth of bilayer layer location of these drugs has been ascertained by CD spectroscopy, as well as the effect of cholesterol within the bilayer⁸⁰.

Fluorescence techniques can be employed to great effect to analyse the way that drugs interact with membranes. The scope of what can be achieved with fluorescence is too broad to be covered in detail in this chapter; a short overview is presented. Fluorescence emission, fluorescence lifetime imaging spectroscopy (FLIM), fluorescence anisotropy, fluorescence resonance energy transfer (FRET), fluorescence correlation spectroscopy (FCS) and single molecule detection techniques are all discussed in detail with relevant references in Lakowicz⁸¹. Recent research includes, however, steady state fluorescence anisotropy measurements (used in parallel with x-ray diffraction and NMR, see later) to study the effects of the non-steroidal anti-inflammatory drug (NSAID) diclofenac on model and erythrocyte membranes⁸². It was found that interaction of diclofenac caused an increase in rigidity in the polar head-group region of the model membranes and an increase in the disorder of the acyl chains. The antioxidant effects of NSAIDs and their interactions with membranes have also been studied using a combination of steady state anisotropy and fluorescence intensity decay⁸³. Quantifiable interactions between the lipid membrane and drug molecules can be achieved using fluorescence intensity measurements of environment-sensitive lipid probes, for example fluorescein-labelled dipalmitoylphosphatidylethanolamine, (FPE)⁸⁴.

The fluorescein head-group is extremely sensitive to electrostatic changes at the lipid/water interface, and so the intercalation of charged drugs leads to changes in fluorescence intensity. However, this can only provide relative quantification unless the partition function and pK_a of the drug is known precisely. Fluorescence techniques can also be applied to cell culture assays; for example, fluorescently labelled phospholipids have been incorporated into cell culture assays, to identify CADs which are implicated in the lipid storage disorder, phospholipidosis⁸⁵.

Often classical lab techniques, and particularly fluorescence are more beneficial in probing drug-membrane interactions when used as part of a multi-technique study. For example, steady-state fluorescence anisotropy, was used in conjunction with quasi electric light scattering, attenuated total reflectance infra red (ATR-FTIR) spectroscopy and ³¹P NMR to measure the interactions of the antibiotic ciprofloxacin with model membranes composed of phosphatidylglycerol (PG) or phosphatidylcholine (PC) head-groups⁸⁶. This work demonstrated a strong preference for the antibiotic binding to the anionic PG lipids, which are major components of the bacterial cell membrane.

The classical techniques for the quantitative analysis of condensed-phase structures are small

angle X-ray scattering (SAXS) and neutron scattering (see Koch *et al.* for a comprehensive review⁸⁷). SAXS in particular allows the direct observation of the stability of lyotropic lipid structures and provides quantitative information on the variation in structure that the addition of a drug molecule can exert upon a lipid sample.

By its nature, any crystal or semi-crystalline material such as those under consideration consists of a periodic lattice, with unit cell dimensions of a similar order of magnitude to the wavelength (λ) of an X-ray. As such, any X-ray incident to such a crystal will be diffracted, with the degree of diffraction dependent upon the separation of the constituent molecules and the arrangement they have adopted. By analysis of the variations in diffraction over time, the effects of the drug partitioning upon the phase behaviour of the lipid sample can be accurately plotted. Since the development of a position-sensitive linear X-ray detector in 1972 by Dupont *et al.*⁸⁸, the measurement of such parameters can be made almost in real-time, permitting the kinetic analysis of phase transitions such as those described in Figure 6 in response to stimuli such as pressure, temperature or intercalation of exogenously applied compounds.

Calorimetry studies, analysing small changes in the heat and/or heat capacity of a system after a minute perturbation, can provide a precise picture structural or morphological changes. Of interest for those wishing to study drug-membrane interactions is isothermal titration calorimetry (ITC)⁸⁹, in which consecutive injections of a known solution (in this case the drug under consideration) are mixed with a known concentration of vesicles or micelles under isothermal conditions. 'Blank' runs are also performed to give the enthalpies of dilution of each analyte, and these heats of dilution are subtracted to give the energy of interaction between the drug and membrane.

Differential scanning calorimetry (DSC) is a technique often applied in conjunction with SAXS to probe the thermodynamic phase transitions of a lipid or mixture of lipids in a sample. This provides a plot of enthalpy versus temperature, effectively recording the isobaric heat capacity of the sample: any sharp changes of the plot indicate a phase transition of some kind. In the case of lipids and liquid crystals in general, this could be indicative of a change in lipid polymorphism or a chain melting process. In this way, the precise phase boundaries for a given lipid system can be identified, reversibly and non-destructively, and coupled with SAXS structural data a well-defined map of phase behaviour can be established. A full overview of the calorimetry of lipid membranes can be found in a review by Heerklotz⁹⁰ and references cited

therein.

Both solid- and solution-state NMR approaches also directly probe interactions between amphiphilic molecules dissolved in a bilayer and their surrounding lipids⁹¹, albeit often at high doping concentrations due to the low sensitivity of NMR spectroscopy. These techniques have been applied in the modelling of the transport of highly lipophilic ligands such as cannabinoids^{91,92}, but also in the pursuit of a mechanistic explanation for a number of lipid-storage disorders, most notably phospholipidosis.

High resolution NMR provides detailed information on the dynamics and conformation of membranes and the effect which the addition of a drug has upon those membranes; conversely it is also possible to determine the effect of a membrane on the orientation and conformation of a drug molecule. Many nuclei can be studied, particularly ¹H, ²H, ¹³C, ¹⁵N, ¹⁹F and ³¹P, meaning that specific isotopic enrichment of probes can yield a range of data including position, orientation and dynamics of a drug within a membrane. Changes in the field at which resonance occurs, the spin-spin coupling and the spin-lattice and spin-spin relaxation rates can all be affected when drug molecules and membranes interact. A comprehensive overview of specific uses of NMR spectroscopy in drug-membrane interactions can be found in Seydel and Wiese and references cited therein⁷⁷. Of particular note is the use of ³¹P NMR to study lipid polymorphism; lamellar, hexagonal and isotropic phases all have distinctive NMR signals which are sensitive to perturbation by drug interactions.

Recent experiments described above in section 3.3^{53,54} have demonstrated that certain classes of drug molecules are able to hydrolyse biological membranes, with this phenomenon being linked to drug translocation and non-specific binding. High pressure liquid chromatography (HPLC) techniques⁹³, normally using light-scattering detection, are able to give an accurate and reproducible breakdown of the components during such processes.

HPLC separates the components of a mixture upon based the differences in their affinities toward a static, solid-phase bed, known as a column. This bed is typically composed of functionalised silica, alumina or polymer granules or around 5µm diameter, exposing a very high surface area to the analytes but consequently generating a very high back-pressure in the eluting solvent. Signals are generated via optical or radiochemical techniques and recorded with respect to retention time, the time that each individual component remained resident upon the

column. This requires precise pump control, as flow rates must be maintained at a constant level of ~1 ml/min at a pressure often in excess of 150 bar for reproducible retention times to be practically achievable.

Light scattering analysis is covered in detail in section 5.6.2, (page 122) and so will only be described briefly here. However, the interested reader is directed to comprehensive reviews on the topic for in-depth analysis^{94,95}. Evaporative light-scattering detection relies upon the evaporation of the eluent stream by a directed gas flow into a heated chamber, resulting in the nebulisation of single molecules of the analyte particles. The gas then acts as a carrier, lifting the aerosol stream past a high-intensity bulb. The analyte causes some scattering of this light, which is typically detected at 90° to the incident beam. The technique is destructive as once nebulised, the analyte is unrecoverable; despite this, it provides absolute quantitation (when compared to a calibration curve) for any sample of lower volatility than the eluent, which is extremely valuable in lipid analyses due to their lack of accessible chromophores or fluorophores.

For complex mixtures such as those obtained from *ex vivo* membrane lipid extracts, HPLC coupled with high resolution mass spectrometry (MS) is sufficient for at least semi-quantitative analysis, but provides by no means the ideal solution. A major problem with this technique is ion-suppression: lipid molecules ionise poorly and those that do tend to compete with one another for the available charges, even at very low concentrations⁹⁶. However, via the use of nanolitre-sources for electrospray ionisation, coupled with MS/MS equipment and a range of stable-isotope internal standards, reasonably reproducible relative quantitation can be achieved^{97,98}. For a recent, practical guide to the MS analysis of complex lipid mixtures, see Seppänen-Laakso and Orešič⁹⁹. One further issue with the study of drug membrane interactions by this technique is it requires a direct chemical interaction between the drug and the membrane to be used.

What none of these techniques address, however, are the differences in behaviour between lipids in condensed phases and those in giant unilamellar vesicles. Condensed-phase systems have much higher lipid concentrations than the more diffuse vesicle systems, but typically exhibit much lower diffusion rates for aqueous species due to the restrictions on solvent flow outlined in section 3.1.3. Similarly, condensed-phase systems are often much closer to their excess-water point, or the lipid:water ratio at which the system is fully hydrated. This poses

issues for studies such as this, where the system is dependent upon both a ready stream of water molecules and an even and rapid distribution of buffer, drug and hydrolysis product molecules.

The differences are also an issue which complicates analyses. Although very simple, vesicle systems are preferable where possible as they are much closer to the lipid environment encountered by drugs *in vivo*. However, both NMR and SAXS require at least moderate sample concentrations and destructive techniques such as HPLC-ELSD require several ~50µg samples for any kind of time-resolved analysis to be possible. This is largely incompatible with the extremely low lipid concentrations and high excess water environments required for biologically relevant membrane models to be created.

3.4.2 Industrial and high-throughput approaches

Drug discovery programmes based upon high-throughput synthesis and screening require rapid identification of potential problems relating to administration and transport of a candidate drug, before large sums are spent on the compound's manual optimisation and testing. As a result, efforts were made to develop a robust predictive model that was amenable to microtitre-plate based assays, allowing the testing of many compounds in parallel. Initial assays included those based around a suspended monolayer of human colon carcinoma (Caco-2) cells, with the rate of transport from one side to the other measured by UV-vis. spectroscopy¹⁰⁰. Although this technique gives reasonable agreement with *in vivo* studies in many cases, it suffers from the same impediments as any other live-cell assays, being relatively slow and cumbersome, and dependent upon the state of the cells in any given assay. Furthermore, when studying slowly absorbed drugs, the model often deviates from *in vivo* results by some two orders of magnitude¹⁰¹.

The net result of these failings was the development of the parallel artificial membrane permeability assay (PAMPA)⁷⁷, where a membrane of a selected lipid or mixture of lipids is suspended across the pores in a hydrophobic filter frit. This frit is then placed in a microtitre plate between two aqueous solutions, one containing the analyte, the other the receiving solution. A range of related assays have been developed using this approach – see the recent reviews by Sugano *et al.*^{102,103} for further information.

3.4.3 *In vitro and in vivo techniques*

In vitro techniques have their limitations when it comes to the more systemic or longer-term effects of drugs upon biological membranes, however, and thus *in vivo* techniques are required. This is particularly true in the study of non-specific binding of drugs to membranes, as the degree of binding and sequestration varies significantly between organs, and even between areas in the same organ. For example, the lungs contain a significant number of acidic tissues and thus can accumulate potentially toxic doses of CADs such as amiodarone very rapidly¹⁰⁴.

The two major techniques used to quantitatively analyse drug distribution and specificity are PET and autoradiography of tissue sections. PET is a non-invasive *in vivo* technique, allowing repeated experiments on the same living human subject and using extremely low (nanomolar to picomolar) doses of candidate drug molecules. It is based upon the intrinsic labelling of drug compounds, tracers or known receptor ligands with “neutron-poor” isotopes of atoms such as carbon (forming ^{11}C), oxygen (^{15}O), fluorine (^{18}F) or nitrogen (^{13}N). A proton in these isotopes decays rapidly to form a neutron, a positron and a neutrino. The positron and neutrino leave the nucleus at high velocity: the neutrino is immediately lost, but the positron decelerates *via* interactions with the normal surrounding matter over a length scale of $\sim 3\text{mm}$ before annihilating with an electron in the vicinity. This event produces two gamma-ray photon of 511keV which are emitted at 180° to one another, and which may be detected co-incidentally, allowing the spatial and temporal localisation of the tracer compound in the patient or subject. For a history of PET development, please see Nutt¹⁰⁵; for a recent review of this and the related technique of single photon emission computed tomography (SPECT), refer to Heiss and Herholz^{106,106}.

The short half-lives of the isotopes used for labelling (e.g. ~ 20 minutes for ^{11}C) presents significant challenges to the synthesis of a carbon-11 labelled drug molecule of interest as this means that compounds must be prepared on-site on an *ad hoc* basis, using a cyclotron and rapid labelling, purification and analytical techniques³⁷ in close proximity to the clinical PET scanner. The high sensitivity and the three-dimensional, functional and kinetic data generated by the technique mean that growing numbers of companies are investing in the technology.

Autoradiography is an *in vitro* technique which requires significantly less investment in terms of equipment, but can only usually be performed in humans on post mortem or biopsied tissues. Animals can however, be administered *in vivo* with a labelled drug or compound of interest and

sacrificed, then the tissue of interest removed, sectioned and quantitatively imaged using x-ray film, phosphor imaging plates or scintillation gas detectors. This can provide the precise location and concentrations of the analyte within each two-dimensional slice, but requires significantly more animals per study in order to study the kinetic profile of a labelled compound and to eliminate inter-subject heterogeneities that can generate artefacts in the data. More fundamentally, if using experimental animals, assumptions have to be made in the translation of the findings to humans. The technique has been used to study the distribution of CADs in a number of animal models and make comment about their effects on the plasma membrane^{107,108}.

3.4.4 More recent approaches

The obvious conclusion from the above is that no one technique covers all the bases: a range of approaches are necessary to characterise even the simplest of drug-membrane systems. Fortunately, techniques such as surface plasmon resonance and linear dichroism which extend beyond the classical ones described are being developed to look at drug-membrane interactions from a new perspective.

Surface plasmon resonance (SPR) works by measuring the change in refractive index near a sensor surface, for example as a protein or drug molecule of interest binds to that surface. SPR can provide a label-free, quantitative analysis of the bulk state of a membrane in a small monolayer or bilayer region of interest, and it has been demonstrated that drug-membrane binding constants across three orders of magnitude can be easily obtained and reproduced¹⁰⁹. It is also amenable to moderate throughput, flow-based analysis with assay timescales of around 10-15 minutes, and thus presents some significant advantages over more traditional techniques. Standard SPR techniques have been taken a step further by the recent development of microfluidic channel-based SPR imaging chips¹¹⁰ which allow simultaneous imaging in parallel microchannels. Alternatives to current high throughput screening are being developed; in particular the combination of printing of lipids onto surfaces (as supported lipid bilayers) and microfluidics may prove to be advantageous. A recent article¹¹¹ describes measurement of the change of membrane structural changes on binding of a non-steroidal anti-inflammatory drug to a series of supported lipid bilayers printed on a glass substrate. This type of miniaturisation and

parallel screening technique, coupled with advances in detection methods may provide a method for rapid, robust and economical high-throughput testing of interaction.

An alternative to the highly labour-intensive NMR techniques for the analysis of conformation and orientation within a membrane, described above, has recently been presented by miniaturised linear dichroism cells. This approach utilises the differing absorbances of plane-polarised light by a chromophore depending upon its orientation, and as such can be used to give quantitative, or at least semi-quantitative, data on the angle a compound assumes relative to the interface of a membrane. This membrane is flattened into a disk or cylinder shape by viscosity shear forces created by the rapid rotation of a vesicle suspension in a small quartz cell. Whilst this technique has been in use for some time, signal-to-noise has proved to be a major issue before the manufacture of miniaturised ($\sim 500\mu\text{m}$) cells^{112,113}. Absolute quantisation can still be problematic with this technique unless the molecule in question is rigid and has a well-defined long axis: however, it is amenable to high-throughput testing and can be used to study almost any compound with some form of chromophore and so has some significant advantages over isotope-enriched NMR studies which present the only real alternative.

Recently a new label free method for the study of the association of drugs with membranes has been developed in the form of ultraviolet visible sum frequency generation (UV-Vis SFG)¹¹⁴: the association constants of drugs with membranes measured with this technique are found to correlate well to known partition coefficients. The drive to develop label free techniques has also led to a high throughput screening method for the interaction of CADs with short chain acidic phospholipids using critical micelle concentrations determined by surface tensiometry. This technique has provided good correlations with previously determined phospholipidosis inducing potential of 53 drug compounds¹¹⁵.

However, it is not just in the development of new techniques where there have been advances in the study of drug membrane interactions. Cyclic voltammetry and AC impedance studies on supported lipid bilayers have demonstrated a rapid degradation of the membrane upon the application of both CADs¹¹⁶ and, interestingly, simple amphiphilic organic acids¹¹⁷, in a manner that may be analogous to that reported above. Although these techniques only report upon the bulk properties of the membrane and so cannot reveal the molecular details of the phenomenon, both studies reported rapid and catastrophic defect formation in the supported bilayers over timescales consistent with those reported in previous experiments⁵³.

3.5 Conclusions

The interaction of drugs with membranes is an area of high importance for the pharmaceutical industry when considering the efficacy and safety of their products. The cell membrane is often overlooked in drug development programmes as it is generally the receptors and enzymes contained within it that are the target of the drug molecule. However, the membrane is not a passive or necessarily benign solvent and directly impacts on the protein molecules and complexes it contains. Furthermore, interactions between exogenous, amphiphilic compounds (including the majority of drug classes) and the membrane can directly affect its structural integrity, which can be critically damaging to the cell. While this may produce a desirable outcome, for example killing a tumour cell, it may also cause unwanted damage to cells leading to disease pathology.

4 *Rationale behind PhD research*

The following thesis details attempts made between 2006-2010 to characterise and comprehend the mechanistic steps through which a range of CAD-like compounds catalyse the hydrolysis of the ester bonds between the glycerol and fatty acid moieties in phospholipid membranes.

Although a significant body of work had previously been conducted into the causes and consequences of the hydrolysis (see section 3.3 and references therein and previous theses by Sebai¹¹⁸, Bate¹¹⁹ (unpublished work) and Ling¹²⁰ (unpublished work)), none has yet conclusively determined the parameters which affect the rates of reaction, or the process(es) through which they do so. It has previously been demonstrated that the rate is dependent upon the make-up of the membrane^{53,118}, and that differing CAD structures resulted in dramatically differing rates of reaction; however, the reasons behind these effects were not understood, although a number of plausible hypotheses have been presented.

The purpose of this study was to attempt to unravel, in a systematic fashion, the various factors influencing the reaction and to attempt to control and utilise it. As noted in section 3.2 above, membrane transport poses a significant problem for medicinal chemists, as the factors which influence and control a compound's bioavailability are not fully understood. As a result, compounds which show extremely promising results *in vitro* often fail to do so in animal or human trials, leading to extremely high attrition rates for novel compounds between candidate stage and the clinic. Although the model systems presented here are several orders of magnitude less complex than the diverse lipid structures encountered in biology, it was hoped that this study might provide the foundations for a more quantitative understanding of inter-cellular and systemic transport of small organic molecules in the absence of specific receptors, pumps, or chaperone proteins to facilitate their movement.

4.1 Previous Findings

4.1.1 Hydrolysis mechanism

HPLC studies of drug-lipid interactions had demonstrated conclusively that hydrolysis of the lipids' acyl groups was taking place orders of magnitude faster than was observed in control samples, in a fashion that was catalytic with respect to the drug molecules. The reaction was observed in a range of compounds which bore resemblance to one another in only very broad-brush terms (for a full list see Baciú *et al.*⁵³; those in use in this study are discussed below in section 4.2, page 55): all were charged at physiological pH, almost all showed low aqueous solubility or at least a strong preference for organic solvents, expressed by their relatively high log P values and all contained significant planar aromatic regions or constrained ring structures. Beyond that, however, the compounds had little or nothing in common, narrowing the list of potential causes.

Perhaps unsurprisingly, these statements apply to almost all successful small molecule drug compounds. The vast majority conform to an empirical law known as the "Rule of Five"¹²¹, first presented by Lipinski *et al.* in 1997. This proposed that as a general rule, ideal solubility and membrane permeability behaviour was found in compounds with a formula weight below 500g/mol, with a log P of 5 or lower, five or fewer hydrogen-bond donors and ten or fewer hydrogen bond acceptors. Although experimentally determined and with little or no theoretical justification, this model has held significant sway over pharmaceutical research since its publication, gathering almost 2,000 citations to date. It is generally true for most classes of small molecule drug (although it can be argued that it becomes a self-fulfilling prophecy once accepted as a filter for candidate compounds in industry), but the membrane-traversing reaction observed by Baciú *et al.*⁵³ may present a plausible mechanism through which the parameters above exert their influence.

The drugs tested in the original paper were all cationic in nature, carrying an amine which became protonated at physiological pH. It was hypothesised that the reaction was due to these protons, coupled with the intrinsic hydrophobicity/amphiphilicity of the molecules: the further the drugs migrated towards a membrane hydrophobic interface, the greater the penalty for carrying an unpaired charge, as described in section 3.2.1. This meant that the protons were only weakly bound, acting in effect as free acids attached to a phase transfer agent. This

allowed them to approach closely to the acid-sensitive glycerol esters which form the core of most lipid molecules (see Table 1), and cause their acid-catalysed hydrolysis, as shown below in Figure 12, below.

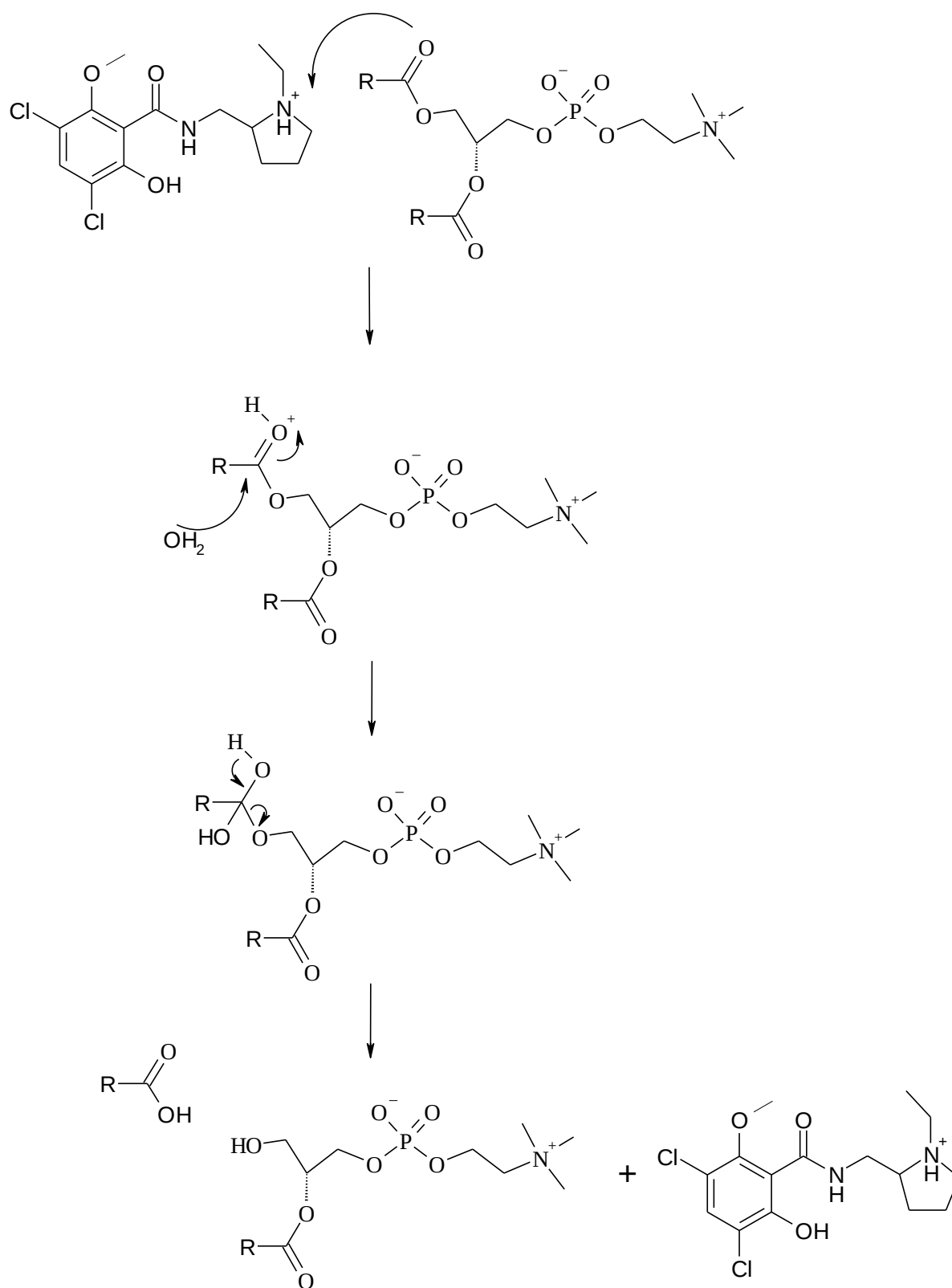


Figure 12: The proposed ester hydrolysis mechanism, with raclopride shown as the proton donor. Note that the drug is unchanged by the process, and how the mono-acyl product of the reaction is in itself a potential substrate.

If the reaction is the root cause of the lipid hydrolysis, then by making small chemical, structural or electrostatic modifications to the catalytic agent it should be possible to adjust the rate at which this occurs. It seems likely that such a process contributes on at least some level to the *in vivo* transport of drug molecules; this means that the rate and degree of a compound's bioavailability can therefore be tuned depending upon its desired application. This has been successfully accomplished in the past by the pharmaceutical industry, although through trial and error rather than design: the transport of salbutamol, the asthma drug, was minimised to avoid the unpleasant side-effects caused by its β_2 -adrenergic agonist action upon systemic receptors by vastly increasing its lipophilicity. This led to the introduction of salmeterol, the same drug but with a long membrane anchor, greatly increasing its membrane binding and thus slowing its release and minimising its systemic (rather than topical) availability¹²². As such, understanding the mechanistic underpinnings of this process is potentially of major interest to the pharmaceutical industry, as a lack of control of systemic availability of a compound is amongst the most significant contributors to attrition rates in drug development programmes.

4.1.2 Hofmeister effects upon drug-mediated hydrolysis

The initial study published on the drug-mediated hydrolysis had demonstrated the effects of drug structure upon the rates of reaction. However, subsequent research suggested that effects could also be observed through the choice of counter-ion in the system, if the drug were administered as a salt⁵⁴. At its most basic level, the Hofmeister effect^{35,36} describes the effect of an ion upon the structure of water around it. Although not as immediately intuitive as the effect of direct structural modifications, it has long been known that salt choice can have major effects upon the stability of colloids and suspensions: indeed, it was the effect upon protein solutions that first led to its discovery, previously described in section 3.2.3, page 27.

Studies investigating the effect of a range of counter-ions to the CAD haloperidol were conducted, with the system showing a strong preference for organic, carboxylate anions⁵⁴. Although this was of some interest, the data did not follow that found in previous studies⁵³, even under identical experimental conditions. Furthermore, the two anions which showed the highest rates of hydrolysis were formed from amongst the strongest acids used: formic acid and trifluoroacetic acid. This raised the possibility that a small excess of free acid could have had a

significant effect upon the observed rates.

4.1.3 Buffer choices

The buffer used in these experiments was 10mM Tris HCl at pH 7.4, in common with the majority of previous studies, allowing direct comparison of the data. However, there were two major problems with this idea. Firstly, the pH of 100mM Tris freebase solution is approximately 10.9. This means that in order to reach pH 7.4, only ≈ 1 in 3,000 Tris molecules remained in a state where they could absorb any further protons generated by the system. Secondly, calculations performed subsequent to publication revealed that the effective concentration of haloperidol in the system was approaching 20mM, or twice that of the buffer. This should have had no impact as the drug was administered in a net neutral salt form. However, it seems likely that the hydrophobic drug ($\log P \approx 4.2$) will migrate to the membrane rapidly leaving its more hydrophilic counter-ion effectively free in solution. This charge separation could potentially cause unpredictable localised charge gradients and counter-ion condensation effects as the system sought to equilibrate. This was compounded by the unknown effects of the generation of fatty acids by the hydrolysis reaction upon the both the local and global pH of the system. The pK_a of a fatty acid within a membrane has been previously shown to be approximately 7.8⁶⁰, implying that around half the fatty acid molecules will be ionised in this system at any given time. After 5% of the original lipid has degraded, then the fatty acid concentration will reach the same as that of the drug, i.e. ≈ 20 mM: if half of these molecules are ionised, then the number of extra free H^+ ions in solution reaches the concentration of the buffer. As such, the pH of the system as a whole has become the topic of much debate within the project team.

Despite this, however, there are precedents: Li *et al.*¹²³ demonstrated that salts of haloperidol remain as a close-ion pair for some time after dissolution, even when chloride ions predominate significantly. This has potential ramifications for drug formulation and initial transport models, although the long residency times of a drug and high chloride concentrations *in vivo* meant that ion conversion would be inevitable. As a result of these observations, buffer and salt effects were made a priority in the investigations detailed below.

4.1.4 Membrane composition

Some preliminary studies had been undertaken to investigate the effect of altering membrane composition upon the observed rates of drug-mediated hydrolysis¹¹⁸. DOPC, the standard lipid model in these studies due to its low spontaneous curvature and relative ubiquity, was substituted with DLPC (a shorter-chained, saturated lipid with significantly lower stored curvature elastic stress) or DOPE, (see Figure 5), whose primary amine head-group leads to it forming inverse hexagonal phase under physiological conditions, meaning that DOPC-DOPE mixtures have a very high curvature stress due to the clash between their preferred packing structures. These results suggested that this stress was the dominating factor: DOPE was hydrolysed faster than DOPC which is turn reacted faster than DLPC. However, this data series was noisy and difficult to interpret, and it was decided that a study in more depth, covering a broader range of lipids, was to be made a priority.

4.1.5 Working hypotheses

As a result of these previous findings, three working hypotheses were generated which were to be investigated during the course of this study. Firstly, it was proposed that the rate of lipid hydrolysis is dependent upon physical state of membrane and physico-chemical parameters of drug. This is important, as it implies that firstly, the rate of hydrolysis can be quantitatively predicted given sufficient knowledge of the structure of the drug or metabolite of interest and the state of the native lipid membrane. It also suggests a physico-chemical basis for the different tissue specificity of different drugs: the lipid makeup of the lungs, for example, is very different to that of the liver and this suggests a mechanism through which that specificity might be controlled.

Secondly, it was theorised that systemic transport is dependent, at least in part, upon this hydrolysis. As outlined previously, there is no overarching theory that explains how and why some drug molecules travel as they do, particularly those with very high partition functions or very low aqueous solubility. Furthermore, considering the vast, intricate and elegant network of biological feed-back processes controlling the body's basic biochemical function, it seems almost beyond credibility that something so apparently fundamental as this might have simply slipped notice. Links have previously been drawn between this process and phospholipidosis⁵³: the

reaction does not necessarily have to have a beneficial influence upon *in vivo* drug transport, but it would seem unlikely that such a process garnered no physiological response whatsoever.

Thirdly and finally, it was theorised that hydrophobic drugs exhibiting low rates of hydrolysis may be sequestered in membranes, dramatically increasing non-specific binding. Of the three hypotheses, this was the one on the firmest ground: it is accepted that hydrophobic drugs are retained in the membrane and indeed, some drugs are designed and optimised to make use of this fact¹²⁴. What again was unclear was how such drugs could ever be persuaded to leave their host membrane: drugs which are sequestered in membrane or adipose tissue are effectively useless unless they contain the receptors of interest. This hydrolysis mechanism fills a potential niche in this regard, supplying a mechanism by which even the most hydrophobic and least soluble drug might effectively be transported through the bloodstream.

4.2 *Experimental Approach*

The study was broken down into two main areas of research. The effects of the drug's steric and electronic structure upon its ability to hydrolyse membranes was probed using a small series of analogues of the dopamine-D₂ antagonist raclopride, a common and well-characterised PET radiotracer in medical imaging studies¹²⁵. This compound was chosen over a range of others due to its synthetic accessibility, presented by the amide bond at its core (see Figure 13, below), its favourable solubility values which simplified handling, as well as its high specificity for the D₂ receptors located in the striatum of the brain and relatively low non-specific binding compared to other such probes¹²⁶.

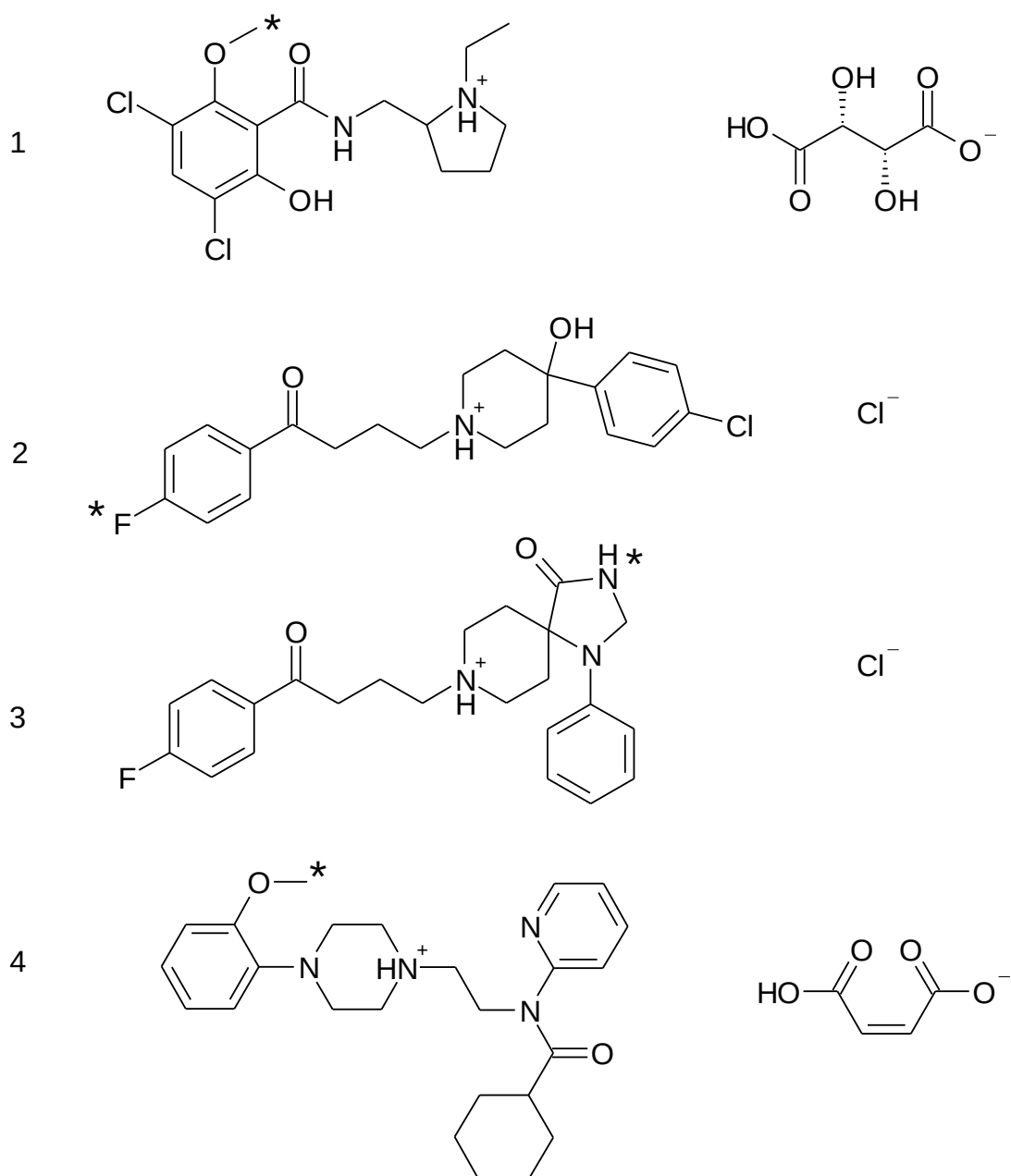


Figure 13: Parent drug compounds used in the study. For PET studies, the atom labelled with a star is substituted for an positron-emitting equivalent. All compounds are shown in the salt form used. 1) The dopamine-D₂ antagonist raclopride tartrate; 2) the D₂-antagonist haloperidol HCl; 3) the D₂-antagonist spiperone HCl; 4) the serotonin-HT_{1A} and dopamine D₄ antagonist WAY 100635 maleate.

The other aspect of the investigation focussed upon the effects of the physico-chemical parameters of the membrane affected the reaction. Previous work had demonstrated that membrane composition had a significant impact upon rates, presumably by changing the effective log P of the drug molecules by altering the ability of the membrane to absorb small molecules. However, there was no clear logic behind the effects: again, hypotheses abounded,

but little was known for certain.

Other physico-chemical variables also required investigation. At their most basic level, lipid molecules in a cell membrane are vastly outnumbered by the surrounding aqueous media and form what are, in effect, giant unilamellar vesicles of some 20-80µm diameter. However, the constraints of SAXS, solid-state NMR and HPLC analysis require significant masses of concentrated sample, meaning that the majority of previous studies were performed in condensed phase. This will inevitably have distorted results to some degree: the water channels in a multilamellar DOPC system are of the order of 3-5nm¹²⁷, meaning reduced rates of diffusion of drug, buffer and hydrolysis product molecules relative to vesicular systems. Similarly, the choice of buffer and salt could potentially play a significant role in the process, giving rise to experimental artefacts. It was recently demonstrated that PIPES buffer (1,4-piperazinediethanesulfonic acid) caused significant perturbation to membranes by acting as a co-surfactant, causing unusual morphological changes and impacting the reliability of research results¹²⁸. Previous experiments^{53,54} were performed using a range of organic buffer molecules, and these may have influenced the findings thereof.

5 Assay development*

5.1 Initial assay conditions

5.1.1 Lipid preparation

In order for these tests to be possible, a thoroughly robust and reproducible assay was required, both for condensed and ideally vesicle-phase models. The starting point for such work was the assay developed by Sebai¹¹⁸ which provided reasonably reproducible rates of hydrolysis of samples in the bulk mesophase and thus formed a good starting point. A typical data plot derived using these conditions is shown below, in Figure 14.

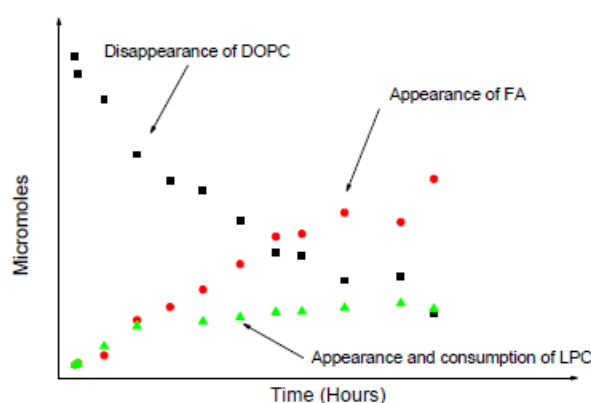


Figure 14: A typical data plot showing the hydrolysis of DOPC by haloperidol under the initial assay conditions, measured by normal-phase HPLC. Reproduced from Sebai¹¹⁸.

In brief, in each vial 20mg (25 μ mol) DOPC with 5 mol% drug were dissolved in a mixture of 1:1

* Error bars have been omitted from this section, as each experiment was an iterative one-off step in the developmental process and both experimental and analytical conditions varied significantly between runs. Furthermore, each experiment required approximately a month of lab and equipment time, thus limiting the opportunity for repeats. This meant that there was neither sufficient data nor any major benefit to be gained by the analysis of the error margins of each dataset, and all data points gathered are shown. However, once analytical conditions had been finalised, relevant results were aggregated to provide a robust statistical analysis – for these data, please see section 5.5, page 90.

chloroform and methanol and the solvent removed under a nitrogen stream. The vials were then dried in a vacuum oven at 40°C overnight, before being hydrated with 10mM Tris HCl at pH 7.4 (Tris(hydroxymethyl)aminomethane hydrochloride), 3:1 buffer/lipid w/w. The assay vials were then vortexed and centrifuged three times to ensure homogeneity, forming a white opaque suspension and incubated at 37°C throughout the course of the experiments. The pH of the buffer and all others described in this text were measured using a Hanna Instruments (Leighton Buzzard, UK) pH210 Microprocessor digital pH meter. Lipids were purchased from Avanti Lipids (AL, USA); all other compounds were purchased from Sigma-Aldrich (UK) unless otherwise specified and were of at least 95% purity. All buffers were made using HPLC grade water (VWR, UK). If SAXS measurements were to be taken in parallel with the HPLC analyses, 8-10µl of the suspension was dispensed into a 1.5mm diameter glass capillary tube (Capillary Tube Supplies Ltd., UK) and was spun to the bottom of the capillary *via* centrifuge. The capillary was then flame sealed and capped with a silicon gum to prevent drying.

6µl samples were taken every 3-4 days using a Gilson pipette and dispensed into a 1ml HPLC sample vial. These vials were dried under vacuum in a desiccator connected to a Thermo Fisher Micromodulyo freeze-dryer to halt any further reaction, and were then redissolved into 1ml methanol for HPLC analysis.

These conditions were taken as a starting point for the development of the assay used throughout this thesis, to allow direct comparison with historical data. The optimisation process and its results are described in full below, in section 5.4 page 72.

5.1.2 HPLC equipment and initial chromatography conditions

Analyses were performed using an all-PEEK (polyetheretherketone), dual-pump system (Model 626 LC, Waters). Initially, measurements were conducted on a Kromasil 60-5 column (Hi-Chrom) and analysed via evaporative light scattering, using an ESA 301 detector. The initial mobile phase gradient used ran from 101/21/2/0.1 hexane/isopropyl alcohol/acetic acid/triethylamine (solvent A) w/w to 155:17.5:2:0.1 isopropyl alcohol/water/acetic acid/triethylamine (solvent B), degassed through a Degassex DG-401 unit (Phenomenex). 100µl of the ethanol solution was injected on each occasion using a Waters 771Plus autosampler module, giving strong ELSD signals with relatively low amplification, meaning a good signal-to-noise ratio. Each HPLC run

ran a gradient from 100% A to 50:50 A and B over the course of 35 minutes. Due to the normal-phase conditions chosen, oleic acid eluted first and was difficult to resolve from the solvent front; DOPC eluted with a retention time of approximately 18 minutes and lyso-OPC eluted after approximately 21 minutes, shown below in Figure 15.

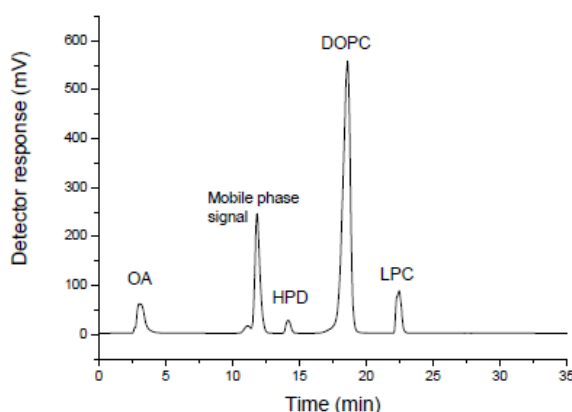


Figure 15: A typical HPLC spectrum showing the hydrolysis of DOPC by haloperidol hydrochloride (HPD) under the legacy analytical conditions used as a starting point in the study. Oleic acid (OA) is eluted first, in a peak which was often difficult to distinguish from the solvent front. Lyso-lipid (LPC), the most polar of the species, was eluted last. Reproduced from Sebai¹¹⁸.

These conditions had a number of drawbacks that required improvement or resolution before truly high-throughput or real-time studies could begin. Firstly, the gradient required for efficient separation meant that each sample took 35 minutes to run, as can be seen above. Previous experiments had shown almost total disruption of lipid vesicles by CADs over this timescale⁵³, which effectively precluded the possibility of real-time analysis. Furthermore, the complex solvent mixtures required meant that reproducibility was problematic, as small errors in component make-up could lead to significant changes in the retention times of the analytes. As a result, a thorough optimisation programme was initiated early in the study to look at both column technologies and elution gradients – see section 5.2 for details.

5.1.3 Small-angle X-ray scattering

SAXS was conducted using custom-built equipment, based around a Bede (Durham, UK), Microsource X-ray generator with integrated optics, an Ealing (Ealing Electro Optics, UK), linear transition stage and a Photonic Science Ltd (Battledie, UK), Gemstar intensified CCD X-ray

detector¹²⁹. X-ray data were typically acquired over a 60-second exposure and spacings were calibrated against a silver behenate standard¹³⁰. Diffraction patterns gained from the samples prepared above were analysed with the IDL-based AXcess software package that was developed by Andrew Heron at Imperial College⁵³.

5.2 HPLC optimisation

Whilst the approaches described in section 5.1.2 yielded data about the initial rates of reaction, they provided few indications as to the eventual fate of the lyso-lipids generated as a result of the hydrolysis due to its inability to quantify the fatty acid peak. Normal phase HPLC, as described above, retains compounds for a time that is (broadly speaking) proportional to their polarity and number of hydrogen bond donors and acceptors⁹³; fatty acids, particularly the 18:1 oleic acid generated from the hydrolysis of DOPC, are almost entirely neutral, hydrophobic species and have very few interactions with the stationary phase. As a result, the retention time of the fatty acid was below 2 minutes, which coupled with its relatively broad peak profile meant it vanished into the solvent front of the HPLC spectra.

Another issue was that the run time was far too long to be used for any real-time analysis of samples: in the fluorescence micrographs taken in the initial study, massive morphological changes had occurred within 30 minutes of drug administration. Using the initial solvent profiles, this would only have allowed one data point, at $t=0$, which would be meaningless. As a result, early efforts were devoted to optimizing the HPLC parameters to improve both resolution and run time.

While lipids are amenable to separation by HPLC and a number of well-characterised approaches exist for their isolation, most techniques depend upon a two stage process. Initially, lipids are separated into head-group classes *via* normal-phase chromatography; each of these peaks is then recovered, dried and redissolved in an organic solvent for reverse-phase chromatography to isolate the individual acyl species^{93,131}. This was both too cumbersome a technique for the purposes required in this study, but was also inhibited by solubility issues: it was extremely challenging to identify a solvent or mixture of solvents that would simultaneously deliver good chromatographic separation, would dissolve the di-acyl, mono-acyl and fatty acid species and was compatible with the all-PEEK HPLC system outlined in section 5.1. The PEEK used in the manufacture of the HPLC was an issue, although it conferred certain advantages with regards to lipid binding, as it effectively eliminated the steel surfaces to which the lipids would otherwise bind strongly and spontaneously, blocking the tubes and causing mechanical failure of the device. However, PEEK is incompatible with tetrahydrofuran (THF), chloroform and dimethylsulphoxide (DMSO), which cause swelling and softening of the polymer which prevents the use of these otherwise extremely strong and versatile solvents. This was a considerable

disadvantage in this case, as many alternatives were not suitable for one reason or another: for example, while methanol rapidly solubilised both mono-acyl and di-acyl phosphocholine, it is largely immiscible with oleic acid except at very low acid concentrations.

5.2.1 Exploration of column and solvent options

The HPLC optimisation process began with the testing of a variety of column techniques and solvents that were employed over approximately a six month period: both normal- (silica, diol, amine and nitrile) and reverse-phase (C_8 , C_{18} , and phenyl) column packings were experimented with in consultation with experts at Waters and Varian (now a part of Agilent), each through a variety of solvents. The major difficulty that presented itself was the vastly different behaviour of the acyl species *versus* the fatty acid. It was possible to resolve the two acyl lipids on most normal phase systems, with varying degrees of success and with differing retention times, but in each case the fatty acid was effectively lost in the solvent front. In contrast, reverse-phase techniques gave excellent resolution of the fatty acid from the acyl lipids, but it was extremely difficult to resolve the acyl species reliably. This process could have been sped significantly had the HPLC been fitted with automated method development software; unfortunately, the unit was too old to be compatible with such a package and so there was little option but to perform the process manually.

Several weeks of development led to the adoption of a 150 x 4.6 mm nitrile column, generously donated by Varian, as the separation medium of choice, with a subsequent reduction in analysis time from 35 minutes to 23 minutes, shown below in Figure 16. Although efforts were made to modify and simplify the solvent system to improve reproducibility, these led to failure and so the same complex solvent mixture was retained. This technique presented a slightly better retention profile for fatty acid species, while still permitting precise resolution of the two acyl classes, although it was still slower than was perhaps ideal.

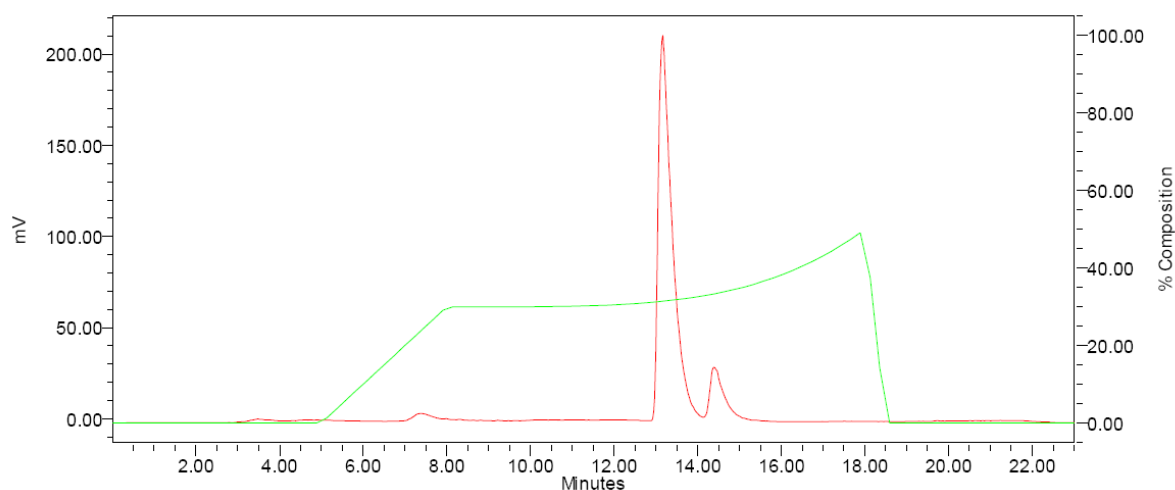


Figure 16: A example of an HPLC spectrum showing DOPC degradation using the nitrile column method described in 5.2.1. Note that timescale is significantly shorter than in Figure 15 and that artefact peaks due to mobile phase have been eliminated, at the cost of a quantifiable fatty acid peak. Green trace shows solvent profile, as % strong solvent.

5.2.2 Final HPLC conditions

After further consultation with Varian, a novel column packing was developed from a Polymer Labs test compound originally designed for the low-pressure separation of protein species. Known as PLRP-AQ, it contained 12 μ m hydrophilic, hydroxylated polymer beads with hydrophobic pores of around 100Å and a pore volume of 45%. This gave a mixed mode selectivity via both partition as in gel phase and more normal adsorption interactions, with a particular affinity for amphiphilic molecules. Successfully trialled as a 45mm column, this was scaled up to a full 150 x 3.9mm size for further analyses and the technique formed the backbone of the study during its final 12 months. This column was used with a water and 0.1% formic acid to methanol gradient as external factors made the sourcing of adequate volumes of acetonitrile, the favoured 'strong' solvent, both difficult and prohibitively expensive. This approach reduced run times to 15 minutes, and importantly allowed on-line, real-time analysis: for the first time, crude samples of lipid and buffer could be injected directly onto the column without pre-treatment. The solvent profile consisted of a short run at 95% water to clear the buffer and any inorganic salts from the column which were otherwise found to co-elute with the lyso-lipids. This was followed by a rapid climb to 70% methanol, followed by a shallow gradient to 95% methanol. Retention times and mass-to-peak-area data are discussed in detail below in section 5.3.

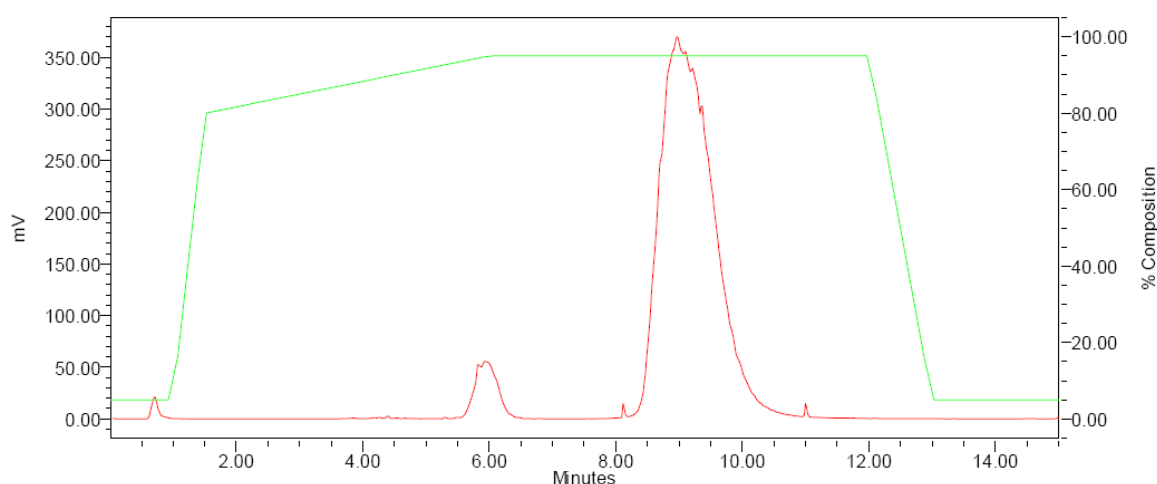


Figure 17: An example of an HPLC spectrum showing the degradation of DOPC analysed using the experimental PLRP column described in section 5.2.2. Although peaks were broadened due to large particle size and the use of methanol rather than acetonitrile as the strong solvent, retention times were reduced and the need for sample pre-treatment was eliminated. Green trace shows elution profile in % methanol; retention times are discussed in section 5.3.

This made vesicle-based assays a real possibility: one of the major stumbling blocks to their usage was the requirement for sample drying before analysis. In a condensed-phase sample, only a small amount of buffer is present and so even under drying, it was unlikely to have a significant effect upon the sample. However, in a vesicle-based assay there is a much greater volume of water to remove in order to quench the reaction – this led to extremely high buffer concentrations towards the end of this process, introducing unpredictable artefacts.

Throughout this process, the detector remained the ESA 301 unit described in section 5.1. As the study progressed, photomultiplier tube voltages, nebuliser and evaporation temperatures and injection volumes were modified, and the machine itself was serviced or repaired several times, altering the absolute signals generated. Fortunately, it was relatively easy to normalise the peaks recorded by the unit, meaning that these changes had little or no impact upon the data recorded, aside from providing a significant improvement to sensitivity and peak shape. This can be observed in the tight spacing of the aggregated dataset, which can be seen in detail in section 5.5.

5.3 *Detector Calibration*

Calibration was conducted using standard solutions of each lipid required at a concentration of 0.3-1mM in methanol, and the volume (and hence number of moles) injected was plotted against the peak area recorded by the ELSD. Initially, precise lipid masses were measured using a five-decimal-place balance (Sartorius), to allow directly quantitative determination of the relative masses of lipid and lyso-lipid present in a given sample. However, the masses involved (1mM DOPC is equivalent to 0.786mg/ml) meant that small drifts in balance calibration or even air pressure could lead to large error propagation, meaning such calibrations had significant error bars. Because the point of each analysis was to assess the degree of hydrolysis as a percentage rather than in absolute units, this proved to be unnecessary: the peak areas were normalised to generate gradient data, and the gradients of different lipids were compared to allow the quantitative calculation of the degree of hydrolysis of any given sample, assuming that the hydrolysis of lyso-PC was negligible relative to that of DOPC. Although this may be thought of as quite a serious approximation, the linear nature of all the data plots generated suggest it is a valid one, as shown in the results of a typical assay containing DOPC with 5 mol% raclopride tartrate, shown below in Figure 18, below.

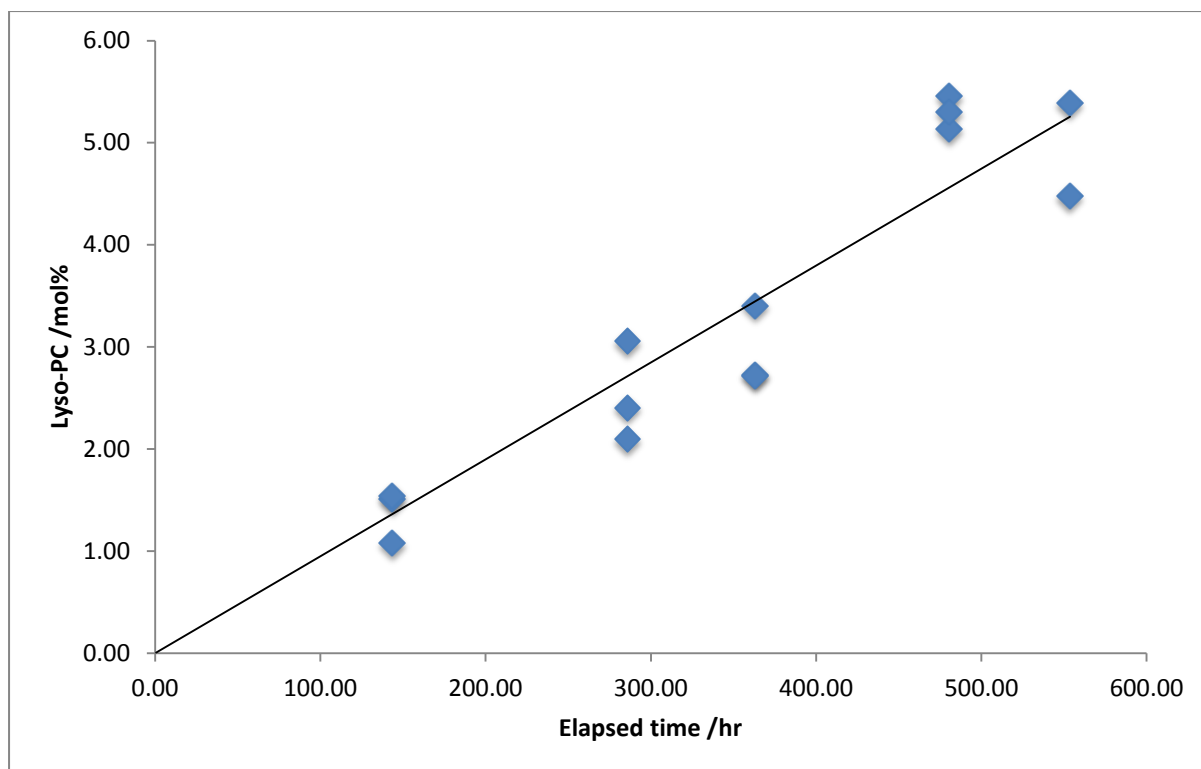


Figure 18: The results of a typical DOPC hydrolysis assay, $n = 3$. The lipid was co-lyophilised with 5 mol% raclopride tartrate and incubated at 37°C. Trend line is added to guide the eye.

This technique generated some interesting results that were of significant interest to the study. Firstly, it was found that at low sample concentrations, the performance of the ELSD unit was non-linear, whereas at higher sample concentrations or greater injection volumes, the signal obtained was directly proportional to the number of moles injected, shown below in Figure 19.

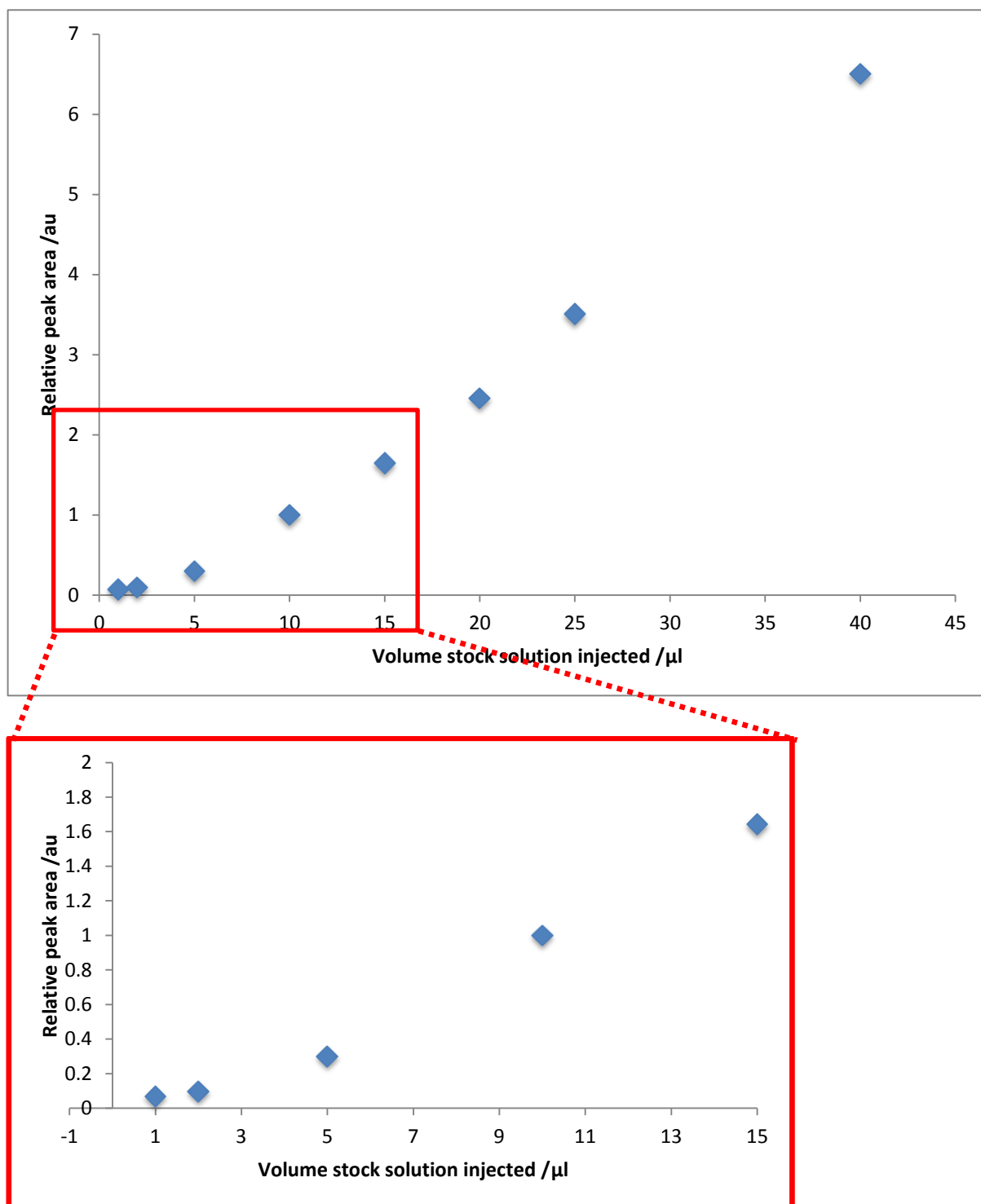


Figure 19: The non-linearity in signal observed at low sample concentration. Although small, this effect was consistent and repeatable. Example shown is from a stock solution of $\sim 0.5\text{mM}$ DMPC in methanol, photomultiplier tube set to 700V. Inset shows the detail at low sample volumes.

This was of note as previous studies had observed a significant lag period of some 30-50 hours in between the administration of a hydrolytic drug compound and the first recorded lyso-PC signal

(unpublished data). This was initially taken to stem from some form of internal re-organisation, as the drug bound to the membrane and undertook some form of conformational change or reached some critical concentration before the reaction began to occur at any significant rate, similar to that observed in the activity of PLA₂ (see section 3.3.1). This was supported in some measure by SAXS data published previously⁵⁴ (discussed at greater length in section 5.5.2), which showed significant, time-dependent phase changes in the long-range organisation of DOPC lamellae which correlated strongly with observed rates of hydrolysis. However, the detail obtained from these calibration experiments suggest a much simpler alternative: that the 'lag' phase was simply an artefact generated by the limited sensitivity of the light-scattering detector at low sample levels. This could potentially have had a significant impact during the early stages of each experiment when lyso-PC signals were low, leading to the non-zero y-intercepts observed in the data plots presented below. Later experiments revealed that the error lay as much with the autosampler as the detector: repeated injections of 5µl samples gave a range of peak areas, suggesting that the unit could not accurately dispense volumes below 10µl. The other finding of the calibration experiments was the discovery that the signal observed was, within error, constant between all lipid molecules, dramatically simplifying analysis, shown below in Figure 20.

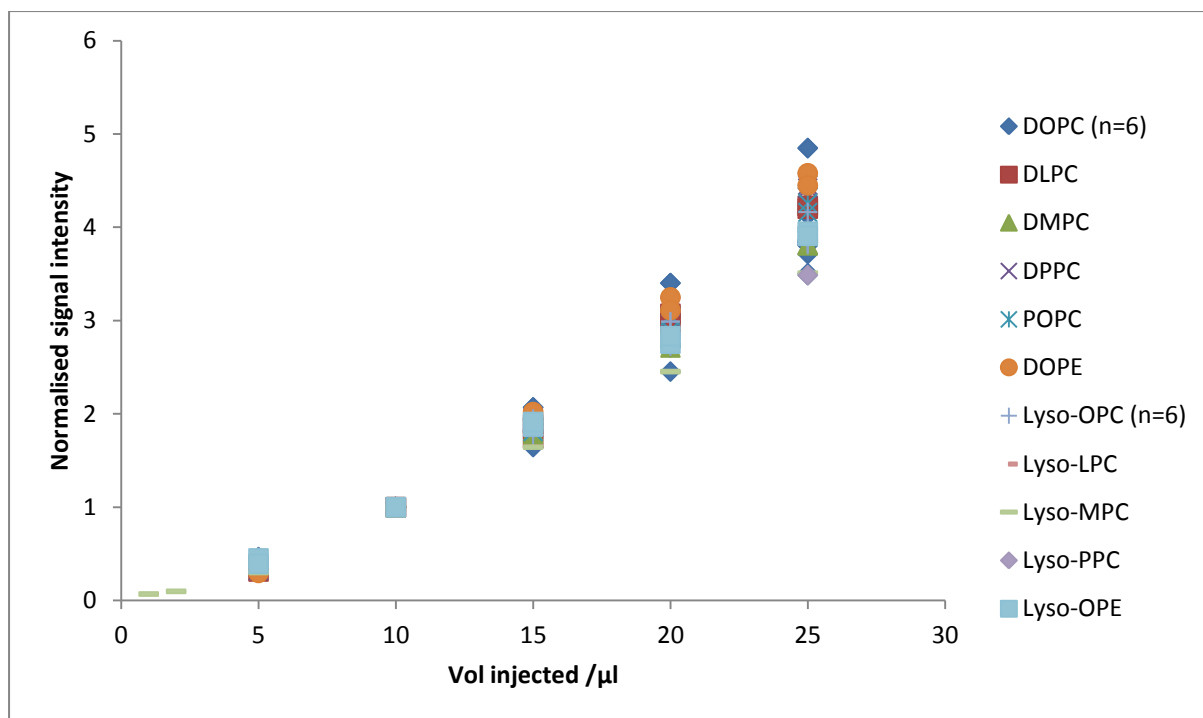


Figure 20: Normalised calibration data from the injection of standard lipid solutions, concentration 0.3-1mM. Data were normalised around the smallest reliable injection volume, 10μl. There was no discernable correlation between molecular weight or degree of unsaturation and detector response in the region of interest. Linear regression analysis gave errors of ± 0.489 a.u. intensity (2 s.d.).

Lipid species	Retention time /min
DOPC	10.9 (17.6)
DLPC	7.5 (12.7)
DMPC	8.8
DPPC	10.2
POPC	11.0
DOPE	11.6
Lyso-OPC	6.1 (9.6)
Lyso-LPC	(5.5)
Lyso-MPC	5.2
Lyso-PPC	6.0
Lyso-OPE	6.5
Cholesterol	11.1

Table 2: The retention time of each lipid species studied in this investigation, *via* the HPLC method described in section 5.2.2. Where two values are given (e.g. for DOPC): the first is the retention time *via* the optimised protocol; the value in brackets describes the result of the extended gradient described in section 6.2.

The retention times for each of the lipid species studies are listed above in Table 2. The standard conditions described above worked well for the majority of PC and PE lipid studies, but it can be seen that the co-elution was observed between DLPC and several lyso-lipid peaks. As a result, an extended version of this system with a shallower gradient step was established to run over 20 minutes: the retention times under these conditions are shown in brackets.

Fatty acid peaks were not quantified under these conditions. Most fatty acids are effectively immiscible with water and methanol: although they came off the column with a retention time of around 12-13 minutes, they did so in a broad and noisy band with a very low mean intensity, to the point where they made little or no contribution to the signals of other species. This meant that, as described above, all the following data is presented on the assumption that raclopride's hydrolytic activity towards di-acyl lipids is significantly greater than that towards lyso-lipids. However, the linear growth of the lyso-lipid signals in all studies suggests that this is a valid assumption, although one that may not be true for all CAD-like compounds.

5.4 Buffers, salts and ionic strength

5.4.1 Experimental issues and potential artefacts

Upon revisiting the data derived from the original assay, several unusual phenomena were observed. Firstly, rates of hydrolysis were extremely slow, reaching only around 10-15% hydrolysis after some three weeks of incubation. This compared very poorly with the extremely rapid morphological changes observed in GUVs via fluorescence microscopy (see Figure 10, above). There was some concern that this was a result of either the choice of buffer, 10mM Tris HCl (Tris HCl), pH 7.4 at 37°C (equating to pH $\approx$ 7.7 at room temperature¹³²), or of the restrictions imposed upon the reaction kinetics by the multiple bilayers of the lamellar structure adopted by the lipid under these conditions, as described above. It was also observed that rates of reaction were not constant: that the rate followed an accelerating curve over time, suggesting some kind of autocatalytic mechanism might be at play.

5.4.2 Incubation conditions

Early experiments showed biphasic results: over relatively short time periods, (<240 hours), the hydrolytic behaviour of the drug was linear and extremely repeatable. Above these timescales, the assays showed significantly different behaviour, demonstrating exponential growth of the lyso-PC mol fraction but with significant variability between runs (see Figure 21 for examples). These assays were stored at 37°C, as described in section 5.1.1, in plastic test-tube racks in an incubating oven. No assay data were used from samples >700hrs old, as after this point significant chain-fission products were observed in the DOPC chromatograms, making results unreliable.

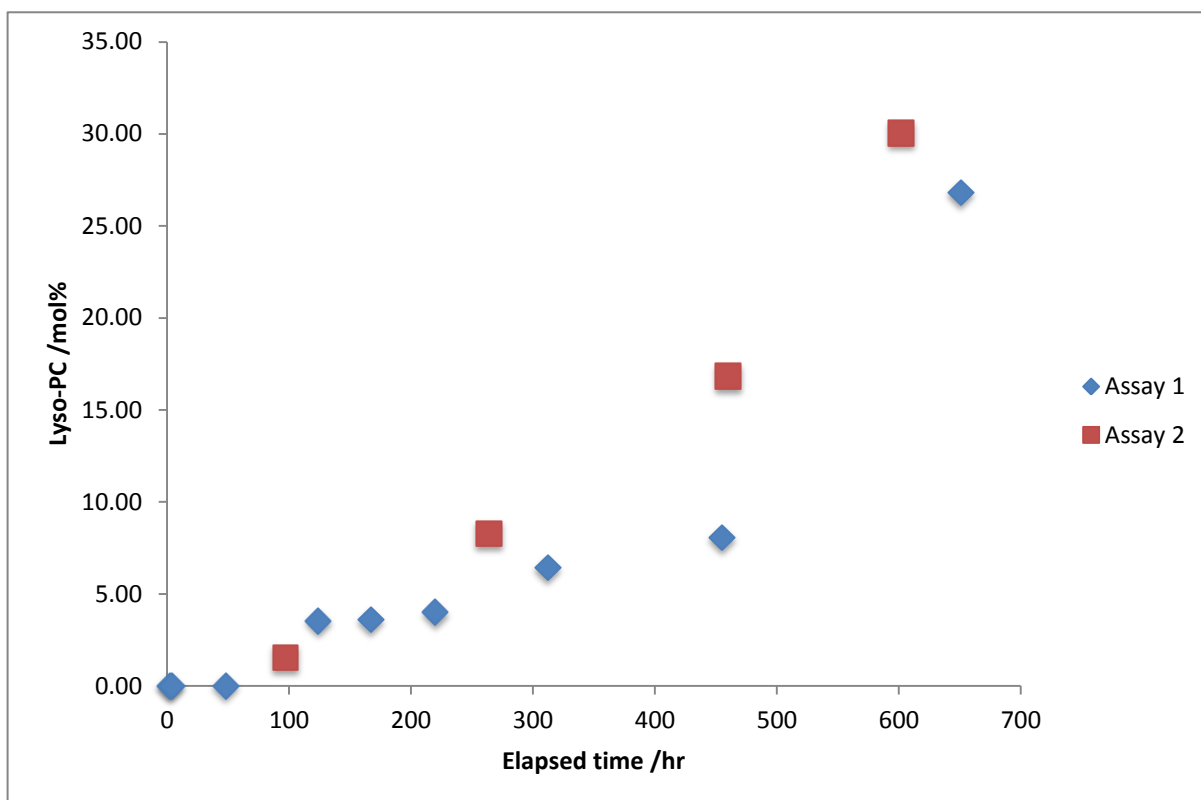


Figure 21: The apparently exponential rates of raclopride-mediated hydrolysis observed in early assays. In these examples, raclopride tartrate was added at 5 mol% relative to lipid and hydrated 3:1 w/w with 10mM Tris HCl. Assays were run with identical batches of lipid and drug, but on different dates.

In between samples, it was observed that a significant percentage of the buffer solution in each vial had evaporated from the lipid at the vial's base and condensed around its neck, due to the thermal gradient caused by the contact of the vial base to the aluminium shelves of the incubator. This led to experimental issues, as the samples had to be re-centrifuged and vortexed to ensure homogeneity before sampling. This meant that the samples were experiencing different conditions over the course of the assay, rendering such results unreliable. Furthermore, although the water from each sample was evaporating away from the lipid, it is very unlikely that the buffer molecules themselves were doing so. This will inevitably have led to high concentrations of buffer in the semi-dehydrated lipid, with unpredictable results.

In order to counter this effect, the vials for subsequent assays were stored in aluminium heating blocks which provided close contact to the glass vials throughout their length. This change had an immediate effect, as can be observed from Figure 22.

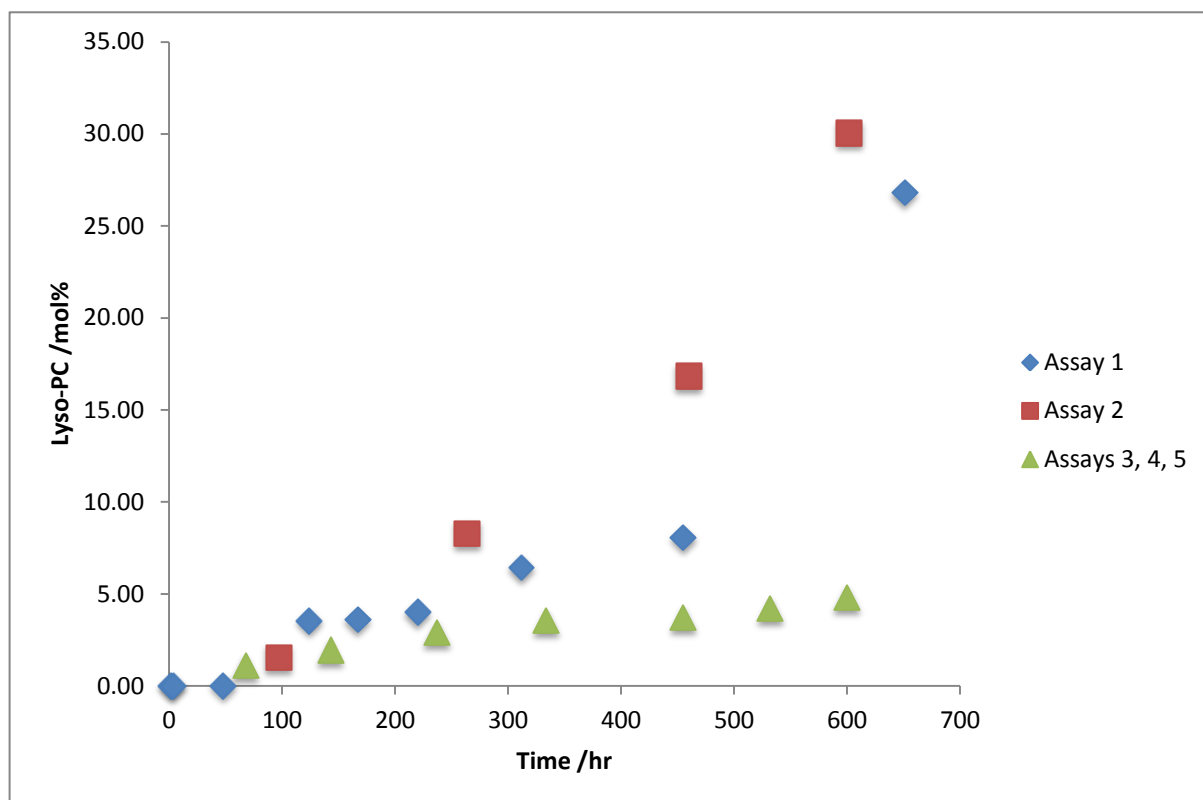


Figure 22: Data from the next three assays showing the hydrolysis of DOPC by 5 mol% raclopride performed under condition identical to those in Figure 21, but incubated in aluminium blocks. Original data is overlaid for illustrative purposes.

This was an unexpected result, in many ways. While some effect upon rate was expected, Le Châtelier's principle would suggest that dehydrating such a system would slow the rate of a hydrolysis reaction, not increase it. It is quite possible, however, that the DOPC remained above its excess water point at all times before the introduction of the heating blocks: DOPC is fully hydrated at any point above 45 weight% water (corresponding to a water:lipid ratio of around 35:1)¹³³, meaning that the increase in rate could possibly be attributed to the relative increase in buffer concentration. In either case, these preliminary experiments raised a number of questions which required resolving before the assay could be relied upon to any significant degree.

5.4.3 Excess water level

Even after the introduction of the heating blocks, the lipid mixture rapidly took on a viscous, translucent appearance as the reaction progressed in an assay vial, more like a putty or a viscous gel than the expected white, opaque fluid. It was proposed that this, too, was due to a shortage of excess free water: the volumes of water involved were sufficiently low that over time, even a slightly imperfect vial seal could lead to significant dehydration and hydrolysis, by its nature, consumes a molecule of water for each reaction step. With a significant percentage of the available water held in the lipid head-groups' hydration shells, it was possible that the reaction was being limited by its availability, or lack thereof. Furthermore, this viscosity made accurate testing challenging, as extracting sufficient lipid to provide a clear signal became an issue.

In order to eliminate this possibility, an assay was run with both raclopride and spiperone (another of the well-characterised compounds¹³⁴ used during the original discovery) as detailed above. However, the volume of 10mM Tris HCl buffer was varied in each vial, between 3:1 and 100:1 w/w relative to lipid, as shown in Table 3. This would not only highlight any rate-limiting effects due to low hydration, but would also provide an excess of buffer relative to drug and/or fatty acid in more dilute samples without directly increasing its concentration. A control vial was also prepared, identical to those with the highest excess water levels but containing no drug, as it was suggested that the equivalent doped sample would react the fastest if hydration was indeed the limiting factor. Five samples were taken from each vial over the course of 28 days and freeze-dried immediately to prevent further reaction, before being analysed as above in section 5.1.

Mass DOPC /mg	DOPC /umol	Drug name	Lipid:drug ratio	Mass drug /mg	Buffer:lipid w/w	Vol Buffer /μl	% water
20	25.44	Raclopride tartrate	20	0.51	3	60	75.00
20	25.44	Raclopride tartrate	20	0.51	10	200	90.91
20	25.44	Raclopride tartrate	20	0.51	25	500	96.15
20	25.44	Raclopride tartrate	20	0.51	50	1000	98.04
20	25.44	Raclopride tartrate	20	0.51	100	2000	99.01
20	25.44	Spiperone	20	0.50	3	60	75.00
20	25.44	Spiperone	20	0.50	10	200	90.91
20	25.44	Spiperone	20	0.50	25	500	96.15
20	25.44	Spiperone	20	0.50	50	1000	98.04
20	25.44	Spiperone	20	0.50	100	2000	99.01

Table 3: The experimental design of the initial experiment to determine the effect of excess water upon rates of hydrolysis.

No hydrolysis was observed in any of the spiperone-doped samples: tubular crystals were

observed in all within seven days of initiation. As these were not observed in the raclopride samples, it was taken that these crystals were formed of precipitated spiperone, which would prevent any reaction from occurring. This may have been due to the drug being administered as a free-base, rather than as a more soluble salt form. More interestingly though, hydrolysis was only observed in the first two raclopride samples, with the 3:1 w/w mixture reacting significantly faster than the 10:1 w/w vial. Furthermore, the approximately threefold increase in dilution led to a similar decrease in rate. This unexpected result led to the experiment being repeated, with DOPC-buffer mixtures between 2:1 and 10:1 w/w, all doped with raclopride tartrate at 5 mol% relative to the lipid. The results of this experiment are shown below, in Figure 23.

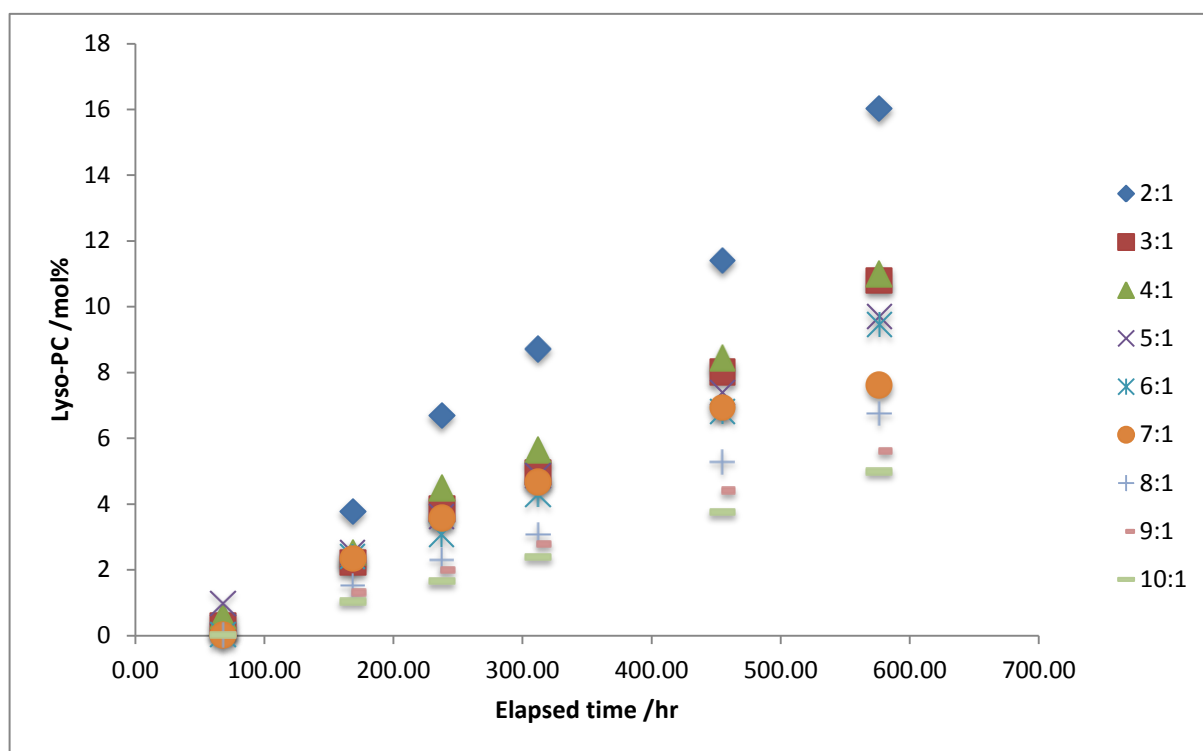


Figure 23:The hydrolysis observed in otherwise-identical DOPC samples doped with 5 mol% raclopride tartrate. Ratios correspond to the amount of 10mM Tris HCl added relative to lipid, w/w.

When the gradients of these data are converted into units of $\text{mol hr}^{-1} \text{mol}_{\text{RAC}}^{-1}$ (where mol_{RAC} is equal to the number of moles of raclopride present) and plotted against buffer:lipid ratio, the following plot emerges (Figure 24).

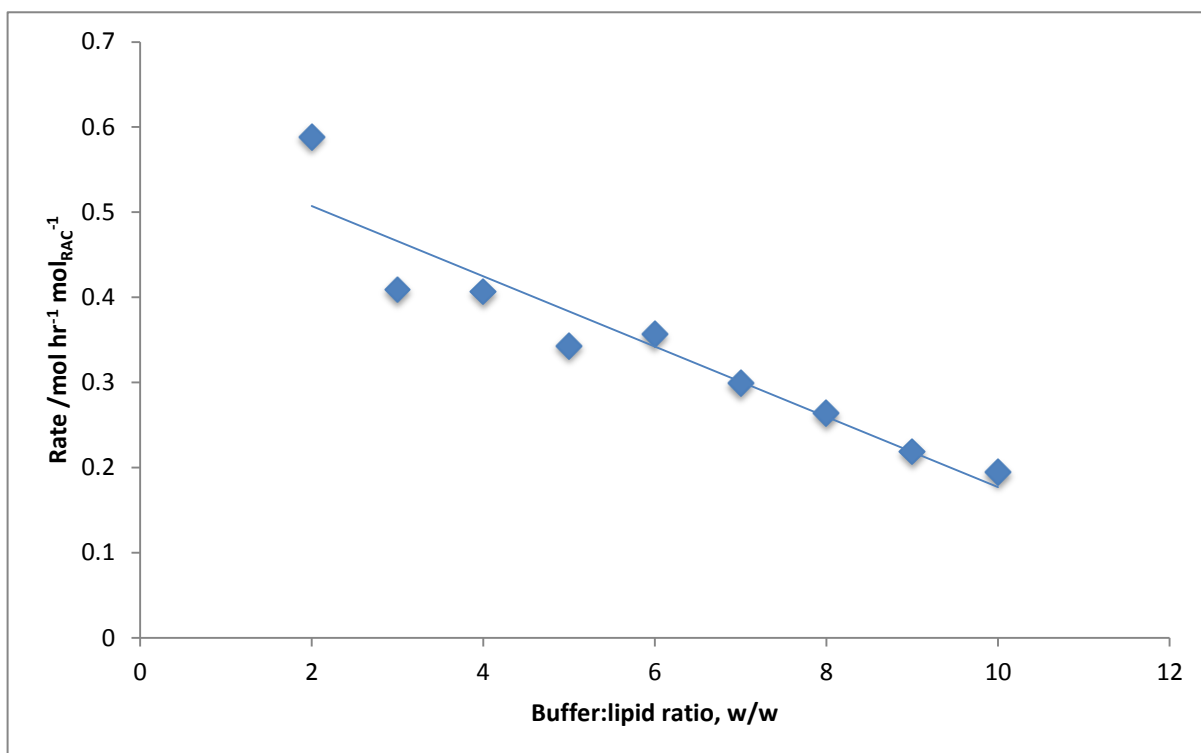


Figure 24: The dependence of the rate of raclopride-mediated DOPC hydrolysis as a function of buffer:lipid ratio. Buffer was 10mM Tris HCl. Equation of trend line is $y = -0.041x + 0.59$. Assuming the trend remains linear until the lipid reaches its excess water point at 45 weight%¹³³, this predicts a maximum rate of $0.556 \text{ mol hr}^{-1} \text{ mol}_{\text{RAC}}^{-1}$. Beyond this point, it seems unwise to speculate.

The linear decrease in rate with dilution could really only be due to one of five things:

- the dilution of the relatively hydrophilic raclopride, ($\log D_{\text{oct}} 1.33$ at pH 7.4¹³⁵) leaving less in the membrane to react
- that despite its theoretically neutral form, the raclopride salt was overwhelming the buffer at low ratios, creating more extreme pH conditions than were realised
- the buffer molecules were migrating to the interface and in some way inhibiting the reaction
- the effect of the excess water upon the average DOPC aggregate size
- the ratio between the number of tartrate to chloride anions in the system led to unusual surface effects at the membrane interface.

Although not immediately intuitive, previous work had shown some dependence of the sample

viscosity¹³⁶ and rate of hydrolysis⁵⁴ upon the choice of counter-ion within the system. As explained in section 4.1.3, at a 3:1 w/w ratio of 10mM buffer to lipid and 5 mol% raclopride tartrate with respect to lipid, there are approximately twice as many tartrate ions as there are chlorides in solution. As the buffer volume increases, so too does the number of chlorides, meaning that at ratios greater than 6:1, chlorides outnumber tartrates. As chloride is a reasonably chaotropic anion relative to the strongly kosmotropic tartrate, it was hypothesised that due to the Hofmeister effect (see section 4.1.2), the movement of this ratio might play a significant role in the conditions at the membrane interface.

5.4.4 Buffer concentration

In order to probe these effects, the experiments shown in Figure 23 were repeated with a more concentrated buffer so as to eliminate any such Hofmeister effects (see section 4.1.2). This would also have the effect of preventing any changes in pH in the system caused by the addition of drug, and could potentially also give some insights into any effects caused by the buffer molecules themselves. As such, the entire experiment was repeated using 100mM Tris HCl as buffer. It was rapidly observed that the viscosity and opalescence described above in the 10mM buffered systems was in some way linked to the buffer strength, as the 100mM samples were significantly whiter and more fluid than previously. However, none of the samples showed any measureable hydrolysis at all over a period of 650 hours and the experiment was abandoned.

This was, in many regards, also unexpected. A higher ionic strength within the buffer should, in theory, make the solution less welcoming to hydrophobic species and thus drive a greater percentage of the drug into the membrane, increasing the observed log D. Similarly, it seems unlikely that this change caused the precipitation of the highly water-soluble drug. This result implied, then, that the effect was either due to the buffer molecules themselves having an inhibitory action, that the counter-ion was involved in some way or that the reaction depended upon the drug in some way overwhelming the buffer and creating more extreme pH conditions than were expected, despite its theoretically net neutral form. The final option was thought to be most likely, supported by the observation that none of the results plots generated showed a line through the origin, suggesting a lag-phase or a period of internal reorganisation before the reaction took hold. However, this was easily explained with low injection calibration of the ELSD

detector, which revealed a non-linearity of signal at low mass levels (see section 5.3), suggesting that these results were simply an artefact of the detector.

In order to confirm these findings, the effect of concentration was probed further, using Tris HCl at 100, 50, 25 and 12.5mM, formed by dilution from 100mM stock using HPLC grade water. All were added at 3:1 w/w, as in previous studies, as it was felt that this gave a quantifiable rate of reaction but simultaneously offered some protection against the samples totally dehydrating during incubation.

The results of this assay are shown below, in Figure 25: what they reveal is that a decrease in buffer concentration led to an increase in rate of reaction in a smooth fashion. There was no obvious 'critical concentration', above which all hydrolysis ceased, simply that as buffer concentration increased the rate slowed until it was no longer detectable using the methods available. This finding eliminated the physical effect of the water upon the lipid aggregate size from the investigation, as these effects were observed at a constant percentage of excess water, meaning that the lipids in each vial had, at least on average, an equal exposure to the bulk medium.

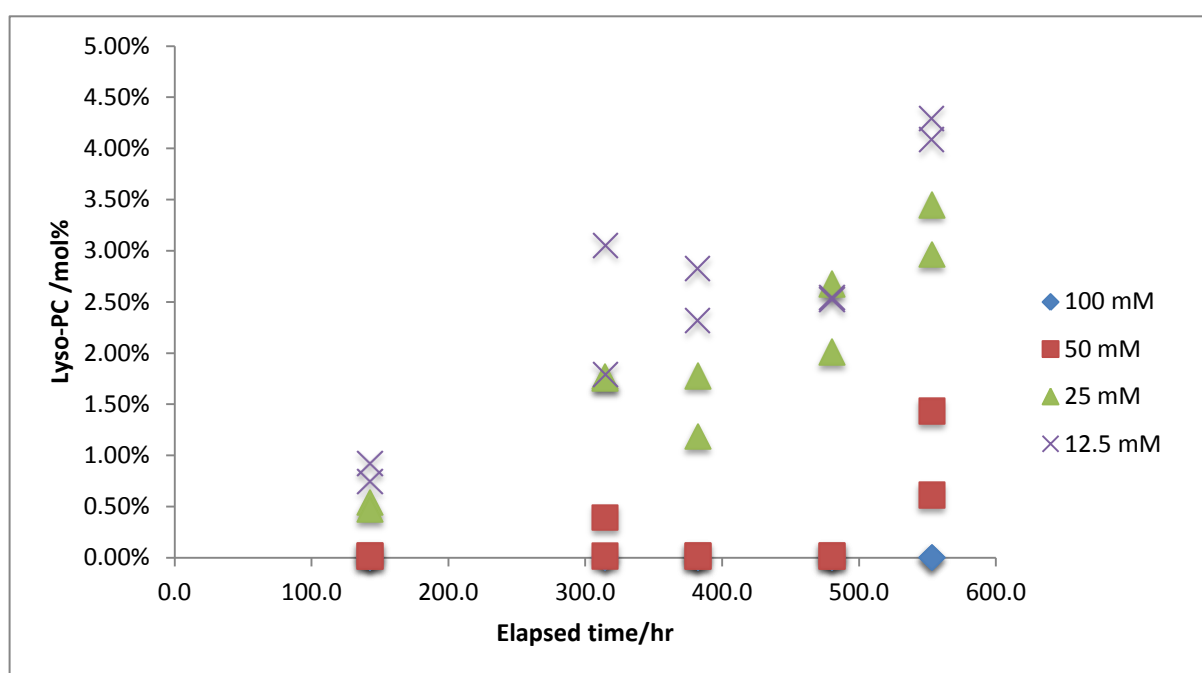


Figure 25: The effects of increasing Tris HCl buffer concentration upon rate of DOPC hydrolysis by raclopride tartrate ($n=2$).

However, it was still not possible to tell from these data which component of the system was

having the effect: the Tris molecules or the chloride counter-ions. The latter posed a serious impediment to the idea that this process may be important *in vivo*: the typical chloride content of a physiological fluid such as blood serum is around 100-110mM¹³⁷ over a volume of many litres, something that no amount of drug formulation could compete with. However, the idea that Tris had an inhibitory effect was also problematic, as the vast majority of previous studies had been undertaken using this buffer or one of its derivatives. If this were in some way sequestering drug molecules or otherwise preventing CAD-lipid contact, many previous results would be rendered unreliable and our understanding of the process as a whole would require a thorough review. As a result, identifying if one or more of these factors was responsible for the observed phenomena became an urgent priority.

5.4.5 Choice of counter-ion

Initially, the effect of the counter-ion upon the system was investigated. Firstly, experiments were run using the sodium salt forms of all the anions studied in place of raclopride: formate, TFA, tartrate and chloride (shown below in Figure 26). None of these assays showed any hydrolytic activity over three weeks' incubation, strongly suggesting that the counter-ion in the bulk medium made little or no difference to the rates of reaction. However, sodium ions are not membrane-permeable without assistance (see section 3.2, page 25 for a full discussion), unlike the amphiphilic CAD molecules which preferentially partition into the lipid bilayer. As a result, the interface itself was not charged under these conditions, meaning that this model could not account for the effects of the condensation of the anions onto the membrane surface.

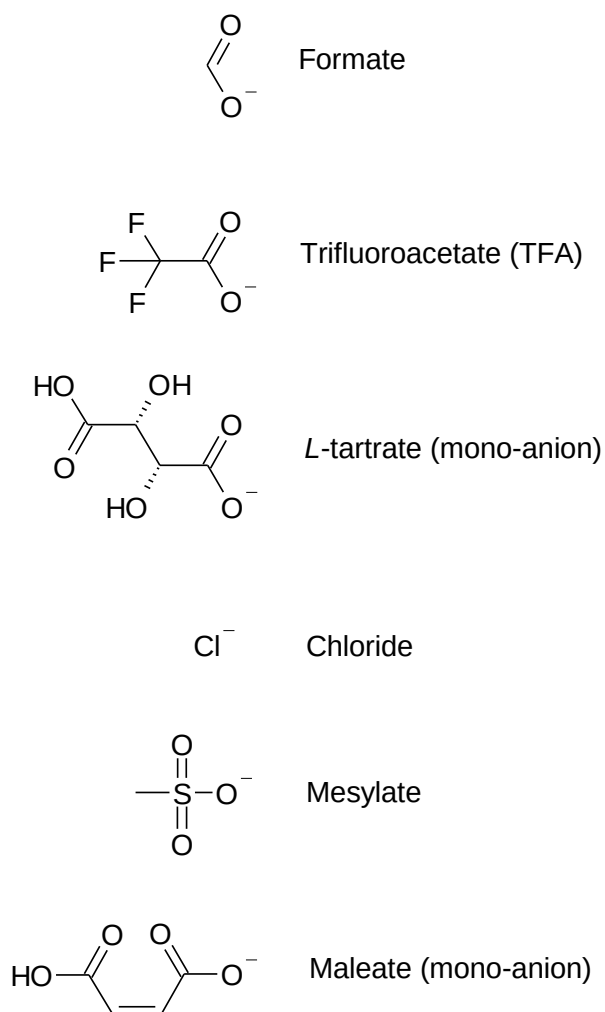


Figure 26: The anions studied to isolate the effects of the Hofmeister series³⁵ upon the rates of CAD-catalysed DOPC hydrolysis.

To investigate any counter-ion effects further, 4 solutions containing 100ml 100mM Tris free-base (pH 10.8-10.9) were made up using HPLC-grade water. These were buffered to pH 7.7 at room temperature (equivalent to pH 7.4 at 37°C¹³²), with a range of organic acids: trifluoroacetic and methanesulphonic acids were added directly; maleic and *L*-tartaric acids were first dissolved in water to form a 2M solution, then added to the Tris. All pH values obtained were in the range 7.6-7.7 at room temperature (approx. 24°C).

The first experiment performed was a repeat of the excess buffer assay shown in Figure 23: identical in every way, except for the substitution of Tris tartrate in place of Tris HCl. This experiment was designed to isolate any Hofmeister effects which may have influenced the system, by eliminating (as far as was possible) any chloride ions within the system, meaning that ion exchange by the raclopride molecules with the bulk solution would have no effect. As a

result, if chloride ions were inhibiting the reaction, the observed rates of hydrolysis for each of the vials in this assay would be equal.

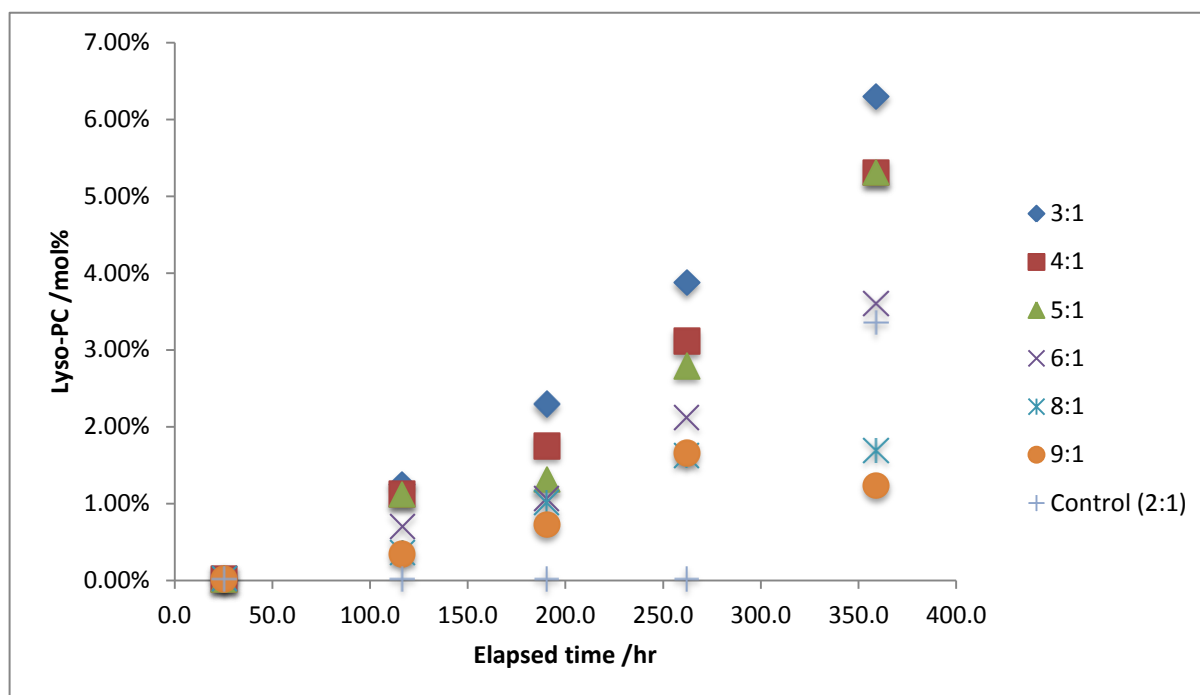


Figure 27: The hydrolysis observed in otherwise-identical DOPC samples doped with 5 mol% raclopride tartrate. Ratios correspond to the volume of 10mM Tris tartrate added relative to lipid, w/w. Control sample contains no drug, but is otherwise identical. Samples containing ratios 2:1, 7:1, 10:1 dehydrated due to faulty vial seals and so are not shown.

This revealed rates that were, within error, identical to those uncovered in section 5.4.3: as the volume of buffer in the system increased, the reaction slowed dramatically. This meant that chloride ions were not responsible for the observed results, and that Hofmeister effects could be assumed negligible. This directly contradicted the earlier, published findings reported to affect the rate of haloperidol-induced hydrolysis⁵⁴ and so the experiment was repeated using 5 mol% haloperidol hydrochloride rather than raclopride tartrate. In this and subsequent haloperidol experiments, no hydrolysis was observed at all despite the appearance of a peak corresponding to haloperidol in the HPLC traces derived from the samples, meaning that the drug had not precipitated out in the way that spiperone was observed to do in section 5.4.3. These results will be discussed below in section 5.5.1.

In order to confirm the findings of Figure 27, similar experiments were performed with 10mM Tris maleate, Tris TFA, Tris mesylate and unbuffered pure water at water-lipid ratios of 3:1, 6:1 and 9:1 in order to give a spread of results. These results (not shown) looked very similar to

those shown above, to within $\pm 1\%$ hydrolysis, with the exception of the maleate data which showed almost no degradation at all over some three weeks of incubation. Similarly, when a range of Tris tartrate concentrations were used, similar to those in Figure 25, the results were effectively indistinguishable from those found using Tris HCl.

One thing that rapidly became obvious about the mixtures was that the viscosity and opalescence observed in the samples (described in section 5.4.3) was significantly more apparent in the pure water samples than in the buffered ones. This observation is of interest, as it was thought up until this point that the viscosity was due to a shortage of excess water due some form of ion-binding or formation of extended hydration shells by the Tris molecules. Instead, these results suggest that the effect is caused by a swelling of the channels between membranes due to electrostatic repulsion of the anionic fatty acids and/or cationic drugs, which will have a significantly greater influence in an unbuffered system with a consequently greater Debye length. Recent studies have suggested that in weakly buffered systems such as the one under investigation, anionic vesicle diffusion rates are significantly slower than calculations predict due to the contribution of longer-range hydrostatic effects upon the vesicles effective cross-sectional area¹³⁸.

Interestingly, this behaviour could be mimicked by co-lyophilising with 5 mol% dimethylditetradecylammonium bromide (DMDTAB) (see Figure 28, below), before hydrating as normal with Tris HCl. This compound provides a permanent cation, thus no potential to carry a proton and no hydrolysis. However, it has an extremely high log P (calculated at 5.96 at pH 7.4³⁴) and thus is almost totally membrane bound, mimicking the electrostatic effects of having high levels of cationic drug within the bilayer. A system containing DMDTAB showed no membrane hydrolysis over the course of more than three weeks, but assumed the viscous and opalescent form described above within seconds of hydration. The phase adopted by the sample was probed by SAXS (described fully in section 5.1.3) but no diffraction could be observed, suggesting an amorphous state rather than the expected lamellar phase.

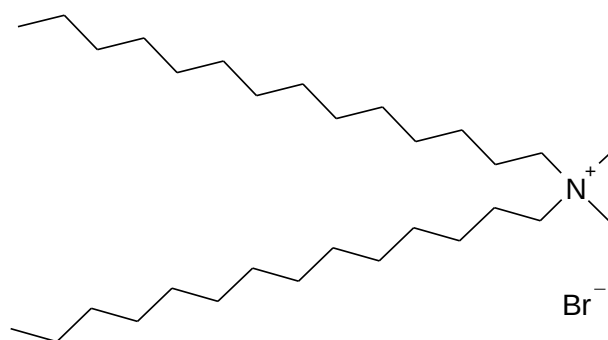


Figure 28: The structure of the bilayer-forming cationic amphiphile, dimethylditetradecylammonium bromide.

That the results for pure water mirrored those of the buffered systems is of little surprise, as the pH of water should be ≈ 7 as opposed to 7.4 in the controlled systems, and as the drug is added as a net neutral salt no changes to it should take place. It does raise some interesting questions about the strength of Tris buffers at these pH values, though: if the pH is affected by the ionisation of a percentage of the fatty acid molecules generated during the hydrolysis reaction (discussed in section 4.1.3), then the fact that results in pure water mirror those in buffered media suggests either that pH plays no role in determining the rate (which seems unlikely given the proposed mechanism), or that the buffer is rapidly becoming overwhelmed. As a result, a range of preliminary investigations were undertaken into the effects of a range of other buffers, described below in section 5.4.6.

The inactivity of the Tris maleate assays remains unexplained, for now: it may have been due to simple experimental error, as the maleate assay diluted 3:1 buffer-lipid dehydrated very rapidly due to a faulty vial seal. However, this was not the case for either of the other two vials and the source of their inactivity remains unclear.

5.4.6 Salt concentration and ionic strength

It had by this point been conclusively demonstrated that the kinetics and rate of the reaction in the condensed phase did not depend on the Hofmeister effect, nor the presence or otherwise of Tris molecules. However, this did not explain why no reaction was observed in 100mM Tris hydrochloride when lower concentrations of the compound gave such repeatable results. It was posited that the effect may have been due to the ionic strength of the solution: that increased screening of charge and of the dielectric constant gradient, (the difference in the capacitance of

the water *versus* the hydrophobic regions, a measure of the changes in polarity between the two regions), between the bulk medium and lipid interface may in some way inhibit the availability of protons to initiate the otherwise-catalytic reaction. This too could pose a significant barrier to the applicability of the research to biological systems, which often have a sodium chloride concentration of >150mM and thus a much higher ionic strength than those previously studied.

In order to isolate any effects of ionic strength upon the system, a number of assay vials were made up containing 3:1 ratios of 10mM Tris hydrochloride to DOPC (w/w) with 5 mol% raclopride tartrate and varying concentrations of NaCl as shown below in Table 4. The NaCl was added before lyophilisation, so made no difference to total buffer volume or concentration. Every other variable was constant between each vial and the mixtures were incubated at 37°C, as standard. The results are shown below in Figure 29.

Table 4: The composition of assay vials to isolate the influence of ionic strength upon the rate of reaction.

Mass DOPC /mg	Drug name	NaCl conc /mM	Vol Buffer / μ l
20	Raclopride tartrate	0	60
20	Raclopride tartrate	10	60
20	Raclopride tartrate	20	60
20	Raclopride tartrate	30	60
20	Raclopride tartrate	40	60
20	Raclopride tartrate	50	60
20	Raclopride tartrate	60	60
20	Raclopride tartrate	70	60
20	Raclopride tartrate	80	60
20	Raclopride tartrate	90	60
20	Raclopride tartrate	100	60

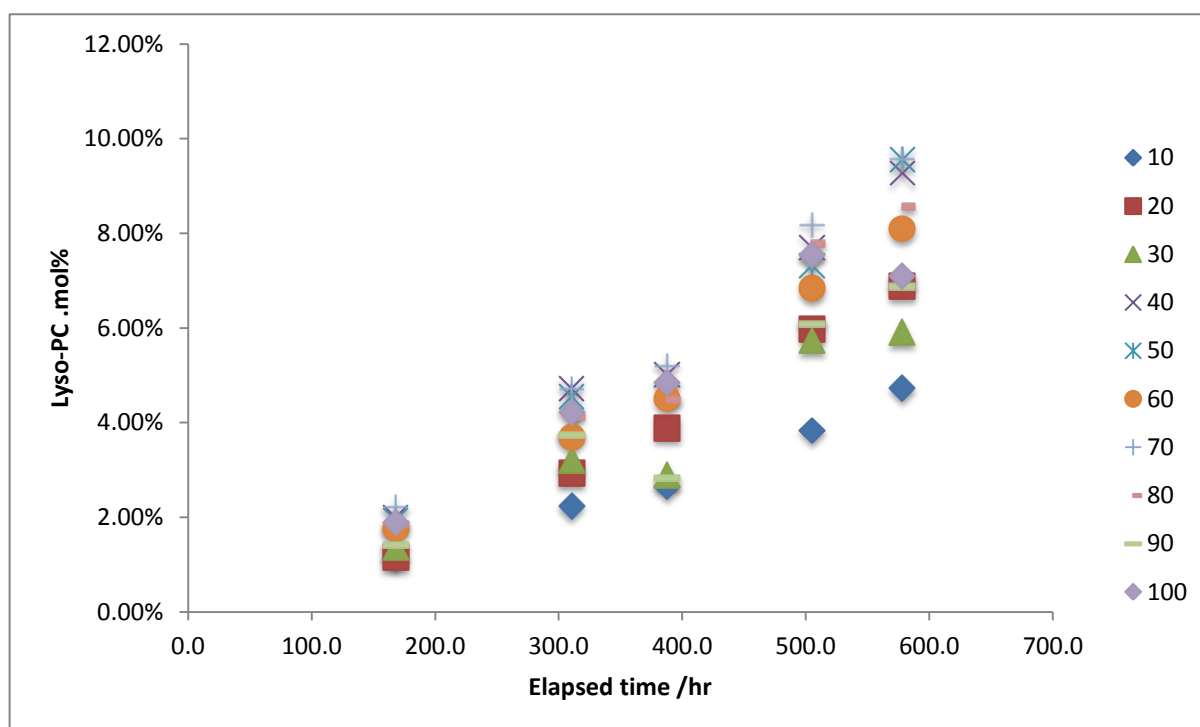


Figure 29: The influence of NaCl concentration upon the rate of DOPC hydrolysis caused by 5 mol% raclopride tartrate, plotted as a function of time. Buffer was 10mM Tris HCl. Key refers to the NaCl concentration in each vial in mM.

Although on first glance this set of results appears very similar to earlier charts, upon closer inspection the situation is not so clear-cut. A plot of $[\text{NaCl}]$ vs. rate of reaction is shown below in Figure 30, showing that there is little if any relationship between the two variables, although there may be some correlation at low concentrations. Furthermore, the rates observed in Figure 30 are comparable with those found in other experiments conducted in 3:1 buffer:lipid mixtures in the absence of any such salt, (cf. Figure 24, page 77), suggesting that the ionic strength of the bulk medium does not play any significant role in determining the rate of drug-induced hydrolysis.

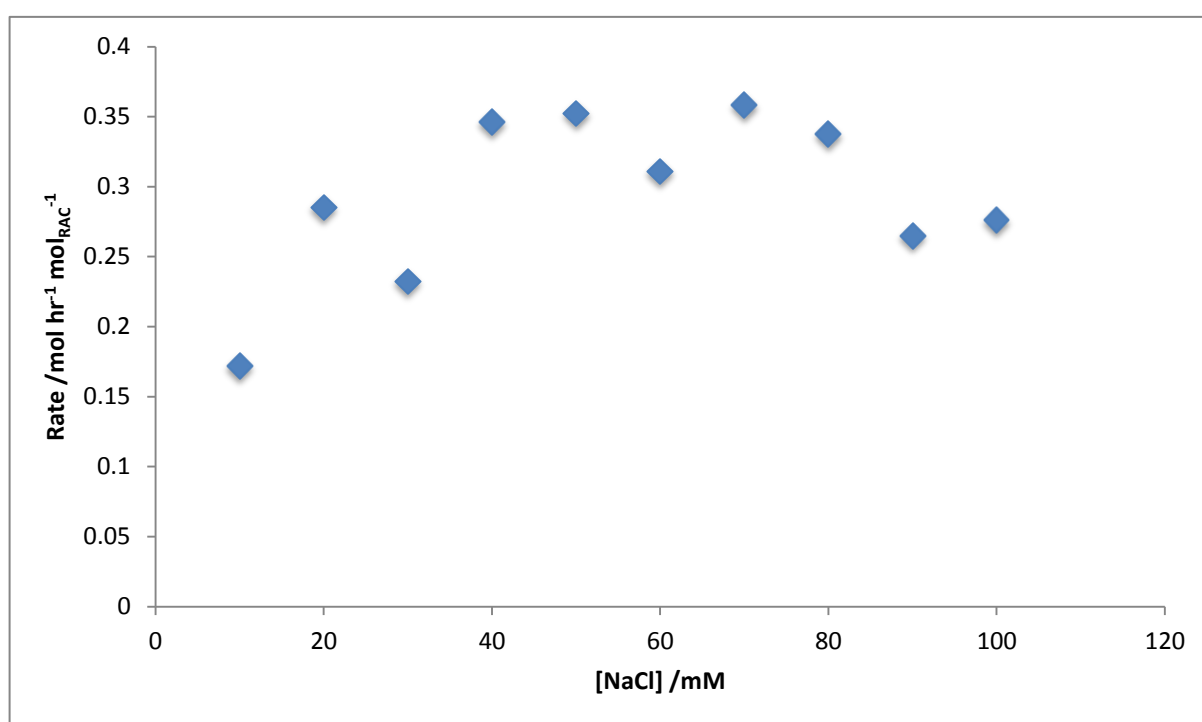


Figure 30: The influence of NaCl concentration upon the rate of DOPC hydrolysis caused by 5 mol% raclopride tartrate, plotted as a function of NaCl concentration. These rates are comparable, within error, to those found at 3:1 buffer:lipid in previous experiments in the absence of any salt.

This discovery provided a significant boost, as it removed one of the major potential stumbling blocks that prevented the application of the discovery onto a physiological environment. In order to develop this further, a number of alternative buffer systems were investigated in an attempt to move closer to a physiologically relevant system.

5.4.7 *Choice of buffer molecule*

In section 4.1.3 it was shown that Tris buffers at pH 7.4, although simple, useful systems for buffering media that may be exposed to basic compounds, are of limited use when acidic molecules are introduced. Originally it was chosen as the drugs of interest were typically basic in nature, but since raclopride is added as a net neutral salt and the course of the reaction produces significant concentrations of fatty acid molecules, research into the suitability of other options was required. Furthermore, it inhibited the rate of reaction as its concentration increased, as demonstrated in section 5.4.4.

The inverse relationship between Tris concentration and rate of lipid hydrolysis suggested that the buffer itself may somehow be poisoning the reaction. It became a priority to identify whether this was an artefact of the condensed phase assay, was due to the choice of buffer or whether the reaction simply could not progress in strongly buffered systems. If the latter was the case, then it became extremely unlikely that the reaction could provide the proposed 'missing' mechanism of drug transport discussed in section 3.3.

In order to probe the matter, two other buffers were selected from the 'Good list'¹³² to investigate whether this was a buffer-specific effect or more of a general issue. Phosphate-buffered saline (PBS) and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) were dissolved in HPLC-grade water to give 10mM concentrations. The PBS was pre-calibrated to give a pH of 7.4 @ 25°C, (confirmed by independent measurement as previously), whereas the HEPES was purchased as a free acid, pH 5.23 @ 21.7°C. This was basified with NaOH to a pH of 7.63 @ 21.9°C, giving a pH of 7.4 @ 37°C.

The relative rates of hydrolysis caused by raclopride tartrate in DOPC hydrated with the three buffers Tris HCl, HEPES sodium and PBS was then probed directly. The PBS assays appeared to remain very fluid and opaque relative to the Tris and HEPES, which became viscous and opalescent as described previously. The results of the assay are shown below in Figure 31, below.

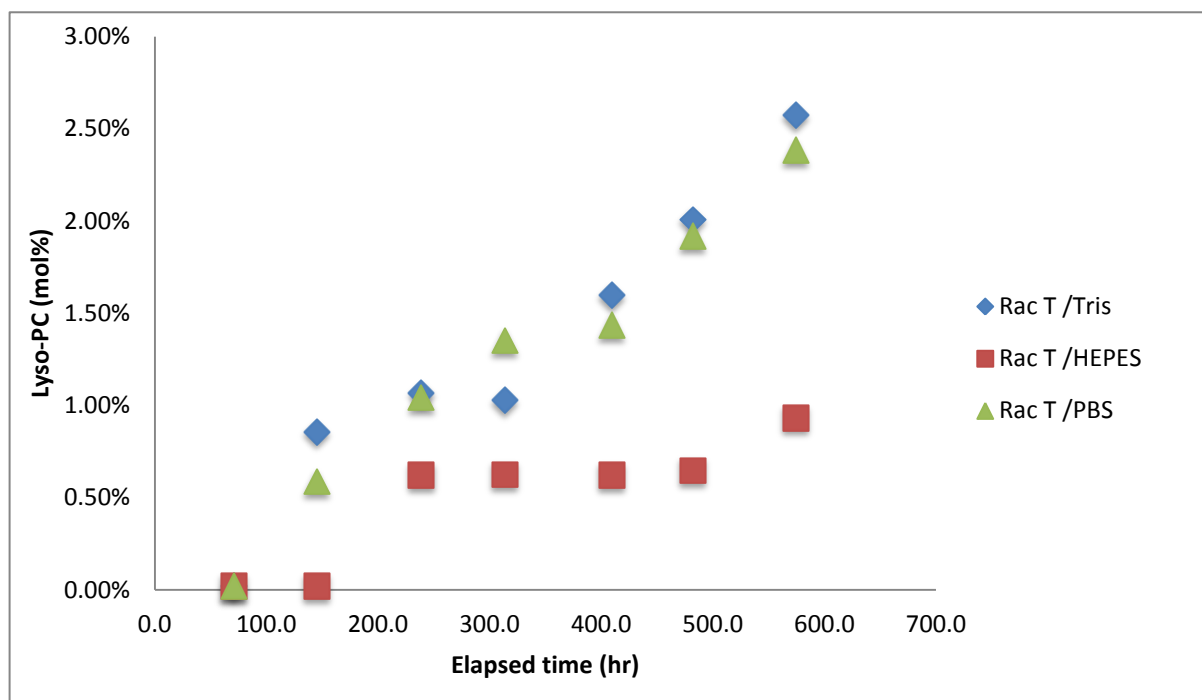


Figure 31: the effect of a range of 10mM buffers upon the rate of raclopride-catalysed DOPC hydrolysis.

Significant care must be taken when analysing these results: a number of the mixtures assayed showed no degradation at all, including some where identical mixtures showed significant hydrolysis, suggesting an experimental error in preparation. Omitting those results, it appears as though Tris does not inhibit the reaction significantly at 10mM concentrations relative to either of the two other buffers tested, nor relative to unbuffered pure water as tested in section 5.4.5. By contrast, the vials containing HEPES showed significantly slower rates of hydrolysis, although they were still detectable and quantifiable. This may have been an artefact of the experiment, although recent research has demonstrated that the closely related buffer 1,4-piperazinediethanesulfonic acid (PIPES) can cause significant fluidisation and perturbation to a membrane by intercalation throughout the interface due to its amphiphilic structure, in a manner analogous to the CADs under study¹²⁸. However, this was not pursued further: the data were sufficiently strong to suggest that even if rates were lower, the reaction occurred in HEPES-buffered systems at a rate that was both observable and, more importantly, quantifiable.

In any case, it was felt that PBS provided a more physiologically relevant system of study and one which eliminated a number of the buffering issues raised previously. As a result, all assays conducted after this point were performed in a PBS buffer, unless otherwise specified.

5.5 Assay reproducibility

In order to probe the reliability of the condensed-phase results, data from 22 assays not known to be erroneous due to experimental error drawn from across the course of the investigation were collated and combined. It was expected that there would be some variability within the data, as assays had been taken using several batches of both DOPC and raclopride tartrate from a number of different suppliers and analysed using several different HPLC approaches. Beyond that, the ELSD detector upon which all the analytical HPLC data depended had failed several times leading to significant repairs and refurbishment, and it was expected that this would have had an effect upon the detection threshold of the data gathered, despite attempts to normalise it as far as possible.

Despite these confounding factors, results from 22 assays performed over the three years of raclopride testing showed a strong correlation suggesting a high degree of reproducibility, as detailed in Figure 32. It can be seen that comparison between assays leads to some spread in the data, due to experimental error, evaporation of stock solutions, pipetting errors etc. and that spread was more significant after long incubation periods as small differences in preparation were magnified over time.

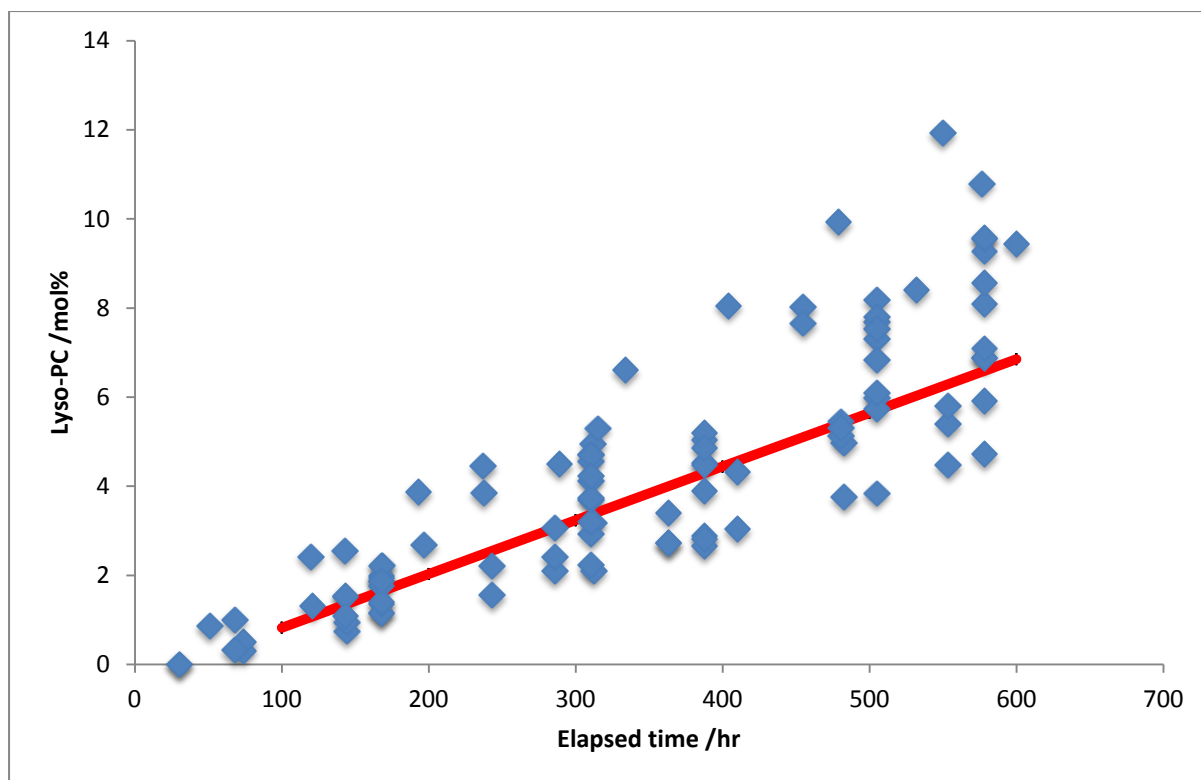


Figure 32: Cumulative data from 22 separate experiments investigating raclopride tartrate mediated DOPC hydrolysis, hydrated at a ratio of 3:1 10mM buffer:lipid w/w. Data includes points using differing buffers, salt concentrations, batches of reagent and HPLC conditions. Line of best fit is equal to a rate of $0.241 \text{ mol hr}^{-1} \text{ mol}_{\text{RAC}}^{-1}$, $p < 1 \times 10^{-4}$, $r^2 = 0.602$.

The spread of results shown in these data are significant and greater, in some cases, than the magnitude of the trends observed in some experiments. In order to obtain useful error bars, a quantitative assessment was required of the variability between vials in the same assay. This was assessed through the preparation of nine identical assay vials, each containing DOPC doped with 5 mol% raclopride tartrate and hydrated in 10mM PBS solution as previously. These vials were sampled over the course of some 600 hours, and the results compared (Figure 33, below). These revealed very tight grouping of data, lending a high degree of confidence to measurements produced via this technique. The error-bars thus derived were used in all subsequent assays to give an indication of precision, as the experiments were so long in duration that few repeats of each were possible.

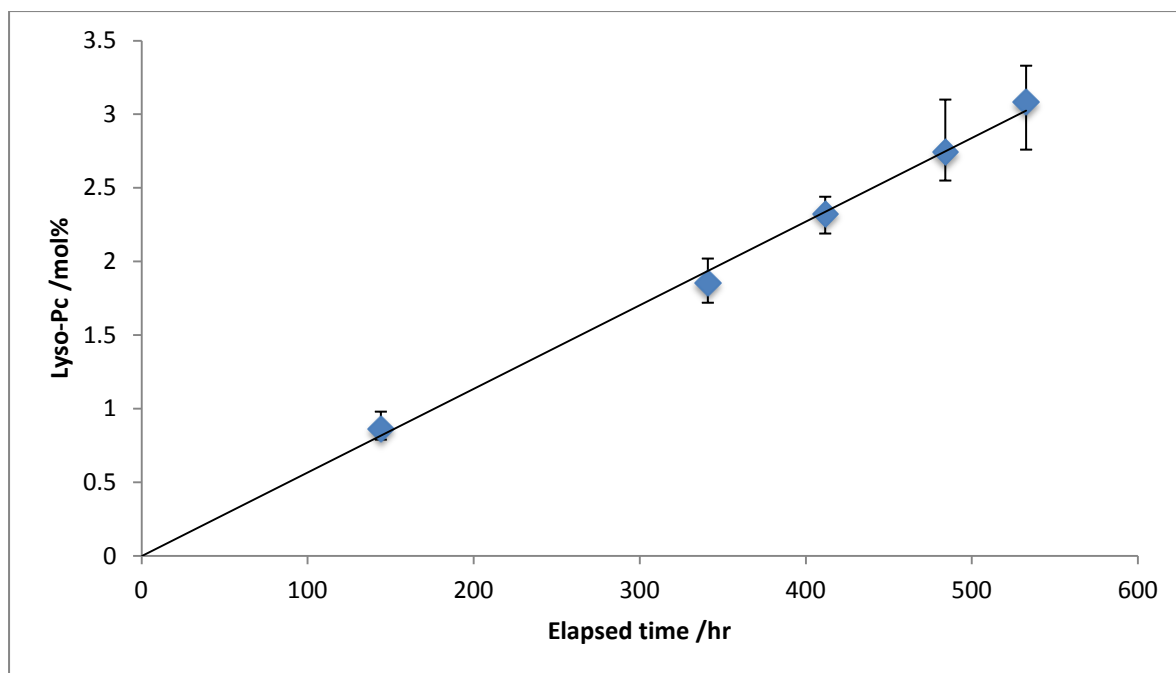


Figure 33: The mean hydrolysis values derived from 9 identical samples of DOPC with 5 mol% raclopride tartrate incubated with 10mM PBS. The trend-line is equal to a rate of $0.114 \text{ mol hr}^{-1} \text{ mol}_{\text{RAC}}^{-1}$; error bars show the actual spread of results. These error bars are used throughout the remainder of this report.

5.5.1 Effects of raclopride concentration

At this point in the investigation, all experiments had been conducted with 5 mol% raclopride tartrate relative to DOPC, to allow direct comparison with earlier studies. However, it was not known whether this was the optimum concentration for the drug: its relatively low log D means that a significant amount of drug, approximately $1/8^{\text{th}}$ of that added, was partitioned in the bulk buffer and the effects of any aggregation such as those observed with spiperone in section 5.4.3 were unknown. Experiments were underway to investigate the phase behaviour of DOPC / lyso-OPC / oleic acid systems both with and without raclopride by other investigators, but this was a time-consuming process and it was felt that a relatively rapid HPLC assay might provide at least a guide to assay design until the phase diagrams had been completed.

It was also important to develop the basis for a calibration set for optical experiments such as those conducted on drug-lipid vesicle systems at the Diamond synchrotron (described in section 5.6.2), which provided information complementary to that derived by HPLC. Although it was accepted that rates in condensed-phase systems such as the one described were orders of

magnitude slower than those observed in giant vesicles⁵³, it was hoped that the data from multi-lamellar systems would act as a trend guide whilst a quantitative vesicle-based assay could be designed.

As a result, an experiment was initiated to analyse the dose-response curve of raclopride in DOPC. DOPC samples were doped with 0.1, 1, 10 and 20 mol% raclopride tartrate, as well as samples doped at 5 mol% to act as an internal standard. It was felt that concentrations higher than 20 mol% would be likely to induce significant morphological disruption to the host membranes; concentrations below 0.1 mol% could not be accurately measured with the available apparatus. These assays were hydrated with 10mM PBS, as above, and incubated at 37°C for some three weeks. The results are shown below, in Figure 34.

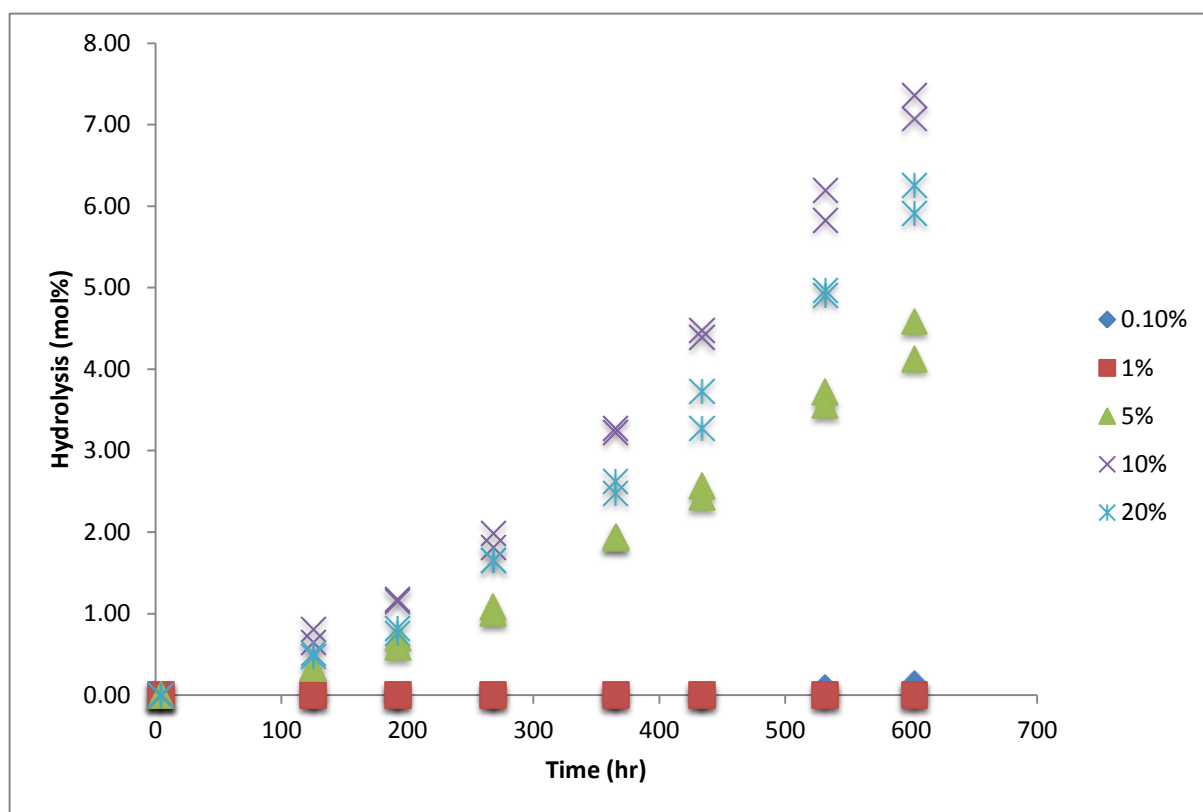


Figure 34: The dose-response data of raclopride tartrate in DOPC ($n=2$, all data points shown). Key refers to the concentration of raclopride tartrate present, in mol% relative to DOPC.

These data reveal a complex relationship between the drug concentration of the system and the observed rates of hydrolysis. Low concentrations of raclopride induced little or no hydrolysis beyond that observed in control experiments: while it has been observed that the performance of the ELS detector in use was non-linear at very low signals (see section 5.3), this does not

explain the total lack of observed lyso-lipid in these assays. While there remains the potential for the findings to be a result of simple experimental error, there is also the plausible possibility that some critical concentration of drug molecules is required in the membrane before hydrolysis can take place. Such a concept has precedent: for example, the small amphiphilic molecule curcumin has been observed to have two distinct binding modes when exposed to a DOPC membrane, corresponding to a saturable, low-energy mode at the membrane surface followed by a higher-energy binding which the authors attributed to sequestration of the molecule deep within the hydrophobic interior of the membrane¹³⁹, and similar behaviour has been observed in a range of CADs¹⁴⁰. It may be that in these cases, ionic interactions between the dopant and the head-group provide a more favourable binding site or a kinetic barrier to prevent the partitioning of the amphiphile beyond the phosphate head-groups on the exterior of the membrane and into its oily core: this is likely to have a perturbing effect upon the membrane, which may offer a tool to study the process. Some of the experiments intended to probe this effect are detailed below in sections 5.5.2 and 5.6.

5.5.2 Results with other drugs

Despite thorough characterisation of the system with raclopride tartrate, limited success was met when attempting to analyse the interactions of other drugs with DOPC using the methods outlined above. Previous experiments by the group had observed rapid and dramatic effects with drugs including WAY-100635 maleate, spiperone HCl, haloperidol HCl and even raclopride's closely-related analogue eticlopride HCl under near-identical conditions, but no detectable hydrolysis was observed by any of these compounds during the course of this study. In the case of spiperone, crystals were observed forming in the base of each of the assay vials, suggesting that the hydrophobic drug molecules had crystallised out of solution, preventing any reaction occurring. However, in the case of haloperidol, a peak was observed on the HPLC traces taken suggesting that, in this case at least, the drug had been retained in the membrane. Some hydrolysis was observed by organic salts of haloperidol, in a repeat of the experiments leading to the publication of the 2008 *Industrial & Engineering Chemistry Research* paper⁵⁴. However, the data was difficult to reproduce accurately due to the drug's low solubility, high toxicity in lipid systems¹⁴¹ and the potential for the introduction of artefacts as explained in section 4.1.2, and it was abandoned in favour of raclopride.

Haloperidol-doped DOPC samples analysed by SAXS showed a significant and reproducible

destabilisation and swelling of the lamellar sheets' d -spacing which, over time, collapsed back to form a still-swollen but significantly better-defined (and therefore more ordered) L_α phase (see Figure 35, below)⁵⁴.

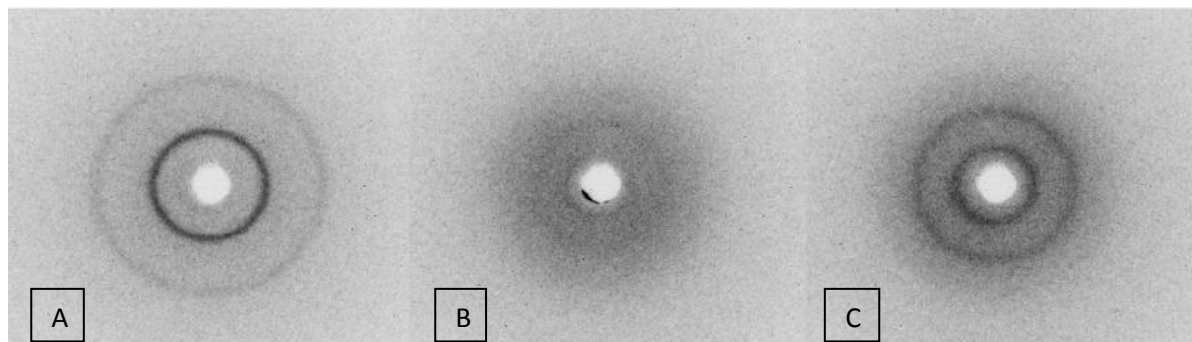


Figure 35: Small-angle X-ray scattering (SAXS) images showing the swelling of an L_α DOPC system through the addition of 5 mol% haloperidol: (a) DOPC in excess water ($61.36 \pm 0.57 \text{ \AA}$), (b) five days after the addition of haloperidol ($107.70 \pm 3.21 \text{ \AA}$), and (c) eight days ($89.46 \pm 1.07 \text{ \AA}$). Reproduced from Casey *et al.* 2008⁵⁴.

This swelling, which is not observed in the fluid lamellar phase formed by the control sample (d -spacing of $61.3 \text{ \AA}$, similar to that in Figure 35a) is the result of repulsion between charged bilayer interfaces¹⁴². This indicates that the drug intercalated into the membrane, forcing apart its constituent lamellar sheets. However, the low resolution of the peaks in image in Figure 35b implies that this was not an even process: not all membrane layers contained the same concentration of haloperidol cations, leading to a range of swelling behaviour and a disruption of the system's long-range order.

This extremely swollen system was not stable for long periods, and the system relaxed to a still-significantly enlarged but much better-resolved system, shown in Figure 35c. This may have been the result of an equilibration, as the haloperidol cations spread equally throughout the lamellae to form an equally-spaced system, or may have been due to a neutralisation of the charge on the haloperidol molecules, as they donated their protons in exchange for a more favourable interaction with the hydrophobic interior of the membrane as described above. The lack of any detectable hydrolysis provides circumstantial evidence for the latter, as neutral molecules sequestered within the oily core of a membrane can play no part in the acid hydrolysis of its acyl bonds at the bulk water interface. However, vesicle-based fluorescence results seem to support the former hypothesis and are discussed below in section 5.6.1. Interestingly, these effects were not observed in samples doped with raclopride tartrate, despite the latter's hydrolytic activity. This may imply that the more hydrophilic raclopride is

only ever a transient visitor to the membrane interface, or alternatively that the internal hydrogen bonding present within raclopride¹³⁵ permits the existence of the net neutral zwitterion species even at the membrane interface, effectively preventing any such charge-charge repulsion between sheets.

In an attempt to identify a minimum pharmacophore capable of causing the hydrolytic effect, perhexiline maleate was added to a series of DOPC systems. Perhexiline is a simple, hydrophobic structure (calculated log $D_{7.4}$ 3.93³⁴) formed of two cyclohexyl and one piperidine moieties connected by an ethyl linker (shown in Figure 36), featuring no other structural groups. As a result, it was hoped that any hydrolytic activity observed in perhexiline-doped systems could be unequivocally assigned to the basic nitrogen in the molecule, if only through a lack of other possible options. Beyond that, perhexiline-induced phospholipidosis has been observed to cause significant membrane lesions in both the liver¹⁴³ and the brain¹⁴⁴: in this way, the drug offered the chance to conclusively link the two phenomena.

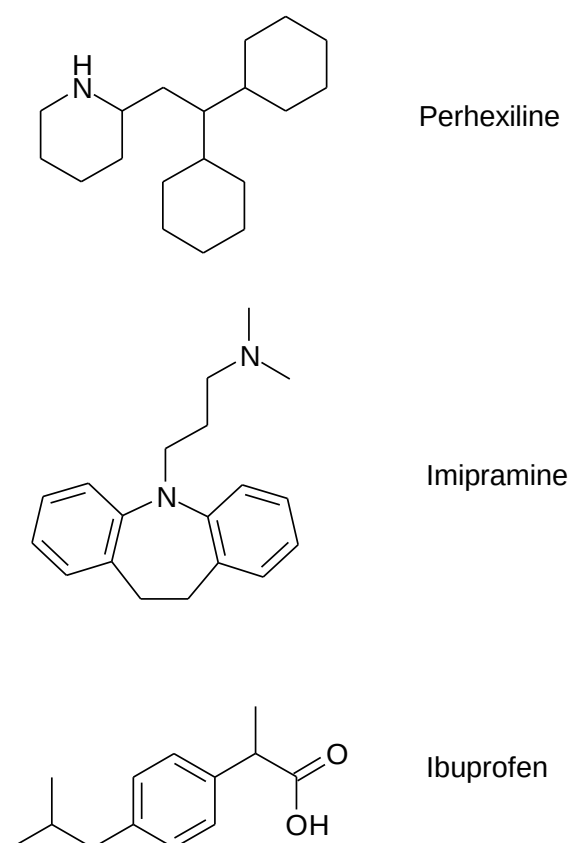


Figure 36: The structures of the anti-anginal drug perhexiline, the 5-HT inhibitor imipramine and the non-steroidal anti-inflammatory drug ibuprofen.

Similarly, testing was carried out on imipramine HCl, a simple tricyclic antidepressant with a high affinity for serotonin (5-HT) receptors. It, too, has strong links to phospholipidosis³⁹, as well as a well-characterised influence on membrane fluidity and leakage in both model systems and erythrocyte membranes¹⁴⁵. Despite repeated testing, however, no hydrolysis beyond that of control was observed by either drug and the experiments were abandoned.

One drug that did show consistent and reproducible hydrolytic action raised more potential questions than it answered. Ibuprofen, (2-(4-(2-methylpropyl)phenyl)propanoic acid), showed a detectable and linear rate of hydrolysis at 5 mol% concentration, as shown below in Figure 37.

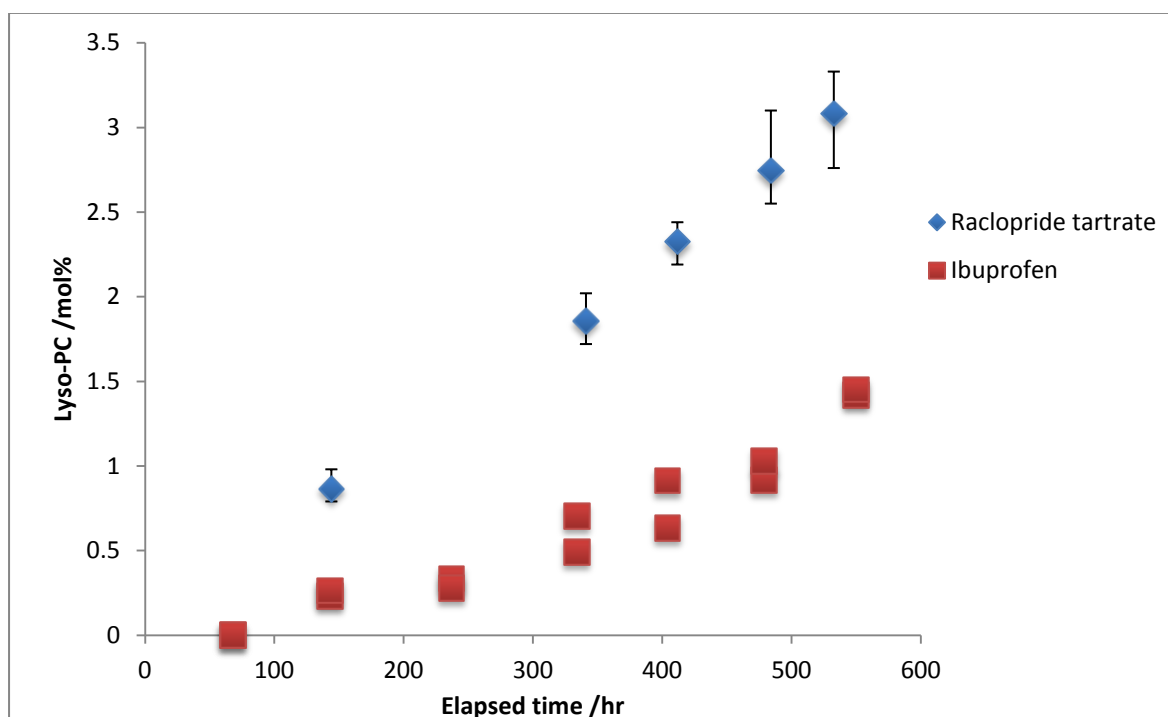


Figure 37: The rate of ibuprofen-mediated DOPC hydrolysis, compared to that of raclopride tartrate (from Figure 33). Although slow, the lyso-lipid signal was reproducible and significant relative to that observed in control samples. Rate is equal to $0.0597 \text{ /mol hr}^{-1} \text{ mol}_{\text{RAC}}^{-1}$, approximately half that observed for raclopride tartrate. All Ibuprofen data points shown.

Ibuprofen is a small, organic acid with a pK_a of 4.45, meaning it is almost entirely ionised at pH 7.4¹⁴⁶. It has a reasonable aqueous solubility in this form, but retains a significant solubility in organic media, particularly in lipid membranes giving a $\log D_{\text{DOPC}, 7.4}$ of 1.89, or half a log unit greater than raclopride¹⁴⁷. However, all other compounds observed to catalyse the ester hydrolysis of lipids have been cationic in nature; ibuprofen is an anion. This means that in its preferred, ionised form it does not carry an acidic proton: paradoxically, it is only as a neutral

species that it can possibly show any activity if the hypothesis behind the reaction mechanism is correct.

Ibuprofen has, interestingly, been previously observed to catalyse defect formation and eventual destruction of supported membranes, via cyclic voltammetry studies¹¹⁷ as well as the destruction of lecithin liposomes to form lipid tubules and small micellar bodies in a manner exactly analogous to that described by Baciú *et al.*, albeit over a slower timescale¹⁴⁸. Although these previous studies assume the effect is purely detergent-based (which seems absurd, considering the effect is time-dependent), a mechanism is proposed which adds some support to the idea of ibuprofen-induced hydrolysis. Avdeef *et al.* suggest a “pH piston”, in which the depth of the binding between the ibuprofen molecule and the host membrane is determined solely by its ionisation state¹⁴⁷, shown schematically below in Figure 38. This model is supported by ¹H NMR data, which shows two different interactions between ibuprofen and unsaturated lipid species, separated by a distance greater than the length of an ibuprofen molecule¹⁴⁹.

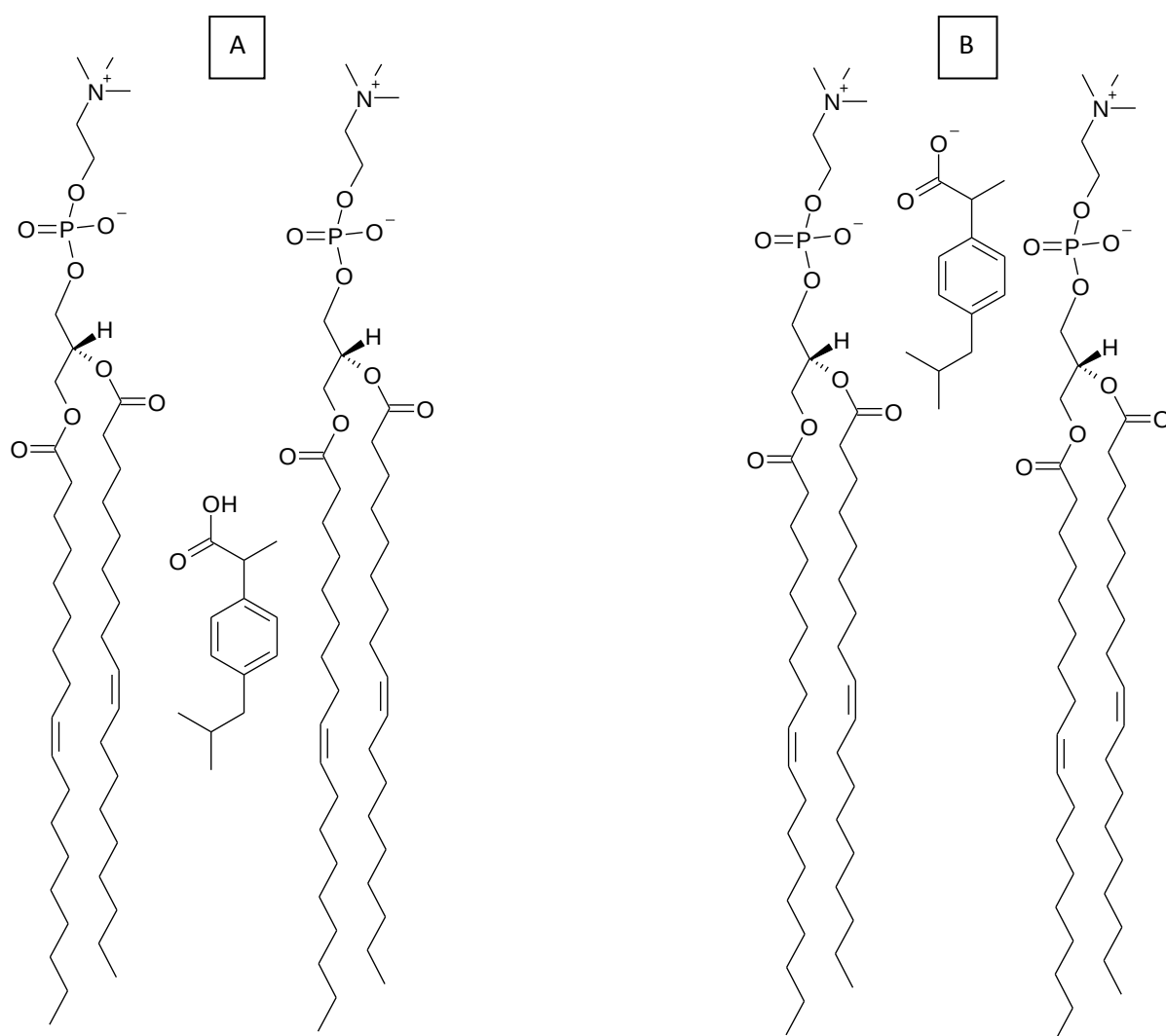


Figure 38: The “pH piston” model proposed by Avdeef *et al.*¹⁴⁷, showing the approximate equilibrium positions of the ibuprofen molecule in its a) neutral and b) ionised state in a DOPC membrane. Support is presented in the form of ¹H NMR data which show significant shifts in both the protons of the quaternary amine and of the methylene groups in the membrane’s hydrophobic core¹⁴⁹.

This leads to a biphasic binding curve, directly analogous to that described for curcumin and others above. In the initial, low energy binding phase, ibuprofen was found to actually stabilise the membrane, reducing the incidence of defects and decreasing current flow in cyclic voltammetry studies¹¹⁷. It was proposed that this is due to an increase in membrane stiffness, and may give a clue as to the lack of hydrolysis observed in low concentration raclopride systems such as the ones described in section 5.5.1. By contrast, in its neutral form the equilibrium position is proposed to be determined by π - π overlap between the drug’s phenyl ring, as observed in raclopride and a number of small drug molecules during *ab initio*

computational studies by Rosso *et al.*¹⁵⁰, and is stabilised by a hydrogen bond between the carboxylic acid of ibuprofen and an acyl carbonyl from one of the surrounding lipids. These interactions present a plausible route by which ibuprofen might catalyse the hydrolysis of such lipids: if the acidic hydrogen is thought of as in equilibrium between binding to the drug and an adjacent lipid acyl bond, ester hydrolysis will inevitably occur given a sufficient supply of available water molecules. As water molecules are predicted to be found in large numbers at the level of a membrane's glycerol moieties by molecular dynamics simulations¹⁵¹, this seems a likely outcome of the above model.

This does not necessarily apply to cationic compounds such as raclopride, however. In the case of raclopride, the electrically neutral form adjacent to the glycerol moieties is the inactive version, and the head-group co-ordinated ionic form is active. One may speculate about the role of the internal salt form described by Tsai *et al.*¹³⁵ in the role of proton donor to the neutral species, which might conveniently explain the lack of activity observed in even such close analogues as eticlopride, whose pK_a is only ≈ 8 and thus too weak to be significant at physiological pH¹⁵². At this stage in the investigation, little more could be said about the model although some speculative proposals were discussed. However, later research shed more light upon the ionisation state of raclopride in the membrane and its influence upon the molecule's depth of residency.

5.6 Vesicle-based assays

The failure of the condensed-phase assay to reliably observe hydrolytic effects in so many systems either previously demonstrated or strongly suspected to be active led to attempts to develop a unilamellar vesicle-based technique, such as that described by Baciú *et al.*⁵³ Although that fluorescence microscopy-based experiment revealed a wealth of data, it was not easily adapted to provide quantitative data regarding the chemistry and rate of reaction in the system and so was of limited value in characterising the process under investigation. As such, techniques capable of directly measuring (rather than simply visualising) the process were required for further progress to be made.

Another reason for the development of a vesicle-based assay lay in the surface activity of all such membrane-bound drugs. Almost all CADs exhibit millimolar critical micelle concentrations (CMC) and begin to exhibit surfactant effects well below this concentration³³. Raclopride is not reported to form micelles in aqueous solution due to its zwitterionic nature¹⁵², but this does not preclude any surfactant effects as the drug will not necessarily remain in this state in a hydrophobic environment such as a membrane interior¹³⁵. Many studies have previously reported major structural and mechanical changes to lipid aggregate structures after the administration of a range of amphiphilic and hydrophobic drugs^{16,51,153,154}, but where these have been explained they have been attributed to simple detergent or intercalation effects, rather than a chemical alteration as observed here. Decoupling any such surfactant effects upon the membrane topography from those caused by the products of the hydrolytic reaction would provide an important foundation for the application of the reaction in systemic transport.

Finally, the attempted development of vesicle models was driven by kinetic considerations. Condensed-phase models such as those outlined above are a long way removed from the micron-scale, broadly unilamellar structures making up cell membranes. Furthermore the formation of multi-lamellar vesicles isolates a relatively large proportion of the lipid system from the bulk solution, with potential impacts upon the local pH and the distribution of drugs or reaction products within the system. As a result, it is very difficult and probably unwise to draw strong conclusions about the absolute rate of these hydrolytic processes *in vivo* from the condensed-phase results described above.

All vesicles were prepared using a LIPEX gas-driven extruder (Northern Lipids, Canada).

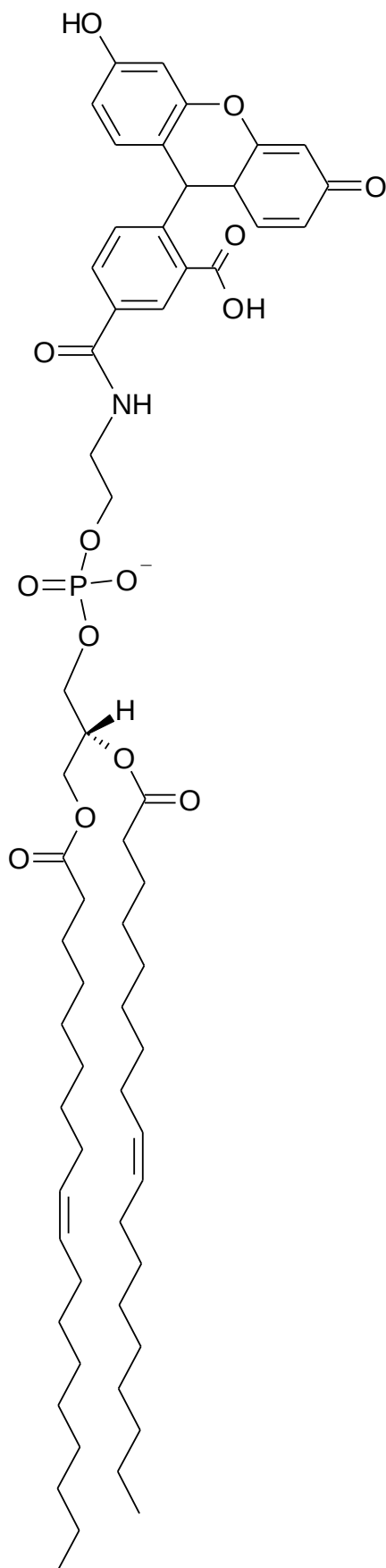
Lyophilised lipid powders or mixtures of lipid and probe were hydrated with the relevant buffer to form 1mM suspensions, which were then extruded 10 times through 2 stacked 200nm polycarbonate membranes (Nucleopore) using compressed nitrogen at approximately 8bar. Lipid suspension recovery was essentially quantitative via this process. These suspensions were transferred to one or more polystyrene or special optical glass microcuvettes (VWR, UK), as PMMA cuvettes were known to induce artefacts in the ADIFAB assays described below. Vesicle size and polydispersity was checked using dynamic light scattering using a Malvern Instruments High Performance Particle Sizer at 37°C. The vesicle populations produced were reasonably consistent, with average diameter $175.4 \pm 10.9\text{nm}$ ($n=8$). All studies were conducted using pure DOPC as the host membrane, unless otherwise specified.

These studies progressed broadly in parallel with the HPLC method development outlined above in section 5.2 and are reported here in chronological order. This had the effect that the vesicle-based HPLC experiments detailed in section 5.6.3 only became available after the experimentation into the optical methods detailed below.

5.6.1 Fluorescence studies

Initial experiments focussed upon optical methods, due to the extremely high sensitivity of fluorescence techniques and the availability of a range of well-characterised probes and protocols for the analysis of everything from fluidity and domain formation¹⁵⁵ to vesicle leakage¹⁵⁶. Furthermore, fluorescence monitoring is an extremely rapid process, allowing sample rates of hundreds or thousands of Hertz if required, meaning that time resolved data could, in theory, be gathered with exquisite resolution using off-the-shelf apparatus.

All fluorescent measurements were taken using a Cary Eclipse Fluorimeter (Varian Inc., now part of Agilent, UK). Vesicle suspensions were analysed in either disposable polystyrene or special optical glass microcuvettes (VWR) in a four-position cuvette holder attached to a temperature controlled water bath. Temperature was monitored through a thermocouple located in the sample holder itself and all measurements were performed at 37°C.



5.6.1.1 FPE

An initial approach was to analyse the rate and degree of membrane binding of different compounds to unilamellar vesicles of DOPC, in an attempt to correlate literature partition coefficients, (normally taken in 1-octanol), with the binding to and hydrolysis of DOPC systems. This was of particular importance, as many sources highlight the difference in affinities of drugs, particularly amphiphilic drugs, for membranes as opposed to standard models¹⁴⁷ and without an understanding of these differences, any relationship drawn would inevitably contain artefacts and a high degree of noise. However, the fact that hydrolysis was expected to occur in these systems added a significant degree of complexity, as the effective partition function of a compound for a membrane is extremely sensitive to its composition¹⁵⁷, stored curvature stress^{158,159} and electrostatic charge¹⁶⁰, all of which were expected to change dynamically as the reaction progressed.

In order to monitor this process, a fluorescein-labelled dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (FPE) probe developed by O'Shea *et al.*¹⁶¹ was used, shown left in Figure 39. This membrane-bound probe sits at the surface of the membrane, its fluorescence dependent upon the degree of delocalisation within the fluorescein moiety, which is in turn dependent upon the ionisation state of the molecule. At the surface of a membrane, this is dictated by the surface potential (ψ), which is a function of the surface charge at the membrane and the bulk electrolyte concentration⁸⁴. As a result, FPE is an extremely sensitive probe into the charge density at the surface of a membrane, allowing (with sufficient knowledge of the

Figure 39: the structure of fluorescein-labelled dioleoyl-*sn*-glycero-3-phosphoethanolamine, (FPE).

partition behaviour of the compound of interest) quantitative and time-resolved analysis of binding behaviour. While the absolute partitioning behaviour was not known for the systems in question, due to the issues outlined above, it was hoped that through the use of well-characterised and hydrolytically inactive cationic standards with extremely high log P values such as DMDTAB, the data gathered could be used to generate at least a qualitative data trend that would be complementary to that available via SAXS measurement, as described in section 5.5.2.

As FPE exhibits what is effectively a zero flip-flop rate¹⁶², it was possible to add the lipid either before extrusion in order to generate at roughly uniform coverage of the probe over both leaflets of the vesicle, or alternatively to add it exogenously after extrusion of the DOPC. This led to the probe being present only in the external leaflet, allowing, in principle, analysis of the rate of permeation of a drug across the membrane. In all experiments, the 1mM DOPC vesicle suspensions were doped at 0.1 mol% with FPE dye, and 1ml of the suspension was analysed as described above. λ_{EXmax} was found at 499nm, λ_{EMMmax} was found to be 529nm, $\pm 2\text{nm}$. Dopant solutions were made up to 10mM concentration in water, where possible; otherwise in methanol or DMSO. This meant that a 1 μl injection of dopant solution equated to a 1mol% addition relative to the lipid concentration. After each injection, the mixture was agitated via the aspiration and release of 300 μl of the suspension. Data collection was continuous, except during these injections, giving what was in effect a micro-scale stopped-flow experiment.

Control samples were run to check for contributions to fluorescence from all buffers, solvents, drugs and lipids, but all showed negligible effects at the wavelengths of interest. This was important as raclopride, in particular, has some intrinsic fluorescence ($\lambda_{\text{EXmax}} \approx 350\text{nm}$) and so the potential existed for energy transfer effects to generate artefacts in the system if the emission spectra overlapped with the probe.

Photobleaching of the probe was analysed by allowing two positive control samples, (those containing DMDTAB) to equilibrate under the excitation beam for extended periods. DMDTAB was also used as the calibration control for the testing: the molecule has a very low intrinsic curvature, forming bilayers under almost all conditions and lying in a stable L_{β} phase at room temperature¹⁶³. This means that it has a minimal effect upon the morphology of the vesicles, unlike the membrane-softening detergent effects of lyso-lipids and many CADs. That, coupled with its extremely high partition function, (the molecule is effectively insoluble in water) and its

permanent cation meant that it could be assumed at all DMDTAB was rapidly absorbed by the lipid vesicles and thus that the signal obtained through this process was the maximum possible for that doping regime. Typical raw data is shown below in Figure 40 for injections up to 20mol% DMDTAB: the experiment was repeated five times under varying conditions, revealing similar trend-wise data in each.

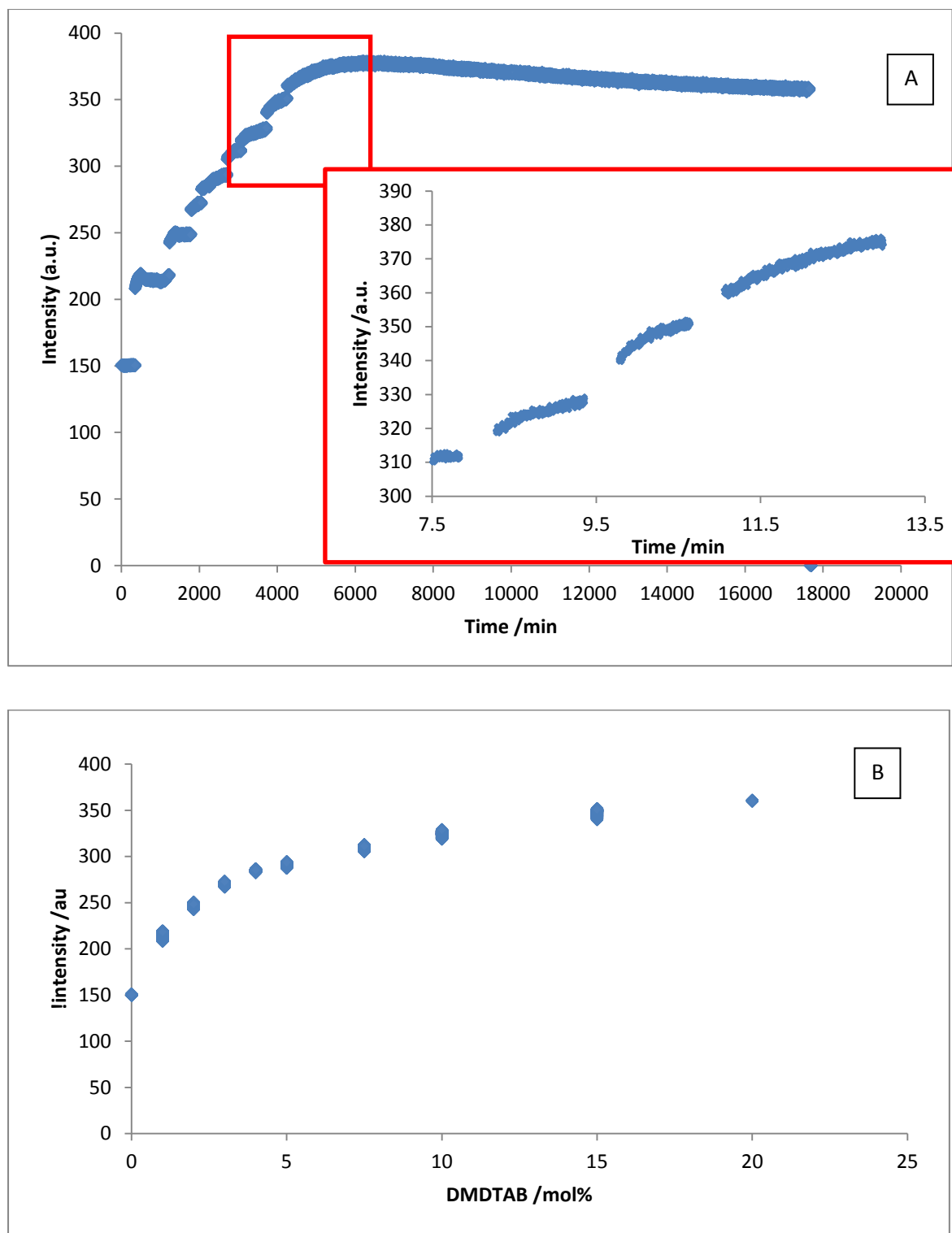


Figure 40: A typical FPE fluorescence curve, obtained through the titration of DMDTAB solution into a population of FPE-doped vesicles. Injections in this experiment were continued until the concentration of DMDTAB reached 0.2mM, or 20mol% relative to the lipid. After this point, fluorescence was monitored for 20 minutes to observe equilibrium kinetics and rates of photobleaching. (a) Raw data plot of intensity vs. time. Inset is the region highlighted in red, showing significant increases in intensity for some minutes after injection. (b) The same data, plotted to show the increase in fluorescence intensity vs. added DMDTAB (photobleaching data removed for clarity).

The above plot reveals two interesting details about the kinetics of the probe. Firstly, the fluorescence intensity of the FPE continued to rise: experiments showed that intensity was not saturated by even 50mol% DMDTAB, well above the compound's CMC value, although increases were greatest during initial, small additions of cationic species. This suggests that either the vesicles' affinity for DMDTAB was effectively unsaturable, which seems unlikely considering its permanent cation and the curve shown above in Figure 40b, or alternatively that even in PBS the additional charge in the system was screened insufficiently well to prevent DMDTAB aggregates from influencing probe molecules in adjacent membranes. Neither was an ideal scenario for the purposes of these experiments, as they made direct quantification of the amount of dopant within a membrane problematic. However, the effects of photobleaching were reasonably low in the system under study, approximately 1 a.u./minute of continuous illumination, meaning that for the initial injections which were of the greatest interest, the effects could largely be ignored.

The second, more subtle point of interest in the above scan is highlighted in the inset on Figure 40a. This shows that the increase in fluorescence intensity was not over immediately: although there was an instantaneous jump in the fluorescence signal after DMDTAB injection and mixing, there was a slower component to the increase which took some 5 minutes to reach equilibrium. This introduced a number of issues, both as to the quantification of dopant in the membrane (as the slow-rising signal often made a contribution towards intensity that was similar to that observed from the instantaneous mixing) and the increased illumination which exacerbated losses to photobleaching, but also regarding the physical mechanism underpinning its effects. Various models were proposed (outlined in Figure 41, below): slow diffusion of DMDTAB into the membrane as the electrostatic repulsion between the cations equilibrated with the hydrophobic attraction between the amphiphiles was supported by the apparent increase in effect with increasing dopant concentration (a), while another model suggested that the method of DMDTAB administration led to a high localised concentration of the compound, saturating FPE molecules (b). This led to the initial rise, which then increased slowly as the DMDTAB equilibrated amongst other vesicles as they collided, exposing more probe molecules to their charged environment. This explained the data well and was supported by some other experiments (discussed in section 5.6.4.1, below), but assumed the reasonably rapid transfer of DMDTAB molecules from one bilayer to another, requiring their energetic (and thus unlikely) rotation through 180°.

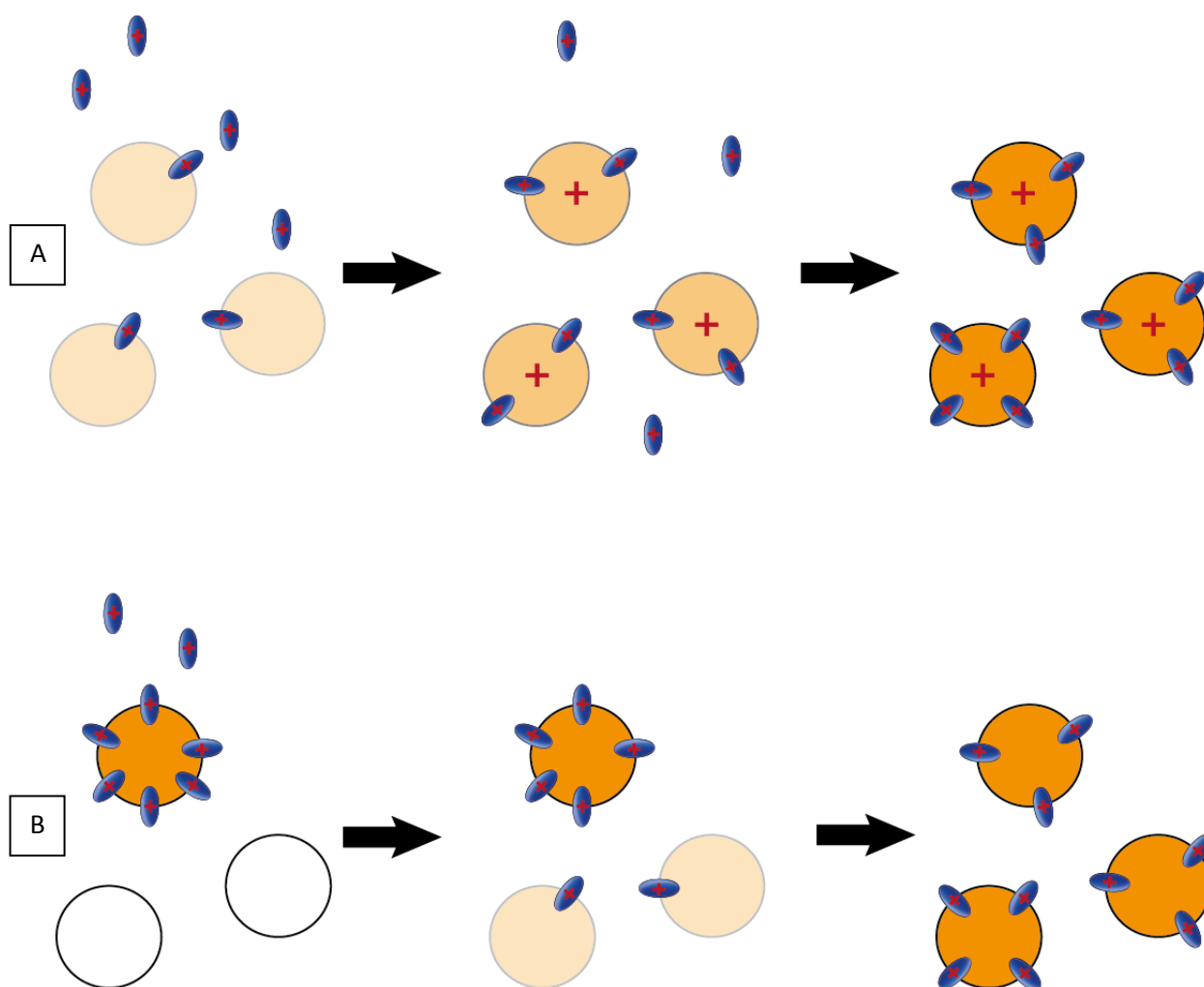


Figure 41: The two proposed models which describe the kinetics of FPE response to DMDTAB. In (a), the DMDTAB molecules (blue ellipsoids) are distributed evenly throughout the vesicle population and initially bind rapidly causing even, low fluorescence. However, as they bind, the surface charge build-up repels other molecules, slowing equilibration. In (b), DMDTAB-vesicle binding is very rapid, causing a high local concentration (and thus fluorescence) to develop and saturating the local FPE molecules. Over time, these dopant molecules equilibrate throughout the system, increasing overall fluorescence.

Experiments were attempted with FPE to measure the binding of a number of CAD-like molecules to vesicle populations. Although outright quantitation was problematic, it was hoped that at the very least the experiments would provide a qualitative framework of trend data that could be used to compare CADs and their analogues to known standards. As a result, experiments were conducted using haloperidol (in methanol), raclopride and two analogues of raclopride (all in buffer) which will be discussed in section 7.3.

FPE exposed to haloperidol behaved, on first glance, in a manner analogous to the DMDTAB experiments described above: the probe increased significantly in intensity with each injection

of haloperidol solution, converging towards a maximum value after about twice the concentration of DMDTAB required, as shown below in Figure 42. However, there are a number of important differences between the haloperidol data and that in Figure 40.

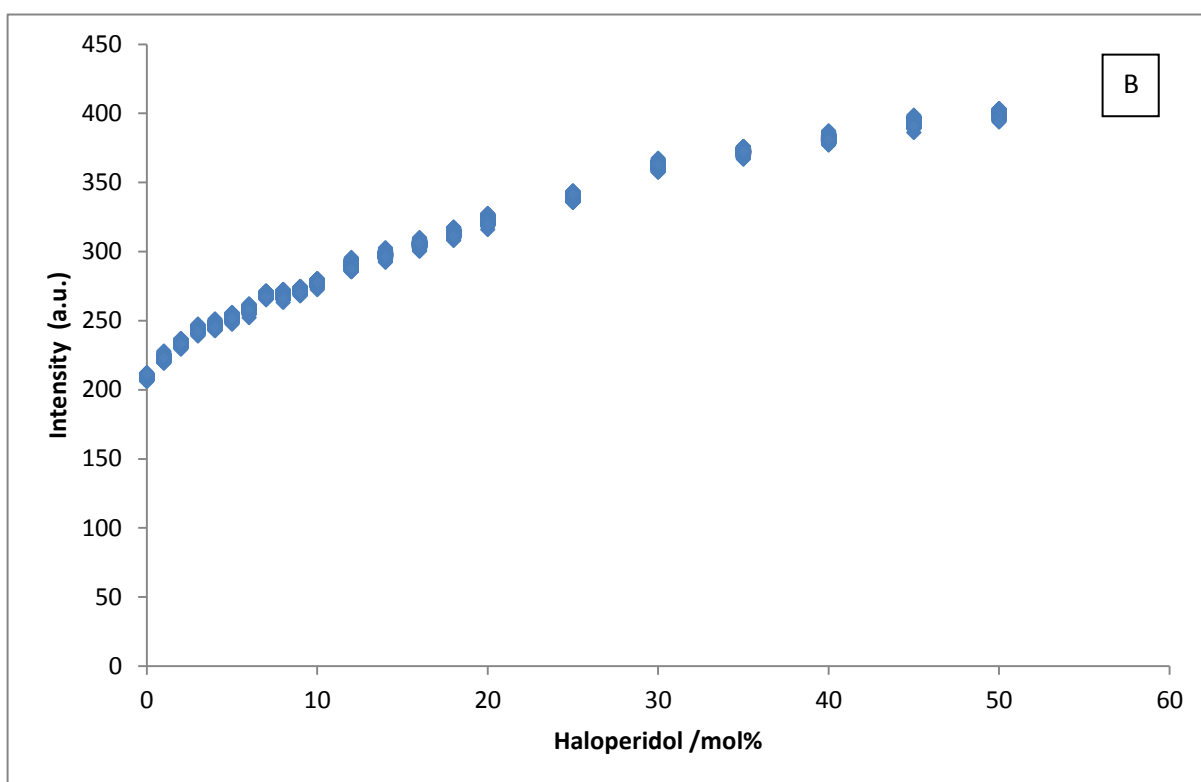
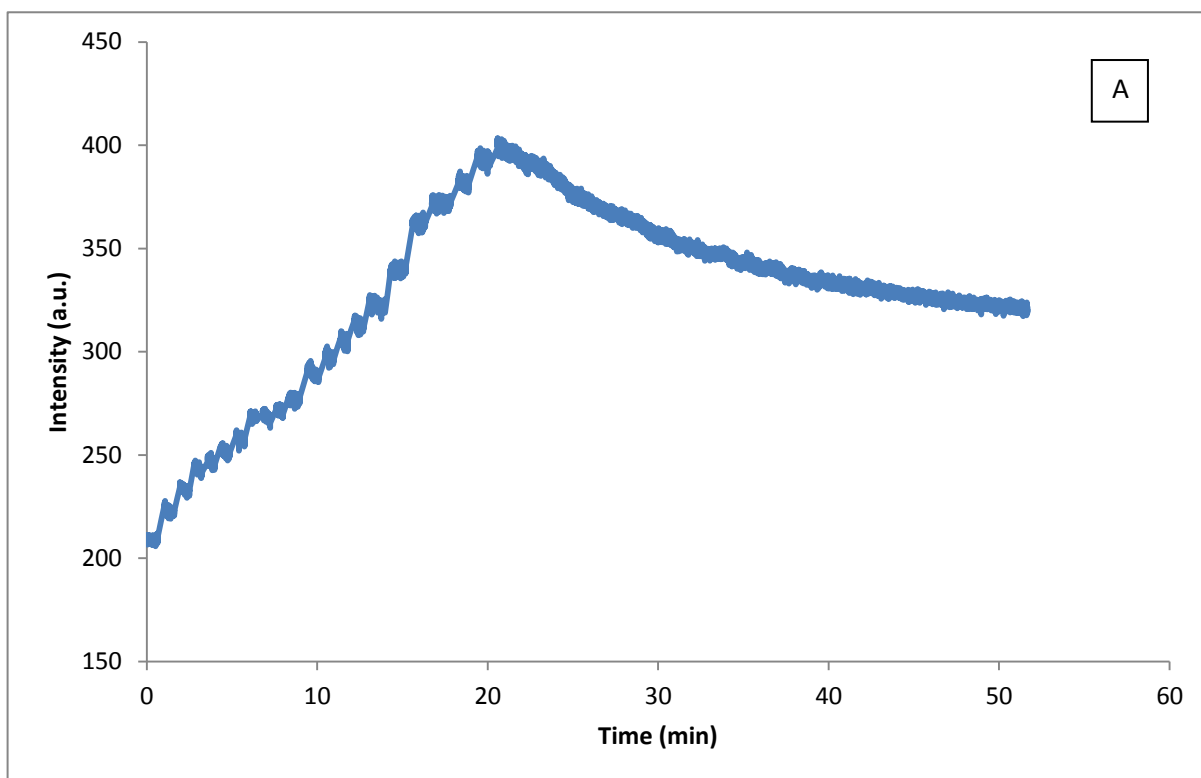


Figure 42: The fluorescence curves found in FPE experiments with haloperidol HCl: (a) the raw data, showing fluorescence signal vs. time; (b) the data when plotted against haloperidol concentration (long tail-off data from >20 mins removed for clarity).

Upon closer examination, it can be observed that the “equilibration” period of a slow rise in intensity of some 5 minutes after each DMDTAB injection is replaced here by a sharp and immediate drop-off, far in excess of the 1 a.u./min photobleaching observed in Figure 40a. Initially, this was assigned to the previously-observed hydrolytic action of the drug carrying itself away for the bound FPE, or neutralising its membrane charge through the introduction of anionic fatty acid species thus dimming the probe’s output. However, neither condensed- or vesicle-phase experiments in this study revealed any reproducible haloperidol-mediated hydrolysis. Instead, then, it must be linked to movement of the compound away from the interface, either towards the membrane interior or, as seems more likely, precipitating out as was previously observed with the closely structurally related spiperone in section 5.4.3 (page 75). This could, in turn, provide an explanation for the SAXS results previously reported⁵⁴ (see Figure 35, page 95), where haloperidol induced significant swelling and disorder of its host membrane, which slowly relaxed back to something approaching its native state over the course of some eight days. The more rapid efflux of haloperidol from the membrane in this experiment suggests a substantial difference in kinetics between the two phases, which may explain some of the disparity in timescales between previously reported data and that above.

This was reinforced by the results gleaned from experiments with raclopride, or rather the lack of similar response triggered by the drug’s addition. The addition of raclopride or any closely structurally-related analogue of the drug actually led to a small decrease in intensity (shown below in Figure 43), as if the drug was present as a neutral species or even an anion. Control experiments with methanol had previously ruled out haloperidol’s carrier solvent as the source of the effect, and repeat experiments produced near-identical results.

This seems unlikely upon first examination, considering the pK_a values of the functional groups present (the phenol and amine have pK_a values of 6.11 and 9.52, respectively¹⁵², giving raclopride a pK_i of 7.82 and suggesting a predominantly cationic form at physiological pH). However, it is unlikely that the formation of a charged species is as favourable in hydrophobic media as in bulk water, meaning that one or other net neutral species, either the zwitterion or the unionised form, may be predicted to be preferred in the membrane interior.

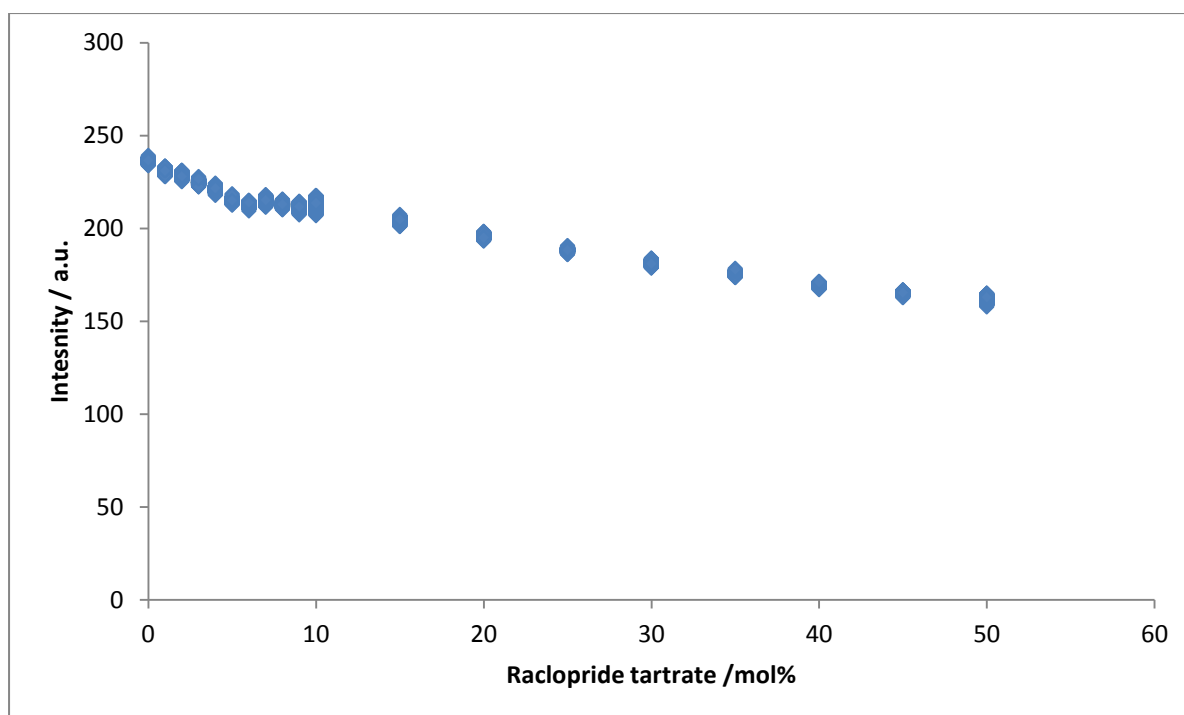


Figure 43: The influence of raclopride tartrate upon the fluorescence of FPE when added exogenously to labelled DOPC vesicles.

It seems improbable that a compound which had been demonstrated to hydrolyse the acyl groups at a lipid interface so effectively could impart its effects without binding to the membrane, and yet the strongly positive data from the haloperidol and DMDTAB trials were not replicated by raclopride. The results mean that there was no increase of cationic charge at the interface: this does not implicitly mean that the raclopride was not bound, but does mean that any so present was not protonated, existing as a net neutral species. Furthermore, this neutral species cannot have significantly altered the surface charge density of the interface⁸⁴: while stable internal salt species have been identified by molecular dynamic simulations¹³⁵, it seems doubtful that zwitterionic species such as they could exist next to the fluorophore without inducing a change in its electronic environment.

However, this presents a conundrum: for raclopride to hydrolyse a membrane efficiently, it must have access to a steady stream of protons. This evidence suggests that raclopride, unlike haloperidol, is only transiently charged within the membrane – that it is only capable of retaining protons for fleeting moments, and that the rate of hydrolysis is then dependent upon the probability of reaction between this briefly available proton and a neighbouring lipid molecule. The small but measurable decrease in fluorescence over the course of the scans may

then be a product of photobleaching coupled with a contribution from anions created by a small number of liberated fatty acids.

The strong response of FPE to haloperidol means, by contrast, that the vast majority of the drug is protonated at the interface and that this may explain its apparent lack of reactivity. By strongly retaining its extra proton, it effectively neutralises its own activity. The effective pK_a must be dependent upon the depth of residency of the CAD in the bilayer: theoretical calculations suggest a drop of up to 4.5 log units of basicity as a compound is dragged through a membrane²⁷. This might also suggest a mechanism of sorts for some of the behaviour observed during the method development phase: a large volume of excess water will inevitably lead to smaller, vesicular aggregates comprising few lamellae, meaning higher curvature and deeper penetration of water into the membrane. Larger, flatter aggregates will instead have curvature and thus head-group exposure determined by thermal fluctuations of an otherwise largely flat system. This will lead to lower water penetration and therefore a more hydrophobic interface, lowering the probability of a CAD retaining its proton when resident.

5.6.1.2 PENN-1 FRET probe

PENN-1 is a lipid-based probe developed by Wichmann *et al.* in 2006 to report upon rates of lipid hydrolysis by enzymes such as PLA2¹⁶⁴. It consists of a phosphatidylethanolamine head-group that is modified to present an unhydrolysable ether linkage in place of the ester moiety at the *sn*-1 position so as to prevent its recognition as a substrate by Phospholipase A₁, while each of the fatty acid chains carries a fluorescent probe to act as a FRET pair. The probes chosen were 7-nitrobenzo-2-oxa-1,3-diazole amine (NBD) in the *sn*-1 position, at the end of a C₁₂ alkyl linker, and the 2-hydroxy derivative of 9-diethylamino-5*H*-benzo[*a*]phenoxazin-5-one (Nile red) in the *sn*-2 position. The dyes have extremely high spectral overlap and both probes are net neutral at physiological pH and so extremely lipophilic: although some disruption to the packing of the host membrane's hydrophobic region was inevitable, it was at least minimised by the insertion of the largely planar, aromatic fluorophores.

The dye presented an optical and directly quantifiable probe into the kinetics of the hydrolysis of the phospholipid ester bonds: by comparing the emission of the green, quenched NBD signal relative to the red emission of the FRET pair, the degree of hydrolysis of the probe could be

accurately measured in real-time and non-destructively. The probe was generously donated by Carsten Schultz of the European Molecular Biology Laboratory in Heidelberg, and was stored in CHCl_3 at -20°C until use. Initially added in a method similar to that outlined above for FPE at 0.1mol% relative to 1mM DOPC vesicles in pure water, it was observed that the much weaker FRET signal was very difficult to distinguish from noise except at very high photomultiplier tube (PMT) voltages; no improvement in signal-to-noise was observed through further dilution. However, by moving to a 1mol% ratio of PENN-1 to DOPC, λ_{EXmax} were found at 520nm (Nile red) and, more weakly, at 440nm (NBD) $\pm 5\text{nm}$, due to high noise. λ_{EMMmax} was easier to locate at approximately 610nm, with no fluorescence signal observed below 550nm implying a near-total transfer of energy between the green NBD dye and the larger Nile red, shown below in Figure 44. Control experiments run in chloroform confirmed that the NBD dye was present and visible as when analysed as a monomer, $\lambda_{\text{EXmax}} \approx 440\text{nm}$, $\lambda_{\text{EMMmax}} \approx 520\text{nm}$, although even under these conditions the intensity of the Nile red fluorophores' absorption and emission seemed much greater than that of NBD.

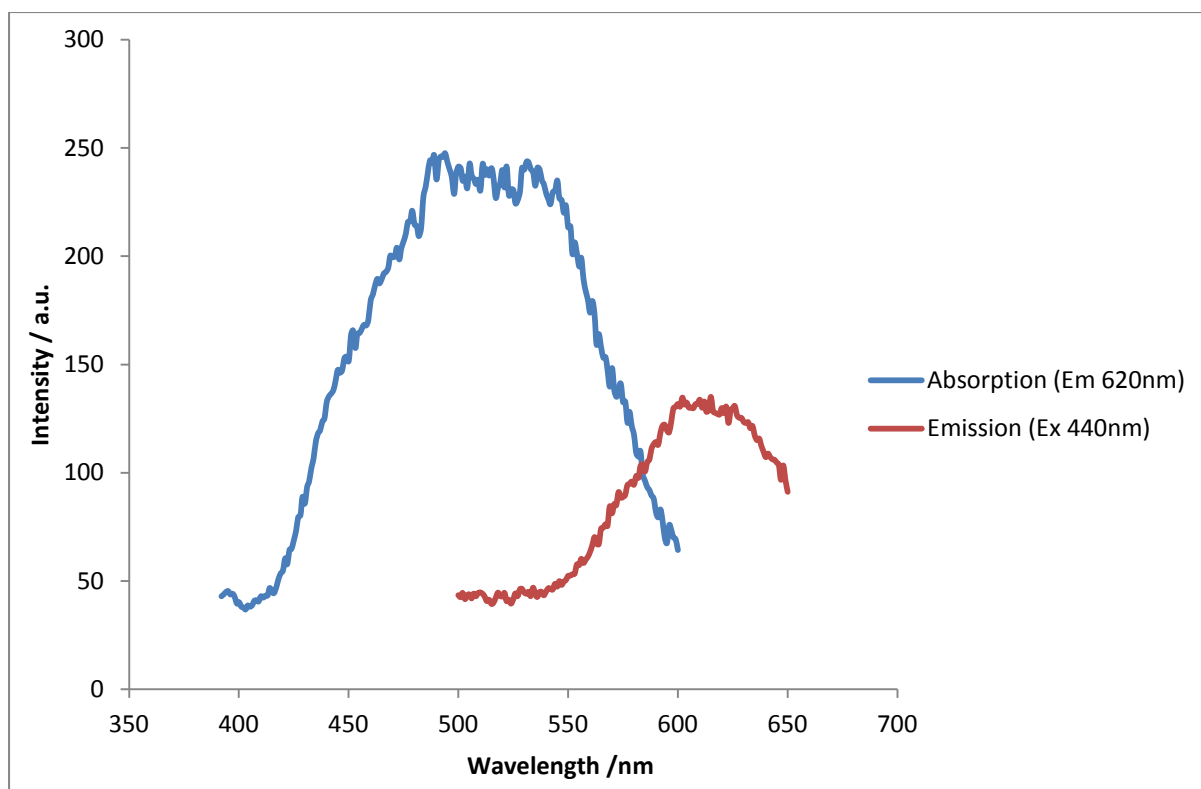


Figure 44: The FRET fluorescence spectra of PENN-1¹⁶⁴, present at 1% in 1mM 100nm DOPC vesicles in water.

In previous studies conducted at Heidelberg using this and related dyes, the probe had either been dosed in relatively high concentrations into monolayers of cells or alternatively had been the only phospholipid present in a dilute solution of Triton-X, meaning that the signal relative to light scattering by the vesicles was relatively high^{164,165}. Furthermore, an unknown mechanism had led to the probe accumulating exclusively into intracellular membranes: no fluorescence was observed in these studies in the plasma membrane. It was felt that neither of these protocols was ideal: PLA₂ is sufficiently ubiquitous and cell membranes are so complex that drawing any meaningful conclusions from cellular studies would have posed significant difficulties, whereas the alternative assay described led to an artificially high enzyme-substrate ratio in a highly strained system, which meant that measurements taken on such a system could not be easily compared to those in a more biologically relevant environment.

Previous studies using PENN-1 had been undertaken at Imperial College, based around fluorescence-monitoring of HPLC fractions using a Waters 474 in-line fluorescence detector coupled to the apparatus and set-up described in section 5.1.2 (L. Setchell, unpublished research). However, due to either the kinetics of the condensed-phase system or the very differing affinities of the probe and its degradation products for the columns in use relative to the native lipids, these had failed to reveal any conclusive data. As a result, vials were prepared in a manner similar to that described above: DOPC and 1 mol% PENN-1 were co-lyophilised from chloroform, then hydrated in water to give a 1mM suspension and extruded as described above. Raclopride tartrate, haloperidol free-base and ibuprofen free-base were dissolved in DMSO to form 10mM solutions. 1ml of lipid vesicle suspension was dispensed into a micro-cuvette along with 5µl drug solution or DMSO control, and the cuvette sealed with Parafilm. The cuvettes were analysed at 37°C, initially sampling very rapidly in order to capture any unusual effects of the drugs partitioning into the membrane.

This technique revealed no response in the 520/610nm emission ratio mediated by any of these compounds over the first 120 minutes of scanning: although absolute intensity at 610nm was lower in the DMSO control than in the other samples, this was not met by a concomitant increase in 544nm emission and so was almost certainly due to simple experimental error. In order to assess whether the reaction was occurring very slowly or was experiencing a latency phase such as that shown in PLA₂ experiments⁵⁹, measurements were continued for some six hours. In this phase, measurements were taken once every ten minutes in order to minimise

photobleaching effects. Again, however, no change in the 520/610nm ratio was observed for any of the compounds tested in this or repeat experiments, and the combination of low signal-to-noise coupled with the extreme scarcity of the probe meant that this avenue of experimentation was abandoned.

With hindsight, the probe was significantly better adapted to measuring the activity of PLA₂ than it was our system: PLA₂ is a cytosolic, globular protein and so does not depend significantly upon the hydrophobic region of the lipids for substrate recognition¹⁶⁶. By contrast, both the empirical data outlined above and the computational results shown by Rosso *et al.*¹⁵⁰ suggest a strong dependence of the rate and degree of hydrolysis upon interactions between the drug molecule and the fatty acid tail of the lipid substrate. The use of PENN-1 as a probe therefore relied upon finding interactions between the CADs and one or other of the large fluorophores which mimicked those of the drugs' interactions with oleoyl chains, and these were not detected.

5.6.1.3 ADIFAB

Acrylodan-labelled intestinal fatty acid binding protein (ADIFAB) is, as its name suggests, a protein isolated from rat intestine covalently modified so that an acrylodan moiety is bound to its Lys²⁷ residue¹⁶⁷. This amino acid changes conformation and environment when the protein binds to a fatty acid, in turn altering the those of the fluorophore and shifting its λ_{EMMmax} from 435nm (unbound) to 487nm (bound) when exciting at the probe's λ_{EXmax} of 386nm (all values $\pm$ 2nm). Monitoring at these wavelengths leads to some overlap of emission, and so for effective analysis of the state of the probe, the ratio between fluorescence at 432 and 505nm was measured. The dissociation constant of the probe for oleic acid, as in this study, has been measured at 0.28 μ M ($\text{pK}_d = 6.55$), meaning that the relationship between the fluorescence ratios and the free fatty acid in the system can be found *via* Equation 8, where $Q = I_{\text{free}}(432\text{nm})/I_{\text{bound}}(432\text{nm})$, R_o = the ratio of intensities at 505nm/432nm in the absence of fatty acid and R_{max} = the ratios when the probe is saturated¹⁶⁷.

$$[\text{FFA}] \approx K_d \times Q \text{ —————}$$

Equation 8

The probe offered the potential of an extremely sensitive tool to analyse the rates of hydrolysis in a rapid and non-destructive manner that did not intrinsically affect the membrane, as the protein is globular in nature and so is not, in theory, membrane bound. It has also been thoroughly characterised, permitting a directly quantitative analysis of the free fatty acid concentration in solution.

However, this in turn poses issues: because the probe is only sensitive to *free* fatty acids, those retained within the membrane are effectively invisible to the protein and the acids' high partition functions (oleic acid has a $\log P > 5$) meant that a significant degree of hydrolysis was required before meaningful signals could be determined. Furthermore, this partition function was expected to vary dynamically during any given experiment as a function of composition, surface charges and curvature, making precise quantification difficult. Finally, in order to minimise any effects due to variations in vesicle size and morphology, it was desirable to use a uniform population of extruded, unilamellar vesicles for testing. This meant that ADIFAB had to be added exogenously after extrusion and yet research has demonstrated that fatty acids equilibrate across a membrane very rapidly⁶¹. It was therefore possible that observed fatty acid concentrations could be significantly below those actually generated as a result of their sequestration both in the membrane and in the vesicles' internal volume, from which the protein was effectively excluded.

Preliminary experiments were undertaken to evaluate the potential of the probe. DOPC vesicles were prepared at 100 μ M concentration in the supplied ADIFAB buffer, containing 20mM HEPES, 140mM NaCl, 5mM KCl and 1mM Na₂HPO₄ according to the protocols described above. 100 μ M ADIFAB (FFA Sciences LLC, USA) was added in its 50mM Tris HCl storage buffer to give a total protein concentration of 0.2 μ M and dispensed into disposable polystyrene microcuvettes. The temperature inside the fluorimeter was controlled as previously and set to 37°C throughout and blank scans were run to correct for the scattering of blank cuvettes containing only HEPES buffer. Standards were run containing HEPES and ADIFAB solutions without lipid to investigate the effects of DMSO and photobleaching upon the probe, as well as with 10 μ M oleic acid in 1 μ l DMSO to provide a positive control sample. Data was recorded from each sample every 20 seconds for 1 hour, during which time photobleaching was negligible after an initial equilibration period. These results showed an R_0 value of 0.365 ± 0.016 , identical to that of R_{DMSO} , while R_{max} was recorded as 3.67 ± 0.63 , although this is reduced to 3.40 ± 0.22 if the

equilibration period is omitted (see Figure 45).

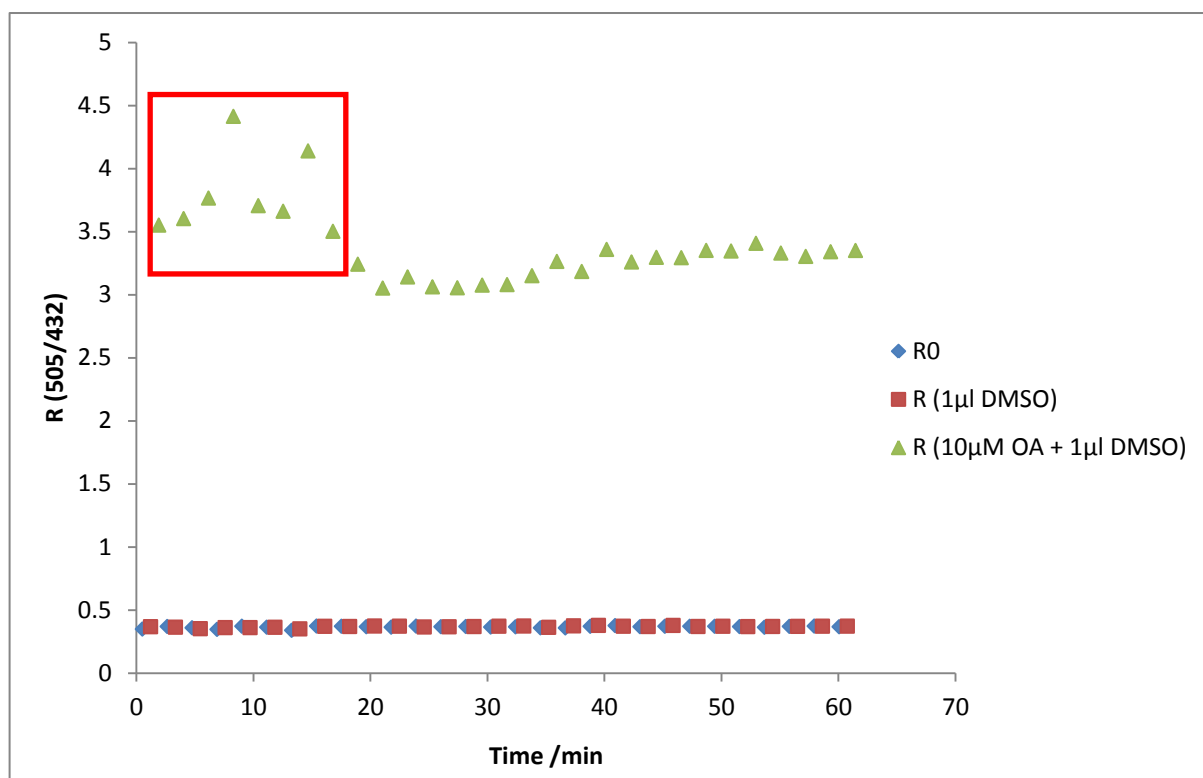


Figure 45: ADIFAB control and photobleaching data, recorded every 20 seconds over 60 minutes. The values are corrected for scattered light from blank scans of buffer alone: small errors in these values propagate to give the large distortions visible in the fatty acid-containing scan above. Red box highlights apparent equilibration period.

Calibration scans were run in both the presence and absence of 100µM 100nm DOPC vesicles (see below), to give a feel for both the sensitivity of the probe and also the extent of hydrolysis that would be required before a signal could be detected. The apparent twenty-minute equilibration period at high signal gave some cause for concern, as it suggested that the probe may not be ideal for the measurement of dynamic systems such as the one in question: however, Figure 46b suggested it was only an issue at very high signal, and it was hoped that the gradual nature of the fatty acid build-up would minimise any impact.

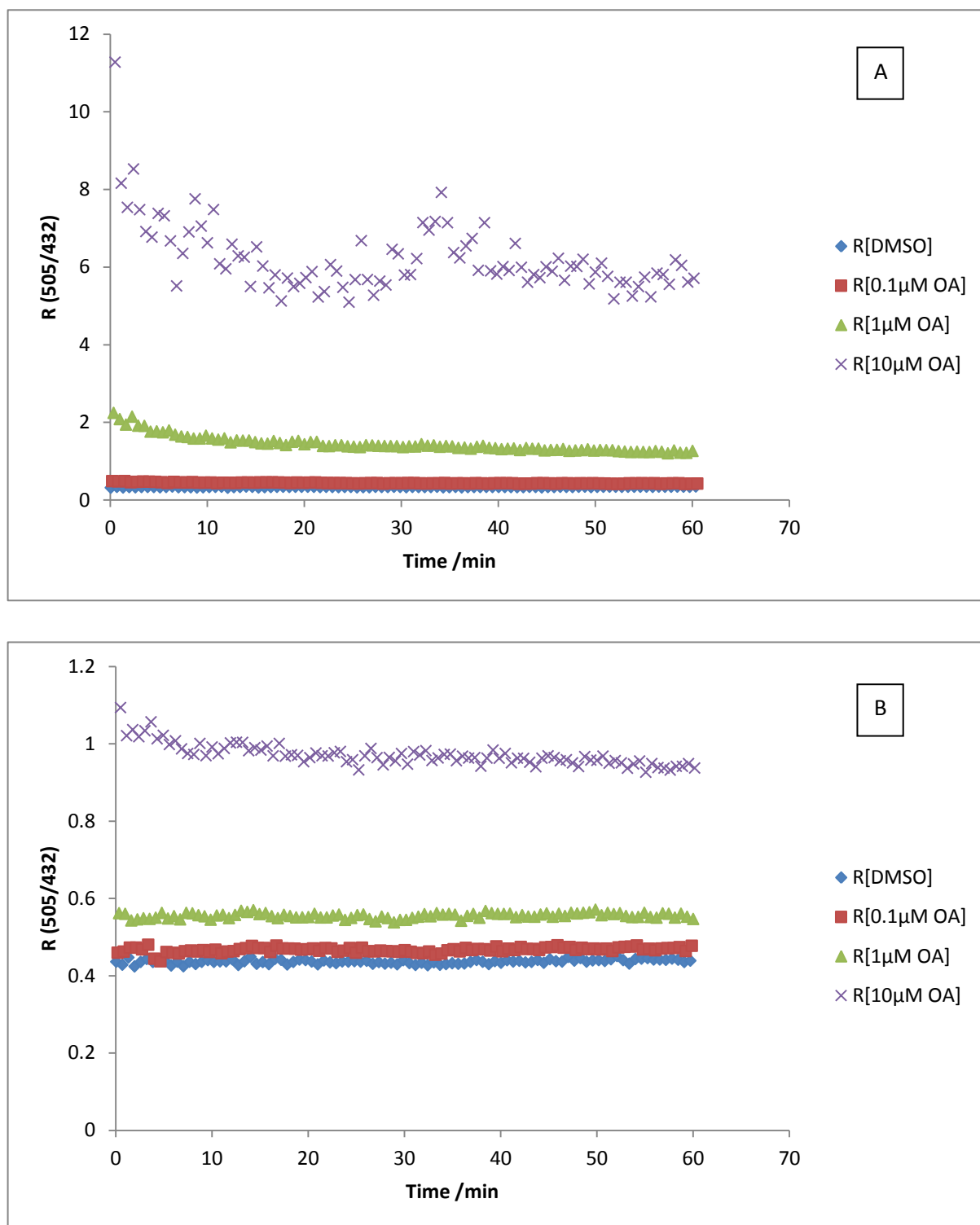


Figure 46: Oleic acid calibration scans in (a) the absence and (b) the presence of 100µM 100nm DOPC vesicles.

These calibrations were run using identical stock solutions of buffer, protein and fatty acid and all fatty acid solutions were made by serial dilution of the 10µM stock so that only 1µl of DMSO

was ever injected. The difference in $R_{[\text{DMSO}]}$ values shown above ($R_{[\text{DMSO}]} = 0.33 \pm 0.010$ in Figure 46a; 0.44 ± 0.011 in Figure 46b) imply that the system is extremely sensitive to the final protein concentration, and therefore required the addition of a significant concentration of fatty acid in order to provide a saturated signal for normalisation at the end of each run. Using the published K_d value¹⁶⁷ and assuming approximately $0.2\mu\text{M}$ ADIFAB, approximately a $0.5\mu\text{M}$ concentration of free acid was required to ensure saturation which could have posed difficulties considering the compound's low aqueous solubility and high partition function. As a result, the empirical approximation for R_{max} and Q in oleic acid binding published by Richieri *et al.*¹⁶⁸ was used in preliminary experiments, as it relied solely upon easily measured R and R_0 values (Equation 9).

$$[\text{FFA}] = K_d \times 19.5 \times \text{————}$$

Equation 9

Despite these limitations, it is clear from the data in Figure 46 that ADIFAB had the potential to detect hydrolysis levels of below 1% in vesicle suspensions and so was potentially a powerful and high-throughput probe into the activity of hydrolytic compounds in the membrane.

An experiment similar to that in Figure 46b was prepared, dosing the $100\mu\text{M}$ 100nm DOPC vesicles with $0.1\text{--}10\mu\text{M}$ raclopride tartrate dissolved in DMSO, again by serial dilution so that in each case $1\mu\text{l}$ DMSO was added to 1ml DOPC suspension. These were analysed on the fluorimeter overnight: rapidly for the first 15 minutes to capture any kinetic effects of binding, then once every ten minutes for the next 12 hours. The results were unexpected, as can be seen below in Figure 47. Illustrated in this example is the system that was doped with $0.1\mu\text{M}$ raclopride, corresponding to $0.1\text{mol}\%$. However, similar behaviour was observed in all four cuvettes including, unexpectedly, the DMSO control.

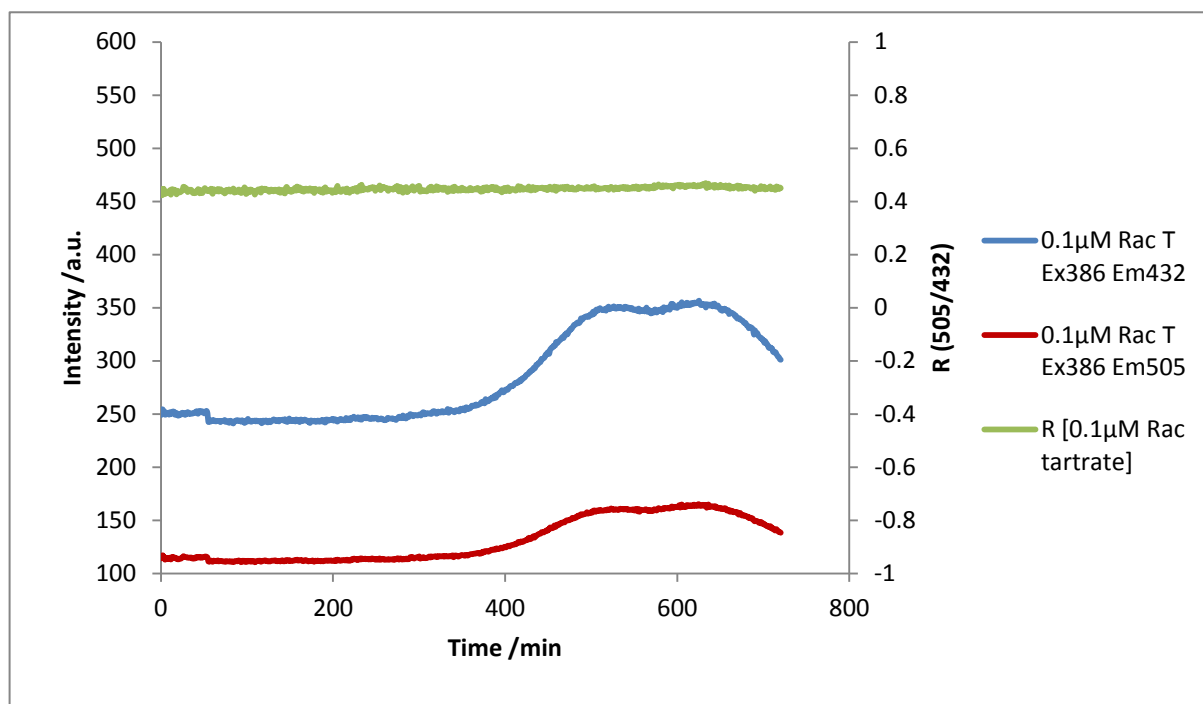


Figure 47: The absolute fluorescence intensity and corrected 505/432nm peak ratios for 100μM 100nm DOPC vesicles doped with 0.1μM raclopride tartrate in 1μl DMSO. Axes are offset for clarity.

These findings were extremely interesting: bulk-phase assays had found what appeared to be a lag phase suggesting some kind of internal reorganisation of the drug-lipid aggregate before hydrolysis took place, and upon first glance this appeared to be the fluorescence manifestation of that phenomenon. However, this could not possibly be the case in the DMSO control vial. A number of repeat experiments were performed to confirm that this was a real result and not that, for example, the DMSO blank had become contaminated. Similarly, control runs were performed to check for any fluorescent interactions between the probe and the drug, or anything unusual happening to the cuvette over time. Repeats were also performed in PBS, which importantly gave a similar ionic strength to the HEPES solution supplied with the protein¹⁶⁸ in case the HEPES was interfering through some mechanism. Each found the same behaviour: after some six hours, the fluorescence intensity began to rise dramatically at both 432 and 505nm, and yet the ratio between the two peaks was always identical within error.

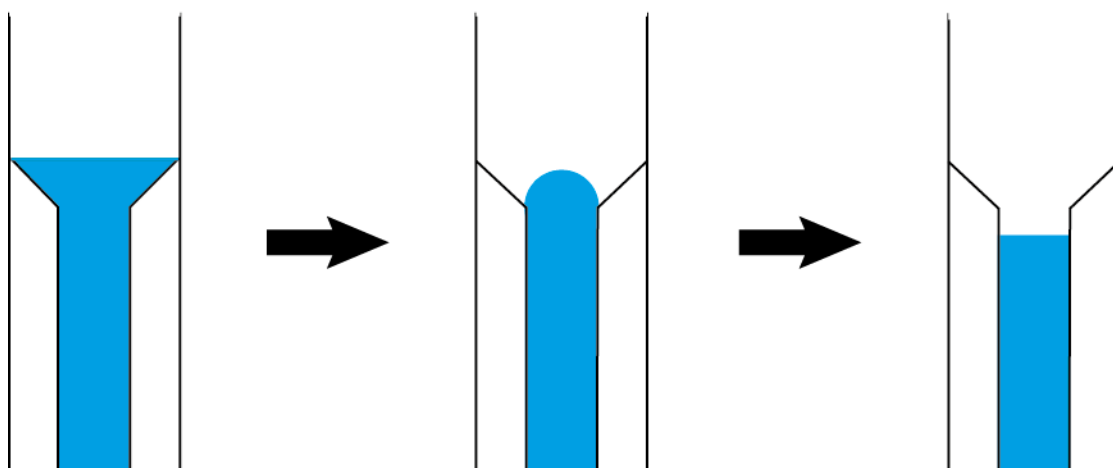


Figure 48: The gradual evaporation of the ADIFAB-labelled vesicle suspension produced a highly curved interface, which led to a significant increase of the quantum yield of the probe suggesting a degree of surface activity.

Eventually, the effect was isolated to the slow evaporation of the buffer from the heated cuvettes. The shape of the microcuvettes meant that after a period, a highly curved interface developed. As evaporation continued, this curvature decreased until eventually a flat interface was restored (shown schematically above in Figure 48) and that despite the protein's apparently cytosolic nature¹⁶⁹, this was reflected in the fluorescence of the probe. Repeat reactions with fully sealed cuvettes showed negligible evaporation over the course of 12 hours and similarly flat fluorescence emission spectra. Although there was a very small rise in R during these experiments, it was also observed in control samples and so was probably due to protein unfolding over the time-course of the experiment. The experiments were repeated under a variety of conditions and with other CAD compounds, but no change in R was observed and the reactions were abandoned.

5.6.2 Dynamic light scattering

5.6.2.1 Lab-based laser scattering

It was hoped that at least a qualitative analysis of the morphological effects of the drugs upon the vesicle systems could be assessed by dynamic light scattering, using the Malvern Instruments High Performance Light Scattering unit described in section 5.6. Dynamic light scattering works by comparing the Rayleigh scattering of coherent light by small particles in a suspension, up to about half the wavelength of the incident light, at different periods over time.

The change in scattering over time is dependent upon the Brownian motion of the particles suspended in the sample. This is a function of the concentration and hydrodynamic radius of the suspension and the time interval between sampling, as well as the viscosity of the suspending medium. Large particles, or those suspended in a viscous environment are only able to travel short distances in between samples, leading to small steps between scattered intensities and rounded, smooth graphs; smaller aggregates can travel much further in the same time period, presenting jagged, saw-tooth data-plots as shown schematically in Figure 49b and c, below.

The difference in signal collected over time by a detector situated at an angle to the incident beam (see Figure 49a). Data is collected and deconvoluted from the resulting patterns of constructive and destructive interference, although quite sophisticated techniques are required to achieve high resolution data except from very dilute samples due to multiple scattering effects, as scattered light is deflected in new directions on its path through a densely-packed suspension. For a full review on the technique and the associated data handling protocols, please see Berne and Pecora (2000)¹⁷⁰: experimentally, almost the entire process was automated by the supplied Malvern HPPS software and little user input was required.

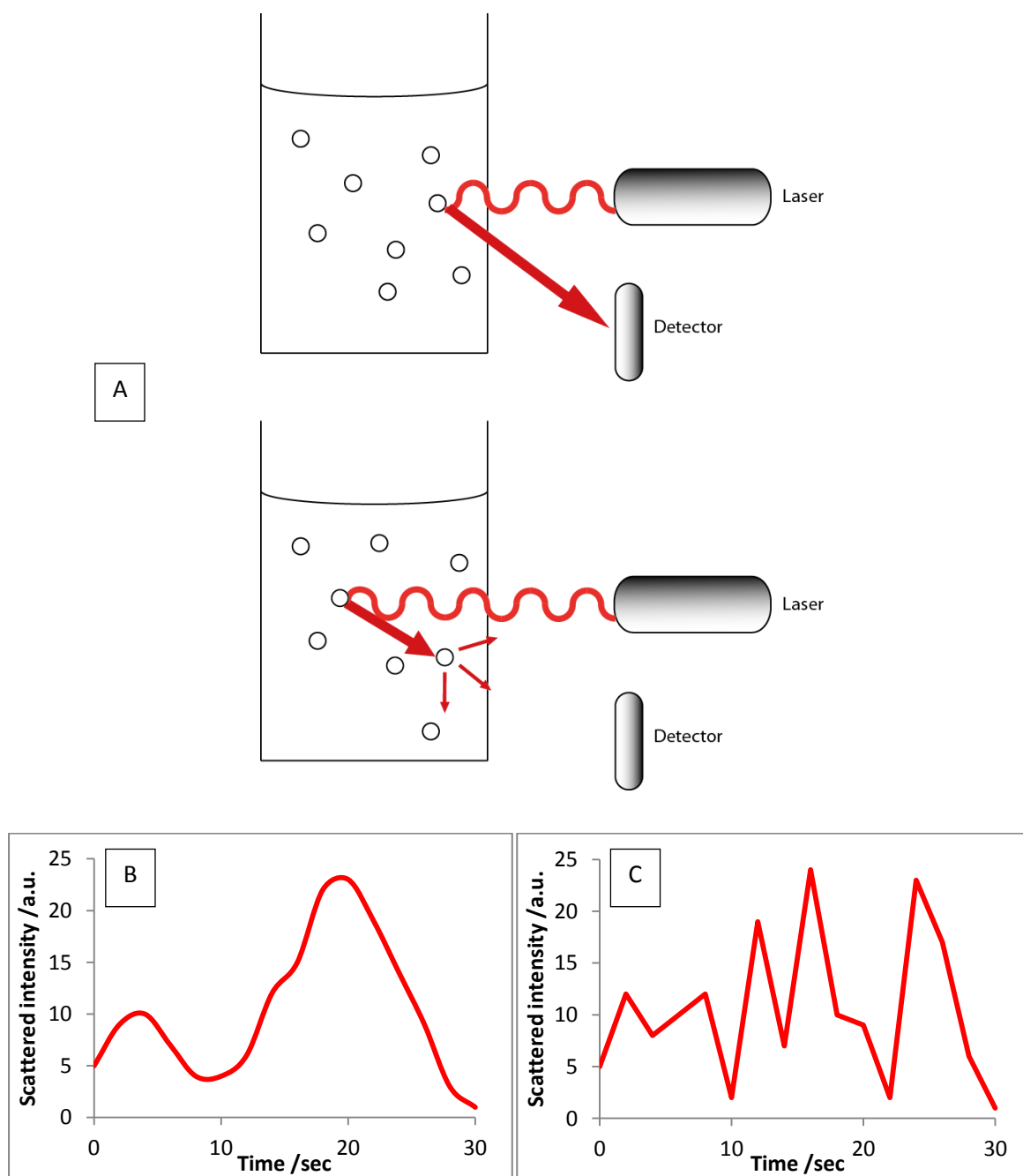


Figure 49: a) Dynamic light scattering records the fluctuations in scattered light from an incident laser beam. The speed and scale of the fluctuations are dependent upon the size of the particles, their concentration (through constructive and destructive interference) and the viscosity of the suspending medium. Large particles, or those in viscous suspensions, move very slowly making the fluctuations in their scatter large and ponderous (b) relative to the rapid, saw-tooth scatter exhibited by suspensions of smaller aggregates or colloids (c), shown here in schematic form.

While this technique could not provide a quantitative view of the state of hydrolysis within the membrane, it could provide data about the average structure of the vesicles within the sample

cuvette. Although such averaged data is inevitably of relatively low resolution, when coupled to the complementary data that was to be derived from the on-line vesicle-based HPLC methods under development, it was hoped that a much fuller picture would emerge.

The technique could also potentially explain the longer-term future of the daughter lipid structures created by the hydrolysis of the parent, diacyl species. If the vesicles behaved in a manner similar to the condensed-phase samples observed in earlier experiments⁵³, it was predicted that the sample would degrade from a largely monodisperse suspension of unilamellar vesicles into a large number of small micelles enriched in lyso-lipid and a much larger, effectively insoluble aggregate enriched in fatty acids in the inverse hexagonal phase. These structures would eventually degrade further, to form highly water-soluble glycerolphosphatidylcholine species and fatty acids that would be almost completely immiscible with water. However, the actual rate of this process remained largely unknown, and it was uncertain whether or not the DLS machine available would have the necessary resolution to observe the process, or whether the original, sharp and monodisperse signal would simply spread out into a broad, low-intensity and data-poor blur (see Figure 50).

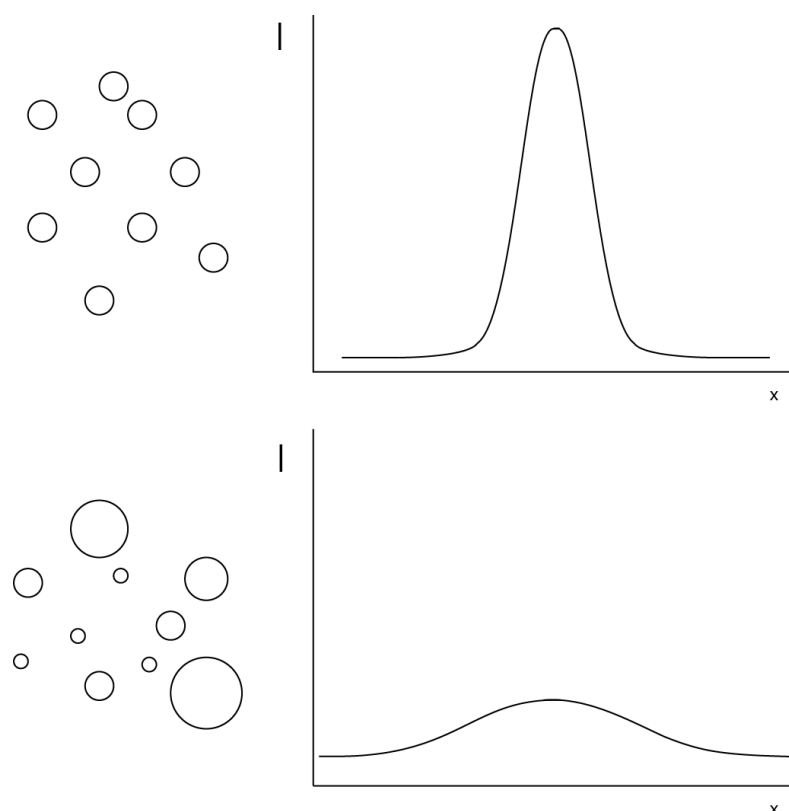


Figure 50: The two likely outcomes of the observation of the effects of hydrolysis *via* DLS. A monodisperse suspension of small vesicles produces a narrow, sharp function (top); a polydisperse mixture produces a broad, flat response of limited quantitative value.

In order to investigate these possibilities, preliminary studies were undertaken using the available in-house DLS. 10mM DOPC vesicles were made in pure water by extrusion using 100nm filters, to give vesicles of average diameter 112.0nm, FWHM 36.7nm, PDI 0.012. These were dispensed into four microcuvettes and two doped with 5mol% raclopride tartrate (added in 10µl water), one haloperidol free-base (in DMSO) and one with 10µl DMSO as a control. In each case the mixtures were agitated by aspiration and dispensing a portion of the suspension using an Eppendorf pipette, then were sealed and inserted into the DLS, the sample chamber of which had been pre-heated to 37°C. Sizing information was recorded in three scans of thirty seconds each, recorded on ten occasions over two hours.

None of the samples showed any significant deviations from initial parameters described above in terms of sizing or scattering intensity over this period. To check for any sign of activity, each cuvette was incubated overnight at 37°C and rescanned three times for thirty seconds each:

again, none showed any changes beyond experimental noise. It appeared that the haloperidol precipitated from the suspension during its overnight incubation, which may not be surprising considering the low aqueous solubility of the free base. However, no such precipitate was observed in the cuvettes containing raclopride.

Although these results constituted a setback, it was quite possible that the reaction was occurring at a reasonable rate in these systems but that the results were not observable with the available apparatus: Rayleigh scatter scales with [particle diameter]⁶, meaning that small, 5-10nm micellar aggregates such as those expected scatter negligibly relative to the 200nm parent vesicles. As a result, a much more flexible experiment was proposed, using custom-built equipment and analysed using in-house, bespoke software based around LabView code (National Instruments, USA) written by N. Brooks.

5.6.2.2 Synchrotron light scattering studies at the Diamond Light Source (UK)

Synchrotron radiation offers complementary information to that obtainable *via* DLS: Soft X-rays with a wavelength around 1 Ångstrom interact with features of a similar length scale and so are ideal for probing the wall structure of lipid bilayer vesicles (which generally have a bilayer thickness on the order of 50 Ångstroms). Scattering proceeds in the same manner as in the classical condensed-phase SAXS experiments described in section 3.4.1, but in this case the scattering is caused by the deflection of the incident beam through interaction with the phosphate groups at the interface between the hydrophilic and hydrophobic regions of the membrane.

The scattering angles used in this type of experiment are small, typically from 0.01-1°, and the scattered intensity is very weak relative to the incident beam. The scattered rays can shed light on the bilayer thickness, the average vesicle radius, the degree of polydispersity (defined by _____) and, for multilamellar systems, the repeat spacing (*d*-spacing) between each layer¹⁷¹. A schematic of this is shown in Figure 51.

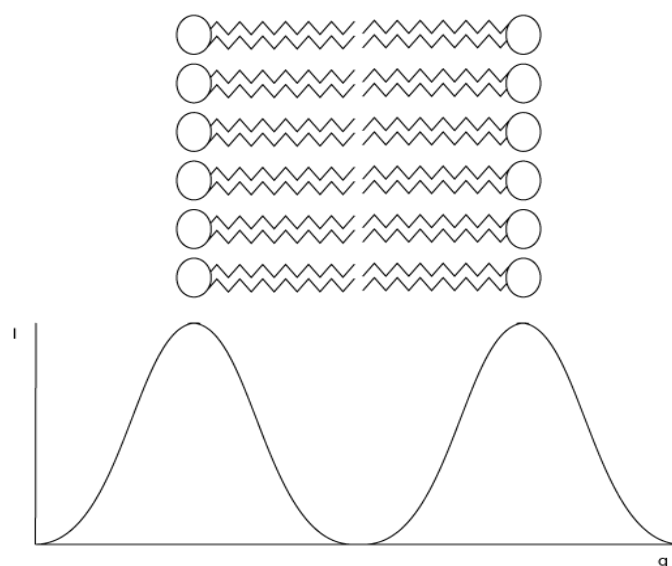


Figure 51: A schematic showing the scattered X-ray intensity as a function of q , and how this relates to the location of the head-groups within the bilayer. Adapted from Brzustowicz and Brunger, 2005¹⁷¹.

Experiments were performed on the I-22 beam-line at the Diamond Light Source at the Harwell Science and Innovation Campus near Didcot, Oxfordshire. I-22 is a high intensity, small angle scattering beam-line with a tuneable X-ray energy from 3.7 keV (3.35Å) to 20 keV (0.6Å), and adjustable detector flight tube length from 1 – 9 metres. X-rays are detected by a 2-dimensional RAPID high sensitivity multi wire gas detector. Vesicle suspensions were made up as previously described in section 5.6 using 200nm-pore membranes, to make a 10mM concentration.

Experiments were initially attempted with 1mM suspensions as in previous experiments, but it was found that the signal was so weak at this concentration that extremely long exposures (>5 minutes) were required to recover any useable images, preventing the capture of any kinetic data. Suspensions were made up in saline solution and in PBS, as described previously; solutions of raclopride tartrate, spiperone HCl and WAY 100635 maleate were prepared to 10mM concentrations in DMSO (see Figure 13, page 56 for structures).

Experiments were performed in a stopped-flow fashion. Three syringe pumps were prepared: Pump A contained a 50ml syringe of the relevant buffer solution, Pump B contained a 20ml syringe of vesicle suspension and Pump C contained 1ml of drug solution in DMSO, as shown below in Figure 52. Each was connected to a three-way mixing tee (IDEX Health & Science, formerly Upchurch Scientific) which led to a 2 mm diameter polycarbonate X-ray capillary (Enki Microtubes, Italy) in the path of the X-ray beam. The capillary tube was held in a temperature-

controlled block which was attached to a circulating water bath, which allowed the temperature to be controlled and measured to within 0.1°C. Pump control was achieved remotely using an in-house developed scripting program written using National Instruments' LabView by Dr Nick Brooks. This software permitted sequential runs of experiments to be performed automatically, meaning that user input was required only to refill reagent reservoirs. The capillary was initially flooded with 5 volumes of buffer, before being loaded with vesicle suspension and either 0.01, 0.1, 1 or 10mol% drug. Data collection began immediately, and 60 exposures of 25 seconds each were recorded, with a 5 second delay between each. Control experiments were performed using pure DMSO instead of drug under identical conditions; positive controls were performed with bee-venom PLA₂, using 5mM DOPC vesicles prepared in PLA₂ buffer consisting of 50mM Tris HCl, 100mM NaCl and 1mM CaCl₂. The scattering images collected generally showed diffuse X-ray scatter with one very broad peak.

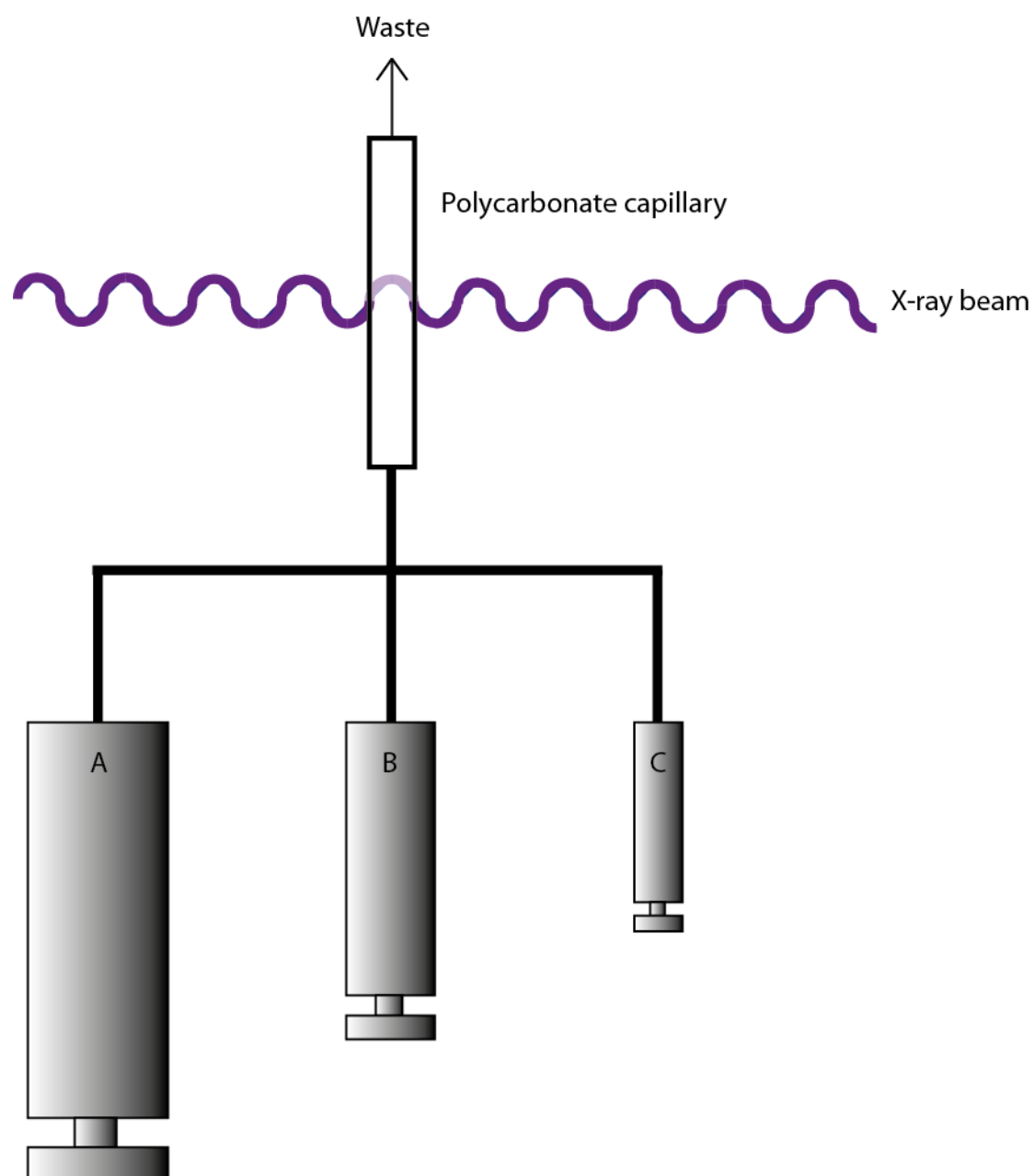


Figure 52: The experimental set-up used during the vesicle scattering studies attempted at the Diamond synchrotron light source in Didcot, Oxfordshire, UK. Syringe A contained 50ml aqueous buffer, B contained 20ml vesicle suspension in the same buffer and C contained 1ml drug solution in DMSO.

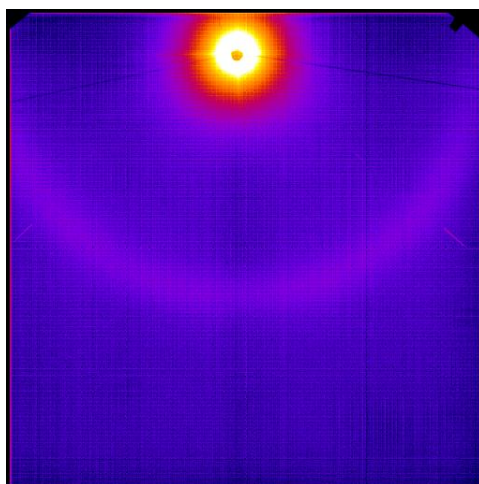


Figure 53: An image captured during a typical synchrotron radiation scattering experiment showing the raw data yielded by the apparatus described above (exposure time = 25 s). 60 of these were taken per experiment and combined to show the time-resolved behaviour of the vesicle populations.

5.6.2.2.1 *Synchrotron scattering theory and modelling*

Data analysis was performed in several iterative steps. Initially, the raw data (see Figure 53) was processed to remove as much background scattering as possible using appropriate background images. Each image was then radially integrated to provide a 1-D diffraction plot of intensity vs. q , where $q = 2\sin(\theta)/\lambda$ (2θ is the scattering angle and λ is the wavelength of the incident beam)¹⁷². The q scale was calibrated against a standard sample of wet rat-tail collagen with a well-defined layer spacing of $676\text{\AA}$ ¹⁷³. These 1-D plots were collated and plotted against time to form a 'stackplot' showing changes in the vesicles' scattering over time. This stackplot was fitted in the region $0.05 < q < 0.2$ pixels to yield data on the average position of the positions of the lipid phosphate groups relative to the bilayer centre and the d -spacing of any multilamellar vesicles formed based upon the model of Brzustowicz and Brunger¹⁷¹ (described below, typical results shown in Figure 54).

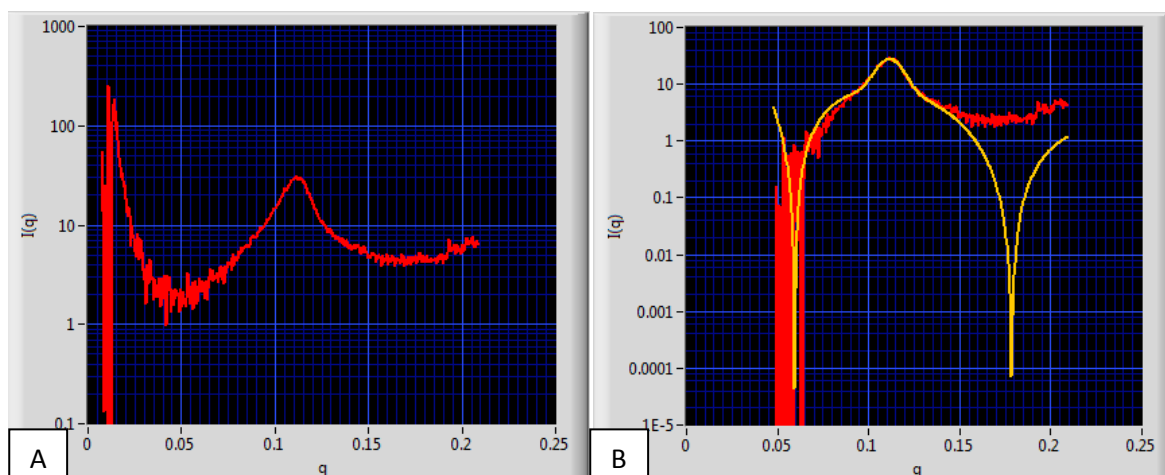


Figure 54: (a) A typical 1-D I vs. q plot, after noise subtraction; (b) the same data, with beam flare excluded and showing the fit of the model described below (yellow line).

The model outlined by Brzustowicz and Brunger¹⁷¹ assumes that no correlation between vesicles is observed in the sample, meaning that the 1-D X-ray intensity $I(q)$ depends only upon the bilayer form factor, $F(q)$, the Fourier transform of the electron density of the bilayer $\rho(r)$ which is shown schematically above in Figure 51. In this model, the bilayer is divided into three regions (k), where at the lipid head-groups $k = 1$ and 3 and at the centre of the hydrophobic core, $k = 2$. The electron density profile may then be modelled as a series of n Gaussian shells, each with amplitude ρ_k , centre position relative to the centre of the vesicle δ_k and uncertainty (essentially, the roughness of the function) σ_k , leading to Equation 10, below.

Equation 10

If it is assumed that each vesicle in the population is spherical and thus radially symmetrical, the form factor $F(q)$ can be found *via* a Fourier transform of Equation 10.

Equation 11

In order to find an expression for the measurable quantity $F(q)^2$, the polydispersity was taken into account, again using the Brzustowicz model using a Gaussian fit to describe a population of

vesicles with average radius R_0 . If

Equation 12

where ε_k = displacement from the vesicle radius, then Equation 13 results.

Equation 13

However, the model outlined in Equation 13 applies only to systems containing only unilamellar vesicles; experience suggested that a significant number of multi-lamellar vesicles would also exist in the suspension, especially after the administration of the DMSO solution. This could potentially lead to significant diffraction, as the intensity of scatter from multi-lamellar bilayers is significantly greater than that from single-walled vesicles. As a result, a further Gaussian term was added to allow for these data, shown below in Equation 14.

Equation 14

5.6.2.2.2 Synchrotron scattering results

Although several thousand images were recorded under a range of conditions, it was difficult to extract meaningful conclusions. In part, this was due to the diffuse nature of the target material: even at 10mM concentrations, the signal-to-noise ratio of the collected data was poor, complicating analysis. As a result, the most reliable information came from the d -spacing data generated by the multi-lamellar vesicles that were inevitably present in the suspensions – an unfortunate finding, considering the purpose of the exercise and the kinetic considerations outlined in section 5.6. Other errors were introduced by the inter-dependence between the

parameters making up the model: in theory, it was possible to find values corresponding to the vesicle average size in a manner analogous to the DLS values above, but in practise the function often generated nonsense values and so the numbers generated were disregarded. However, all experiments returned an initial d -spacing of around 56Å suggesting good reproducibility and showed rapid increases in this distance with 10% DMSO solutions, as might be expected. A smaller initial increase in d -spacing of 1-2Å, (exemplar data shown below in Figure 55) was observed in all systems, presumably due to the relaxation of the vesicles as the flow speed through the fluidic system dropped, leading to a concomitant drop in shear forces. This may not reflect an actual change in d -spacing, but instead be a result of the change in morphology as the vesicles returned to a spherical shape, due to the inter-dependences described above.

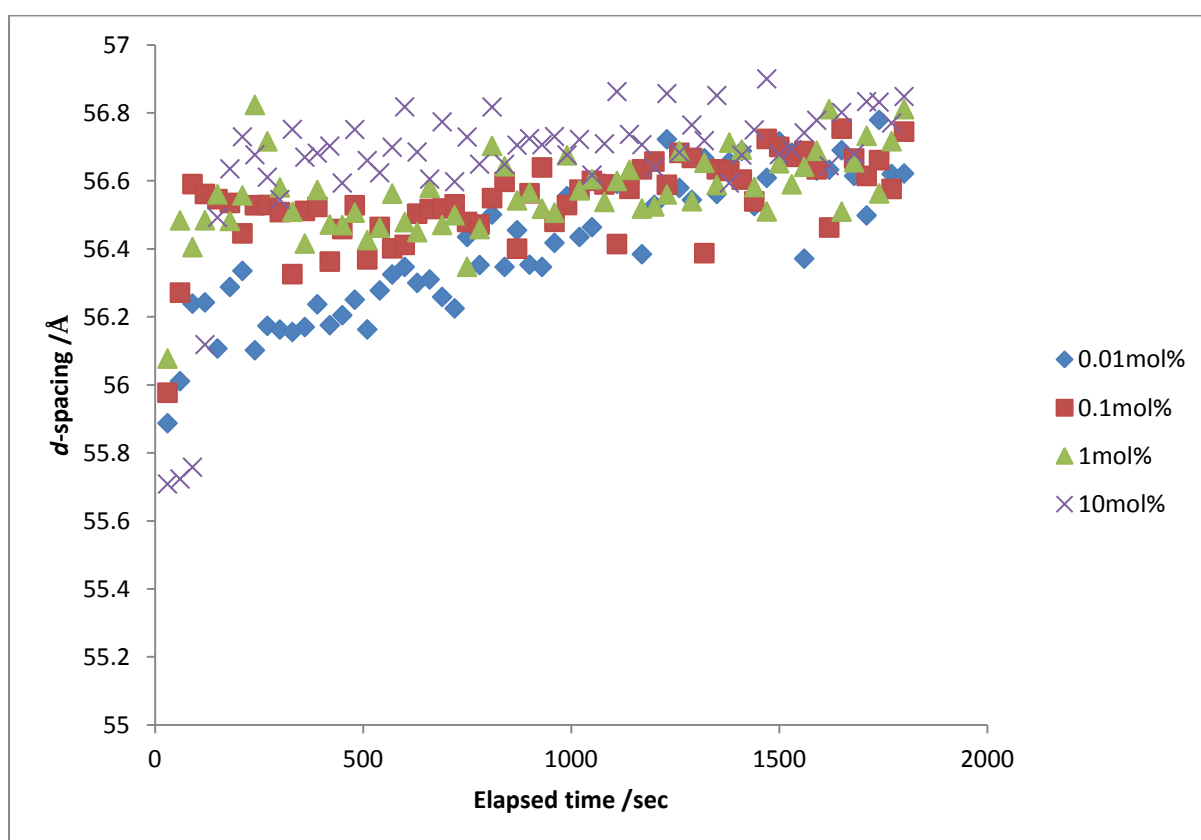


Figure 55: Exemplar data gathered during X-ray scattering experiments on 200nm DOPC vesicles containing varying proportions of, in this case, spiperone HCl added in DMSO. Note the initial rise in d -spacing as the vesicles ‘relaxed’ as the flow was stopped.

The PLA₂ data which provided the positive control data for the experiment revealed the expected dose-dependent increase in bilayer thickness and d -spacing over the time-course of the experiments, (as shown below in Figure 56), triggered by the accumulation of negative

charge in the membrane due to fatty acid. During this period, the water channel thickness (found as the difference in location between the two maxima, representing the phosphate moieties at the lipid-water interfaces) remained effectively constant in all fits, at a steady 4Å, comparing well with literature values of around 3-5Å¹²⁷. This implies that the expansion was due to hydrolysis occurring, as no DMSO or other co-surfactants were present and the protein is only transiently bound to the membrane, minimising its contribution.

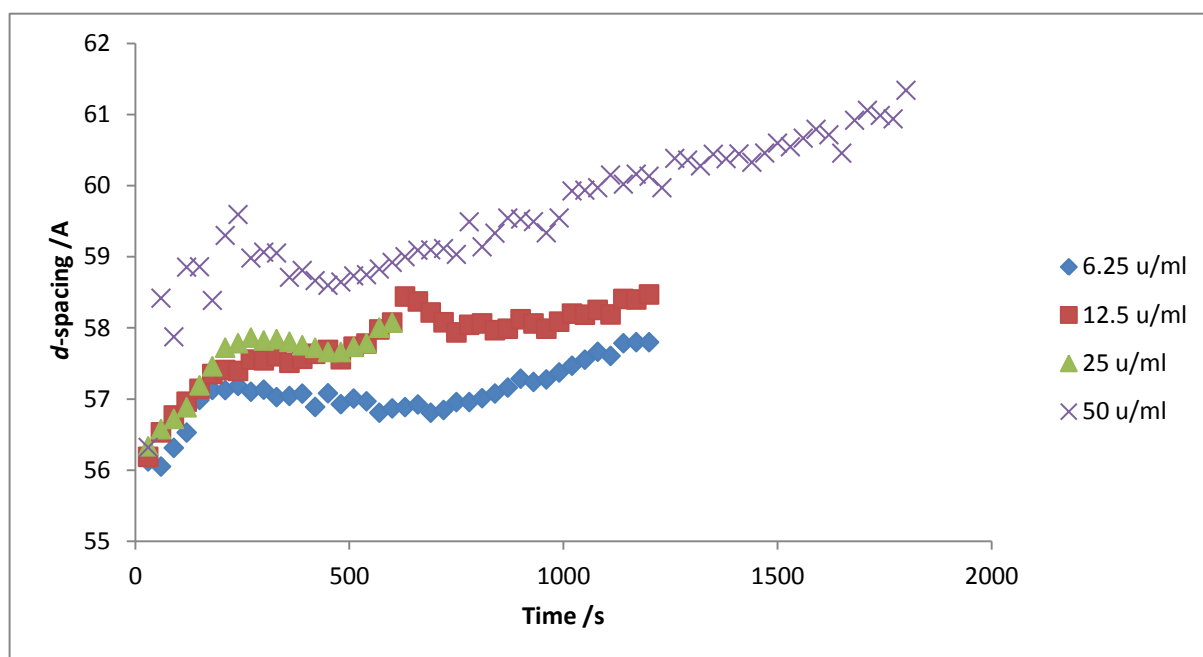


Figure 56: *d*-spacing data recorded for 5mM DOPC vesicle systems containing differing concentrations of PLA₂ enzyme. One unit of enzyme will hydrolyse 1.0 µmole of *L*-α-phosphatidylcholine to *L*-α-lysophosphatidylcholine and a fatty acid per min at pH 8.9 at 25 °C, although observed rates were significantly below these values in both these experiments and others conducted by other researchers.

Unfortunately, no other results showed a change in vesicle morphology over the course of the experiments. Data similar to the above were recorded for WAY 100635 maleate, spiperone HCl and raclopride tartrate (all in DMSO), but none revealed anything significant beyond the DMSO spectra recorded that could not be attributed to experimental artefacts. Membrane swelling was observed in most samples after doping, with two to three times the change in *d*-spacing observed in saline systems than in PBS-buffered samples observed even in DMSO control experiments (for exemplar data, see Figure 57, below).

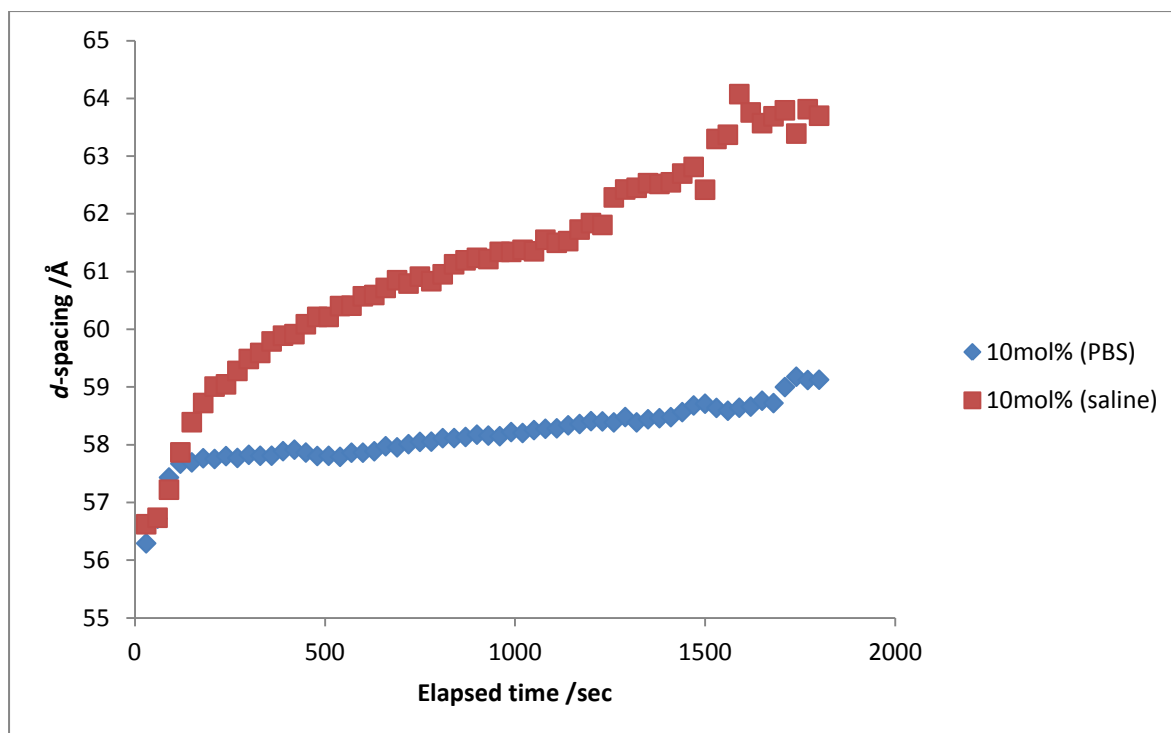


Figure 57: The increase in *d*-spacing exhibited by 200nm DOPC vesicles upon addition of 10% DMSO to the buffer. Although both systems experience some swelling after injection, the vesicles in the unbuffered solution exhibited greater than twice that shown by the buffered system.

This is of passing interest, as the Debye lengths in both cases were effectively identical and all drugs were added as neutral salts in DMSO. This suggests that the phosphate ions had a role to play in controlling dopant distribution within the system, although the mechanism for this is unknown. Again though, due to the interdependence between the variables of the model as outlined above, it was difficult to attribute this to any one effect. Microscopy studies demonstrated that the administration of DMSO caused significant vesicle rupture in the immediate vicinity of the injection (experiments described in section 5.6.4.1), and it may be that the observed data simply reflects the formation of many more multi-lamellar bodies in saline than in PBS, although this remains speculation.

Little more of value was identifiable from the experiments: significant data was lost in the experiments due to beam flare, which obliterated the linear region of signal which might have provided much more precise vesicle size information. Major issues were also experienced with beam damage to the polycarbonate capillaries – this led to distortions in the gathered data, producing whiskered tails of beam-flare which led to the loss of many data points.

The reasons for the failure of such different drugs with partition values spread across three orders of magnitude to show any significant effects upon the membranes remains unknown. All have previously been demonstrated to hydrolyse membranes rapidly; although some non-specific binding to the fluidic system was inevitable, this was as likely to be an issue for the much more plentiful lipids as it was for the drugs and so any such effects should have averaged out.

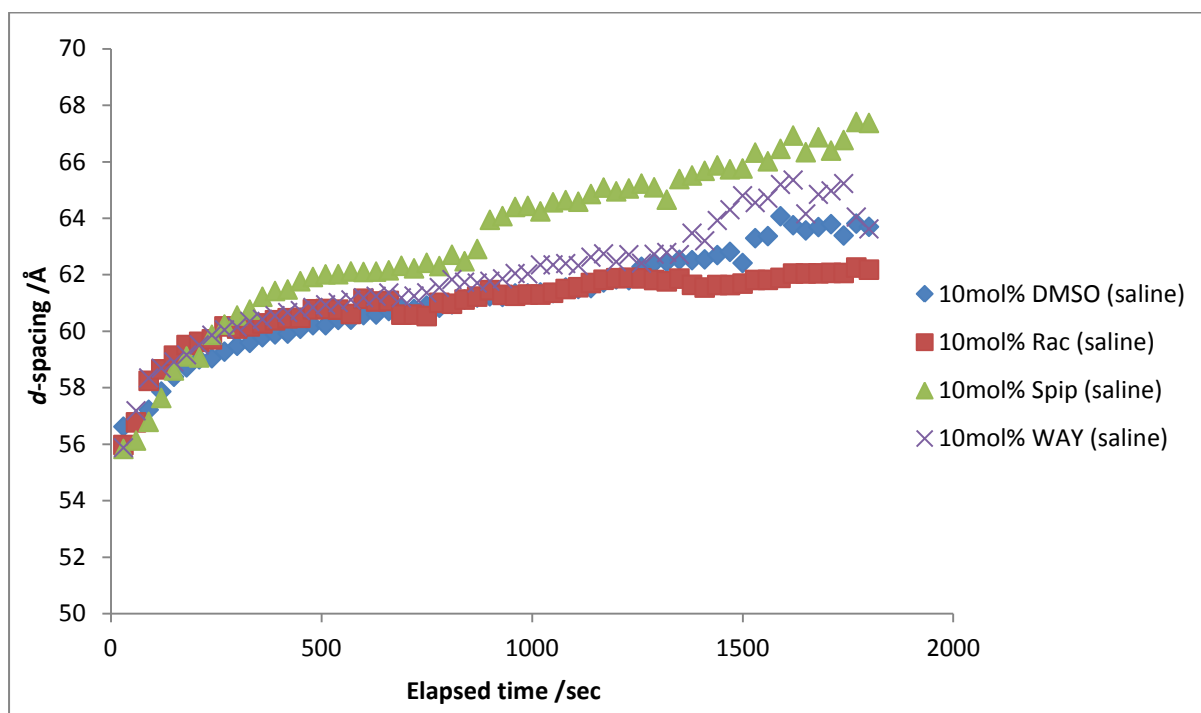


Figure 58: The increase in *d*-spacing caused by the addition of 10mol% of several CADs to the vesicle suspensions. It can clearly be observed that most if not all the contribution after the vesicles' initial relaxation is caused by the carrier solvent.

What was genuinely surprising is that the introduction of charged species had little or no more effect than the membrane-softening DMSO (see above): the Debye length within both systems was of the order of $7\text{\AA}^{174}$, meaning that electrostatic effects were expected in a way analogous to the SAXS images shown in Figure 35 (page 95). Those experiments also served to define a typical *d*-spacing for a DOPC lamellar stack of around $61.4\text{\AA}^{54}$, similar to those values shown in Figure 58. Those results were observed in a condensed-phase system with a high degree of long-range order – it is possible that in a more chaotic system dominated by single unilamellar vesicles such effects should average out, but one might still expect the uneven distribution of the exogenously-applied drug across the membrane leaflets to have some effect.

5.6.3 HPLC assay

By this point, the direct-injection HPLC assay had become available, making vesicle-based chromatographic experiments available for the first time (see section 5.2.2). This had the potential to add significantly to our understanding of the kinetics of the system: data points could be gathered with a temporal resolution of 15 minutes without sample preparation, allowing reasonably closely-spaced analyses to be conducted even on samples reacting as fast as that in Figure 10.

Initially, an isocratic technique with a high percentage of organic solvents was developed which yielded good quality results. Unfortunately, this led to the co-elution of the inorganic salts and buffers in the samples with many of the lyso-lipid signals of interest, meaning that the more complex gradient was required. A number of alternative techniques and tweaks to the method were also suggested (personal communication with James Stratta, Varian) including column heating or doping the eluent with trifluoroethanol: unfortunately, the combined restrictions of an all-PEEK system and financial constraints made such optimisations impractical. However, the gradient method described above gave sufficient resolution for the purposes of this study and with a high degree of reproducibility, becoming the workhorse technique for many important experiments.

Vesicle suspensions were prepared for HPLC testing in the same way as described previously: lyophilised lipids were hydrated with water or buffer to form an opaque and milky 1mM mixture, which was vortexed and extruded ten times through 200nm-pore polycarbonate membranes to form a largely homogenous suspension of unilamellar vesicles, average diameter $\approx 180\text{nm}$ as described above. 1ml of this suspension was dispensed into a glass HPLC vial and doped with a drug or control substance dissolved in water or DMSO, depending upon the compound. The drugs were added in 1 μl solvent (0.1% of total medium) to minimise any dilution or solvent effects, (final concentrations were 10^{-7} , 10^{-6} , 10^{-5} or 10^{-4}M , corresponding to 0.01, 0.1, 1 or 10 mol% relative to the lipids) and the suspensions agitated to promote homogenous mixing. This was one significant departure from the assay conditions in the condensed-phase experiments: in this case, drugs were added exogenously to complete lamellae; previously, drugs had been co-lyophilised with the lipids, meaning that their incorporation into the bilayers was instantaneous and even across the system. The autosampler module of the HPLC had a temperature controlled sample chamber, meaning that all

experiments could otherwise be run under identical conditions to those in condensed phase described above. This chamber and the vesicle suspensions were pre-heated to 37°C before the drug solutions were added so that temperature was constant throughout the analyses.

This sweep of concentrations was designed to bridge the gaps between the relatively high dose regimes created in the condensed phase assays and the very low, nanomolar doses experienced by such lipid structures under physiological and pharmacological conditions. Hydrolytic effects had been observed very rapidly in previous vesicle studies: it was hoped that these experiments would demonstrate the contribution of the reaction to *in vivo* transport and bioavailability under low-dosage conditions as well as providing a calibration set for the Diamond studies[†].

Preliminary experiments were conducted using WAY 100636 maleate (in DMSO solution) in DOPC vesicles in an unbuffered saline solution, as adding the compound under these conditions had caused some form of response in the raw synchrotron data described above. However, when analysed by HPLC they showed zero hydrolysis over the course of 12 hours at all concentrations. However, this was not unexpected: in contrast to previous work¹¹⁸ at no point in this study were any hydrolysis effects observed which could be ascribed to WAY activity. As a result, the experiments were repeated using DOPC vesicles in a pure water medium, using raclopride tartrate dissolved in water to create the full range of drug concentrations outlined above.

These four vials were analysed for twelve hours after the point of drug injection, meaning that each vial was sampled once per hour. Again, over this period no lyso-OPC signal was observed at any point, suggesting that either no reaction was occurring or that it was far too slow for the products to be quantifiable, in contrast to previous studies⁵³. However, a white precipitate was observed in the vial doped with 10⁻⁴M (10 mol%) raclopride. This had been observed previously in some of the light-scattering and fluorescence studies outlined above but dismissed as an artefact caused by lipid aggregation on, for example, a small flaw in the surface of micro-cuvettes. However, it was observed in this study in what appeared to be a dose-dependent manner: over the next 48 hours of incubation, all four vials began to show the formation of a precipitate far in excess of the volumes of raclopride tartrate added, although no lyso-OPC

[†] These studies were undertaken during the development and refinement of the software platforms required to analyse the data from the Diamond synchrotron experiments: as a result, the failure of those experiments to show any activity was not at this point known.

peaks were observed at any point.

This was the cause of some excitement – if some unknown mechanism was causing the precipitation of the lyso-lipid or some drug-lipid complex, the lack of measured lyso-PC could be neatly explained and the result could potentially provide direct evidence that the drug was being expelled from the bilayer as part of the process. This seemed odd, however: lyso-OPC is a reasonably soluble compound in water as is raclopride and although the inverse is true for oleic acid, it is a liquid at 37°C, forming an oily layer on the surface of bulk water rather than a crystalline aggregate. Furthermore, there was no obvious driving force for precipitation: lyso-OPC is soluble up to its CMC of 5 μ M¹⁷⁵ and even if the highly lipophilic fatty acid could be persuaded to leave the remaining membrane, any mixture of DOPC, oleic acid and L-OPC not heavily enriched in the acid will form an easily-dispersed liquid crystal phase (R. Macey, unpublished work).

This experiment was repeated a number of times, varying the type of drug and the lipid concentration, but to no avail. On no occasion was any lyso-OPC peak observed, but in many the white aggregate or precipitate formed. Positive control experiments were conducted using bee venom PLA₂ enzyme, similar to those conducted at Diamond (in conjunction with Claire Stanley). Experiments were conducted using 5 and 10 units/ml PLA₂ with 100nm DOPC vesicles in PLA₂ buffer as described previously at 25°C. These experiments were conducted using dilute 100 μ M DOPC suspensions in order to tie in with other experiments in progress and thus required high signal amplification for meaningful data to be extracted. However, they showed a rapid increase in the lyso-lipid signal as the DOPC peak decreased in area, reaching an equilibrium at approximately 25% DOPC-75% L-OPC within 60 minutes (for exemplar data, see Figure 59).

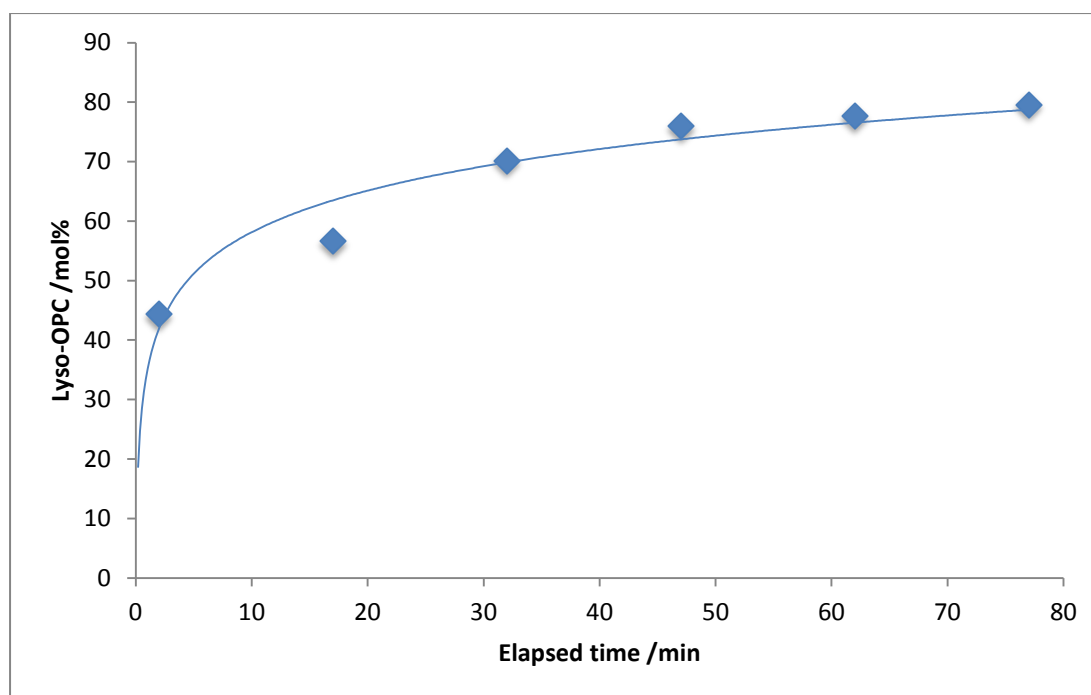


Figure 59: A typical data plot showing the rate of hydrolysis caused by the addition of 10u/ml bee-venom PLA₂ to a suspension of 100µM DOPC vesicles. Data fits well to a logarithmic plot, ($R^2 = 0.94$), as demonstrated by Richieri and Burack¹⁷⁶.

This was an important result, in many regards: it proved that the vesicle-based HPLC system really could function as an on-line, preparation-free experiment, delivering quantitative, relatively highly temporally resolved data in an automated fashion. However, the ease with which the activity of PLA₂ could be monitored with the HPLC assay meant that whatever was causing the precipitate in drug-doped samples was very unlikely to be hydrolytic in nature, as lyso-OPC signals were observed below 1 mol%, corresponding to a 1µM concentration. A number of attempts were made to characterise the precipitate: a 20mM suspension was sent for solid-state ³¹P NMR and samples were analysed *via* polarising microscopy. However, all results were inconclusive, showing nothing more than multilamellar bodies and attempts to consistently reproduce the aggregates under clean, controlled conditions met with failure. As a result, the effect was attributed to simple aggregation of unilamellar vesicles into multilamellar stacks which sank due to their relatively high density.

5.6.4 Possible reasons for experimental failures

There are a number of possible reasons for the failures, not least that the reaction may simply be extremely slow, meaning that the rates of hydrolysis exhibited in condensed-phase systems are in fact representative of those in any system. However, both previous experimental findings and the rapid partitioning behaviour observed with the FPE probe relative to similar condensed-phase SAXS experiments suggest that this is not the case. Earlier experiments showed significant and rapid membrane degradation by compounds which have shown no activity at all under any of the conditions described above, bulk-phase or in vesicles, despite the broadly identical experimental conditions. It seems unlikely that this is due to batch variability between lipid or drug stocks, especially as the results above have been collected over a four year period; similarly, it is improbable that all the previously reported results (or all those above) are the products of experimental artefacts induced by carelessness or contamination, as both sets exhibit such consistent behaviour.

One possible suspect behind the failure of the vesicle studies is that of drug administration, or more accurately of the homogeneity of drug concentration across the vesicle population. In condensed-phase samples, the drug is co-lyophilised with the lipid, leading to an even spread of the compound throughout each leaflet of the lamellae and in roughly equal concentration throughout. Such an administration technique is not possible in vesicle experiments: the data gathered during a previously reported study⁵³ showed that dramatic morphological changes could be induced by lipid hydrolysis over a timescale shorter than that required to extrude a homogenous population of unilamellar vesicles, and giant vesicles grown in the presence of CAD compounds were much smaller than expected and filled with small daughter micelles and mixed vesicular bodies (see Figure 10b). Because of this, drugs were added exogenously to pre-formed vesicles, and it was hoped that a combination of electrostatic repulsion and entropy-driven migration would rapidly lead to an averaged drug concentration across the entire vesicle population.

If this were not the case, it may explain a proportion of the results outlined above. CAD compounds, by their very nature, are surface active and show a marked preference for hydrophobic or interfacial environments. Should a high local concentration form in a small population of the vesicles present, it is quite possible that they would cause either the vesicles' rupture through detergent effects or could alternatively form meta-stable mixed micelles and

small vesicles, highly enriched in CAD. If the former is the case, the ruptured fragments would begin to act as sites of nucleation about which other vesicles would coalesce, forming bulky, multilamellar aggregates; in the latter case, little hydrolysis would occur due to the low numbers of acyl species exposed to the drugs and it is very unlikely that a detectable level of lyso-lipid would be formed.

5.6.4.1 Experimental evidence to support hypotheses

Although hypothetical explanations, both are backed to some degree by experimental findings. A number of proof-of-concept microscopy experiments were conducted in collaboration with Claire Stanley and Dr Nick Brooks in an attempt to recreate the results of the experiments in Figure 10 in the much more complex environment of a living cell or population of cells.

The bio-mechanical microscope (BMM) system was a custom-built design built by Dr Nick Brooks, based around a Nikon TE2000-E inverted microscope utilising Hoffman modulation optics¹⁷⁷ to enhance the contrast between a P-815 mouse tumour cell immobilised upon a micro-pipette and the bulk 50mM HEPES buffer. CAD solutions were introduced to this buffer at physiological concentrations (10^{-5} – 10^{-15} M) in either DMSO or water depending upon the solubility of the drug in question. The system was maintained at a temperature of 37°C using a circulating water bath (Grant LTD6G, -20 to 100 °C), connected to the sample holder using flexible plastic tubing and controlled using a PT 100 thermocouple (TC Ltd, Uxbridge UK).

These experiments were very complex and the data derived was difficult to quantify and so they were discontinued, but not before two significant phenomena were observed. Firstly, in successful experiments membrane budding was observed, with membrane fragments of the P-815 mouse tumour cell becoming sequestered in the micro-pipette used to prevent the cell drifting out of the plane of the microscope, as shown below in Figure 60, below.

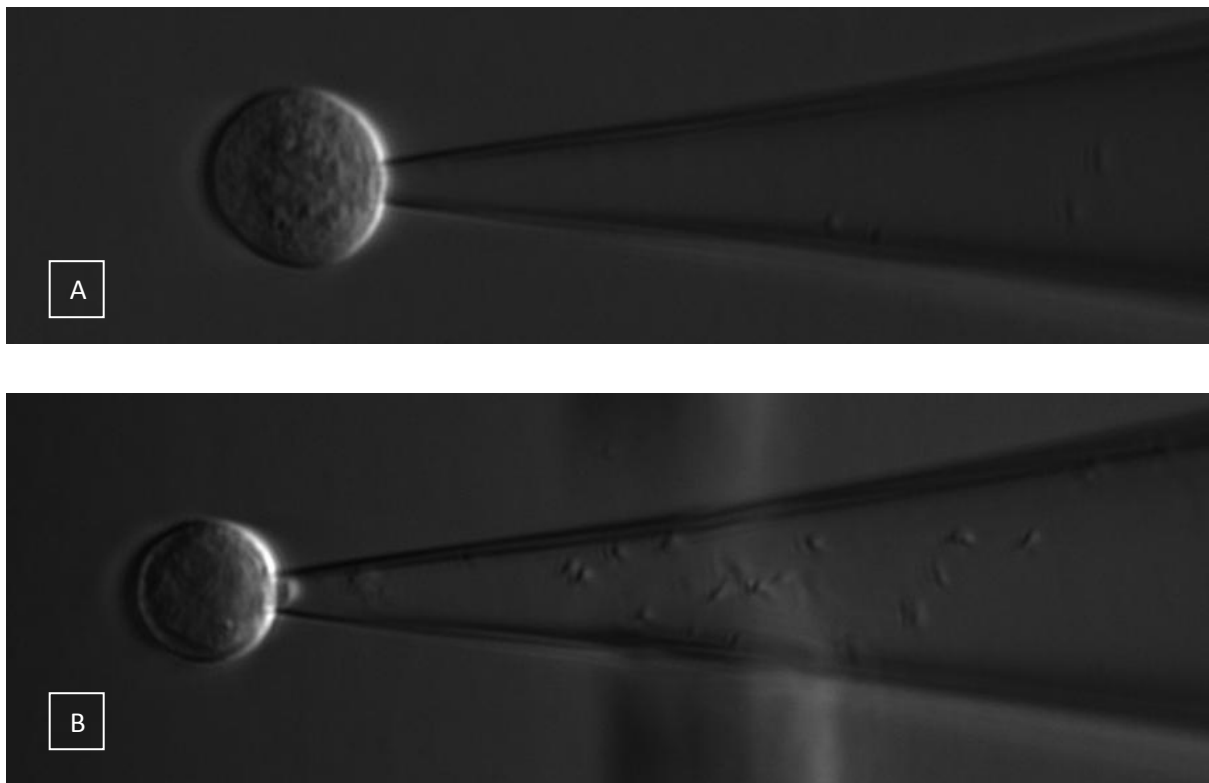


Figure 60: Membrane budding observed in a P-815 mouse tumour cell after administration of 2 μ l spiperone HCl solution into the HEPES buffer solution, creating a global drug concentration of 6×10^{-13} M. (a) shows the cell at $t = 0$, (b) shows the same cell at $t = 90$ minutes. Fragments similar to those observed in image (b) above were found throughout the microscope slide after the experiment. However, only those retained in the microscope's narrow field of focus are visible.

The second observation was that the drugs did not distribute equally throughout the buffer medium: cells close to the point of administration suffered a near-instantaneous collapse, puckering and shrinking very rapidly, followed by a slow recovery to a about two-thirds of their original diameter and the formation of large 'blebs' of cellular lipid material, empty of cytoplasm as shown below in Figure 61. Those more than a millimetre or two from the site of drug injection showed no reaction at all – it was this effect that led to the abandonment of the experiments, as it was impossible to consistently induce the same level of effect in cells in order to reproduce the data. This also meant that there was no effective technique for determining the absolute concentration of drug in any cell: although the global average concentration was known, the observed effects indicated strongly this was not representative of the cell population.

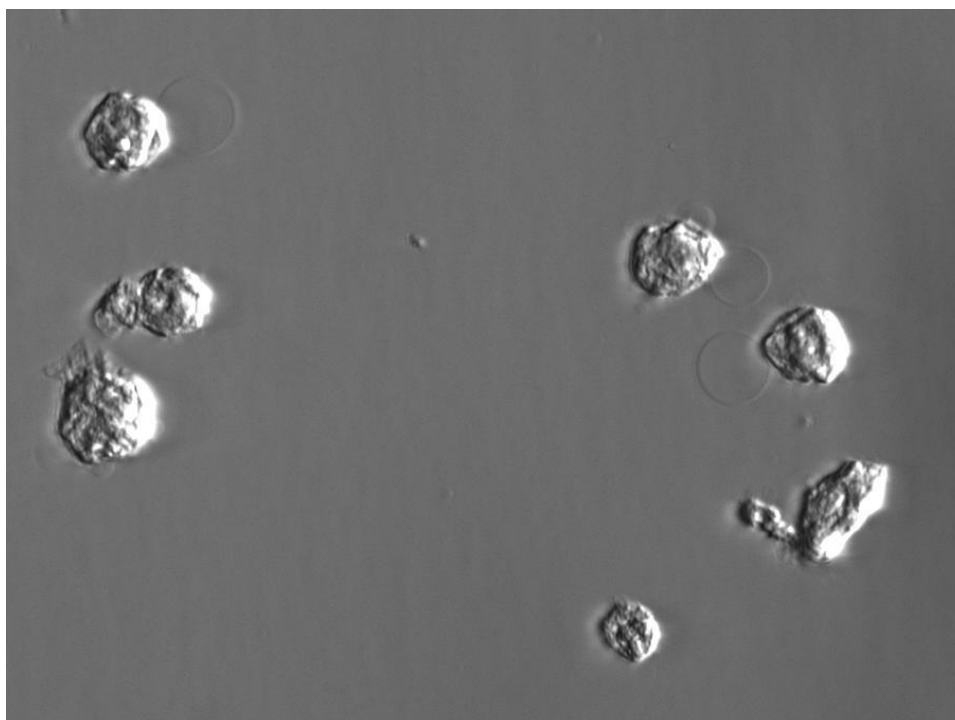


Figure 61: The ‘blebbing’ of cells following the administration of 10^{-7} M haloperidol HCl in 2 μ l DMSO to 200 μ l cell suspension in HEPES buffer. Cells ≥ 1 mm from the site of injection were unaffected.

These observations make sense in the light of the previously reported data and those presented above. The initial, rapid binding to a nearby membrane followed by a slow equilibration phase may be reflected in the unusual fluorescence behaviour observed when haloperidol bound to FPE-doped membranes as described in section 5.6.1.1 and similarly the previous SAXS analyses described in section 5.5.2. Similarly, this offers an explanation as to the effectiveness of the initial fluorescence experiment that was reported by the group⁵³ (Figure 10): in this experiment, the lipids present were arrayed as a series of anchored vesicles on a flat platinum electrode, some centimetres from the site of drug administration. This means that the drug solution would have had the opportunity to diffuse a lot further and distribute itself across a much wider region than would be the case in a free-floating suspension of vesicles or cells, where drug-membrane contact would be almost instantaneous (see Figure 62, below).

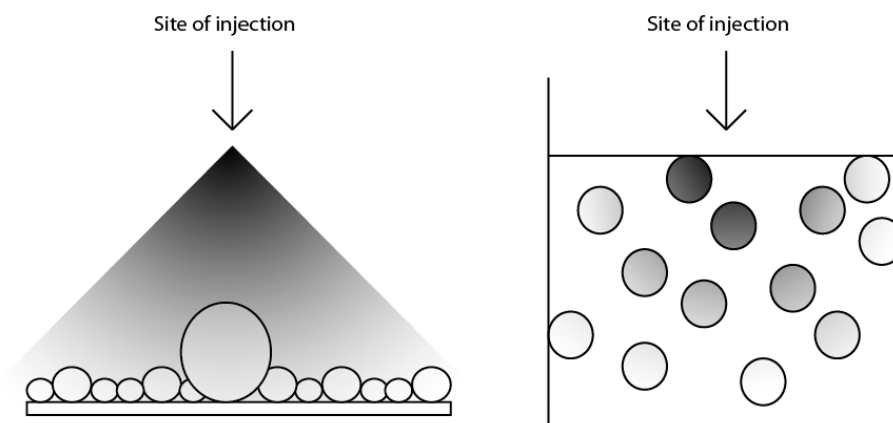


Figure 62: Schematic representations of the drug concentrations (shown as the shaded gradient) in microscopy experiments such as those in Figure 10(left) and in vesicle suspensions such as those described in this chapter (right). In microscopy experiments, each vesicle is roughly equidistant from the site of injection and so receives an equal dose; in suspension, the high log P of the drug means it is rapidly absorbed within a few mm of administration and so many vesicles experience no exposure at all.

The fact remains, however, that these fluorescence and SAXS effects were not observed in raclopride-doped systems, and yet raclopride is the only compound (aside from ibuprofen) to have shown consistent, reproducible rates of hydrolytic activity. Indeed, raclopride caused no effects upon the fluorescence of FPE at all within error, once dilution effects had been eliminated; although raclopride-induced hydrolysis induced some small changes in *d*-spacing and packing in condensed-phase systems (discussed in more detail in section 5.5.2), these were in no way as significant or as rapid as those induced by haloperidol. As it is vanishingly unlikely that raclopride can induce the hydrolysis of a glycerol ester bond in a membrane without in some way penetrating the interface of that system, its ability to do so with little or no detectable footprint is confusing, especially when combined with partitioning data (see section 7.3, page 193). The experimental evidence above demonstrates conclusively that it is doing so as a neutral species, either as a genuinely unionised molecule due to the reduced ability of the compound to retain its charge in the membrane, or as some form of internal salt or zwitterion. Potential explanations for this will be discussed in section 7.3.

6 Effects of membrane composition

The failure of the vesicle assay to produce the high-throughput, real-time results hoped for led to a change of approach as it meant that large-scale investigations of the effects of drug structure became impractical. Instead, the investigation turned to focus upon the influence of the membrane composition (and thus stress, curvature etc.) exerted upon the reaction, and to unpick the mechanistic detail of the process. Under the dynamic and rapidly-changing environments observed in previous experiments⁵³, this would have been very challenging due to the high degree of flux within the membrane composition. However, the slow-reacting and reproducible condensed-phase assay provided the ideal platform from which to probe these effects.

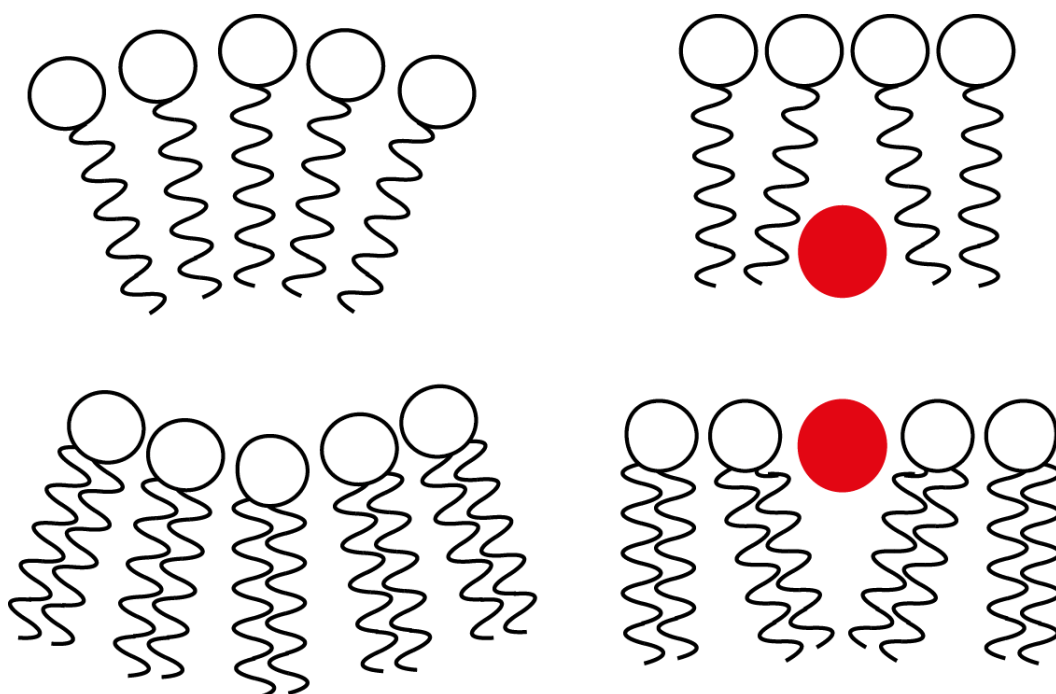


Figure 63: The intercalation of drugs into a stressed bilayer can lead to a free energy gain. In the examples above, a Type I monolayer is made to lie flat through the intercalation of a drug molecule (red) between its fatty acid tails; a Type II membrane is made to lie flat *via* intercalation between the head-groups, allowing increased space for chain splay.

Previous studies had posited that the rate of hydrolysis was proportional to the curvature elastic stress within the membrane⁵³: that the drug compounds would have an increased affinity for highly stressed membranes, deriving a free-energy gain as they intercalated between the component molecules and thus allowing more room for chain-splay or screen head-group repulsions (see Figure 63). Such a concept has precedent: it was shown by Attard *et al.* in 2000 that membrane biosynthesis is at least in part regulated through such processes¹², and a range

of enzyme activity has since been observed to be dependent on such forces^{178,179}. It has also been investigated theoretically, with studies demonstrating the interplay between vesicle morphology and the local *versus* global concentration of co-surfactants or other amphiphilic inclusions¹⁸⁰, with results matching those predicted by experiment¹⁸¹. However, research has always pursued the impact of the binding of exogenously applied amphiphiles on the shape of the host membrane, rather than the other way round as is of interest here and to the best of our knowledge, this is the first study undertaken from such a direction.

The model proposed by Attard and later researchers suggests that the partition function of a compound into a vesicle or bilayer is strongly dependent upon the local mechanical state of the membrane, which may in turn be affected by global factors such as the composition and mixing characteristics of its constituents. As can be seen in Figure 63, these factors influence both the extent of the partitioning but also the depth at which the compound resides. This, then, implies that a modified version of the crude octanol-water log P assay used so extensively by the pharmaceutical industry may actually prove one of the more useful predictors into a candidate compound's eventual bioavailability.

6.1 DOPC-DOPE mixtures

In order to probe the effects of membrane composition a range of experiments were initiated that were designed to look at not just the ingredients of each system, but to isolate the assorted physico-chemical parameters which altered with composition and to identify which, if any, was responsible for the observed changes in rate. The first experiment investigated the influence of DOPE within a DOPC membrane, which has been demonstrated to add significantly to the curvature stress of a membrane as it attempts to drive the bilayer towards the H_{II} phase¹⁸².

Under excess water conditions DOPC and DOPE mix almost ideally, showing negligible de-mixing at any composition¹⁸³, preventing any effects due to phase separation. In this state, the system remains lamellar from 100% DOPC until around 75-80% DOPE^{184,185}, when the concentration of the strongly type II PE reaches a temperature-dependent critical point and drives the system into the inverse hexagonal phase as described in Figure 6, (page 23). However, DOPE also introduces free amines into the membrane interface: hydrogen-bond donors which could have a confounding effect by dominating the binding between the drug and the lipid, which has been demonstrated computationally to depend in DOPC upon the π - π interactions between the drug and the lipids' fatty acid tails¹⁵⁰.

Regardless of any intermediates, this phase change would exclude water from all but the narrow channels characterising the inverse hexagonal phase, thus slowing the diffusion of drug molecules into and lyso-lipids away from the interface. It was therefore expected that as the system changed from the lamellar to the inverse hexagonal state at the phase boundary, a major change in the rate of hydrolysis would be observed. There have been reports of unusual rhombohedral and phases observed near the phase transition boundary under certain conditions¹⁸⁴: however, there remains some controversy regarding their existence in suspension as opposed to supported on solid surfaces.

This L_{α} - H_{II} phase boundary was to be found somewhere around 80mol% DOPE¹⁸⁵: although 20mol% DOPC is reported to be sufficient for the system to retain a metastable lamellar structure¹⁸⁶, it forms intermediate structures tentatively identified as cubic before progressing to H_{II} over a matter of hours. The effect of raclopride upon these structures was unknown, and so SAXS studies were performed in parallel with the HPLC trials.

In order to probe the contribution of these factors, an experiment broadly similar to those

outlined in section 5.1.1 was set up as shown in Table 5, below. Due to experimental error, raclopride tartrate was added at approximately 6.66 mol% relative to lipid rather than the 5 mol% that was standard: however, the inclusion of two vials containing only DOPC lipids provided an internal standard meaning that the data was not wasted.

Vial	DOPC /mol%	DOPE /mol%	DOPC /mg	DOPE /mg	Lipid:Rac ratio	Mass Rac /mg
A	100	0	30	0.00	15	1.27
B	100	0	30	0.00	15	1.27
C	80	20	24	5.68	15	1.27
D	80	20	24	5.68	15	1.27
E	60	40	18	11.36	15	1.27
F	60	40	18	11.36	15	1.27
G	40	60	12	17.04	15	1.27
H	40	60	12	17.04	15	1.27
I	20	80	6	22.72	15	1.27
J	20	80	6	22.72	15	1.27
K	0	100	0	28.39	15	1.27
L	0	100	0	28.39	15	1.27

Table 5: The experimental set-up for the experiment to probe the influence of DOPE upon the rates of raclopride mediated hydrolysis. Due to experimental error, raclopride tartrate was added at a 15:1 lipid:drug ratio, equating to 6.66mol%.

After drying under vacuum overnight, the lipids were hydrated using 10mM PBS buffer and homogenised as normal. Approximately 10µl of each suspension was dispensed into a 1.5mm diameter X-ray capillary tube and sealed as described in section 5.1.3: these capillaries were incubated under the same conditions as the bulk sample vials and so hopefully exhibited identical behaviour over time. Samples were analysed repeatedly over a period of some 500 hours from incubation – the results of the HPLC measurements are displayed below in Figure 64. Unsurprisingly, DOPE co-eluted with DOPC and lyso-OPE with lyso-OPC due to the reverse-phase chromatography conditions, meaning that hydrophobic effects dominated the separations observed (see Table 2, page 70). This had the virtue of simplifying the analyses as calibration plots for the PC and PE lipids were essentially identical, albeit at the loss of direct information on any chemoselectivity in the reaction. Data was collated as previously, and is displayed below in Figure 64.

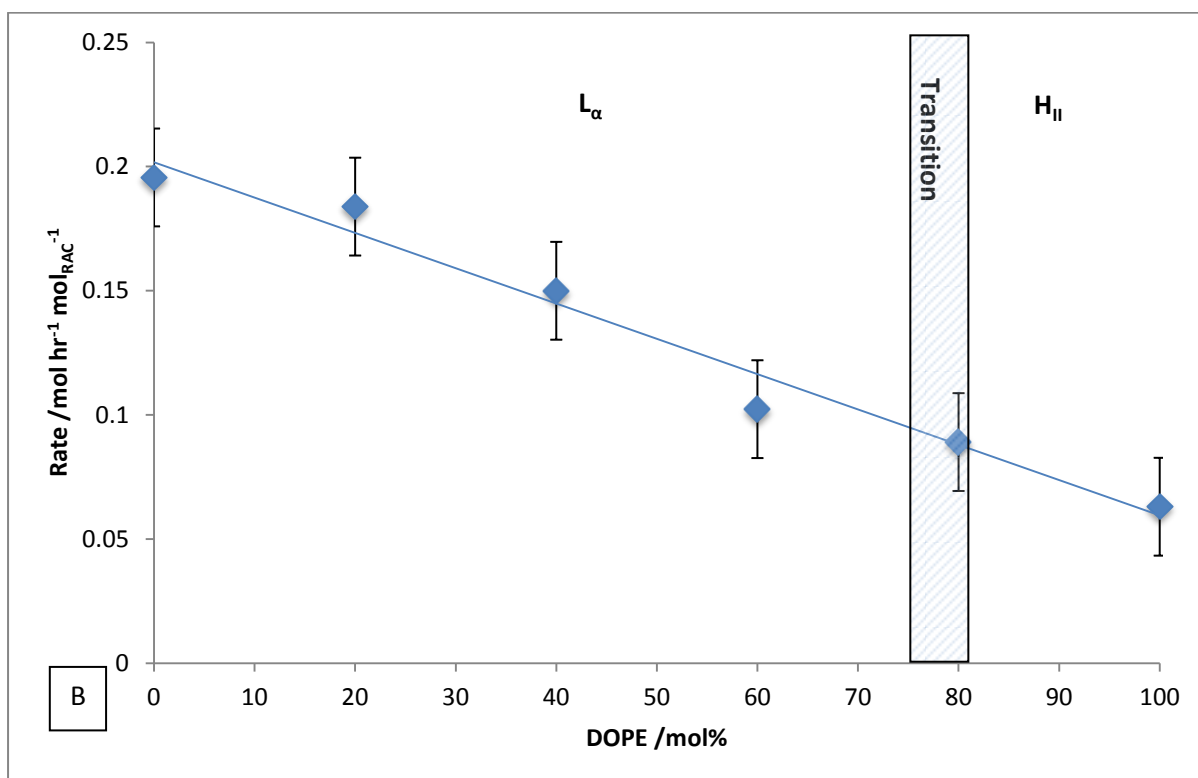
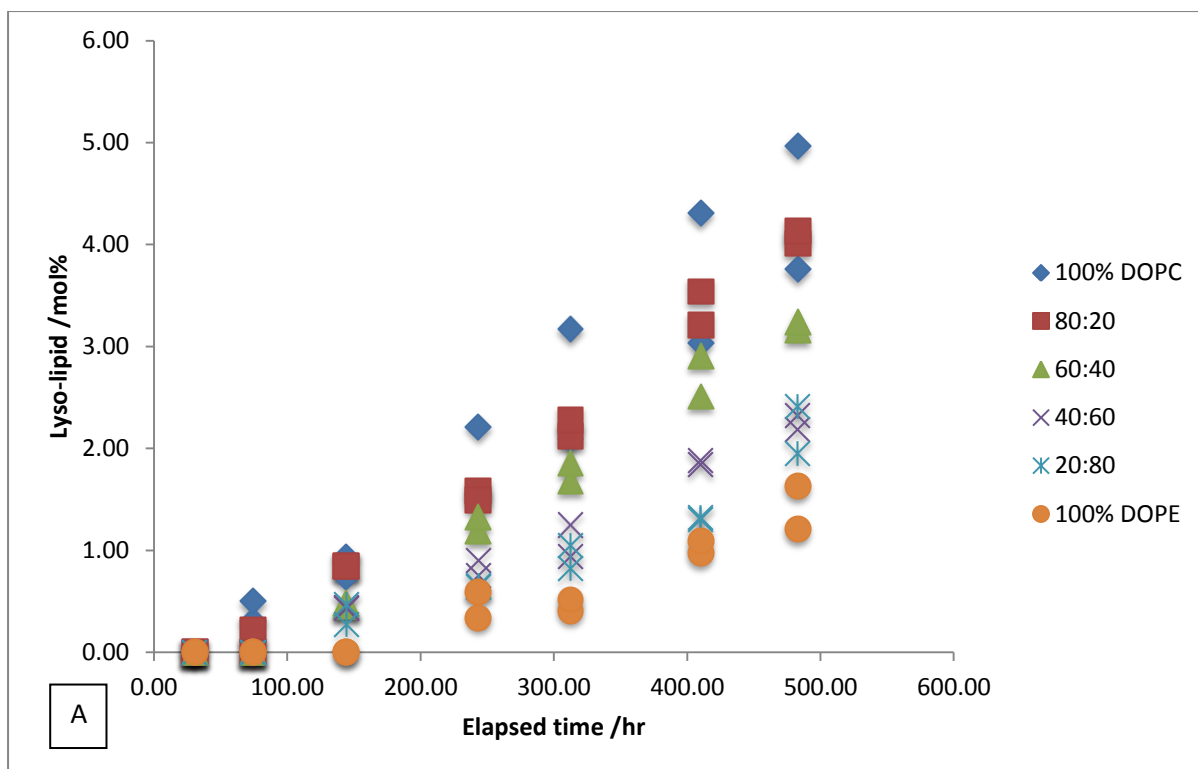


Figure 64: The time-resolved degradation of mixtures of DOPC and DOPE by 6.66mol% raclopride tartrate. (a) the total rates of lyso-lipid formation as a function of time ($n=2$); (b) the absolute rates plotted as a function of membrane composition. Error bars are 2 s.d., as data from Figure 33 is not valid in this experiment. Phase behaviour is shown as a function of composition.

These data showed an unexpected trend: in contrast to previous experiments^{53,118}, not only did DOPE appear to inhibit the reaction but it did so in a manner that varied linearly with concentration, whereas the phase of the system had little if any role. The data gathered from the samples of 100% DOPE fitted the trend very closely, and even samples which exhibited phase changes over the course of the reaction period continued to show linear rates of generation of lyso-lipid. The samples composed of 80% DOPE 20% DOPC (shown above in Figure 64b as the transition zone) experienced a catastrophic phase change after approx. 250 hours after which they showed negligible diffraction, meaning no long-range order existed within the lipid aggregate. The appearance of these samples also changed, forming a viscous, transparent gel. Despite this, they showed an identical rate of generation of lyso-lipids before and after this transformation.

In many regards, this is a confusing finding: the transition from $L_{\alpha} \rightarrow H_{II}$ phase creates large, hydrophobic voids in between the packed lipid cylinders, (as discussed in section 3.1.3, page 18), which leads to big free energy gains when the system absorbs molecules to fill them. In the experiments detailed previously, (see Figure 34), at least some link was observed between the concentration of drug within the system and the observed rate of hydrolysis, whereas the evidence above gives it a much more limited role. What it does clearly demonstrate, though, is that bulk phase properties have little influence upon rates in this system: the linearity of the plot in Figure 64 as it crossed at least one phase boundary means that the rates must be determined by drug-lipid intermolecular parameters, rather than through interplay between the drug and the membrane as a whole. This could, however, be due to the formation of strong hydrogen bonds between the raclopride molecules and the primary amine head-group of the DOPE – this would sequester the drug molecules some distance from the interface (as shown for the case of acetic acid by Hitchcock *et al.*¹⁸⁷), and could prevent their exposure to the changing environment in the membrane core.

Another definite result is that the curvature elastic stress of a membrane is not a reliable predictor of the hydrolytic activity of a drug within it. Due to its inter-molecular hydrogen bonds, DOPE has more than three times the negative spontaneous curvature of DOPC (-0.351nm^{-1} versus -0.115nm^{-1})¹⁸⁸ and a mixture of the two varies linearly with composition¹⁸⁹: maintaining a bilayer structure containing any significant concentration of such components must come at a major energetic cost. Although this network of inter-lipid hydrogen bonds may

be disrupted by the drugs' presence, they are present in far too low a concentration to prevent significant lipid-lipid bonding occurring. It would appear then that this stored mechanical energy has little or no role in the regulation of hydrolysis rates in this system, or at least that in the case of PC-PE mixtures any contribution is dominated instead by direct intermolecular forces.

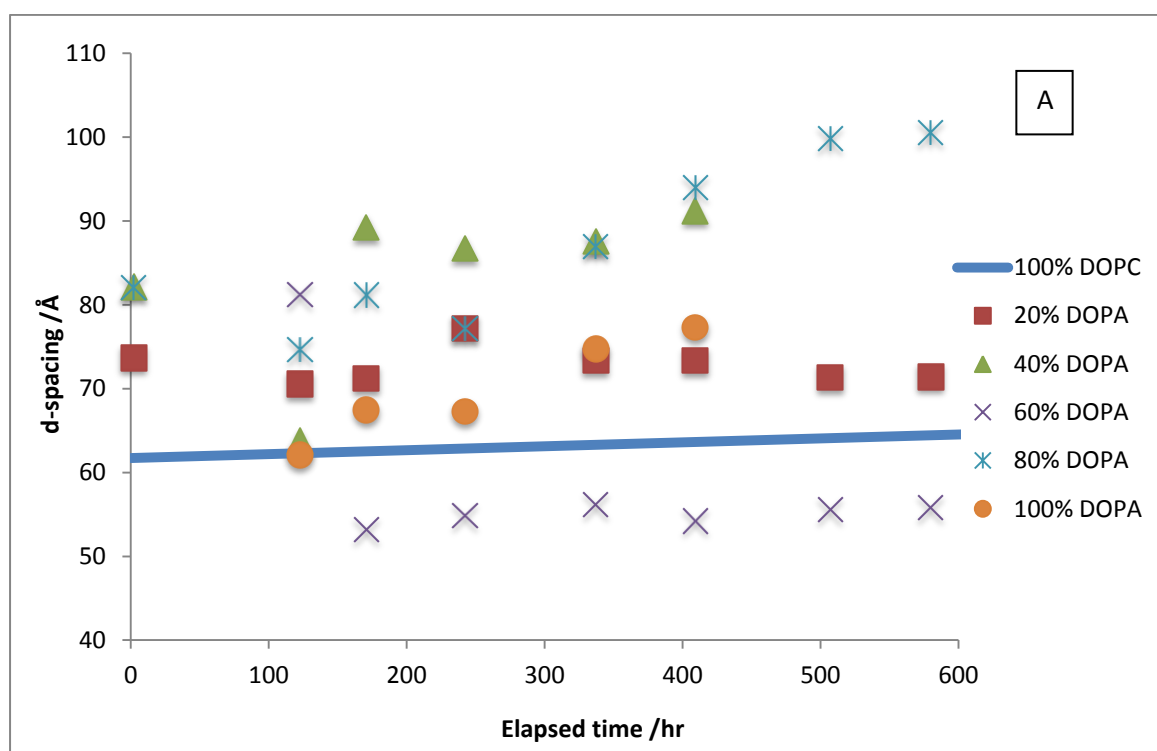
6.1.1 Other lipid head-groups

The same experiment was conducted using a range of charged phospholipids with the same dioleoyl acyl chains. Samples were prepared using mixtures of DOPC with 1,2-dioleoyl-*sn*-glycero-3-phosphate (sodium salt) (DOPA), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (DOPS). All of these lipids contain an effectively permanent anion, and so had the potential to significantly increase the partitioning of raclopride into the membrane both through simple electrostatic attraction but also through the potential formation of hydrogen bonds between the drug and individual lipid molecules. Unlike the net neutral PC and PE species, this bond would be much more likely to form between the drug and the phosphate anion, locating the drug much closer to the sensitive acyl interface. Previous experiments with DOPC-DOPG mixtures (D. Casey, unpublished research) suggested that although the charged lipid had a roughly identical rate of hydrolysis whatever its concentration, increasing its mole fraction led to a concomitant rise in the rate of DOPC hydrolysis which might be attributed to this effect. However, this data was extremely noisy and unclear due to the problematic solubility of the DOPG under the HPLC conditions at the time, and so the experiment was repeated and broadened using the new column media and conditions described in section 5.2.2.

Unfortunately, the new conditions provided no better resolution of the broad, diffuse charged lipid peaks despite chromatographic experiments with a range of reverse-phase solvents such as acetonitrile and *iso*-propyl alcohol. As previously, the lipids were released by the column before the eluent phase was able to dissolve them fully, leading to broad, jagged peaks with very low resolution. The restrictions imposed by an all-PEEK HPLC system meant that stronger solvents which may have alleviated this issue were not suitable and chromatographic quantification was abandoned.

Instead, analysis was attempted using ^{31}P NMR via a 400MHz Bruker system. Dried samples, approx. total mass 1-2mg, were redissolved in 0.75ml CDCl_3 and analysed for periods of up to an hour each (>1200 scans). However, resolution was too low for confident analysis even over these extended scanning periods, showing little or no detail above background. As a result, the ≈ 200 samples taken over the course of the experiment were stored under high vacuum until such a point as a suitably high throughput analytical technique becomes available.

Despite the failure of the direct quantification, samples were still available for SAXS analysis and images were taken throughout the course of the experiment. As in previous studies, these were prepared at the point of lipid hydration and, once sealed in their capillaries, were stored under conditions identical to the bulk suspensions. As in the case of DOPC, all three charged lipid species are reported to form L_α phases at full hydration¹⁹⁰, thus any departure from this form must have been due to hydrolysis effects.



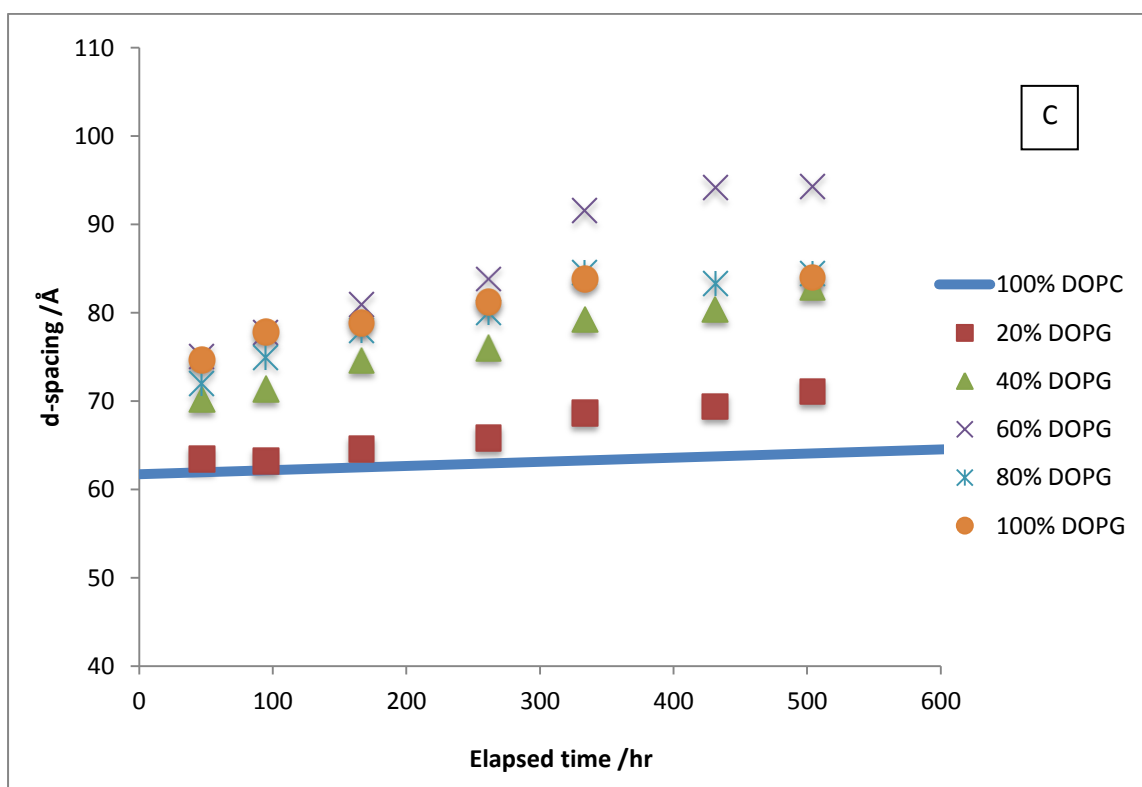
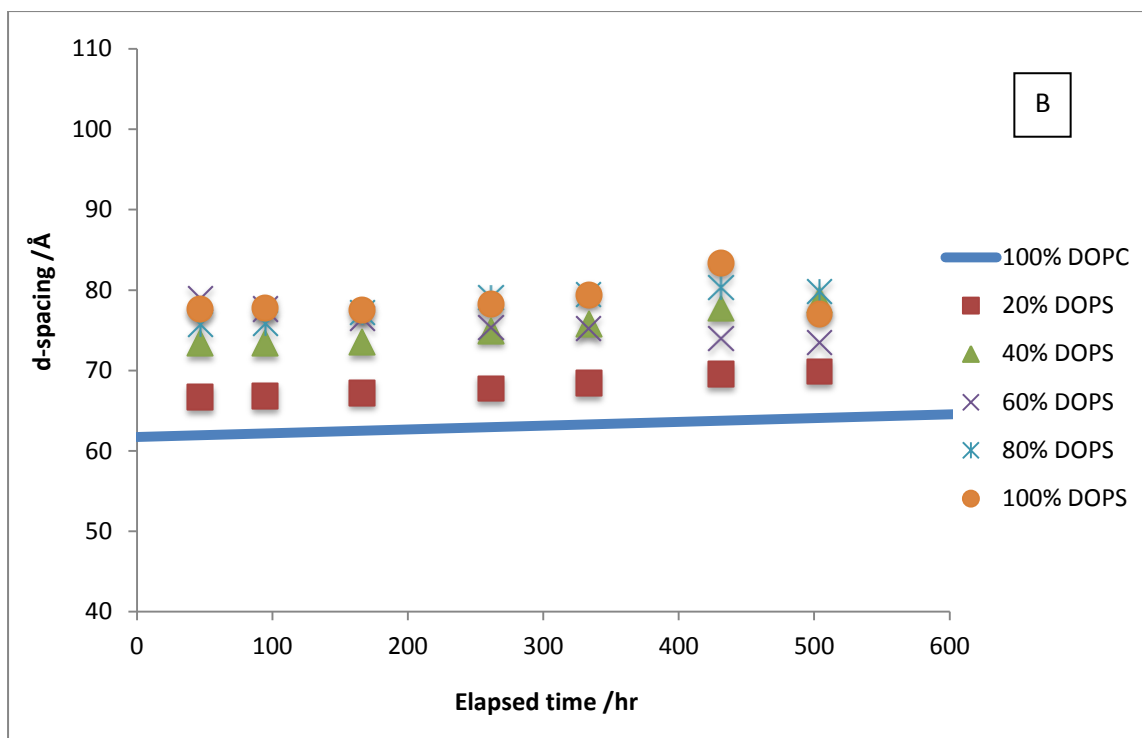


Figure 65: The SAXS data generated from mixtures of DOPC with charged lipids: (a) DOPA; (b) DOPS; (c) DOPG. All data are plotted on identical axes for clarity. Suspected phase coexistence was observed in the samples containing 40% DOPS 60% DOPC, but diffraction was too diffuse for accurate identification. DOPC data added for reference from calibrated phase diagram data (R. Macey, unpublished data), recorded in the absence of raclopride.

As a general rule, d -spacing increased in most systems by around $10\text{\AA}$, although this was not consistent throughout. The DOPA data was particularly noisy, showing no discernable trends at all, and the data from the 60% DOPS 40% DOPC sample shows a consistent and regular downward trend in d -spacing, in contrast with all other plots in the table. A suspected phase coexistence was observed in the samples containing 40% DOPS 60% DOPC, but diffraction was too diffuse for an precise identification.

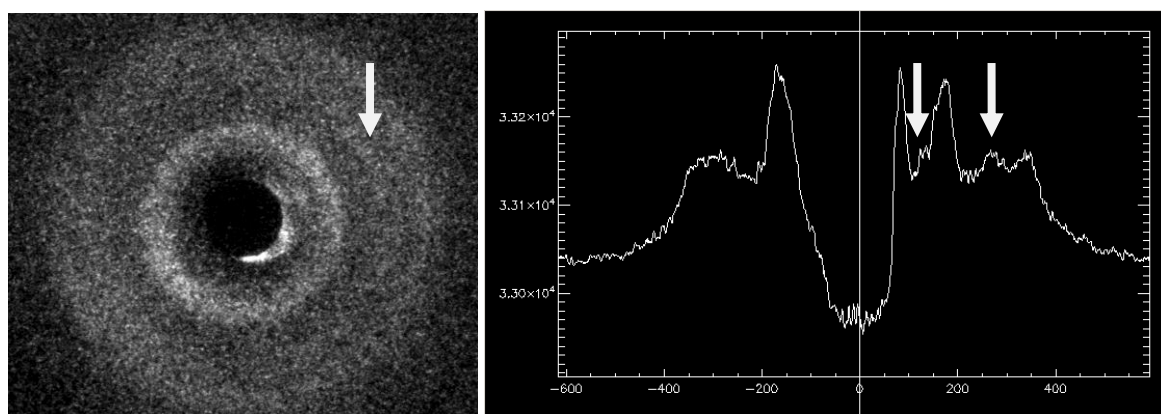


Figure 66: The X-ray scatter from a sample of 40% DOPS 60% DOPC with raclopride as a raw image (left) and as an I vs. q plot (right). White arrows show features indicating possible phase coexistence.

There is also generally a greater degree of swelling observed in the data above than in that from DOPC alone: however, this is not unexpected. Even in PBS solution, the Debye length is $\approx 7\text{\AA}^{174}$; vesicle scattering experiments (see section 5.6.2.2) showed an average bilayer thickness of $\approx 53\text{\AA}$; for a charged system such as those above, a reasonable estimate of minimum water channel thickness would be around two Debye lengths, giving a total d -spacing of $68\text{--}70\text{\AA}$, in good agreement with the data recorded above. Any effects of raclopride upon the membrane macro-structure are likely to be lost within this effect, as even at the lowest concentration of charged lipids studied there are four times the number of anionic species to drug molecules.

As can be seen above, the X-ray scattering data in isolation is of limited value without coupling to precise composition data at each point. While solid-state NMR offers a potential alternative probe into the phase behaviour of each system, solution-state experiments failed to provide the required rate data from the samples available and HPLC experiments were unable to shed light upon the issue. Higher flux sources such as the Diamond synchrotron described in section 5.6.2.2 may have improved our understanding of the systems, particularly the poorly-resolved or ambiguous images such as those outlined above.

6.2 Saturated lipids in the membrane

In order to investigate further the parameters governing the rates of hydrolysis, a range of experiments were conducted in a similar vein to those outlined in Table 5, mixing DOPC with a series of saturated phosphocholine lipids. 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-palmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) were all mixed with DOPC and prepared as previously with 5mol% raclopride tartrate. These molecules feature a head-group identical to that of DOPC, but with fully-saturated fatty acid tails of 12, 14 and 16 carbons each, respectively.

This promised to isolate a number of variables in the space of one experiment. The *cis*-unsaturated bonds in the tails of DOPC reduce their effective length, making them almost exactly the all-*trans* length of those in DPPC. By mixing DOPC with DLPC and DMPC, a degree of hydrophobic mismatch would be introduced with the potential to influence hydrolytic rates. However, the long, fully saturated chains are able to pack around one another very closely, giving the lipids relatively high chain-melting transition temperatures. DLPC becomes fully liquid at around 8°C, and DMPC at 22-24°C, whereas pure DPPC melts slightly above 40°C¹⁹¹ and while their elastic bending constants are difficult to relate directly due to these differing melting temperatures, the elastic bending constants for the lipids increases with increasing chain length¹⁸⁸. In common with the other assays performed, these experiments were incubated at 37°C, meaning that the DPPC was constantly below its transition temperature. In order to investigate the phase behaviour of these DOPC-DPPC-raclopride mixtures, each was analysed by DSC using a PerkinElmer Instruments Diamond DSC, but no significant peaks were observed for any transitions above 0°C except in the case of pure DPPC where the chain melting transition was found at 42°C.

These relatively high transition temperatures had a number of implications. It has been demonstrated through ITC experiments that amphiphilic species such as lyso-lipids compounds experience a log P lower by an order of magnitude or more when host membranes are in the gel phase compared to the fluid lamellar, although this effect is not observed when the added species is extremely hydrophobic as in the case of, for example, fatty acids¹⁹². There is also, unexpectedly, evidence that shorter chained-lipids are better at sequestering small amphiphilic molecules than do longer lipids even when both are in the fluid phase¹⁵⁷, although this experiment was performed at a constant temperature meaning that the shorter-chain lipid was

further above its melting temperature (and thus more disordered and fluid) than was the longer-chained species. However, this effect was limited in intensity (the log P of chlorpromazine dropped from 3.91 in DLPC to 3.53 in DPPC at 50°C) and so is unlikely to significantly influence the results of this investigation.

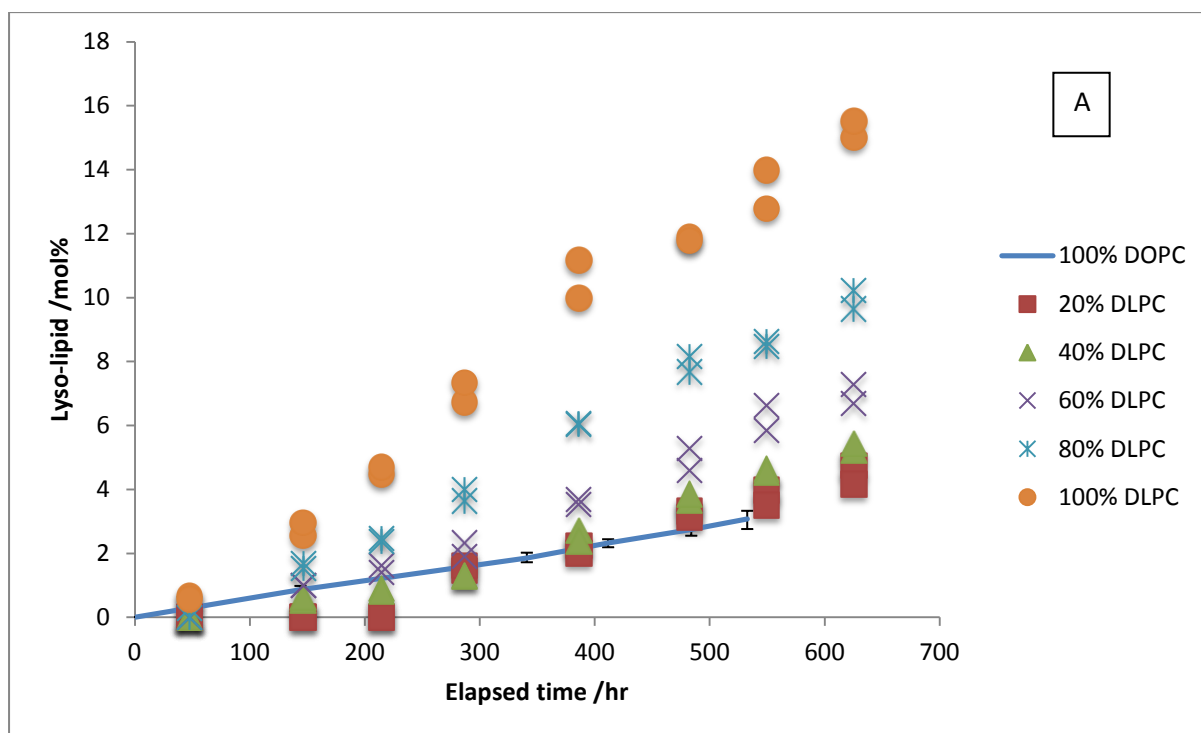
It has been reported that the introduction of CAD-like compounds into a membrane can both decrease and broaden its phase transition temperature in a dose-dependent manner¹⁹³. However, the effect was limited to a drop of only about 2°C at 5 mol% raclopride in the DSC experiments above, meaning that DPPC will still be above its phase boundary at the start of the experimental protocol. It has also been demonstrated that the addition of fatty acids to such membranes will increase the transition temperature under all pH conditions below their pK_a in the membrane (around 7.7¹⁹⁴) by intercalating between lipid molecules, limiting inter-head-group repulsion¹⁹⁵. This, in turn, will change the partition function and membrane topography experienced by any amphiphile such as raclopride, setting in motion what might be considered as a purely physico-chemical feedback loop.

Even through this brief summary, it can be seen that the hydrolysis of lipid acyl bonds by a CAD-like molecule will unfold in a dynamic and extremely complex environment, and one that is affected by many interdependent factors. In this regard, it is in some ways reassuring that the evidence from section 6.1 demonstrates (at least in the case of the $L_\alpha \rightarrow H_{II}$ transition) that the effects of complex composition changes may still lead to relatively simple results.

In order to accurately quantify the extent of hydrolysis in the DLPC-containing samples, the HPLC conditions described in section 5.2.2 (page 66) was required, as the DLPC peak co-eluted with that of the lyso-OPC under the standard protocol. As a result, a longer, shallower gradient was identified running over some 20 minutes. The retention times under these conditions are shown on page 70 in Table 2.

6.2.1 Results with DLPC, DMPC and DPPC

The experiments were run exactly as in section 6.1, although with 5 mol% raclopride tartrate added as in all previous experiments. HPLC and SAXS analyses were conducted over a 625 hour period from hydration and the chromatography results plotted against the calibration factors derived previously in section 5.3. HPLC analyses were conducted on all samples; the large number of assay vials made SAXS under all conditions impractical, so instead images were taken using samples from 20, 60 and 100mol% saturated lipid mixtures. Mechanical failure meant that SAXS facilities were not available until ≈ 200 hours after sample hydration and so early data was lost – however, four SAXS images of each capillary were recorded after this point. In common with the above charged lipid experiments this data revealed little of value, though, revealing no clear trends. HPLC were much more fruitful, though, and results are presented below in Figure 67.



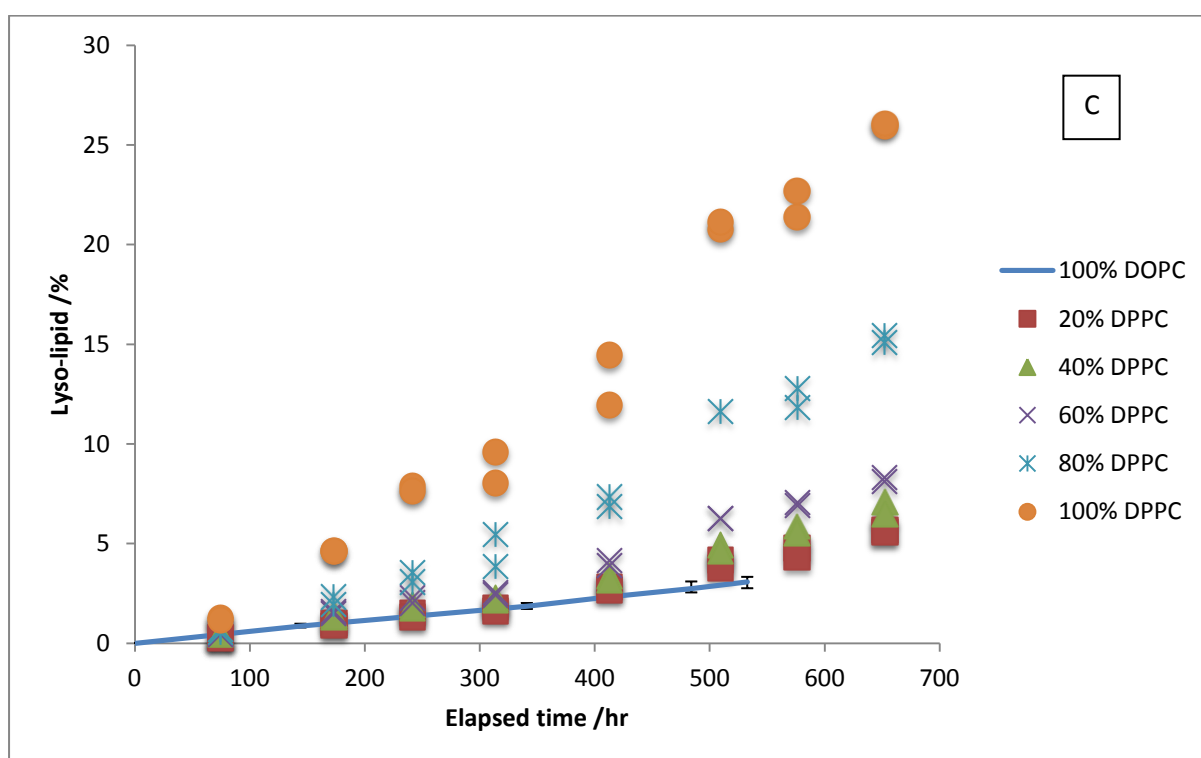
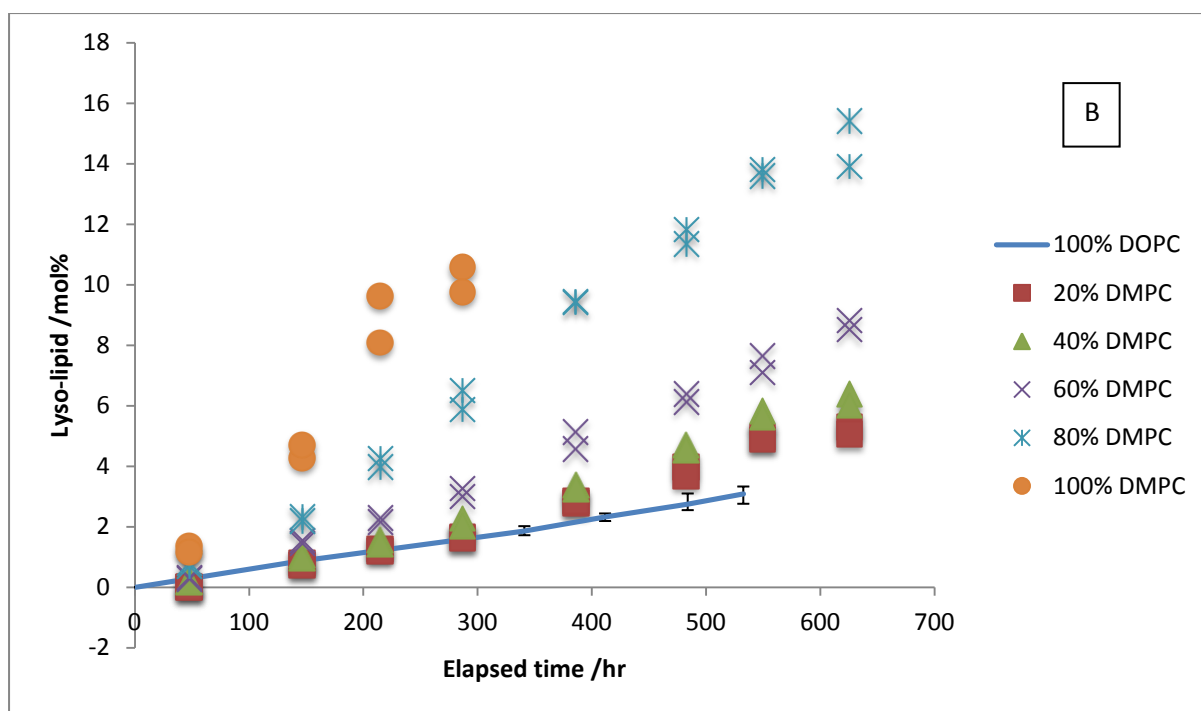


Figure 67: The influence of a range of fully saturated lipids upon the rate of total lyso-PC formation catalysed by 5 mol% raclopride tartrate, showing the effects of (a) DLPC, (b) DMPC, (c) DPPC, $n = 2$. Data are plotted against cumulative DOPC data shown in Figure 33, (blue solid lines), which also indicate typical errors from data derived in Figure 33. After ≈ 300 hours, pure DMPC samples began to show wildly fluctuating data, suggesting some form of contamination or sampling error had occurred: as such, the erroneous data points are omitted.

In each of the above plots, the same trends appear: hydrolysis in mixtures containing 20, 40 and 60mol% saturated lipid is, within error, identical to that observed in pure DOPC. However, above this threshold, hydrolysis begins to occur very rapidly and in contrast to the DOPE experiment described previously, the increase is not linear with saturated lipid concentration (illustrated in

Figure 68, below), instead presenting a sigmoidal relationship, $p < 0.001$.

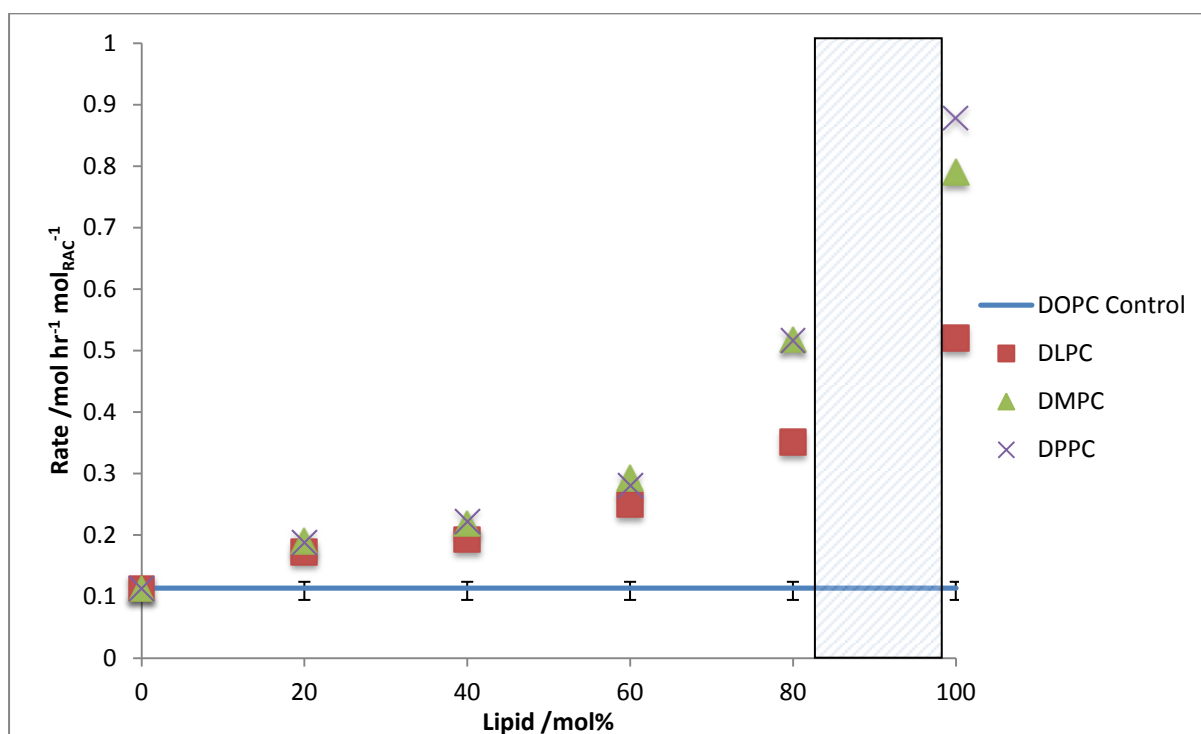


Figure 68: The relationship between saturated-chain lipid concentrations in the membrane and the rate of its hydrolysis by raclopride tartrate. Remaining proportion of membrane was composed of DOPC. Blue shaded area shows region of phase co-existence as identified by fluorescence studies¹⁹⁶. Indicative error bars are shown for 100% DOPC data, and are derived from Figure 33.

This relationship means that the earlier hypothesis that rates are controlled by direct drug-lipid forces must be discounted: there appears to be a discontinuity shown here between those mixtures below 60% saturated lipid and those above that concentration. The fact that this behaviour was repeated in all three experimental systems means that the effect is probably due to the imperfect mixing of high concentrations of saturated lipids with DOPC. In contrast to the mixing of DOPC with DOPE, a temperature and composition-dependent phase coexistence region is observed between ordered domains enriched in saturated lipids and fluid disordered regions enriched in unsaturated molecules. In the case of DOPC-DPPC mixtures at 37°C, the co-existence region has been found by fluorescence methods¹⁹⁶ and X-ray diffraction¹⁹⁷ (see below) to form at shortly below 80% DPPC, tying in neatly with the experimental data presented above;

DLPC or DMPC systems do not show the same obvious discontinuities as they are both fluid at 37°C, but it is not unreasonable to suggest that a similar mechanism may be at play.

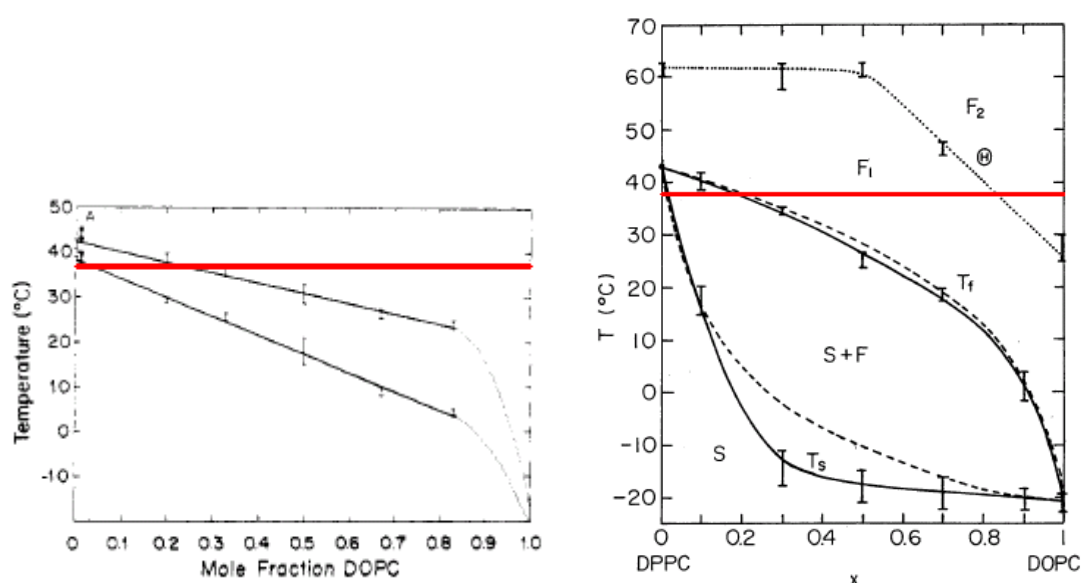


Figure 69: Phase diagrams published showing the de-mixing of DOPC and DPPC through fluorescence methods by Lentz *et al.*¹⁹⁶ (left) and through SAXS analysis by Furuya and Mitsui¹⁹⁷ (right, where S denotes a solid or gel phase and F denotes a fluid system). Red lines are added to show the relevant temperature in the current study.

As described in section 6.2, these ordered, viscous domains typically exhibit a much lower capacity to absorb amphiphilic species than an equivalent-sized fluid-disordered region¹⁹², although in the case of strongly hydrophobic, planar species such as cholesterol this trend is reversed¹⁹⁸. This is likely to be due to the close packing of the acyl chains in the gel phase, limiting the space that can be occupied by exogenous amphiphiles without causing energetically costly flaws in the membrane lattice. This makes the above data all the more surprising: it appears that the formation of liquid-ordered domains in the membrane significantly increases the rate of hydrolysis.

Although unexpected, parallels can be drawn with the activity of PLA₂ in membranes of different phase behaviour, in a manner that may shed some light upon the situation. It has been observed that the “lag-burst” kinetics of PLA₂’s activity are very much a feature of the liquid-disordered phase; by contrast, when PLA₂ is added to lipids such as DPPC in the gel phase, rapid hydrolysis occurs almost immediately unless inhibited by the addition of delipidated serine albumin¹⁹⁹. This was explained by the activation of PLA₂ by anionic domains: during the “lag” phase of PLA₂ in a disordered membrane, all the anionic fatty acid generated is free to diffuse

laterally away from the enzyme, driven by a combination of Brownian motion and charge-charge repulsion. By contrast, the diffusion rates in gel-phase lipids are some 70 times slower²⁰⁰, meaning that an activating local concentration of carboxylate groups can be developed much more rapidly. Although not activated in the same way, CADs have previously been proven to have higher affinities and activities in anionic than neutral membranes (D. Casey, unpublished work): it does not seem unfeasible that a similar effect may be contributing under these circumstances, counter-acting the lower log P experienced by the drugs that was discussed previously. On first glance, this would suggest that the reaction becomes self-promoting: that as the reaction progresses, the increase in anionic fatty acids within the host membrane would attract more and more drug, leading to exponentially increasing rates which are conspicuously absent in all the data presented. However, this scenario is only important in cases where large excesses of cationic amphiphile are available: in the experimental models studied in this report, the high partition function of the drug means that it is effectively >90% membrane-bound under initial conditions, meaning that an homogenous distribution of anions across the membrane interface after this point can have a limited impact.

6.2.2 POPC

While the hydrolysis data presented above in Figure 67 indicates a trend of increase in rate with increasing acyl chain length, (presented more thoroughly in Figure 72, below), there is an obvious and significant discontinuity between the rates of formation of lyso-lipid species in mixtures dominated by unsaturated lipids relative to saturated species. While this is probably phase-dependent, based upon the data above, it was important to eliminate any direct effects of the unsaturation of the acyl chains: to prove that there was no significant differences between the observed rates of reaction due to any affinity between, for example, the phenyl ring of raclopride with the 18:1 *cis*-double bond of the oleoyl chains, as predicted by Rosso *et al*¹⁵⁰.

To confirm this hypothesis, an experiment similar to those above was conducted using mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) with DOPC. This lipid carries one saturated C₁₆ chain and one mono-unsaturated C₁₈ chain meaning it can act as a lateral surfactant, preventing the formation of domains such as those described above. It mixes ideally

with DOPC at all ratios, forming a stable fluid lamellar structure at essentially all temperatures above its chain-melting point of -5°C^{201} . However, under these circumstances it was capable of isolating the contribution of the fatty acid tail group in determining the rate of the reaction: if the choice of acyl group was of significant import, one might expect to find an activity mid-way between that of DOPC and DPPC. If, as theorised above, the fluidity of the membrane is the rate-determining factor, POPC would be expected to cause a much smaller difference to the measured rates of hydrolysis.

The experiment was prepared in a similar way to those above: POPC and DOPC were mixed at ratios from 20:80 respectively through to 100% POPC and co-lyophilised with 5 mol% raclopride. These samples were hydrated with PBS and samples were taken over the course of some 600 hours. The data obtained is displayed below (Figure 70), alongside the averaged rate observed in pure DOPC experiments obtained from Figure 32 for reference. Unfortunately, the similarity in chemical structure between the two potential lyso-lipids coupled with their propensity to isomerise into the *sn*-1 form⁵⁵ meant that the reaction products co-eluted, destroying any information into the reaction's chemoselectivity. However, the data presents a strong indication that the process is effectively random, with both chains experiencing an equal probability of being digested.

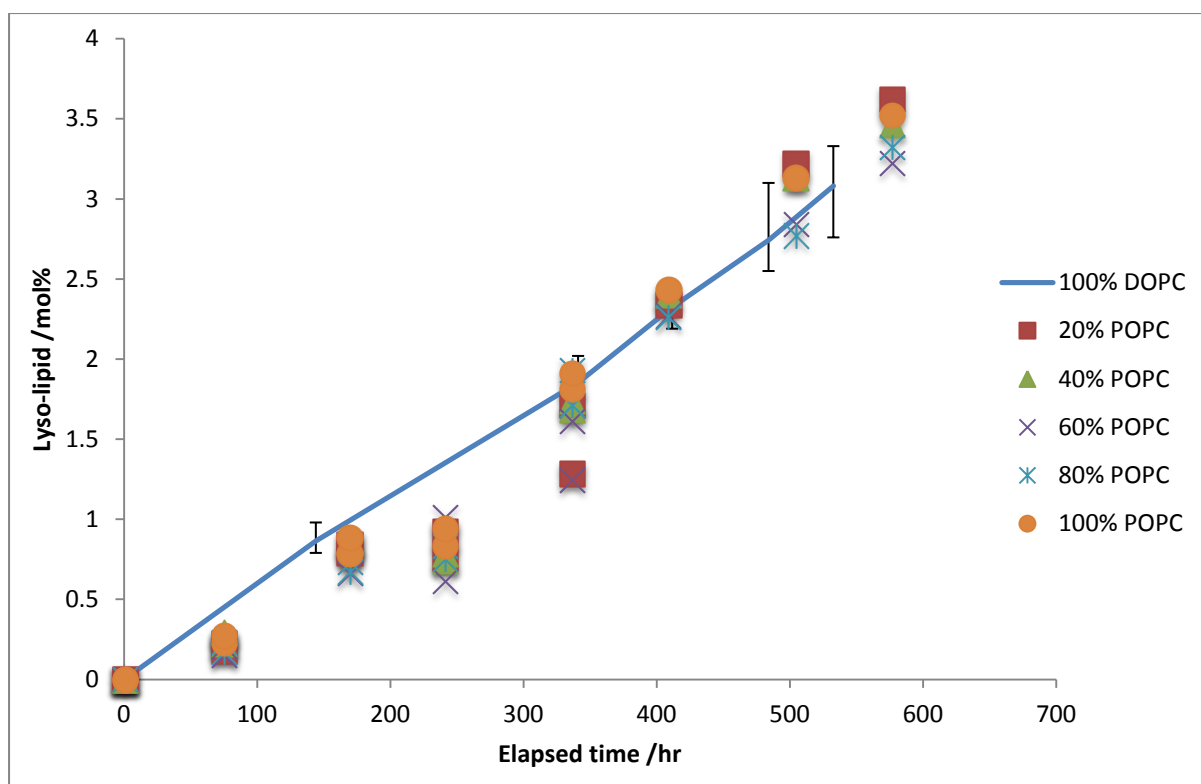


Figure 70: The rates of lyso-lipid formation in a range of mixtures of DOPC and POPC. 100% DOPC data is added for reference, derived from Figure 33. This is also the source of the error bars, as previously.

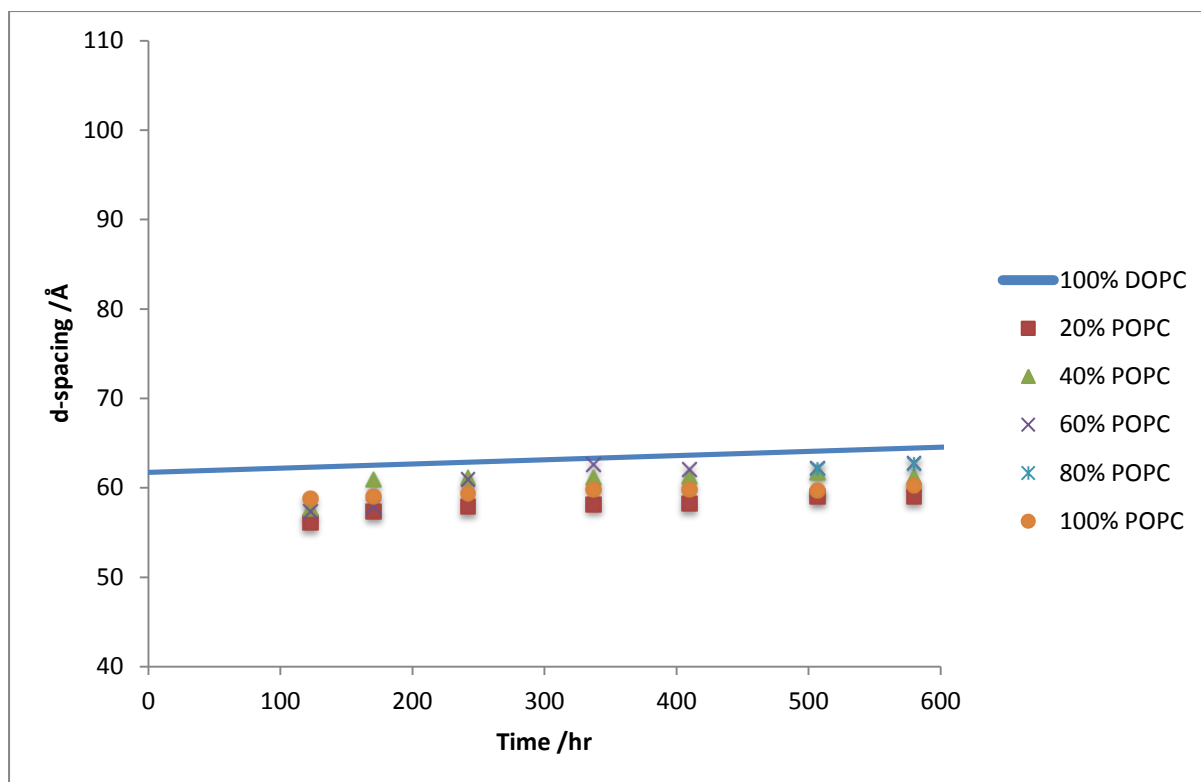


Figure 71: The *d*-spacing exhibited by each of the samples as a function of time, plotted on the same axes as in Figure 65. All values are within experimental error limits of one another, with a slight upwards trend over the course of the measurements. DOPC data are included from calibrated phase diagram studies (R Macey, unpublished data), and were recorded in the absence of raclopride.

The absolute rates identified are within error of those from Figure 33 and extremely consistent within the experiment, giving a rate of $0.132 \pm 0.0159 \text{ mol hr}^{-1} \text{ mol}_{\text{RAC}}^{-1}$, if pure DOPC data is excluded. It is also apparent from the SAXS data above that no substantial physical changes are taking place in the membrane structure during this period: the data is extremely close-grouped when compared to that in Figure 65 (data above is plotted on identical axes for ease of comparison). This lies in stark contrast to the effects that more hydrophobic drugs like haloperidol wreak upon the structure of the host bilayers (see Figure 35, page 95), and raises a number of questions about how the effects of raclopride are mediated if there is little or no sign of membrane insertion by the drug, an idea supported by the fluorescence data in section 5.6.1.1.

This strongly indicates that there is a negligible contribution towards rates of reaction caused by direct drug-fatty tail interactions, suggesting that the observed trend in rates of reaction from DLPC→DMPC→DPPC is due more to microdomain formation and thus restricted lateral diffusion

timescales than it is to any kind of direct drug-lipid complex formation. This is particularly evident when the rates of hydrolysis of the pure lipids studied are compared side-by-side, as shown below in Figure 72.

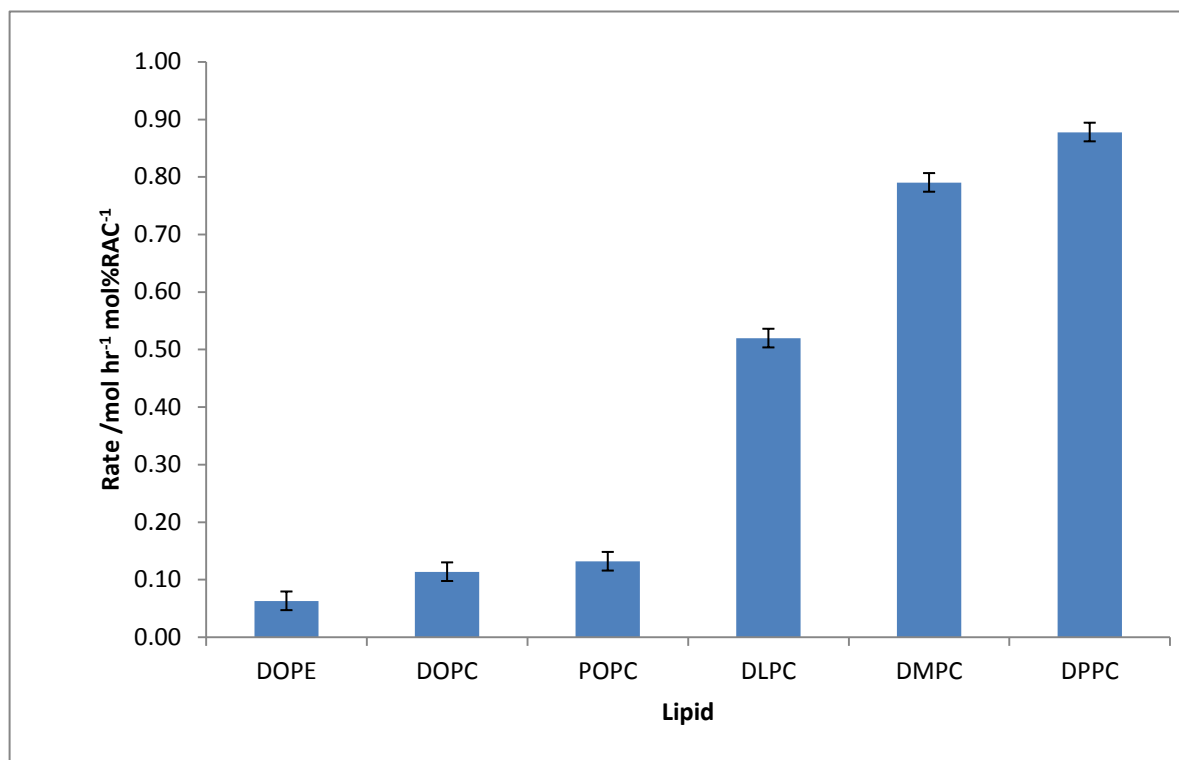


Figure 72: The relative rates of hydrolysis of the pure lipid species analysed. Error bars are calculated from the standard deviation of all POPC data points, assuming that all DOPC-POPC mixtures have identical rates of hydrolysis under identical conditions. Error bars = 2 s.d. derived from Figure 33.

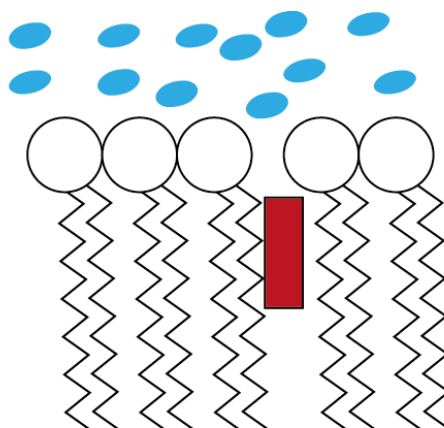
Interestingly, these changes in lateral diffusion are not necessarily linked to changes in sample surface viscosity: at 37°C, it has been demonstrated in electron spin resonance experiments that DOPC and POPC both have higher surface order parameters at 37°C than do DMPC or DLPC²⁰². However, these data do correlate reasonably strongly with the bilayer elastic bending constants – accurate Gaussian curvature modulus values exist for very few lipids or lipid mixtures²⁰³, but although there are considerable variations in the absolute values recorded depending on the technique used for analysis, there is a consistent trend showing rigidity in the order DOPC ≈ POPC < DLPC < DMPC < DPPC¹⁸⁸. This makes physical sense if it is assumed, as a first approximation, that the rate-limiting step of the reaction is the slow migration of protons from the strongly basic drug molecules toward the weakly nucleophilic acyl bond. This will be driven by the change in dielectric environment that the drug experiences as it approaches the hydrophobic interface, decreasing the favourability of the charged relative to the neutral

species. However, in the same way as every other chemical reaction, the probability of this transfer occurring will be dependent upon both the contact time of the two reagents (in this case, the basic centre of raclopride and an adjacent lipid carbonyl bond) and the relative orientation of the two.

For the proton to be transferred to the weakly nucleophilic carbonyl bond, it is likely to require extended exposure to both the hydrophobic region and the target acyl bond linkage. In a gel-phase bilayer, diffusional timescales are sufficiently slow that this may take place efficiently; by contrast, CAD-like compounds experiencing a turbulent and undulating fluid interface have neither the same sustained contact with the acyl interface nor the stable hydrophobic environment, leading to a much lower probability of reaction.

Another way in which this fluidity and thermally-induced turbulence may affect the rate is more indirect. It seems likely that the probability of a CAD triggering hydrolysis will depend strongly upon its depth within the bilayer, which in turn depends upon its effective pK_a . This is influenced strongly by the depth of water penetration into the interface (shown schematically below in Figure 73), as charged species such as the protonated form of the drugs are only favourable in a hydrophilic environment. However, it is a delicate balancing act: the acyl moieties of the phospholipid backbone are not strongly nucleophilic, meaning that rates of reaction will be very slow, yet in the hydrophobic environments that promote the drugs' release of the captured protons, the lipids' ester form is preferred. That suggests the idea that there may only be a narrow window of conditions under which the reaction may be observed at a meaningful rate under these very simple conditions, but also suggests potential approaches by which this may be probed. For example, NMR experiments can readily identify the depth of water penetration into a membrane, whereas the addition or total removal of chelating metals which would interfere with the head-group environment may prove to form the basis of the differences between the results presented here and those previously reported⁵³. Alternatively, the depth of water penetration into a membrane can be directly visualised using membrane bound, environment-sensitive probes such as LAURDAN (6-acyl-2-dimethylamino-naphthalene), which shift their emission spectra in response to the degree of water penetration into a membrane⁸¹.

Ordered membrane,
limited water penetration,
drug floats at interface.



Fluid, disordered membrane:
deeper water penetration leads
to expanded hydrophilic region.
Amphiphilic drug retreats deeper
into membrane core.

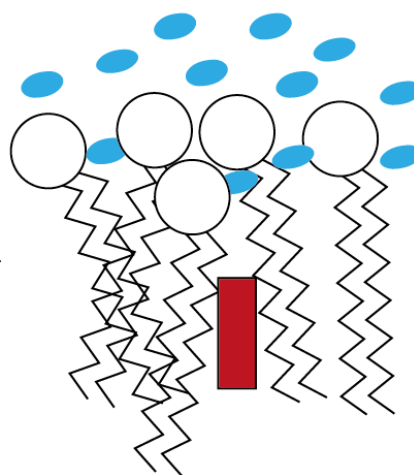


Figure 73: The depth that an amphiphile resides in the membrane will depend upon its physico-chemical parameters but also those of its host membrane. In the example above, a drug molecule (in red) is co-localised with the lipids' acyl bonds when the membrane effectively excludes water; as the membrane becomes disordered, a greater volume of water penetrates beyond the head-groups, expanding the hydrophilic region. As a result, it is hypothesised that the drug retreats deeper into the membrane, preventing hydrolysis.

These results also demonstrate that the partition coefficient of a compound is not as vital to the rate of hydrolysis as was previously thought. As previously discussed, Luxnat and Galla have found that at a given temperature, a short-chain lipid can solubilise a higher concentration of drug than a longer chain structure even when both are in the L_{α} phase, presumably because it is easier to intercalate between the molecules of the more weakly bound short tails¹⁵⁷. This disparity is still greater when the long-chained lipid is in the gel phase, and yet in exactly this situation the above results show significantly faster hydrolysis in DPPC, the gel-phase aggregate, than in any of the fluid systems.

It is difficult to compare the activity of DOPE using this trend due to its hydrogen-bonding capacity and its inverse hexagonal arrangement opening a range of potential confounding

factors. The inverse hexagonal phase limits the applicability of membrane bilayer bending moduli, rendering such comparisons meaningless. Furthermore, the lack of obvious discontinuity in rate at the phase boundary makes it appear likely that the contribution of the hydrogen-bond forming head-group dominates the drug-lipid interactions in this case. If a drug's tertiary nitrogen is in effect sharing its acidic proton with a second amine distant from the target acyl moieties, the probability of a hydrolytic reaction will become almost negligible.

Biological membranes are almost exclusively fluid under normal conditions. However, a range of CADs have been observed to cause rapid morphological and physico-chemical alterations in erythrocytes (for a full review, see Schreier 2000¹⁶): although CADs are often associated with membrane fluidisation, at lower concentrations they can increase membrane stiffness, create meta-stable, crenelated domains or even trigger phase transitions. There also exist natural regions of constrained lateral diffusion in the native cell membrane. The original stable, micron-sized "lipid rafts" hypothesised by Simons and Ikonen²⁰⁴ have been revised so that the sphingomyelin-rich domains are now thought to be around 20nm diameter and with a lifetime measured in tens of milliseconds²⁰⁵: however, this is quite a long time on the scale of chemical reactions, and these may yet have some relevance to drug transport models.

6.3 Influence of cholesterol

Both erythrocyte membranes and lipid nanodomains in live cells are strongly enriched in cholesterol: the strong affinity between ceramide-based lipids and cholesterol was originally mooted as the basis for the lipid raft hypothesis²⁰⁴, and red blood cells are up to 45% cholesterol¹⁸², amongst the highest in human tissue. Furthermore, the introduction of cholesterol into a phospholipid membrane has the effect of dramatically stiffening the membrane¹⁸⁸ and thickening it via chain ordering²⁰⁶, which should promote the lipid hydrolysis reaction if the above hypothesis is correct.

Unfortunately, it has also been demonstrated to prevent drugs from binding to lipid membranes, presumably *via* a competitive process due to its very low aqueous solubility^{157,207}. It induces significant changes in the head-group orientation of its host membranes, the “umbrella” effect (shown below in Figure 74), where the phosphate group of an adjacent phospholipid alters its conformation to minimise water contact with the cholesterol molecule^{208,209}. These strong interactions, honed through evolutionary processes, are significantly greater than those between CADs and similar lipids and so the drugs are displaced and may precipitate out.

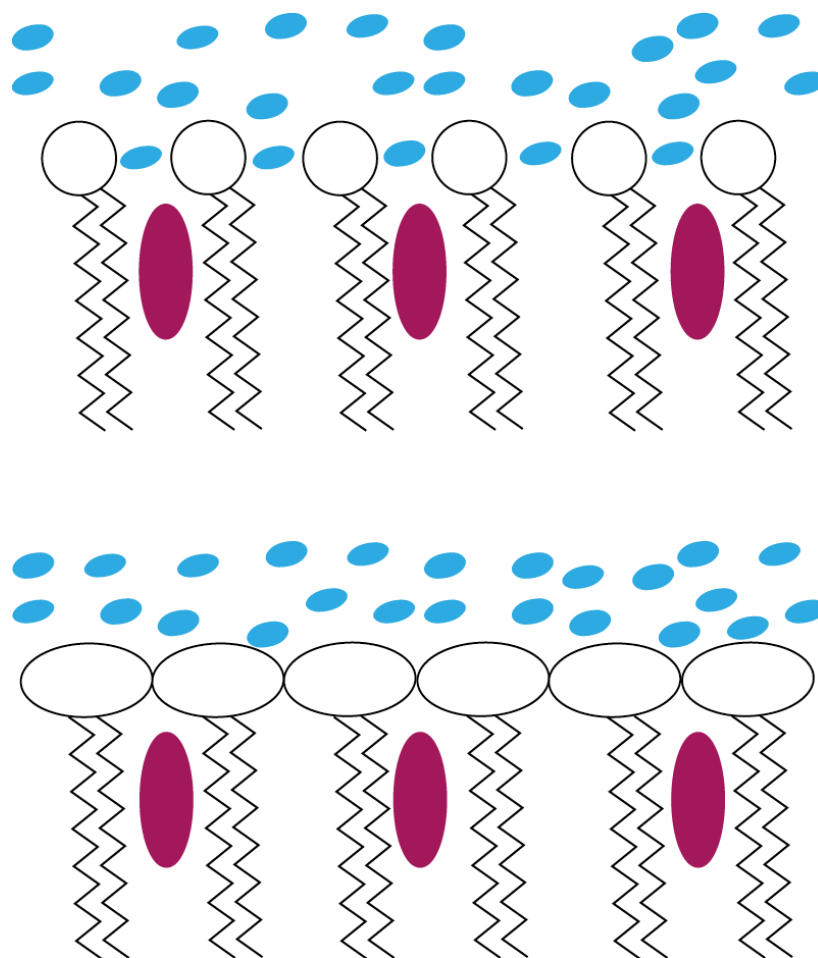


Figure 74: The “umbrella model” of cholesterol integration (purple) into membranes. Initially, the intercalation of cholesterol increases order within the membrane, but exposes the hydrophobic sterol molecules to water which penetrates beyond the lipid head-groups (top). To ameliorate this hydrophobic stress, the lipid head-groups distort to shield the sterols, (lower), but have a finite capacity to do so, setting an effective maximum concentration of cholesterol in the membrane.

The very fact that cholesterol and many phospholipids have such strong interactions effectively limits the solubility of the sterol in a membrane, however. Above concentrations of 45-50 mol%, the cholesterol begins to precipitate out of the bilayer²¹⁰, as the sterol:phospholipid ratio exceeds 1:1 and there are no longer sufficient lipids to prevent its contact with water. As a result, it was not possible to experiment with cholesterol concentrations greater than 50 mol% and even these showed the formation of precipitated cholesterol crystals.

In order to probe and quantify these effects upon the rates of hydrolysis, DOPC was co-lyophilised with cholesterol to form mixtures of 10-50 mol%. These lipid films were hydrated and homogenised as normal and were analysed by HPLC over the course of some 600 hours. Samples were removed and dispensed into 1.5mm diameter capillaries for SAXS analysis, but

despite careful sealing all samples dehydrated very rapidly under incubation and so that aspect of the experiment was abandoned.

As can be seen from Table 2 on page 70, the retention times of cholesterol and DOPC run very close to one another and prevented their independent resolution. In order to calibrate the data, the contributions of cholesterol and DOPC to each peak were calculated with reference to standard solutions and the cholesterol signal was removed algorithmically. However, the values thus derived were found under the assumption that the levels of lyso-PC formed were small, which may have induced errors during the later stages of the experiment. The processed results are shown below in Figure 75.

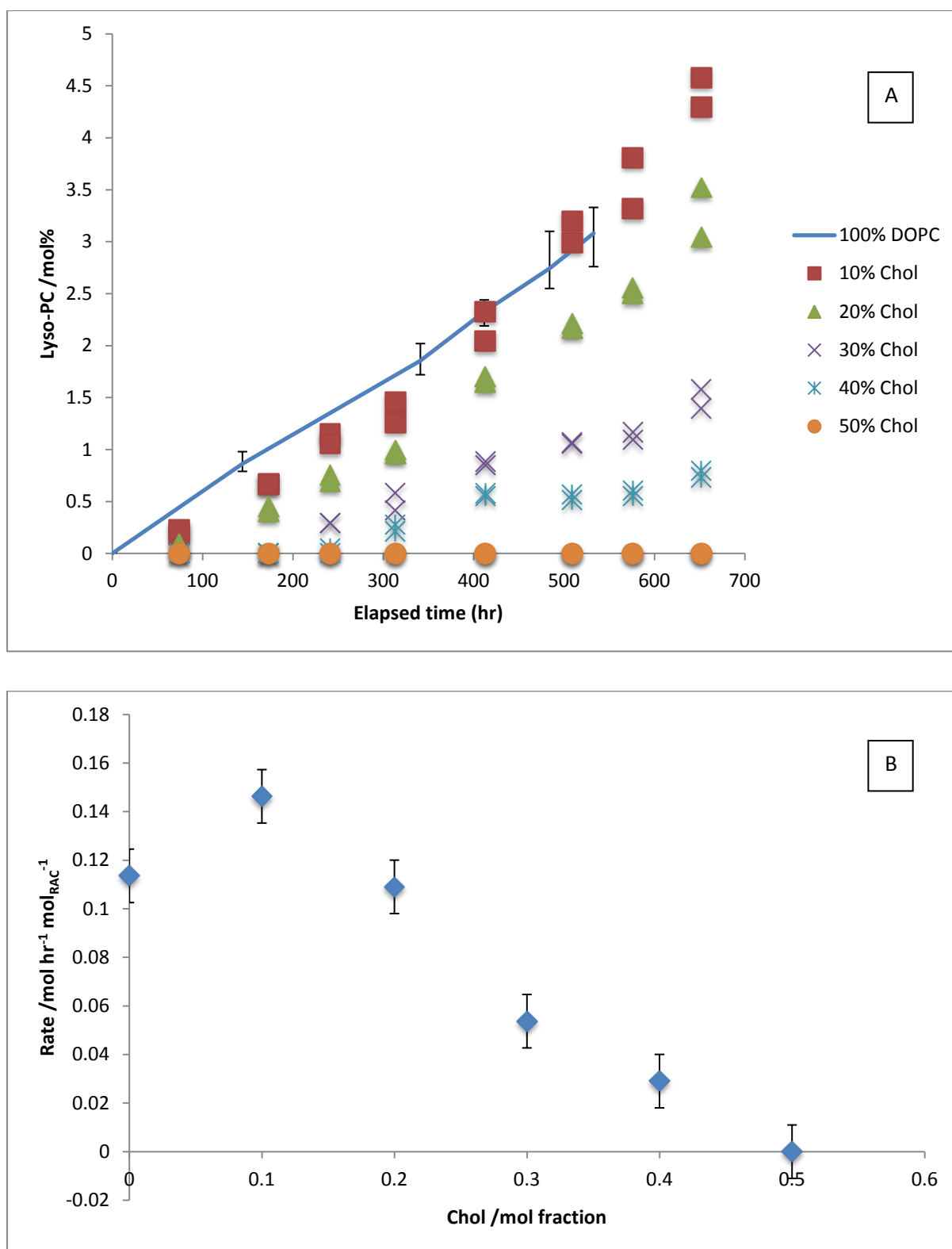


Figure 75: The hydrolysis of DOPC in the presence of varying mole fractions of cholesterol: (a) the formation of lyso-OPC as a function of time; (b) the rate of formation as a function of cholesterol mole fraction. This suggests a straight competitive displacement between raclopride and cholesterol. 100% DOPC data and error bars from Figure 33.

These results demonstrate either that raclopride and cholesterol are competing for the same binding sites at the lipid membrane, or that the conformational shifts induced in nearby lipids by the presence of cholesterol effectively destroys the preferred habitat of the drug within the membrane. The former seems more likely, both by virtue of simplicity but also based upon the findings of NMR experiments probing the orientation of amphiphilic drugs at the membrane, which have shown them occupying positions at the interface identical to that shown schematically above in Figure 74²¹¹.

This finding has some interesting implications for drug binding to biological membranes. Most obviously, it suggests that red blood cells (which have membranes comprising up to 45% cholesterol¹⁸²) may not be effective transport media for drugs *in vivo*. Proteins such as A-glycoprotein and Serum Albumin collect and transport the vast majority of such lipophilic species in the blood and are widely studied as a result, as their actions can lead to a range of unpredictable outcomes for the *in vivo* efficacy of an otherwise promising candidate drug compound²¹². However, a number of amphiphilic compounds including CADs like imipramine have been observed to induce membrane deformations in red blood cells and so it would seem likely that even under very high-cholesterol conditions, a proportion of the drug molecules are absorbed into the membrane.

However, raclopride is not amongst those compounds inducing detectable erythrocyte deformation. At approximately the concentration of cholesterol found in red blood cells, no hydrolysis was observed at all. If it is assumed as a first approximation that cholesterol is not directly preventing the hydrolytic activity of raclopride, then it is acting only as a steric block. If this is the case, reference to the dose-response data plotted in Figure 34 (page 93) suggests that little or any of the administered raclopride has been absorbed by the membrane. While this effect may disappear when considering the extremely large number of erythrocytes available *in vivo*, it still provides food for thought, suggesting an exquisite relationship between CAD structure and their uptake by red blood cells.

Of more direct interest is the observation that cholesterol levels vary significantly between even otherwise-healthy individuals, between 4-8mmol/l blood serum²¹³, a not inconsiderable amount when considering the compound's high log P. This level varies rapidly and dynamically with diet²¹⁴, meaning that uptake and transport of an amphiphilic or hydrophobic drug could potentially be significantly removed from baseline figures depending on a patient's choice of

breakfast.

7 *The future*

In order to achieve the goals set out in section 4, a broader range of drug analogues will require investigation, necessitating a number of different experimental methods depending on the throughput required. One of the over-riding conclusions of the whole project to date has been that no one technique covers all the bases: in order to generate useful models, a combination of high and low throughput approaches will be required, working in synch with one another across an integrated programme. High throughput techniques such as plate-based fluorimetry, HPLC and surface plasmon resonance provide important, scatter-gun data – while they can only report upon the bulk, average properties of a population of cells or vesicles, they fulfil an important trailblazer role for the lower throughput, highly spatially and temporally resolved techniques which will pick out the finer points of the mechanistic detail.

Lower throughput techniques have the potential to generate huge volumes of data about individual data points on the above results, covering the precise behaviour of each system in exquisite detail. In order to probe the tissue specificity of the drug-lipid interactions as proposed in the working hypotheses (page 53), a combination of mass spectrometry (MS) driven lipidomics²¹⁵ and spatially resolved techniques such as fluorescence microscopy and MS imaging will be required. MS imaging in particular shows great potential: by imaging *ex vivo* tissue before and after treatment with a compound, changes in the spatial distribution of specific lipid species can be monitored at a micron resolution via direct MALDI-MS of tissue slices²¹⁶.

The data presented above has all been fed into the computational modelling work-stream which runs in parallel with the non-specific binding initiative, and some of the early products of their labours have already been alluded to¹⁵⁰. Work is continuing on modelling both individual drug-lipid binding interactions, which may be of extreme value if the results in section 6 (page 147 onwards) are to be believed, and also into coarse-grain modelling of the membrane as a whole. Although this technique obviously lacks the resolution of the molecule-molecule interaction modelling, in some regards it provides a much more useful picture of the mechanical processes occurring at the lipid interface in terms of molecule depth, orientation and diffusion rates which have become increasingly important over the course of this investigation. The eventual aim is to coalesce all the various, disparate data strands into one vast, coherent data-set, allowing the production of a computational model that will be able to predict the biological transport of any given molecule from structure alone. That, however, remains a long way off.

Until then, the gold standard for measuring *in vivo* distribution remains PET: despite its extremely low throughput, it permits sequential imaging of the same animal model, thus eliminating inter-subject heterogeneity encountered in techniques which require post-mortem analysis such as autoradiography. It also provides real-time, quantitative monitoring of plasma drug levels both in and outside the region of interest, meaning it can supply accurate figures not just for receptor localisation but also non-specific binding and total volume of distribution. In order to maximise the use of PET techniques, collaboration is set to continue with the GSK Clinical Imaging Centre and their new small animal PET facility. Large animal studies will become available in due course, regulatory affairs permitting, which will greatly simplify the access and availability of this, most powerful of techniques.

7.1 *Synthetic research towards future PET models of non-specific binding[‡]*

In order to explore the influence of drug structure on the measured rates of hydrolysis, the syntheses of a small number of raclopride analogues was attempted. A spread of these compounds could then be radiolabelled and used for PET analysis *in vivo*. These were also to be analysed with the best available biophysical assays developed, to place them into some kind of rank order of hydrolytic activity in an attempt to correlate the *in vitro* measurements with those from the clinic. This could potentially point towards the development of a predictive model for lipid transport, the ideal result of the NSB project as a whole.

Raclopride, with its central amide bond (see Figure 13, page 56), offered great flexibility and relatively simple synthetic accessibility. This allowed the development of a small range of bimodal imaging agents prepared to allow the potential analysis of *in* and *ex vivo* models with the same probe: raclopride-like molecules were synthesised with an intrinsic fluorophore, permitting their direct imaging in cell culture and tissue section as well as *via* much lower-throughput but potentially higher quality PET techniques.

[‡] Adapted in part from thesis submitted for Master of Research award, 2007.

7.2 *Synthesis*

The compounds synthesised fell into two distinct families, as two different roles were required. Initially, an array of raclopride analogues was generated to form structures as closely related as was practical to the parent compound's aromatic region. Literature from the suppliers of raclopride along with experimental findings suggested that the benzylamide region of the compound was photosensitive, which appeared in keeping with its intrinsic fluorescence, absorbing strongly under UV light ($\lambda \approx 360\text{nm}$). As a result, attempts to synthesise analogues retaining all the chemical features of the 3,5-dichloro-2-hydroxy-6-methoxy-benzoic acid met with failure. Rather than becoming heavily involved in specialist techniques and facilities to perform the chemistry in the dark, analogues were instead made using 2, 6-dimethoxy, 2-hydroxy-6-methoxy or 2, 6-dihydroxybenzoic acid building blocks, as shown below in Figure 76. Analogues of each of these are labelled **A**, **B** and **C** throughout.

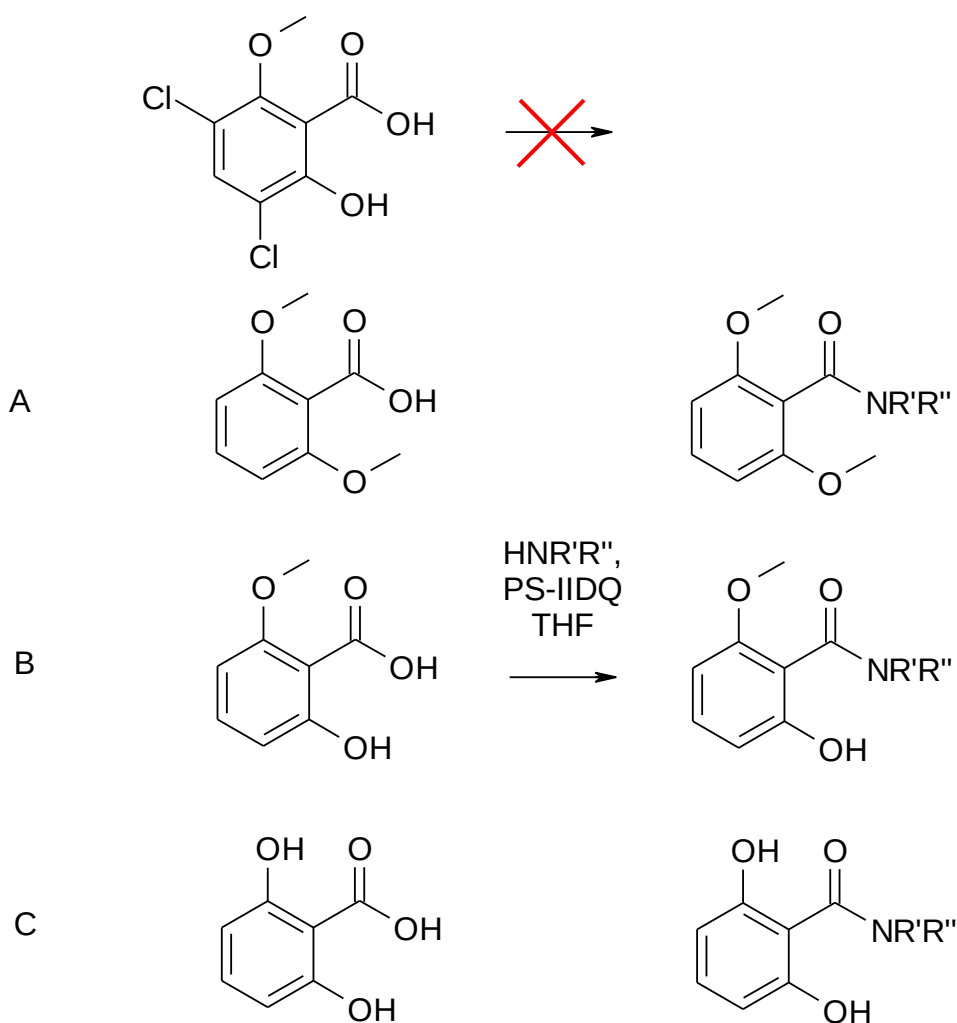


Figure 76: The photosensitivity of 3,5-dichloro-2-hydroxy-6-methoxy-benzoic acid products made them less than ideal for array-based synthesis. As a result, less sensitive analogues were prepared based upon the benzoic acids shown above. For the reaction mechanism of polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (PS-IIDQ), please see Figure 78.

This made syntheses significantly easier, although yields were found in the order **A** > **B** > **C** due to the electron-donating effect of the substituent methoxy groups in **A** and **B** which helped increase the nucleophilicity of the benzoic acid. While this approach greatly simplified the synthesis, it did so at the cost of the zwitterionic behaviour of the analogues at neutral pH which was maintained only by the two strongly electron-withdrawing chloride groups (in comparison to raclopride, eticlopride which only features one chloride group is always cationic at neutral pH¹⁵²). Predicted pK_a values (using Chemicalize physico-chemical prediction software, ChemAxon UK) of the 2-hydroxy group change from 6.26 in raclopride to 7.97 in **B1** while little change is observed in the pK_a of the basic nitrogen, meaning the compound moves from being almost entirely zwitterionic at pH 7.4 to being almost entirely cationic.

These compounds featured only a low intrinsic fluorescence, however, which limited their use in tissue section or cell culture assays. As a result, a second range of compounds was generated, replacing these aromatic regions with *N*-methyl anthranoyl (MANT) amides²¹⁷, shown in Figure 77. These MANT species form the smallest available organic fluorophores, ($\lambda_{\text{Exmax}} \approx 350\text{nm}$, $\lambda_{\text{Emmax}} \approx 446\text{nm}$), but give high, stable fluorescent yields as high as 0.95 in organic solvents and approximately 0.25 in aqueous media²¹⁸. As a result, these compounds were potentially the perfect choice to repeat and build upon the fluorescence microscopy performed in previous studies⁵³, which had been based upon a commercially available 7-nitrobenz-2-oxa-1,3-diazole (NBD) labelled spiperone analogue.

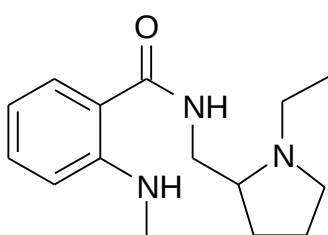


Figure 77: The *N*-methyl anthranoyl amide analogue of raclopride (*D1*), synthesised for its high fluorescent yield and relatively close structural resemblance. The non-methylated analogue of this compound has been demonstrated to show some D_2 antagonism, similar to that of raclopride²¹⁹.

7.2.1 Synthetic techniques, purification and characterisation

Extremely high purity was required for all compounds to be tested: because the reaction under consideration is slow (at least in the condensed phase) and catalytic, a small percentage of high active contaminant could totally dwarf the contribution from the drug itself and lead to misleading results. PET trials have even more stringent purity requirements – because the volumes of positron-emitting label are so small, in the pico-or atto-molar range, the unlabelled substrate must be extremely clean to prevent the labels being mopped up by trace contaminants. This level of purity is very difficult to achieve manually without the sacrifice of significant percentages of otherwise-viable product, and so a range of UV and mass-triggered automated purification techniques were utilised.

Mass-direct automated reverse phase preparative HPLC (MDAP) of methoxy/hydroxy species was conducted at GSK Stevenage on a Waters Sunfire C_{18} column, 150 x 30mm internal diameter

using a mobile phase gradient from water/0.1% vol. formic acid to acetonitrile/0.1% vol. formic acid over 15 minutes. Quantification was via a Waters diode array (220-330nm, averaged) and peak identification by Waters electrospray MS (switching +/-). As a result, most compounds were isolated in the formate salt form.

MANT amide products were isolated using a Combiflash Companion preparative flash chromatography device (Teledyne Isco, USA), using the appropriate pre-packed C₁₈ RediSep R_f column cartridges. The mobile phase gradient ran from 18MΩ water/0.1% formic acid through to methanol, due to the low availability of acetonitrile during this period. Detection was performed at 254 and 350nm, with collection triggered using 350nm absorbance so as to only capture MANT-containing species. As previously, most compounds were isolated as formate salts.

7.2.1.1 Methoxy/hydroxy species

Synthesis of the non-labelled raclopride analogues was conducted using solid phase amide-coupling reagents from the relevant benzoic acid and amine precursors. Previous attempts had demonstrated the relative inactivity of the 2,6-methoxy- and 2,6-hydroxy-benzoic acids in amide synthesis leading to low yields with common reagents such as dicyclohexylcarbodiimide, and more direct syntheses involving the use of thionyl chloride had proved difficult to purify. As a result, polymer-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (PS-IIDQ) was chosen as a coupling reagent²²⁰. This powerful reagent gave rapid, clean formation of all the 2,6-methoxybenzoic acid derivatives synthesised under very mild conditions, with somewhat lower yields for the hydroxy analogues. Furthermore, the side-products of the reaction are all either volatile or are retained on the resin bead, as shown below in Figure 78, allowing purification in some cases by simply filtering the reaction mixture and evaporating the solvents²²¹.

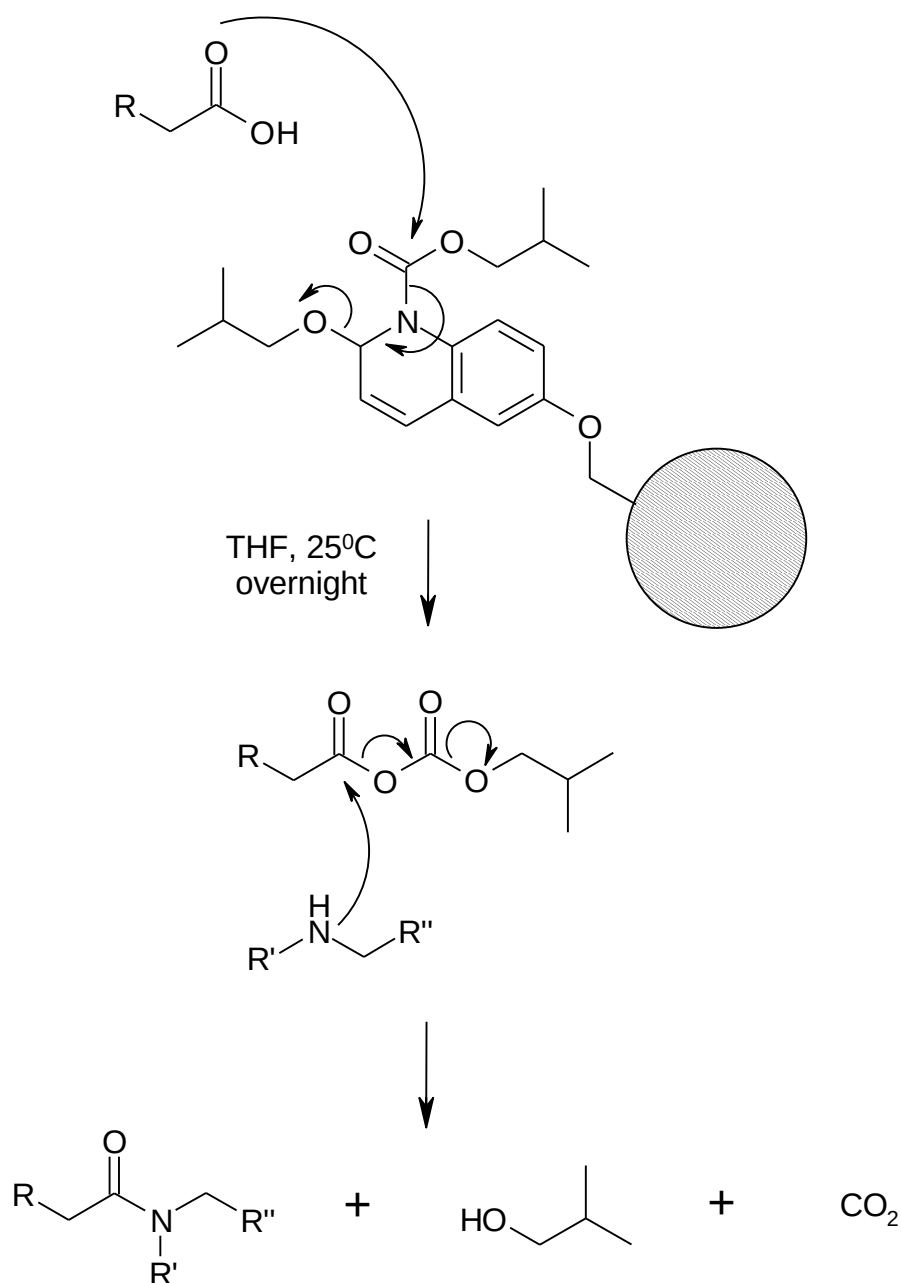


Figure 78: The reaction mechanism of polystyrene-supported 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (PS-IIDQ). All side-products are, under ideal circumstances, either volatile or retained upon the polystyrene bead allowing for extremely simple purifications.

For illustrative purposes, this procedure will be described for the first compound to be successfully isolated, 2,6-dimethoxy-*N*-(2-piperidin-1-yl-ethyl)-benzamide, (**A1**): other syntheses were identical in all respects except for the amine species involved.

2,6-dimethoxy-*N*-(2-piperidin-1-yl-ethyl)-benzamide, (free base) **A1**, (shown below in section 7.2.2). 60mg (0.33mmol) 2,6-dimethoxybenzoic acid was dissolved in 10ml tetrahydrofuran

(THF) with 45mg (0.33mmol) 2-piperidin-1-yl-ethylamine in a glass jar with a fermentation cap. 0.4125g (1.6mmol/g, 2 equivalents) PS-IIDQ was added and the reaction mixture was shaken at 300rpm overnight at room temperature.

A white precipitate was observed in the flask after reaction: this dissolved when dichloromethane (DCM) was added to the vessel. The mixture was filtered under gravity and the solvent removed under reduced pressure to leave an off-white product. 10ml cold THF was added and the mixture shaken briefly to leave the white precipitate observed previously. This was isolated by vacuum filtration and dried in a vacuum oven at 40 °C overnight to give 54.1mg (0.185 mmol, 56.1% yield). Subsequent ¹H NMR analysis in CDCl₃ showed the product to be >95% pure. ¹H NMR [CDCl₃] δ = 1.28 (2H, m); 1.50 (3H, m); 2.42 (3H, m, broad); 2.54 (2H, t); 2.93 (2H, t); 6.41 (2H, d); 7.06 (H, t) ppm.

For those reactions showing lower yields, further purification steps were required. Solid-phase scavenging, using polymer-supported isocyanate and trisamine resins, was disregarded as impractical in this case due to the potential for the scavenging agents to remove significant quantities of target compound at the same time as the impurities. The level of purity required was such that at the milligram scales being worked upon, isolation by hand using normal-phase silica would lead to significant losses of yield. As such, purification was undertaken on an automated reverse-phase column using mass-spectrometry and/or diode array peak identification on industrial facilities generously provided by GlaxoSmithKline plc, described fully below in section 7.2.1. Typical purities were in the region of 95-100% and were confirmed by ¹H NMR.

Some side-product formation was observed using the PS-IIDQ technique. ¹H NMR and mass spectrometry analyses identified the compounds as the urethane structure shown in Figure 79. It was the preferential formation of this species that prevent the isolation of any significant amount of compound **A4** (see Table 6).

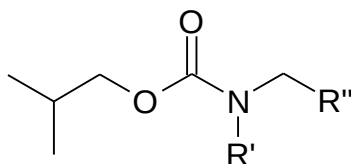


Figure 79: The urethane structure formed when the amine in the last step of the reaction mechanism shown in Figure 78 attacks from the more hindered side.

7.2.1.2 *N*-methyl anthranoyl species

N-methyl anthranoyl analogues of raclopride (labelled **D**), (as well as their *des*-methyl counterparts to allow for potential PET imaging, labelled **E**), were prepared in a straightforward, one-step acylation reaction combining the *N*-methyl isatoic anhydride with the relevant amine under anhydrous reflux conditions. High temperatures were not entirely necessary, as production of carbon dioxide was observed almost immediately after the combination of the reagents (see Figure 80), but it was found to improve yields after testing.

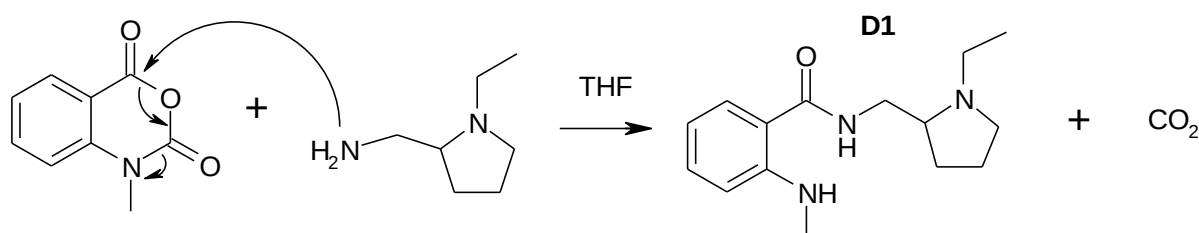


Figure 80: The reaction mechanism of *N*-methyl isatoic anhydride, combining here with 2-aminomethyl-1-ethylpyrrolidine to form compound **D1**. The reagents were combined in 10ml dry THF and boiled under anhydrous reflux conditions for 1 hour, forming good yields.

In the absence of water, this reaction proceeded in good yield; water caused the hydrolysis of the anhydride forming 2-*N*-methyl benzoic acid, which fluoresced at a lower wavelength of around 400nm. The reaction to form the *des*-methyl product was similar, but had a greater propensity to form side-products due to the formation of a free aniline during the process, which could then combine with another anhydride molecule in a potential chain reaction, as shown in Figure 81.

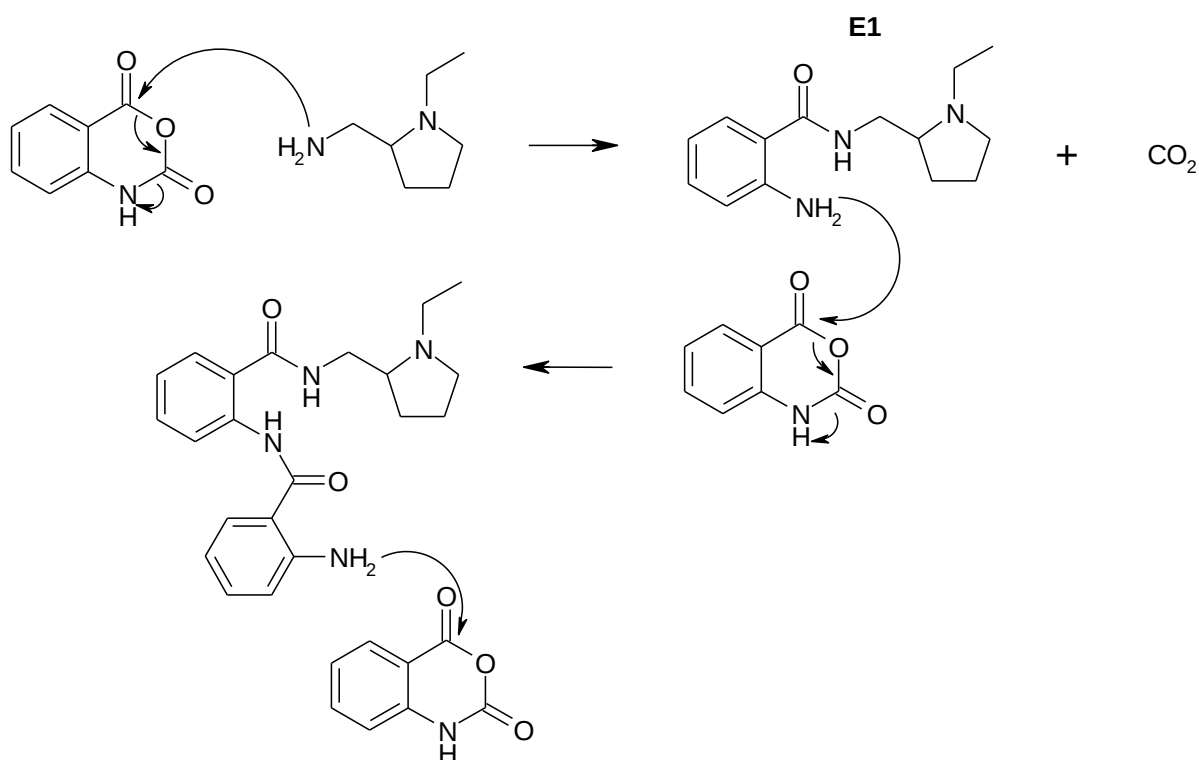


Figure 81: The mechanism of side-product formation when using methyl isatoic anhydride as an acylating agent.

N-(1-Ethyl-pyrrolidin-2-ylmethyl)-2-methylamino-benzamide, (formate salt) **D1**, and *N*-(1-Ethyl-pyrrolidin-2-ylmethyl)-2-amino-benzamide, (formate salt) **E1**

177mg (1mmol) *N*-methyl isatoic anhydride and 256mg (2mmol) 2-aminoethyl-1-ethylpyrrolidine were dissolved in 10ml THF and heated to 60°C under a reflux condenser and a nitrogen atmosphere for one hour. TLC analysis on silica plates (95% DCM, 5% MeOH) showed 2 spots under UV λ 365nm: $R_f \approx 0.7$ (purple, side-product²²²) and $R_f \approx 0.1$ (blue, product). The desired product was isolated by Combiflash, as outlined above. 178.5mg (0.581mmol, 58.1%) was collected. ¹H NMR showed >95% purity, [CDCl₃] δ = 1.36 (3H, t); 1.91 (H, m); 2.04 (2H, m); 2.21 (H, m); 2.71 (3H, s); 2.94 (2H, m); 3.31 (H, m); 3.68 (2H, m); 3.85 (2H, m); 6.66 (2H, m); 7.33 (H, t); 7.47 (H, d); 8.38 (formate); 8.79 (H, amide) ppm.

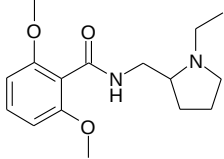
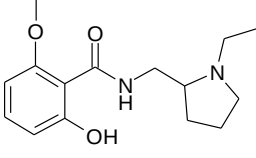
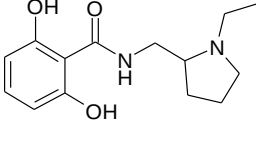
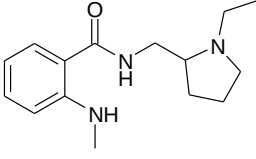
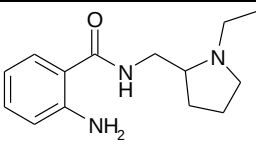
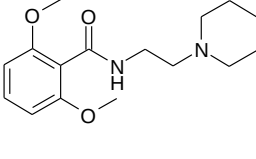
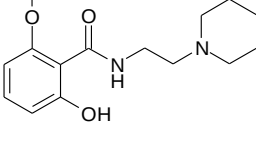
The *des*-methyl analogue, **E1**, was synthesised similarly, using 163mg (1mmol) isatoic anhydride in place of the *N*-substituted reagent. After purification, 118mg (0.412mmol, 41.2% yield) was returned and was shown to be >95% pure by ¹H NMR [CDCl₃] δ = 1.28 (3H, t); 1.81 (H, m); 1.98 (2H, m); 2.17 (2H, m); 2.72 (2H, m); 3.18 (H, m); 3.45 (H, m); 3.64 (2H, m); 3.76 (H, dd); 6.70 (2H, dt); 7.24 (H, t); 7.63 (H, d); 8.80 (formate) ppm.

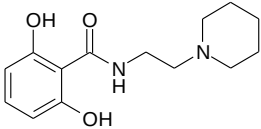
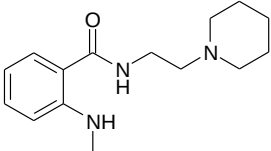
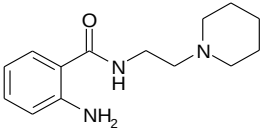
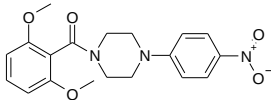
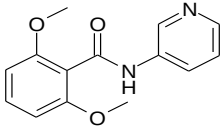
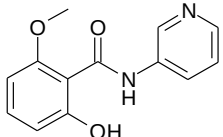
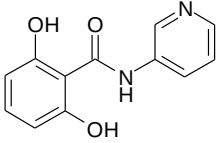
Purities were confirmed by analytical HPLC using an Agilent 1200 series C₁₈ system analysed *via* diode array at 254, 360 and 446nm and by LCMS using a Waters 1525 system through a reverse phase Waters Atlantis DC₁₈ 30mm x 4.6mm column and a Waters LCT Premier quadrupole MS. In both cases, results showed a single peak.

7.2.2 Results

The results of the synthetic programme are shown below in Table 6. Wherever possible, coherent families of compounds were created to allow direct comparisons across the data-set. However, not all compounds were tested, due to experimental issues at varying stages and shifting priorities: some of this synthetic work was undertaken early in the project, and as it was discovered that the previous theories regarding the factors controlling hydrolysis were incomplete, research moved towards a more mechanistic study leading to the results in sections 5 and 6. In some regards, these results are included for completeness, and to form the basis of future studies should this topic be investigated further.

Almost all of the compounds below were purified by automated, reverse-phase chromatography techniques. As such, those containing basic centres were isolated as formate salts; the formula weights below correspond only to the parent molecule in its free-base form.

Compound identifier	Structure	FW	Mass available /mg	Purity /%
A1		292.38	24.7	>95
B1		278.35	41.6	>95
C1		264.33	75.4	>95
D1		261.37	178.5	>95
E1		247.34	65.3	>90
A2		292.38	54.1	>95
B2		278.35	88.0	>95

Compound identifier	Structure	FW	Mass available /mg	Purity /%
C2		264.33	80.3	>95
D2		261.37	258.88	>95
E2		247.34	127.8	>95
A3		371.40	19.5	>95
A4		258.28	<1	N/A
B4		244.25	74.3	>95
C4		230.23	8.4	>95

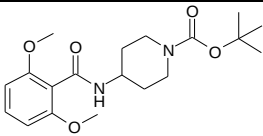
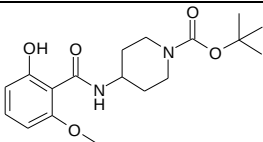
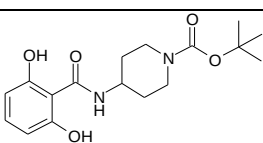
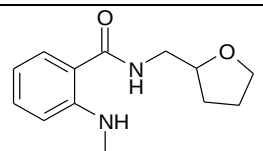
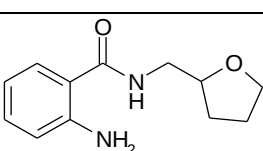
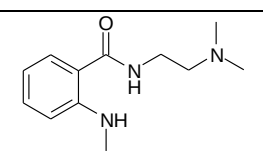
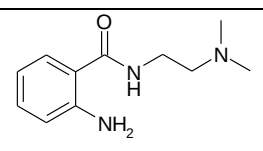
Compound identifier	Structure	FW	Mass available /mg	Purity /%
A5		364.45	75.1	>95
B5		350.42	63.6	>95
C5		336.39	7.7	>85
D6		234.30	21.6	>95
E6		220.27	122.27	>95
D7		221.30	233.86	>95
E7		207.28	194.55	>95

Table 6: The results of the synthetic programme undertaken to probe the dependence of the hydrolytic activity observed upon the structural parameters of a number of raclopride analogues. Not all compounds were tested, due to changing priorities and experimental issues.

A number of these compounds had prior art describing either their synthesis or, in some cases, their activity. For example, **B1** is a precursor in radiolabelling studies²²³, **D3** was previously produced by TimTec Compound Libraries (USA), CAS number 348612-09-9. As previously mentioned, **E1** has been patented for its dopamine-*D*₂ antagonist activity²¹⁹, while **E2** and **E3** were identified as potential drug candidates in 1970 by Bonola and Sianesi²²⁴ and Jacobs²²⁵, respectively. **E7** has been patented as a herbicide in France²²⁶. The other compounds are novel, or at least unpublished as far as can be ascertained.

7.3 Physico-chemical data

The physico-chemical parameters for a number of compounds intended for PET analysis were analysed at the GSK Clinical Imaging Centre (CIC) at Hammersmith Hospital by Chloe Child, using GSK's standard high-throughput, HPLC-based profile. The log D was determined, with reference to well-characterised standard compounds, at pH 2.2, 7.4 and 10.5 using the chromatographic hydrophobicity index (CHI)²²⁷, as was each compound's affinity toward immobilised artificial membranes (IAM), human serum albumin (HSA) (which binds anionic, hydrophobic compounds such as fatty acids in the blood) and α_1 -acid glycoprotein (AGP), which acts as a hydrophobic cation transporter. This process was carried out for compounds **D1**, **2**, **6**, and **7**, which were finally selected to go forward for ¹¹C labelling and PET distribution measurement in porcine models.

All HPLC studies were conducted using the Agilent 1200 series models based at the CIC. Log D was measured using a Luna 50 x 3mm C₁₈ column (Phenomenex, USA), while IAM assays were conducted using a specialised column containing immobilised DLPC molecules bound to an amine-functionalised surface. AGP measurements were taken using a 50 x 3.0 mm AGP column (Chrom Tech, USA), while HSA measurements were taken using a similar HSA-functionalised column. Samples were eluted using a gradient of 50mM ammonium acetate (10mM phosphoric acid for pH2.2 studies) into acetonitrile (Log D and IAM) or propan-2-ol (AGP and HSA). IAM, AGP and HSA assays were all run at pH 7.4.

Compound	CHI Log D pH 2.2	CHI Log D pH 7.4	CHI Log D pH 10.5	Log K IAM
<i>Raclopride</i>	1.01	2.26	1.47	2.07
<i>Haloperidol</i>	1.05	2.18	3.21	2.84
<i>D1</i>	-0.10	1.18	2.50	2.51
<i>D2</i>	-0.18	1.30	2.61	2.50
<i>D6</i>	1.23	2.14	2.03	1.49
<i>D7</i>	-0.70	0.80	1.60	2.24
Compound	% AGP	Log K AGP	%HSA	Log K HSA
<i>Raclopride</i>	52.44		75.39	
<i>Haloperidol</i>	88.63		91.05	
<i>D1</i>	66.29	0.28	50.25	0.00
<i>D2</i>	68.36	0.32	53.77	0.06
<i>D6</i>	40.00	-0.18	57.09	0.11
<i>D7</i>	58.27	0.13	45.53	-0.09

Table 7: The results of the HPLC-based physical chemistry assays run at GSK's Clinical Imaging Centre by Chloe Child. Log D values were calculated from each compound's chromatographic hydrophobicity index (CHI)²²⁷; immobilised artificial membrane affinity (IAM), α_1 -acid glycoprotein (AGP) and human serum albumin (HSA) binding were determined *via* specialist column coatings.

The values derived from these processes illuminate a number of interesting points raised during the attempts to generate fluorescence assays detailed previously in section 5.6.1. Each of the basic compounds, **D1**, **2** and **7**, show significantly higher affinities for the amphiphilic membrane environment of the IAM assay than they do for the more purely hydrophobic one of the reverse-phase, C₁₈ CHI assay. The binding values they display are closely related to those displayed by the fully neutral species at high pH: in the case of **D7**, even more so. This suggests that the free energy that is released by an amine in one of these compounds when it binds a proton is exceeded by the cost of dragging that charge into the hydrophobic interior of a membrane: that these close structural analogues of raclopride dwell sufficiently deeply within the membrane that there are insufficient polar interactions to stabilise a long-term bond to a proton.

Raclopride itself is most hydrophobic when at neutral pH, which is at first counter-intuitive as these are the conditions when the molecule is most charged. However, in this state the molecule forms a zwitterionic internal salt which is net neutral, thus may reside deep within the membrane in a similar manner to the compounds described above. This explains the lack of response of the FPE probe upon exposure to raclopride, described in section 5.6.1.1 (page 103): the introduction of a neutral, hydrophobic species in amongst many others has effectively no impact upon the surface charge function and similarly prompts no build-up of counter-ions upon the surface.

By contrast, this trend is reversed in the neutral **D6**, which aside from its lack of charge is almost isosteric with **D1**. This may be due to it suspending at a different orientation in an amphiphilic membrane than in the more uniform hydrophobic environment, as the most polar bonds in the molecule **D6** are found in the aniline moiety at the other end of the compound to the aliphatic region carrying the charge in **D1**, **2** and **7**.

The data derived from haloperidol testing reveals a crucial difference between its behaviour in the membrane and that of raclopride. By virtue of either its free hydroxyl or its greater molecular length, haloperidol retains significantly more of its charge in the membrane, suggesting that the basic region floats much closer to the polar interface of the lipids. This can be seen from the differences in affinity between the C₁₈ experiments at pH 10.5 and the artificial membrane assay, revealing the causes behind its strong interactions with FPE, especially when compared to those of raclopride.

7.4 Hydrolytic activity

The hydrolytic activity of the compounds described in Table 6 was assessed in the bulk phase, according to the protocols developed earlier in section 5: briefly, each compound was co-lyophilised at a 1:20 molecular ratio with DOPC and hydrated with 10mM PBS buffer, before being homogenised *via* vortexing and centrifugation. These samples were sealed and incubated at 37°C and analysed over a period of some 600 hours. In early studies, samples were simply stored in a plastic test-tube holder, but as described in section 5.4.2 (page 72) these were found to give misleadingly high rates due to the partial dehydration of samples due to the thermal gradients formed in each vial. As a result, the data shown in Figure 82 cannot be used to generate more than trend data, as the internal raclopride standard is shown to hydrolyse DOPC significantly faster than was observed in later, isothermal studies. However, it can be concluded that small modifications to the basic moiety of the parent compound can be used to create significant changes in the rate of observed hydrolysis. Furthermore, this data suggests that the change from a zwitterionic species at physiological pH to a cationic species has little effect upon its behaviour in the membrane, promoting the theory that the drug is effectively neutral when sequestered in the bilayer.

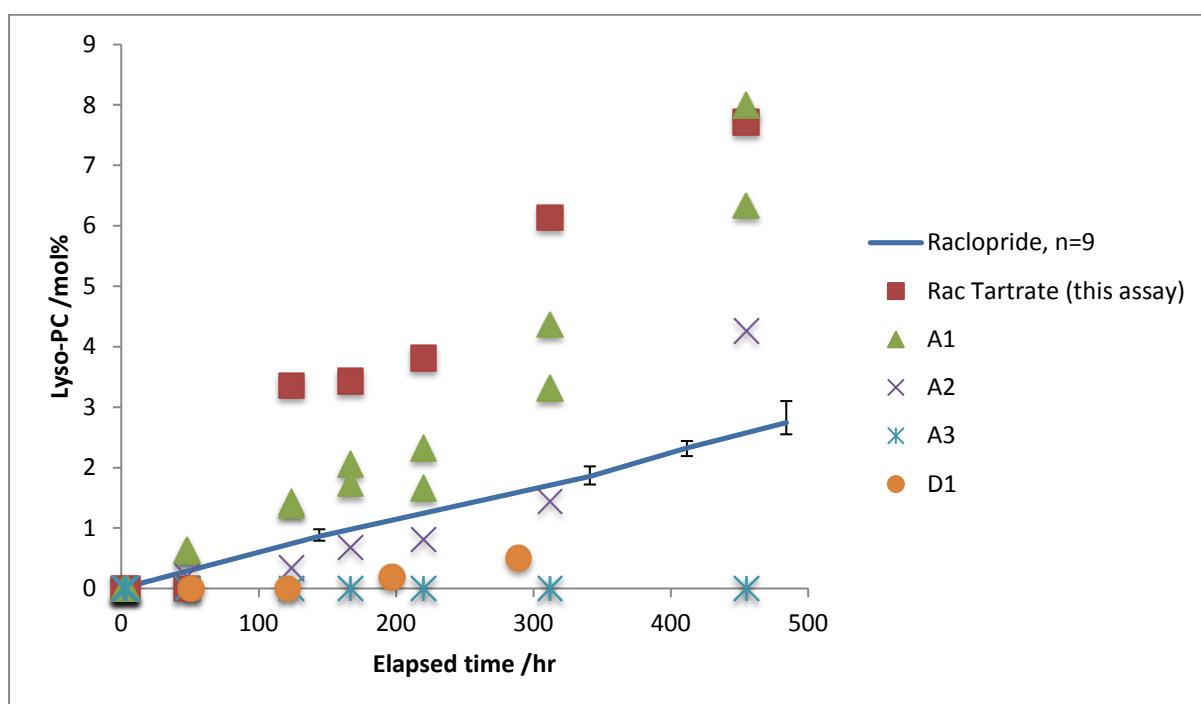


Figure 82: Hydrolysis of DOPC catalysed by compounds A1, A2 and D1, compared to both the standard raclopride data (Figure 33, blue line) and the data gathered using raclopride under identical conditions to the two test compounds (red squares). No other di-methoxy compound demonstrated any hydrolytic activity: A3 is shown for illustrative purposes.

Under similar conditions, **D2** was the only MANT compound to demonstrate any measureable hydrolytic activity, at a rate approximately half as fast as that of **A2** in the example above. Despite repeated testing, no lyso-PC was detected in the presence of any other MANT amide, suggesting that the additional basicity of the probe led to a change in orientation of the molecule relative to the bilayer normal. This is supported by preliminary modelling studies by Callum Dickson (unpublished work) which appear to show the probe compounds lying parallel to the membrane surface at the interface. However, these results are at a very early stage and caution should be exercised when drawing conclusions based upon them.

SAXS data, prepared from samples as described above and flame-and-silicone sealed into 1.5 mm diameter glass capillaries showed no changes of interest in the membrane packing of any of the MANT analogues tested over a period of some three weeks (see Figure 83, below). This is unsurprising considering the lack of hydrolytic activity displayed above, but further reinforces the notion that all these species, including those containing strongly basic tertiary amines, are net neutral when sequestered in a membrane at physiological pH. If this were not the case, significant swelling of the lamellae would be observed, analogous to that demonstrated in Figure 35 in the presence of haloperidol.

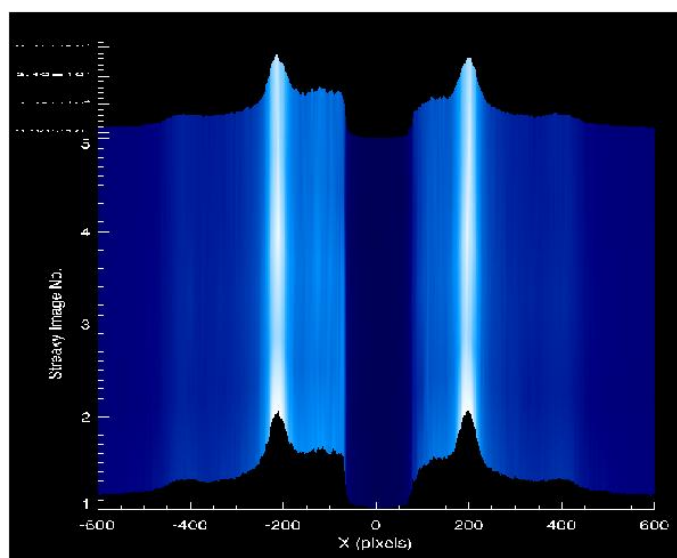


Figure 83: Collated data showing the small-angle X-ray scattering of a DOPC system containing 5 mol% **D1** analysed over the course of 600 hours. No measurable change in long-range packing was observed.

The implications for these findings and those from section 5.5.2 when compared to the drugs' respective hydrolytic activity suggests that previous hypotheses were to a large degree

incorrect: while the affinity of a CAD for its proton (its effective pK_a) does have a major impact upon its ability to trigger hydrolysis, the assumption that higher basicity $\rightarrow$ higher activity has been proven to be too simplistic. This data further supports the existence of a “Goldilocks zone” as described previously – an insufficiently basic compound resides too deeply within the membrane and does not cause hydrolysis, whereas if it is too basic the CAD is unwilling to relinquish its proton to the ester groups and so no reaction occurs.

7.5 Medical imaging applications

In the event, no PET labelling was achieved during the time of the study: a combination of experimental difficulties and logistical issues meant that no successful labelling experiments were performed upon the compounds listed in Table 6. Experimental difficulties revolved around the very low nucleophilicity of the target site chosen for ^{11}C labelling, due to its internally hydrogen-bonded resonance forms (shown below in Figure 84), similar to those found in keto-enol structures²²⁸ such as those found in curcumin²²⁹. These resonance forms were important as the source of the compounds' fluorescence, as the hydrogen-bonded structure forms a second delocalised ring leading to anthracene-like absorption and emission spectra²³⁰. However, by delocalising the electrons on the free aniline, its nucleophilicity was substantially reduced, making its attack by $[\text{}^{11}\text{C}]$ -methyl iodide or $[\text{}^{11}\text{C}]$ -methyl triflate unfavourable even when activated with a hydride reagent such as sodium hydride (see Figure 84).

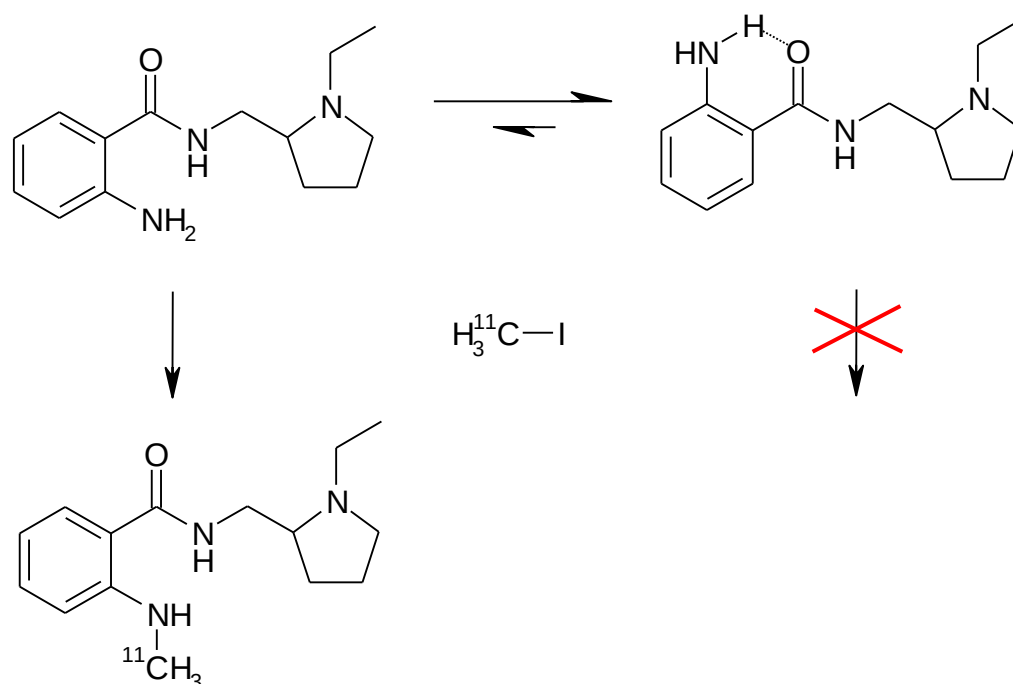


Figure 84: One of the resonance forms of **E1** that prevented efficient labelling with $[\text{}^{11}\text{C}]$ methyl iodide. Internal hydrogen bonding reduced the nucleophilicity of the free aniline to the point that reactions were so slow that they became unsuitable for PET study.

Repeated attempts at Aarhus University Hospital with compounds **E1**, **2**, **6** and **7** above 95% purity all showed similar results: labelling could be made to proceed, but at radiochemical yields in single digits making them insufficient for study. While this reaction is certainly feasible under

standard laboratory conditions, under the low reagent/short timescale regime of PET chemistry the labelling became impractical without significant optimisation work and the study was postponed. In the future, however, it should be perfectly possible to create such dual-modality imaging agents: work is currently on-going into the creation of amide bonds directly, using an aryl halide, an amine and a [^{11}C]-labelled carbon monoxide molecule over a palladium catalyst²³¹. This work will allow the direct, one-pot synthesis of compounds of interest such as the one above, greatly simplifying labelling protocols and allowing the direct comparison of *in vivo*, *ex vivo* and *in vitro* experimental techniques.

8 Conclusions

This study has made significant steps into understanding the mechanistic processes which underpin the hydrolysis of lipid membranes by raclopride, a model compound which bears strong structural resemblance to a wide range of commonly administered drugs. Significant development has been achieved in the identification and optimisation of a robust assay to quantify the phenomenon, and the data derived with these new techniques proved to be extremely reproducible across different batches of lipid, reagent and analytical conditions.

The investigation has overturned a number of the group's previous assumptions regarding the dependencies and mechanisms of reaction, leading to a very different experimental programme to the one originally envisioned. While no study into such a complex environment can realistically hope to identify every dependency of a process, the list of potential influencing factors has been significantly narrowed: the results in section 6 strongly suggest that simple geometric and curvature stress considerations do not adequately describe the behaviour observed, whereas the discovery that more ordered lipid arrangements promote raclopride's hydrolytic activity suggest that either diffusional timescales, drug-lipid residency or water penetration depth may play a significant role in determining how fast the drug can digest its way through a membrane. In that regard, the first of the three working hypotheses described in section 4.1.5 has been fully validated, and these findings are currently being prepared in a format suitable for publication.

It has also been demonstrated that, despite earlier theories to the contrary, having a fully protonated or very high pK_a basic group on the CAD molecule is not necessarily a predictor of potency. The evidence presented in sections 0, 5.6.2.2 and 7.3 strongly suggest that raclopride does its work as a predominantly neutral species, whereas more basic and thus more fully-protonated compounds have shown no activity at all under the assay conditions devised. This also has implications for the leaflet selectivity of the reaction: previously, it was thought that drugs added exogenously could only really influence the outer leaflet, as the flip-flop time for charged amphiphiles is so slow. However, the equilibration time for even very large neutral species such as long-chain fatty acids is very rapid⁶¹, meaning that raclopride could potentially be distributed equally throughout its host membrane over a timescale of seconds.

These findings contribute to suggestions that an ideal range of overlapping physico-chemical

parameters, a so-called “Goldilocks” zone, exists for drug hydrolysis and therefore, if the working hypotheses of the project (section 4.1.5), are to be believed, for drug transport as a whole. Such a concept has precedent – Lipinski’s rule of 5 has been the basis to a greater or lesser extent of drug discovery for a decade¹²¹. However, the understanding of this phenomenon offers the prospect of a genuine theoretical framework about which to hang our understanding of drug transport and bioavailability, rather than the current empirical rule of thumb.

Despite this promise, there is still no real consensus over whether the reaction has any influence over *in vivo* transport, or whether it is simply an interesting but fundamentally insignificant phenomenon when its influence is compared to diffusion and organic transporter pumps described in section 3.2. If the rates observed in the bulk phase studies revealed in sections 5.5 and 6 really are representative of those in a genuine cell membrane, then the study above can be nothing more than a footnote, describing an unusual and unexpected effect but one of only academic interest. However, the direct microscopy results from both in previous experiments⁵³ and touched upon in cell imaging experiments presented briefly in section 5.6.4.1 suggest that, by whatever mechanism, the membrane disruption caused by CADs in giant vesicles and native cell membranes is significantly faster and more universal than in condensed phase experiments. In both these cases, membrane shedding was catalysed by drugs which did not reveal their effects during the bulk-phase assays in this study, suggesting that the kinetics of reaction are very different between the two systems.

This sheds light upon the third of the working hypotheses discussed: these experiments and others like them have demonstrated that drugs sequestered within a membrane have no particular inclination to leave of their own accord and must be propelled by some external force or agent. In the case of raclopride and a number of other CADs, the evidence suggests that agent is of their own making, a detergent droplet formed from the degradation products of their original host. In those compounds where hydrolysis was not observed or was impossible by design, no such movement was observed and instead the compounds were content to reside in the membrane until their eventual precipitation or metabolism.

This provides hope that further study into the phenomenon will confirm not just whether or not and to what degree the process contributes to systemic transport, but also how it might be profitably used by pharmaceutical researchers and companies to minimise the drug dose

required by a patient to reach effective tissue concentrations. In this way, research following on from the above might make a genuine difference to patient healthcare, shrinking the volumes of drug that the body must metabolise and therefore expanding each compound's therapeutic window, limiting toxicity and minimising side effects.

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