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DEDICATION

For my family, especially G.T.K. and M.T.L.

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CHAPTER 1

INTRODUCTION

Biological membranes are a complex and essential component of living cells. These flexible membranes serve as a semipermeable barrier that allows the flow of essential nutrients into and out of the cell. In addition, they have been implicated in a variety of physiological processes (Kazuo Emoto 1996, Fadok, Bratton et al. 1998, Di Paolo and De Camilli 2006) through their ability to serve as signaling platforms. Biomolecules known as lipids in addition to proteins are a prominent component of the plasma membrane. Lipids provide proteins in these signaling platforms with a specific physicochemical environment required for their function and in addition, they can act as second messengers, which aid in both the activation of signaling cascades and the transfer of information from one cell to another. In 1972, Singer and Nicholson developed the Fluid Mosaic model of the plasma membrane. (Singer and Nicholson 1972) In the years since, the model has evolved but remains relevant. In the original model, the membrane consisted of a fluid phospholipid bilayer with mobile proteins distributed throughout. Additions to the model now include the high density of transmembrane proteins, lateral heterogeneity of the membrane (domains), curved membranes, non-lamellar structures, physiological impact of lipid phases, transbilayer lipid motion, and the idea that some proteins only bind the membrane occasionally. (Nicholson 2014) The plasma membrane is a bilayer composed of phospholipids, glycolipids, sphingolipids, proteins, cholesterol, and an array of minor constituents. The compositional diversity of cellular membranes is extensive, and relies heavily on the location of lipid synthesis. (van Meer, Voelker et al.

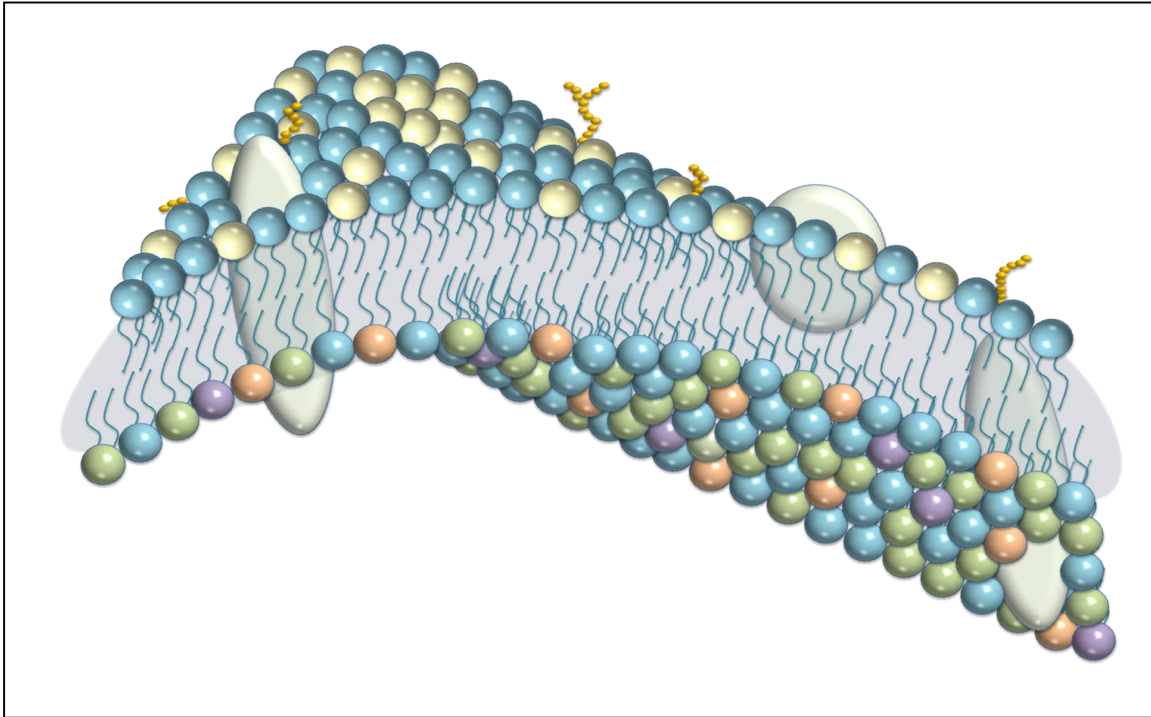


Figure 1: Schematic Representation of the Plasma Membrane. The outer leaflet of the plasma membrane is composed mainly of phosphatidylcholine, and sphingomyelin while the inner leaflet is predominately composed of phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol.

2008) Each organelle has a specific lipid composition, which impacts both the morphology and the function of the organelle. (van Meer, Voelker et al. 2008) For example, the endoplasmic reticulum is enriched in phosphatidylcholine and phosphatidylinositol while the outer leaflet of the mitochondrial membrane is enriched in phosphatidylcholine and phosphatidylethanolamine. (van Meer and de Kroon 2011) The plasma membrane bilayer is vertically asymmetric meaning that the composition of lipids in the inner leaflet differs from the lipid composition in the outer leaflet. While phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), account for more than half of the total phospholipids in most cell membranes PE, PS, and phosphatidylinositol (PI) predominantly comprise the lipids found in the cytosolic facing leaflet of the plasma

membrane, while PC and sphingomyelin compose a majority of the outer leaflet. (Figure 1) Due to the prevalence of PS and PI, which both have an overall negative charge, the inner leaflet of the plasma membrane has a net negative charge. In addition to being vertically asymmetric, the plasma membrane is also laterally heterogeneous. Lipid-lipid, lipid-bivalent cation, and lipid-protein interactions often lead to differences in the lateral homogeneity of the plasma membrane. Several factors are important for the modulation of plasma membrane fluidity and heterogeneity: degree and type of the unsaturation of the lipid tail groups, charges of the head groups, presence of bivalent cations, cholesterol, salt concentration, and opportunity for hydrogen bonding. The interior of the membrane is composed of the chain or tail groups of lipids, which can be both saturated and unsaturated.

Glycerphospholipids, also known as phospholipids are amphipathic. They exhibit a polar headgroup and a non-polar tail group, which leads to the formation of a lipid bilayer with polar and non-polar regions. The glycerol backbone of the phospholipid is linked to fatty acid chains through esterification to form the tail group, while the headgroup is bound to the glycerol backbone through a phosphate group. Due to the chirality of these molecules, the glycerol backbone is denoted as *sn*-glycerol 3-phosphate, where the *sn* indicates the stereochemical numbering. The nomenclature of the lipid is also dependent upon the saturation and length of the acyl chains that are linked to the glycerol backbone at positions *sn*-1 and *sn*-2. The most common acyl chain composition in the plasma membrane is a saturated acyl chain at the *sn*-1 position that is typically 16 or 18 carbons in length and an unsaturated acyl chain at the *sn*-2 position. The addition of one or more *cis* oriented double bonds in the unsaturated acyl chain leads to a “kink” in the chain, which impairs the packing of the lipid leading to a lowering of the phase transition temperature

and in many cases a fluid membrane at physiological temperatures. Through alterations of chemistries of the headgroup, saturation of and length of the acyl chains, more than 1,000 different lipid species can be obtained. (Fahy, Sud et al. 2007) Some common examples of phospholipids that are produced are phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, phosphoinositides, and phosphatidic acid.

Phospholipids have many properties that impact their behavior and the physiochemical properties of biological membranes. The polar and non-polar (interior) portions of the plasma membrane are defined by the polar head groups and the non-polar tail groups of the phospholipids. Based upon the chemistries attached to the glycerol backbone, the phospholipids can be defined as either anionic or zwitterionic. At physiological pH, the zwitterionic lipids will remain neutral, while the anionic lipids will have a negative charge. Additionally, the headgroup moiety of the lipid can alter the hydrogen bonding capabilities of the respective lipid. The differences in both charge and hydrogen bonding ability directly influence the lipid-lipid interactions within the bilayer as well as with other molecular species.

One particularly interesting class of lipids is phosphoinositides. Although phosphoinositides (PIPs) only comprise approximately 1% of the total lipids in the inner leaflet of the plasma membrane, they are important signaling molecules in many physiological processes. The inositol ring of PIPs can be phosphorylated at the 3, 4, and/or 5 position, giving seven different phosphorylated phosphatidylinositol species found in nature. (Figure 2) The monophosphates are represented by phosphatidylinositol-3-monophosphate (PI(3)P), phosphatidylinositol-4-monophosphate (PI(4)P), and

phosphatidylinositol-5-monophosphate (PI(5)P). Phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂), phosphatidylinositol-3,5-bisphosphate (PI(3,5)P₂), and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) comprise the bisphosphates, and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃) represents the only trisphosphate. Although PIPs may only make up a small portion of the plasma membrane, they play an integral role in many signaling events including cell metabolism and cell death (Kisseleva, Cao et al. 2002), cell proliferation (Carney, Scott et al. 1985), cell motility (Derman, Toker et al. 1997, Di Paolo and De Camilli 2006), and cytoskeletal rearrangement (Takenawa and Itoh 2001). In contrast to the low plasma membrane concentrations of phosphoinositides, it has been shown that phosphoinositide concentrations in the nuclear envelope can reach 10 – 15% (depending on the species). (Zhendre, Grelard et al. 2011) This suggests that PIPs in the nuclear envelope assume a structural/morphological rather than a signaling role. (Dumas, Byrne et al. 2010, Domart and Larijani 2012, Larijani and Poccia 2012, Larijani, Hamati et al. 2014) It has been shown that PIPs are not uniformly distributed throughout the inner leaflet of the plasma membrane, but instead can be found enriched in domains. (Gao, Lowry et al. 2011, van den Bogaart, Meyenberg et al. 2011, Posor, Eichhorn-Gruenig et al. 2013, Rosenhouse-Dantsker, Epshtein et al. 2014) These phosphoinositide pools have been shown to be dynamically regulated, which leads to an intricate spatiotemporal control of protein function and hence cell physiology. (Antal and Newton 2013, Posor, Eichhorn-Gruenig et al. 2013) The rich chemical functionality of the phosphoinositide headgroups allows phosphoinositides to interact with proteins in a highly specific manner and to recruit them to distinct cellular sites. It is therefore not surprising that for all phosphoinositide derivatives distinct proteins have been identified as binding partners. For example, PIPs

often regulate signaling events through specific binding to protein domains such as Pleckstrin Homology (PH) domains (Lemmon 2007), Bin-Amphiphysin-Rvs (BAR) domains (Itoh and De Camilli 2006) and Fab1, YOTB, Vac1p, and EEA1 (FYVE) domains (Gaullier, Simonsen et al. 1998) as well as a range of other domains that specifically bind a certain phosphoinositide headgroup. (Lemmon 2008).

The specificity in which PIPs function is remarkable. One example is the enzyme phospholipase C (PLC). PLC selectively hydrolyzes PI(4,5)P₂ to form two important

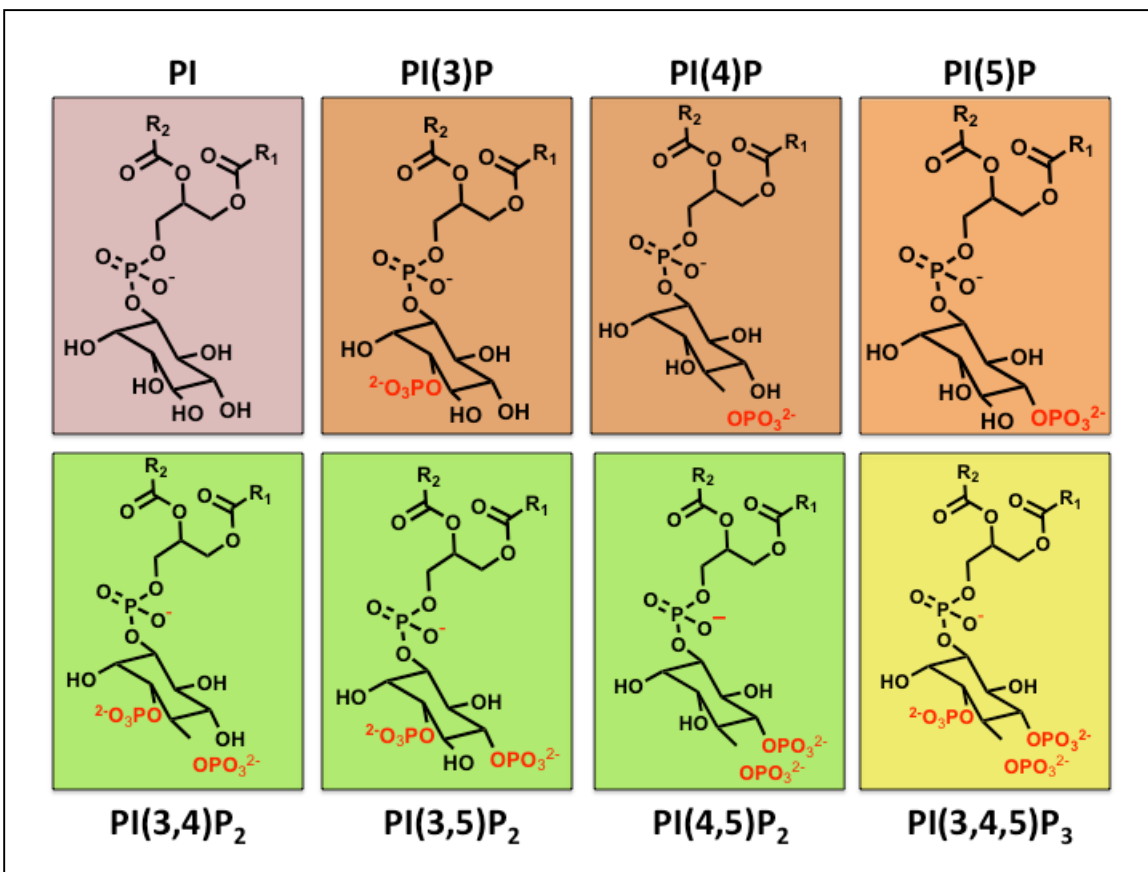


Figure 2: Structures of Naturally Occuring Phosphoinositides. There are seven naturally occurring phosphoinositides: phosphatidylinositol (PI) (pink), phosphatidylinositol monophosphates (orange, phosphatidylinositol bisphosphates (green), and phosphatidylinositol trisphosphate. Phosphatidylinositol monophosphates are PI(3)P, PI(4)P, and PI(5)P. The phosphatidylinositol bisphosphates are PI(3,4)P₂, PI(3,5)P₂, and PI(4,5)P₂. The phosphatidylinositol trisphosphate is PI(3,4,5)P₃.

second messengers, inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG). Once formed, IP3 is able to bind the inositol 1,4,5 trisphosphate receptor (IP3R) receptor causing the Ca²⁺ channels within the endoplasmic reticulum to open, resulting in an influx of Ca²⁺ into the cytosol. The rapid increase in calcium concentration triggers several intercellular responses including cytoskeletal rearrangement, and exocytosis in neurons. (Streb, Irvine et al. 1983, Yoshida and Imai 1997)

While the global plasma membrane concentration of PI(4,5)P₂ within the bilayer is less than 1% of the total lipids, it has been shown to bind almost 280 intracellular proteins. (Catimel, Schieber et al. 2008) In cells, PI(4,5)P₂ binds its protein partners in a specific manner at a particular time and place to provide spatiotemporal control of protein function. The question then arises as to how a lipid in such low supply within the bilayer is able to bind so many proteins with high specificity and provide functional control. A significant amount of evidence suggests that PI(4,5)P₂ is able to enrich in domains, thereby increasing its local concentration and creating a distinct environment for the respective protein to function by participating in the formation of compositionally distinct lipid/protein platforms. Many phosphoinositide binding proteins require for robust binding the presence of other anionic lipids that bind synergistically the respective protein. (Moravcevic, Oxley et al. 2012) The requirement for other lipids to be present for the protein to bind, adds an additional level of control for the protein function since all lipid binding partners need to be in close proximity for the binding event to occur. It is currently hypothesized that specific proteins from the PIP interactome are selected for membrane binding with the help of these multi-contact lipid/protein interactions. However, the mechanisms that lead to the formation of PIP enriched domains (platforms) is still elusive. (Leventhal, Christian et al.

2009, Ellenbroek, Wang et al. 2011, Graber, Jiang et al. 2012, Jiang, Redfern et al. 2014, Wang, Slochower et al. 2014) Owing to the highly negative PIP headgroup, it was initially expected that mutual repulsion would impair the formation of domains enriched in phosphoinositides. However, for distinct physiochemical conditions, evidence supporting the formation of phosphoinositide domains is substantial. Theories for the formation of PIP enriched domains include the formation of a hydrogen bond network (Redfern and Gericke 2004, Leventhal, Cebers et al. 2008), partitioning of PI(4,5)P₂ into cholesterol rich phases (Pike and Casey 1996) such as rafts, electrostatic sequestering (Golebiewska, Gambhir et al. 2006), partitioning out of cholesterol rich phases (Leventhal, Byfield et al. 2009, Leventhal, Christian et al. 2009), an increase in local production of PI(4,5)P₂ (Janmey and Lindberg 2004), and protein fence models (Vanmeer and Simons 1986, Golebiewska, Kay et al. 2011). The formation of phosphoinositide enriched domains in simple binary and ternary lipid mixtures even in the absence of positively charged clustering agents like bivalent cations or cationic proteins, was initially surprising.

The evidence supporting a hydrogen bond network formation for the stabilization of phosphoinositide domains is expanding. Initial studies by Redfern and Gericke suggested that hydrogen bonding played an important role in the formation of phosphoinositide monophosphate (PI(x)P) microdomains in binary PI(x)P/PC model membrane systems. (Redfern and Gericke 2004) They suggested that the hydroxyl groups of the inositol ring participated in the hydrogen bond network by acting as hydrogen donors, while the phosphomonoester, phosphodiester and other hydroxyl groups from adjacent molecules could behave as hydrogen bond acceptors. This argument was further strengthened with evidence that the phosphoinositide polyphosphates were also able to form domains.

However, it was interesting that PI(4,5)P₂ had less of a tendency than PI(x)P to form domains, suggesting that the presence of another molecule and/or lipid may be important for domain formation in a physiologically more relevant context. (Redfern and Gericke 2005) To further investigate the impact of hydrogen bonding in phosphoinositide domain formation, Kooijman et al. utilized magic angle spinning ³¹P nuclear magnetic resonance spectroscopy (NMR). Their data showed that phosphoinositides participate in a hydrogen bond network through both inter- and intramolecular hydrogen bonding, which leads to a “smearing” out of the phosphomonoester group charge. (Kooijman, King et al. 2009) More recent studies have been extended to study how cholesterol and calcium impact this hydrogen bond network. The addition of calcium or cholesterol independently results in a deshielding of the phosphomonoester group and an increase in the negative charge of PI(4,5)P₂. The addition of both cholesterol and calcium is additive, leading to a further increased ionization of PI(4,5)P₂. (Graber, Jiang et al. 2012, Graber, Gericke et al. 2014) These studies are an indication that the complexity of the PIP model membrane systems needs to be increased in order to understand the predominant interactions of PIPs in biological membranes.

In addition to phospholipids and proteins, cholesterol is also a prominent component of the plasma membrane. Cholesterol has been shown to act as a modulator of plasma membrane fluidity, often causing a condensation of the surrounding phospholipids and/or sphingolipids. Cholesterol is typically found in quantities of 30-40 mole% within the PM. The relative cholesterol concentrations in the two PM leaflets have not been satisfactorily determined. While cholesterol is largely hydrophobic, the hydroxyl group, which is generally oriented toward the aqueous environment of the cytoplasm, allows for a slight

amphipathic nature. Cholesterol has two fundamentally different effects on membrane fluidity. The polar hydroxyl group is typically found near the acyl chain ester bond of the surrounding phospholipids, allowing it to alter the membrane fluidity based on temperature and the local phospholipid environment. For a fluid lipid phase, the rigid ring structure of cholesterol leads to a slight increase of the lipid acyl chain order and hence a slight decrease of the plasma membrane fluidity. (Lindblom and Oradd 2009, Nicolson 2014) In contrast, lipids that form a gel phase at the respective temperature, will form a liquid ordered (l_o) phase in the presence of cholesterol. Studies have shown that not all lipids are able to move freely throughout the biomembrane, but instead reside in clustered regions called domains or rafts. Rafts are typically referred to when discussing clusters at the plasma membrane that are in the more dense l_o phase and are enriched in cholesterol and sphingolipids in the outer leaflet of the membrane. In contrast, a domain refers to any cluster of lipids. The concept of lipid rafts has been controversial in the field due to the lack of evidence in cells to support the theory. The transient nature of the rafts as well as the resolution limitations of traditional fluorescence microscopy makes them difficult to investigate. A recent study from Eggeling et al. supports the theory that lipid rafts exist in cells (Eggeling, Ringemann et al. 2009). In their study, Stimulated Emission Depletion (STED) microscopy, a single molecule fluorescence nanoscopy technique, was coupled with Fluorescence Correlation Spectroscopy (FCS) to monitor the diffusion of select lipids in living cells. They found for those lipids and anchors that have been theorized to be raft-resident a slowed diffusion when compared to PE and PS, both lipids that are not thought to be raft-resident. This is one of the first direct observations that lipid rafts may indeed be present in living cells. Even though these types of experiments are becoming more feasible, they are still limited

due to the expense and limited availability of fluorophores. While the general concept for lipid rafts in the outer leaflet of the plasma membrane is well established (gel phase sphingolipids interact with cholesterol to form an l_o phase), it is unclear at this point how the outer leaflet raft domain is linked to the inner leaflet raft domain, what composition such an inner leaflet raft domain would have, and how a liquid ordered phase is obtained. In contrast to the outer leaflet of the plasma membrane, the inner leaflet does not exhibit lipid species in sufficient quantities that form ordered phases at physiological temperatures, i.e., in general, the physiochemical foundation for the formation of an l_o phase domain as described above does not exist in the inner leaflet of the plasma membrane. It is important to note that several phosphoinositide mediated signaling events have been linked to lipid rafts. (Gao, Lowry et al. 2011, Koushik, Powell et al. 2013, Lin, Lu et al. 2014, Rosenhouse-Dantsker, Epshtein et al. 2014) However, the vast majority of these studies employ cholesterol depletion experiments with a physiological response as output. While it is tempting to link cholesterol dependent signaling to raft formation, this link is not necessarily correct since cholesterol impacts also in the absence of rafts phosphoinositide domain formation and hence protein binding.(Jiang, Redfern et al. 2014) It is therefore paramount to determine the conditions that lead to the formation of inner leaflet domains and identify the mechanisms that potentially link such domains to outer leaflet liquid ordered domains. The observation that cholesterol promotes clustering of both phosphatidylinositol and polyphosphorylated phosphoinositides in simple model membrane systems, provides a first glimpse at the mechanisms associated with cholesterol dependent inner leaflet plasma membrane domain formation. Previous data from our lab suggest that the headgroups of PI(4,5)P₂ form a hydrogen bond network in which

cholesterol can participate through its hydroxyl group (Jiang, Redfern et al. 2014). We currently hypothesize that cholesterol inserts between the phosphoinositide headgroups. This leads to a reduction of the electrostatic repulsion between the phosphoinositide headgroups. At the same time, cholesterol apparently participates in the hydrogen bond network formed between the phosphoinositide headgroups. Overall, this leads to a stabilization of phosphoinositide enriched domains. The stabilization of phosphoinositide enriched phases by cholesterol is so pronounced that 20% cholesterol is sufficient to stabilize phosphoinositide vesicles (phosphoinositides by themselves don't form vesicles). (Jiang, Redfern et al. 2014) It is an important question, to be explored in this thesis, whether the PIP/cholesterol interaction is modulated by the presence of other lipids or bivalent cations.

The tumor suppressor protein, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was initially identified as a tumor suppressor locus that is mutated in glioblastomas, breast, and prostate cancers. In addition to being frequently mutated in human cancers, PTEN also plays a role in diseases such as Bannayan-Riley-Ruvalcaba syndrome and Cowden disease. (Myers, Stolarov et al. 1997, Vazquez and Sellers 2000, Simpson and Parsons 2001) The protein consists of 403 amino acids and is composed of an N-terminal phosphatase domain, a C2 domain, and a C-terminal tail that contains a PDZ (Drosophila disc large tumor suppressor (Dlg1) and zona occludens 1 (ZO-1)) domain. PTEN has the ability to act as both a protein and lipid phosphatase. As a protein phosphatase, PTEN can selectively dephosphorylate acidic substrates such as tyrosine, but has also shown activity against threonine and serine to provide regulation. (Maehama T 1998) The lipid phosphatase activity comes from PTEN's ability to dephosphorylate

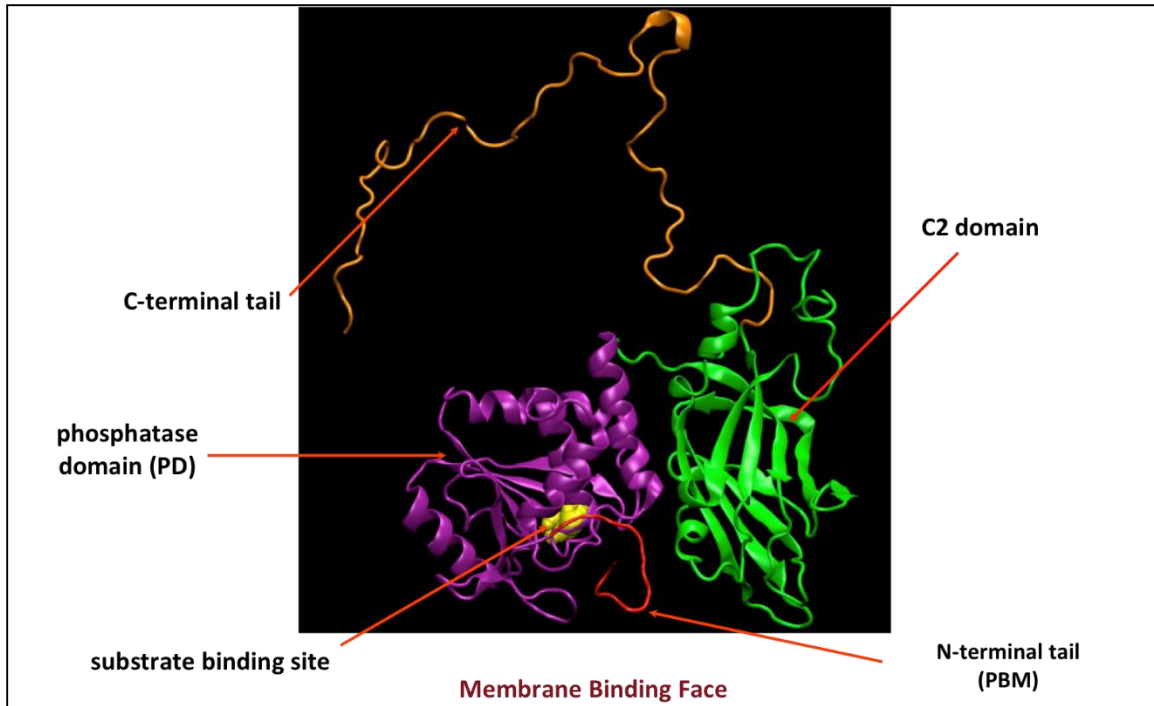


Figure 3: PTEN structure. Structure of PTEN based on the crystal structure and neutron scattering data.

phosphoinositides at the 3 position of the inositol ring. (Lee, Yang et al. 1999) PTEN's protein phosphatase activity is primarily associated with nuclear functions. Although it has several motifs which are found in protein tyrosine phosphatases (PTPs), it has been demonstrated that PTEN functions primarily as a lipid phosphatase. As a lipid phosphatase, PTEN has been shown to play a significant role as a tumor suppressor, namely as an antagonist to PI3K in the PI3K/Akt signaling pathway. A partial crystal structure of PTEN solved in 1999 by Lee et. al., revealed that the phosphatase domain is composed of a five β -stranded β -sheet that packs with two α -helices on one side and four on the other. (Lee, Yang et al. 1999) (Figure 3) The catalytic motif in PTEN (HCKAGKGR) forms the phosphate binding loop (P-Loop), which is located at the bottom of the active site. Unlike other PTPs and dual specificity protein phosphatases (DUSPs), the active site of PTEN is both broad and deep. The nature of this binding pocket in

addition to its positive charge makes it ideal for binding phosphoinositide substrates. The C-terminal end of PTEN consists of two antiparallel β -sheets with two α -helices between the strands to form an overall β -sandwich. This C-terminal region is responsible for binding the plasma membrane. In addition, PTEN also has a short N-terminal PI(4,5)P₂ binding domain (PBD) and a PDZ domain that sits within the C2 domain.

PTEN plays an important role in the regulation of the PI3K/Akt pathway. (Figure 4) In the Phosphoinositide-3-Kinase (PI3K) signaling pathway, a receptor such as receptor tyrosine kinase activates one of the PI3K isoforms. As a result, PI3K phosphorylates the 3 position of PI(4,5)P₂ to form PI(3,4,5)P₃. The proteins Akt, a serine/threonine kinase, and PDK1 (Phosphoinositide Dependent Kinase-1) both have

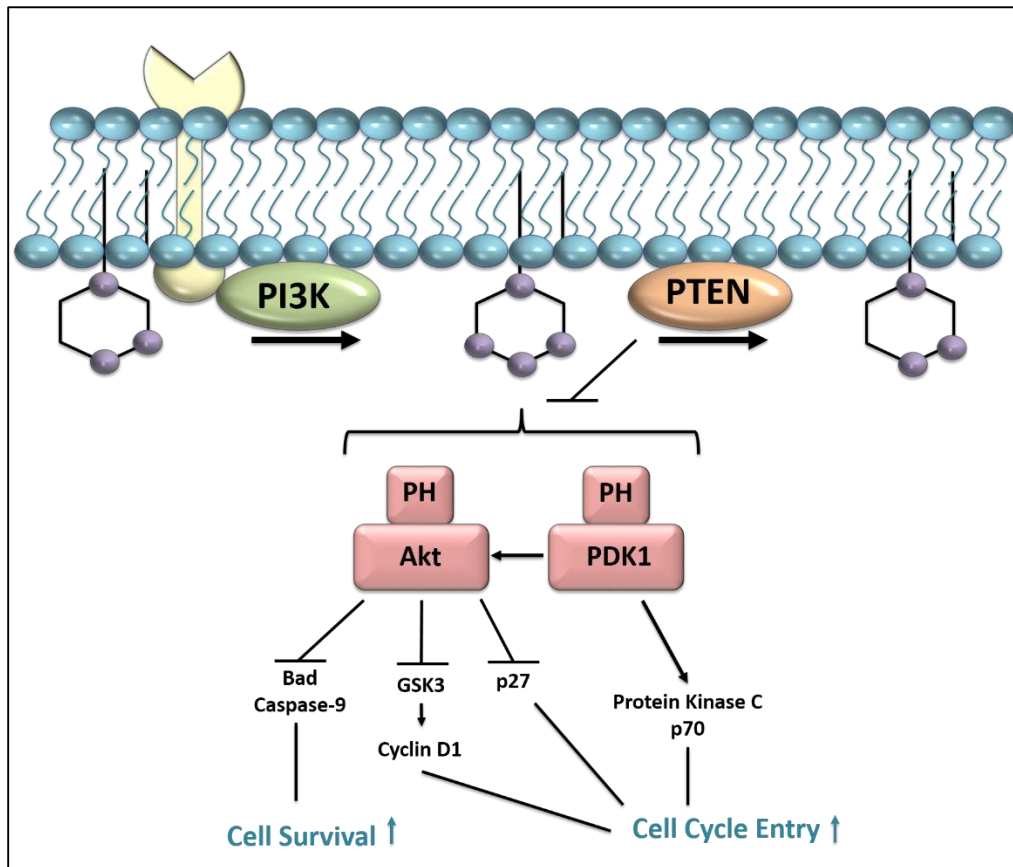


Figure 4: Schematic Representation of the PI-3 Kinase Pathway. The PI3K signaling pathway as a representation of the role that phosphoinositides play in spatiotemporal activation.

specific binding pockets for phosphoinositides, which causes the two cytoplasmic proteins to colocalize at the plasma membrane where PDK1 then activates Akt through phosphorylation. Akt subsequently inhibits several members of the apoptotic pathway through phosphorylation resulting in an inhibition of several proteins involved in apoptosis (cell survival signal) and an activation of proteins that promote cellular proliferation. The tumor suppressor, PTEN, acts as an important regulator of this pathway because it antagonizes PI3K by dephosphorylating the 3 position of PI(3,4,5)P₃ to form PI(4,5)P₂. Understanding PTEN function is an important goal of the scientific effort in our lab. The observation that PTEN binds synergistically PI(4,5)P₂ and PS (or another anionic lipid) makes it necessary to delineate the conditions that lead to the colocalization of these lipids. This is a major motivation of this thesis.

1.1 Objectives and Methodologies

Biological membranes are extremely complex, but lipid model membrane systems can be used to explore particular aspects of their properties and functions. Monolayers at the air/water interface (Langmuir films) are a particularly well-suited model system to mimic the inner leaflet of the plasma membrane because the composition can be varied widely (in contrast, many lipid compositions are not suitable for lipid bilayer formation), a range of powerful experimental techniques are available to investigate them and the interaction with bivalent cations and cationic proteins can be studied comfortably. Surface pressure/area (Π/A) isotherms provide thermodynamic information that can be used to investigate the phase state of the monolayer and obtain information about lipid-lipid interactions. In addition, epifluorescence microscopy can be used in tandem with

surface pressure/area (Π/A) measurements to gain insight into the morphology of the lipid monolayer.

The development of the Langmuir trough in 1917 by Dr. Irving Langmuir and Agnes Pockels allowed for the study of phospholipid monolayers at the air/water interface. Typically, these monolayers are characterized by surface pressure/area isotherms. Using these thermodynamic measurements, information about lipid-lipid interactions for different lipid compositions and subphase conditions can be inferred. By spreading a known amount of the lipid or lipid mixture of interest, the isotherm obtained can provide information about lipid packing densities and lipid phases.

While surface pressure measurements of Langmuir monolayers can provide some information about how the lipids are interacting with one another, epifluorescence microscopy is able to provide information about the morphology of the coexisting monolayer states and the regions in which they are present. (Knobbler 1990) Typical features of the plasma membrane such as lipid clusters, lipid domains, and lipid rafts can be studied using this model system and their properties can be studied based on lipid composition, pH, temperature, and the presence of bivalent cations or proteins.

Epifluorescence microscopy allows for the direct visualization of the changes in the lipid packing, phase transitions, and coexisting states through the incorporation of fluorophores. The fluorophore is able to partition into a distinct lipid phase based upon its chemical and physical properties, for example, a range of fluorescently labeled lipids are available that partition into either ordered or liquid disordered phases which allows for the direct visualization of such domains. In this technique a small amount, 1% or less, of a fluorescently tagged lipid is added to the lipid monolayer of interest. Once the

probe is excited, images of the monolayer can be taken. The partitioning and difference in intensity can provide information on the different lipid densities. The use of fluorophores is somewhat controversial as they can be seen as an impurity in the monolayer, which could lead to false information about the monolayer; however, other techniques such as Brewster Angle Microscopy (BAM), which do not use fluorescently labeled probes, have provided similar information as to that gained with epifluorescence microscopy. (Knobbler 1990)

The overall goal of this thesis is to study the conditions that lead to the formation of phosphoinositide enriched domains in increasingly complex lipid mixtures. We hypothesize that some of the chemical species found in the inner leaflet of the plasma membrane promote phosphoinositide domain formation, while others will inhibit the formation of such domains. Furthermore, we hypothesize that bivalent cations like calcium will further modulate the in-plane interactions among the different lipid species by interacting with their headgroups. In contrast to many other studies, we will focus on lipid systems that are in the fluid phase. While this is significantly more challenging than studying lipid monolayers in a more ordered phase state, it is a better representation of the physiologically relevant case.

We will begin our study by investigating the mutual interactions of PI and PI(4,5)P₂ individually at the air/water interface in the presence of Ca²⁺. We are choosing PI and PI(4,5)P₂ for our studies because they are the most prevalent phosphoinositide lipids in the PM. While previous studies from our group have focused on comparing the physicochemical behavior of the various PIP derivatives, this study focuses on varying the interaction partners of PIPs. We hypothesize that the interaction of the highly

negatively charged head group of PI(4,5)P₂ with calcium will lead to a greater condensation of the monolayer in comparison to the calcium free case than what is observed for PI monolayers in the absence and presence of calcium. These experiments should provide us with basic information about how PI and PI(4,5)P₂ behave individually in the presence of a divalent cation. In addition, we will visualize the PI and PI(4,5)P₂ monolayers utilizing epifluorescence microscopy. For these experiments, it is our hypothesis that we will be able to observe domain formation in the presence of calcium.

Next, we will study the impact of membrane fluidity modulators such as calcium and cholesterol on mixtures of PE/PI and PE/PI(4,5)P₂ in order to better mimic the inner leaflet of the plasma membrane. While there have been studies that investigated the effect of calcium on PI(4,5)P₂ domain formation in mixed monolayers with PC, studies that utilized a lipid mixture better representative of the inner leaflet of the plasma membrane have yet to be carried out. (Leventhal, Christian et al. 2009, Ellenbroek, Wang et al. 2011) In the presence of PC, PI(4,5)P₂ has been shown to form domains; however, it is our hypothesis that Ca²⁺ induced PI(4,5)P₂ domain formation will be altered by the presence of PE since PE and phosphoinositides are expected to form hydrogen bonds. Both PI and PI(4,5)P₂ have been shown to form domains in the presence of cholesterol in giant unilamellar vesicles and monolayers at the air/water interface. (Jiang, Redfern et al. 2014) The question arises whether this will still hold true in the presence of PE, since PE potentially interferes with the cholesterol/phosphoinositide interaction. Therefore, we will add PE to PI/cholesterol and PI(4,5)P₂/cholesterol mixtures to investigate PE's influence on the morphology and phase behavior of the monolayers.

In the next step, we will use Langmuir monolayers to better understand the lipid-lipid interactions occurring between PI and PI(4,5)P₂ in the presence and absence of the modulating factors mentioned above. Previous data from our lab showed that PI/PI(4,5)P₂ form domains in mixed model systems with PC. (Graber, Jiang et al. 2012) From a physiological point of view, formation of PI/PI(4,5)P₂ mixed domains is of major importance because PI is found at about 6-10% of the total plasma membrane lipids, a percentage significantly higher than that of the other phosphoinositides. We hypothesize that PI/phosphoinositide domains are the foundation for platforms that allow proteins to interact with phosphoinositides in an environment that enables not only the specific protein/phosphoinositide interaction, but also provides a protein with a negatively charged environment it is attracted to through non-specific electrostatic interactions. Initially, these studies will be performed in the absence of calcium; however, calcium will be added at later steps to increase the complexity of the system as well as to investigate how these modulators impact the morphology of the membrane. We hypothesize that PI/PI(4,5)P₂ mixtures will form domains in the presence of calcium.

CHAPTER 2

TECHNIQUES

2.1 Introduction to surface pressure/area isotherms

The Langmuir trough is a measurement device that can be used to investigate lipid monolayers at the air/water interface. The surface pressure/area isotherms obtained from these experiments provide valuable information about the thermodynamic properties of the monolayer and hence lipid-lipid interactions can be analyzed. The Langmuir trough set up consists of two movable barriers placed above a rectangular Teflon trough on either side. The barriers are used to compress the lipid monolayer, and are controlled by the NimaTR516 computer program. In addition to controlling the movement and speed of the barriers, the computer program is also used to record the data in the form of a pressure/area isotherm. To begin an experiment, the lipid mixture of interest is dissolved in an organic solvent and this solution is spread on the water surface in a dropwise manner using a 25 μ L syringe. The organic solvent is then allowed to evaporate, leaving the lipid monolayer of interest on the buffer surface.

The surface tension between the barriers decreases in the presence of a lipid monolayer. The surface pressure can be described by the following equation:

$$\pi = \gamma_0 - \gamma$$

Where π is the surface pressure, γ_0 is the surface tension of the subphase in the absence of the monolayer, and γ is the surface pressure when the lipid monolayer is present at the air/water interface. The change in surface pressure between the two barriers is monitored

by using a Whilhelmy plate. As the barriers are compressed, the area is reduced, and the surface density of the lipid molecules increases, resulting in a decrease in γ and an increase in π . The resulting data is a plot of the surface pressure (π) verses area ($\text{\AA}^2/\text{molecule}$) where the area decreases as the surface pressure increases.

Each specific region of an isotherm gives information regarding the phase state of the monolayer. (Figure 5) After spreading, the monolayer is in a gaseous phase state where the area/molecule is large, and hence the molecular density is lower, and the surface pressure is low. Upon compression, the monolayer enters a liquid-expanded/gaseous (LE/G) coexistence phase, which is characterized by a surface pressure $< \sim 0.2\text{mN/m}$. Upon further compression of the monolayer, the phase of the Langmuir film changes from a liquid-expanded phase to a liquid-expanded/liquid-condensed (LE/LC) coexistence region, and finally to a solid (S) state. Each state (LE, LC, and S) corresponds to a decrease in the area/molecule and an increase in the surface pressure (π). Surface/pressure isotherms offer a wealth of information, making them an ideal technique to study the impact of chemical and physical conditions on the lipid monolayer characteristics. Variations in the thermodynamic behavior of the monolayer affect the shape of the π/A -isotherm, indicating changes in the molecular packing of the lipid interactions between the lipid molecules. It should be noted not all Langmuir films will go through all of the states described here during compression of the monolayer.

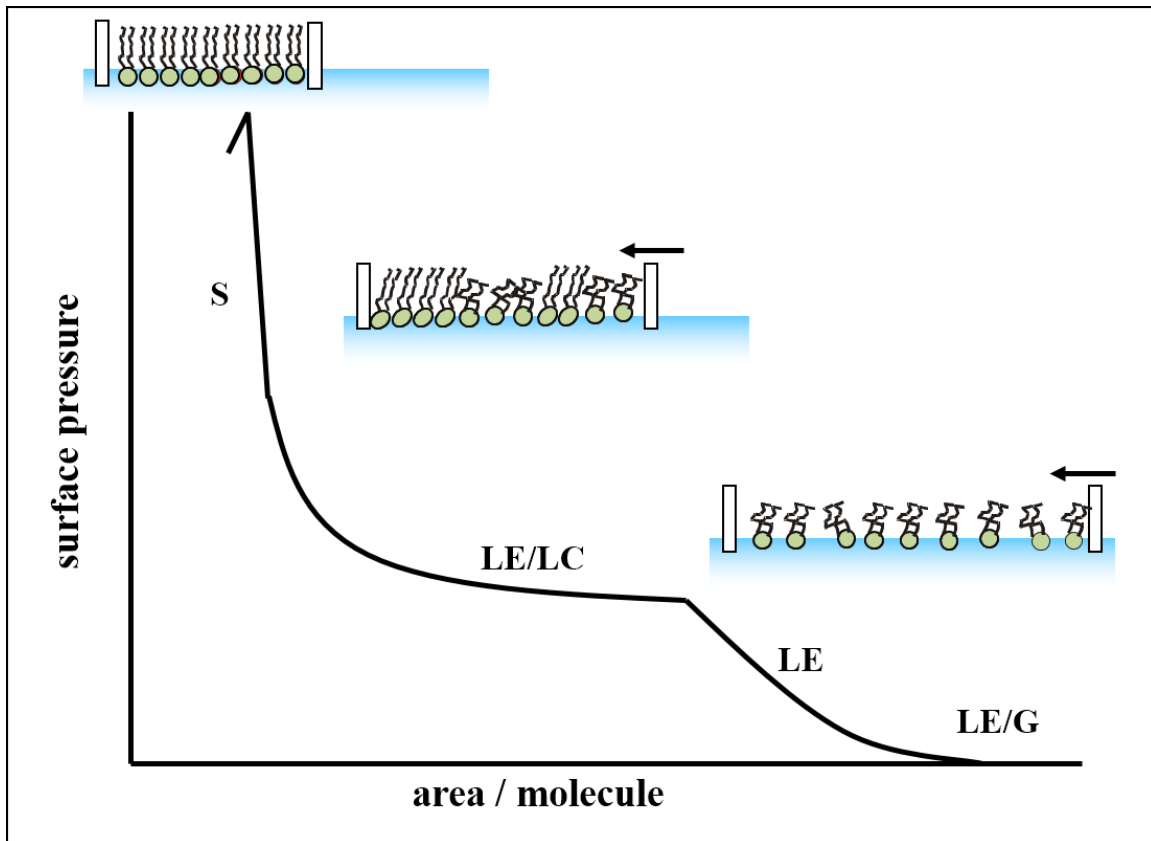


Figure 5: Schematic Representation of Surface Pressure/Area Isotherm. The surface pressure/area isotherm is representative of the different states in which the monolayer can exist.

2.1.1 Aim of surface pressure/area isotherm measurements

The phospholipids used in this study aim to mimic significant aspects of the plasma membrane inner leaflet. In the majority, lipids with acyl chain compositions that lead to fluid phases at room temperature were used for our studies. i.e., either one or both acyl chains of the respective lipids exhibited a single or multiple cis bonds. These lipids were chosen because their physiochemical behavior mimics the behavior of lipids at the inner leaflet of the plasma membrane. In some instances, lipids with two saturated acyl chains were used in order to validate conclusions drawn for the unsaturated, fluid phase forming lipids. In general, experiments with unsaturated acyl chain lipids are more challenging

because of a reduced monolayer stability and data reproducibility. Due to the highly unsaturated nature of the majority of phospholipids used in this study, we hypothesize that most pressure/area isotherms will be characteristic for a monolayer in a more expanded form. In the case of PI(4,5)P₂, we suggest that this expanded form will also be a result of the highly negatively charged headgroup that gives rise to repulsive forces. We expect that PE and PI affect in different ways PI(4,5)P₂ phase behavior through the formation of hydrogen bonds. This interaction is expected to be further modulated when Ca²⁺ ions are present in the subphase. To investigate these interactions, we use Langmuir monolayer films at the air/water interface since changes in the lipid-lipid interaction result in π/A -isotherms that are, depending on the nature of the interaction, characteristic of more expanded or condensed monolayers. By incorporating additional lipids, we are able to gradually increase the complexity of the monolayer, which results in films that mimic more accurately the conditions in the inner leaflet of the plasma membrane, and at the same time, this stepwise approach makes it possible to interpret data from a rigorous physical chemistry point of view.

In addition to studying the interaction of PE with both PI and PI(4,5)P₂, we will incorporate calcium and cholesterol to investigate their impact on the monolayer properties. Based upon the shape analysis of the π/A -isotherms, we will obtain information about how these additives modulate the lipid-lipid interaction.

2.1.2 Methodology of surface pressure/area isotherm measurements

A buffer solution of 10mM Tris, 150mM NaCl, 0.1mM EDTA, at pH 7.4 was used for all initial experiments. For experiments using divalent cations, the buffer composition

was 10mM Tris, 150mM NaCl, and with varying concentrations of Ca^{2+} (0.1mM, 1mM, and 2mM) at pH 7.4. The lipids were received in powder form from Avanti Polar Lipids. (Alabaster, AL) Upon arrival, lipids were dissolved in an appropriate organic solvent mixture, typically 20:9:1 chloroform:methanol:water for PI(4,5)P₂. To determine the accurate lipid concentration of these solutions, a phosphate assay adapted from previous works was performed in triplicate. (Rouser G 1970) For more detail regarding this protocol, please see Appendix (A2). The experiments were performed on a Nima 601M trough (Coventry, UK) that was temperature controlled at $25 \pm 0.2^\circ\text{C}$ for all experiments. (Figure 6)

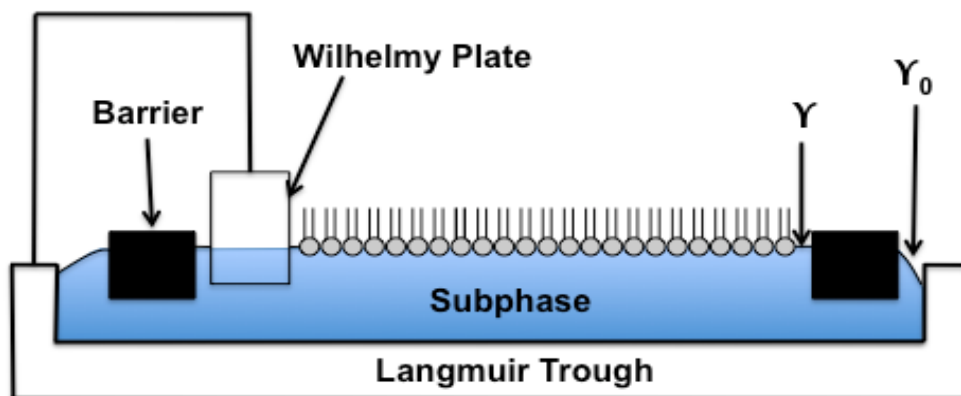


Figure 6: Schematic of a Langmuir Trough. The trough is filled with a buffer subphase of interest, and the lipid monolayer is applied using a 25 μL syringe. A Wilhelmy plate measures the change in surface pressure as the barriers on either side of the trough are moved toward each other.

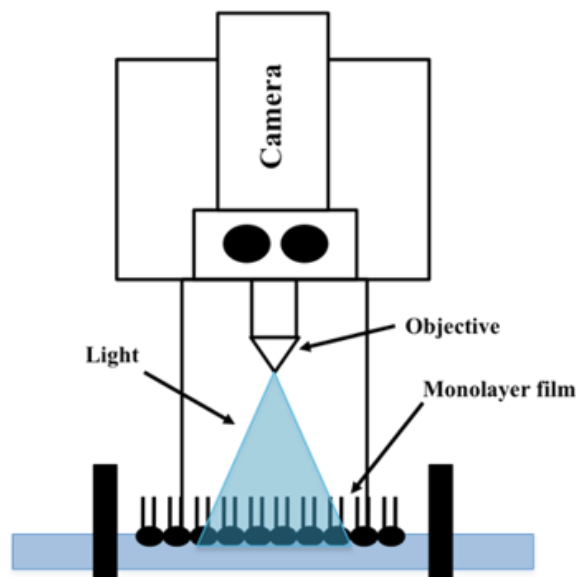
The trough and barriers were cleaned thoroughly between experimental runs with a mixture of hexane and ethanol. The trough was then filled with the buffer subphase of interest. Before each experiment, the barriers were subjected to a series of compression/expansion cycles to ensure there was no pressure increase upon compression (e.g., due to the transfer

of surface active impurities from the bulk to the buffer surface). If the pressure increased, any particulates present at the surface were removed by suction. Once the compression of the barriers resulted in a lack of pressure increase, the barriers were moved to the fully open position, and the surface pressure was set to zero. The calculated volume of lipid stock solution was then deposited onto the subphase using a 25 μ L Hamilton syringe. After 10-15 minutes, in which the organic solvent was given time to evaporate, the barriers were compressed at a rate of 7.3 $\text{\AA}^2 \text{ molecule}^{-1} \text{ min}^{-1}$, and the resulting surface pressure/area isotherm was recorded. For the experiments performed in this thesis, the total area/molecule is calculated based on the total moles of only the phospholipid(s) that are present in the monolayer. These experiments were repeated a minimum of three times for each lipid composition, and the area of the isotherms had to be within 1-2 \AA stroms to be considered reproducible. The error for the isotherms is 2 $\text{\AA}^2/\text{molecule}$ except for the isotherm of PI(4,5)P₂ in the absence of calcium, where the error is larger (5 $\text{\AA}^2/\text{molecule}$) due to instability of the monolayer.

2.2 Introduction to epifluorescence microscopy of Langmuir films

In addition to studying lipid monolayers at the air/water interface through surface pressure/area experiments, epifluorescence microscopy can also be used in tandem to visualize morphological changes in the monolayer. (Figure 7) Epifluorescence microscopy has been used frequently to image lipid monolayers in addition to studying the interaction of lipids and proteins. (Knobbler 1990) Fluorescently labeled lipid probes are incorporated into the lipid monolayer at concentrations less than 1mol%. The distribution of the fluorescent dye is dependent on its physiochemical properties in relation to the

physiochemical properties of the monolayer constituents. Most commonly, lipid fluorophores that either partition into ordered or disordered phases are used to visualize monolayer domains of different order. Since in our study some of the observed demixing processes were associated with fluid/fluid demixing processes, this strategy failed to highlight the morphological differences in some instances. In these cases, we used a fluorescently chain labeled lipid that matched the headgroup of the lipid species we wished to investigate with respect to its distribution. (e.g., if the lipid of interest is PI(4,5)P₂, we used Top Fluor™ PI(4,5)P₂). Incorporation of the probe allows for the study of alterations in the morphology of the monolayer due to subphase modulators like pH, ionic strength or the presence of divalent cations or modulators that are present in the monolayer like cholesterol. The morphology of a monolayer is a reflection of the interaction between the film constituents. Epifluorescence microscopy of Langmuir films was the first method that



was capable of visualizing the morphology in a lipid model system and it remains an extraordinarily powerful tool for the characterization of domains and to identify the conditions that lead to their formation. (Tschärner and McConnell 1981, Stottrup, Nguyen et al. 2010)

2.2.1 Aim of epifluorescence microscopy experiments

The aim of our epifluorescence experiments is to visualize the presence or absence of macroscopically discernible lipid domains. Although our surface pressure/area experiments can tell us much about the interactions of our lipids of interest via thermodynamic measurements, epifluorescence microscopy will allow us to visualize changes in the morphology of the monolayer.

By comparing single component lipid monolayers to binary and ternary mixtures we will gain information about how the additional lipid components influence domain formation. The addition of calcium to the subphase will also provide information about changes to the lipid morphology of the membrane in both single component lipids monolayers and mixtures.

2.2.2 Methodology of epifluorescence microscopy

For the epifluorescence microscopy experiments, the lipid was prepared as previously described for the surface pressure/area isotherm measurements. The experiment is performed the same way as the surface pressure/area experiments; however, for epifluorescence experiments, as the barriers are compressed, a series of fluorescent micrographs are taken, and the corresponding pressures are recorded.

For the epifluorescence microscopic studies, 0.1-0.2mol% of fluorescently labeled

Figure 7: Schematic Representation of Epifluorescence Microscopy Set Up. The upright microscope set up allows the focused beam to excite the fluorescently labeled lipid from above, allowing for the direct observation of morphological changes in the monolayer.

lipid (NBD-PC, Top Fluor PI(4,5)P₂, Bodipy PI, or NBD-PE) with the remaining mol% consisting of the unlabeled lipid(s) of interest was used. Experiments were carried out using an Olympus BX51 upright microscope (Center Valley, PA) equipped with a Hamamatsu EM-CCD camera (Bridgewater, NJ). A 100-watt Hg short arc lamp (Augsburg, Germany) was used as a light source. The microscope also houses a dichroic mirror, excitation and emission filters as well as an aperture diaphragm. A Nima 601M trough (Coventry, UK) temperature controlled to $25\pm 0.2^{\circ}\text{C}$ was added to the microscope below the objective to allow for simultaneous acquisition of both pressure/area isotherms and fluorescence micrographs. The fluorescent images were obtained using a 40X long working distance objective and an EM-CCD camera with an exposure of 30 frames per second. For all experiments a FITC filter cube (Semrock Rochester, NY) was used.

The lipid monolayers were compressed to the desired pressure and images were taken. The monolayer was then compressed further to the next surface pressure of interest. This process was repeated until the collapse pressure was reached. Because the monolayer compression was stopped during the compression to take images, the corresponding surface pressure/area isotherms were not used. Instead, separate isotherms were recorded and the isotherms are used as a reference for the epifluorescence images obtained.

CHAPTER 3

MIXED PHOSPHATIDYLINOSITOL/PHOSPHATIDYLETHANOLAMINE MONOLAYERS AT THE AIR/WATER INTERFACE

3.1 Introduction

Phosphoinositides play a significant role in many physiological processes by providing spatiotemporal control of protein function. The lateral distribution of phosphoinositides within the cytoplasmic leaflet of the plasma membrane is partially dictated by their ability to form clusters/domains under particular physiochemical conditions such as pH, presence of divalent cations, and temperature. At physiological pH, phosphoinositides are both highly negatively charged and are able to form hydrogen bonds. The ability of phosphatidylinositol (PI) and phosphoinositides (PIPs) to interact with cationic species is rooted in the negative charge of the headgroup. At the same time, the hydrogen bond capabilities of PI and PIPs further modulate their mutual interactions as well as their ability to interact with other membranous entities. The distinct stereochemical properties of PIPs enable specific interactions of their headgroups with protein targets that exhibit PIP specific binding domains (e.g. PH, ENTH, FYVE) (Gaullier, Simonsen et al. 1998, Lemmon 2007, Lemmon 2008) and at the same time, cationic proteins and peptides like MARCKS are able to strongly affect the lateral distribution of PI and PIPs. (McLaughlin and Murray 2005)

As the precursor to phosphorylated phosphoinositides, PI is of particular interest. Phosphorylation at the 3, 4, or 5 hydroxyl group of the inositol ring leads to three monophosphorylated PIPs, which participate in a broad range of signaling pathways. PI3-

Kinase (PI3K) can specifically phosphorylate the 3 position of PI to form PI(3)P. Synthesis of PI(3)P enriched pools have been linked to endocytotic and vesicular trafficking events. (Herman and Emr 1990, Odorizzi, Babst et al. 2000) The formation of PI(4)P through PI4-Kinase (PI4K) is essential for cellular signaling, but is particularly important in the Golgi and the trans-Golgi network where it plays a role in vesicle and endosomal trafficking. (Wang 2007, D'Angelo, Vicinanza et al. 2012, Tan and Brill 2014) The other monophosphate, PI(5)P, which is produced via PI5-Kinase (PI5K) is responsible in part for maintaining T-cell homeostasis. (Guittard, Mortier et al. 2010) Central aspects of how phosphoinositide production is controlled and spatially organized are still elusive. PI transfer proteins (PITP) are emerging as important temporal regulators of localized phosphoinositide pools and disruption of PITP function leads to a broad array of disease states. (Grabon, Khan et al. 2015) For example, PITP function and phospholipase C (PL-C) activity are directly linked via feedback loop. Phosphatidic acid (PA), produced during PL-C activation, transported by PITP to the ER where is converted to PI. (Cockcroft and Garner 2013) The transport of PI to the PM allows for a replenishment of PI(4,5)P₂ pools through 4- and 5-PI kinase action. This illustrates how important it is to understand how PI organizes in the PM or other intracellular membranes, which is a factor currently strongly underexplored.

PI accounts for about 6-10% of the lipids in the inner leaflet of the plasma membrane. The overall negative charge of PI is a result of the phosphodiester group, which links the glycerol backbone and the inositol ring. (Figure 8A) The negatively charged PI contributes to the overall negative charge of the plasma membrane. For our studies we use liver PI, which has an acyl chain composition that is typical for PI throughout human tissues (only

GPI anchors, the PI moiety displays a double saturated acyl chain composition). The majority of fatty acid chains represented in the natural occurring PI are stearyl (C_{18}) arachidonyl (C_{20} quadruple unsaturated). Based on the unsaturated nature of the fatty acid chains and the negative charge of PI, it would be expected that repulsive forces would lead to a fluid phase. In Langmuir film experiments, fluid monolayers are characterized by a featureless, expanded surface pressure/area isotherm. From a morphological point of view, such monolayers don't show domains even at low surface pressures. However, there may be some intramolecular and/or intermolecular hydrogen bond formation, which may enable a tighter packing of the lipid molecules. In PI, the intramolecular hydrogen bonding could occur between the inositol ring hydroxyl groups and the phosphodiester group (direct or water-mediated), which would lead to a reduction of the charge density since the charge would be spread out around the ring and would not be localized solely at the phosphodiester group.

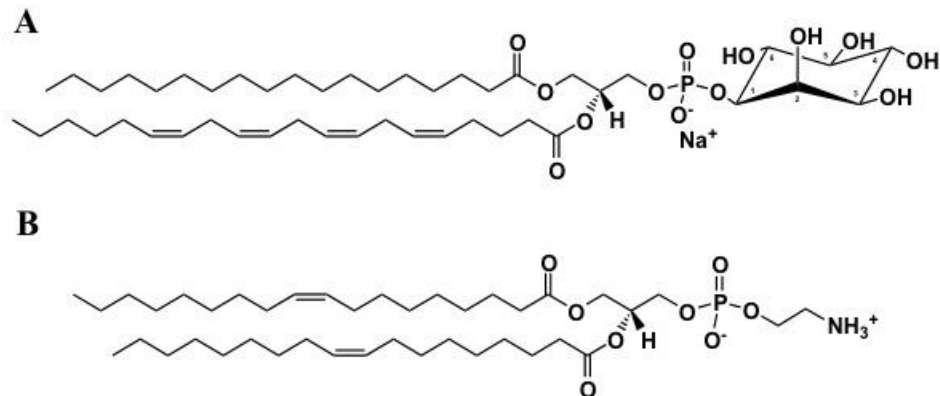


Figure 8: Structures of Phosphatidylinositol and Dioleoylphosphatidylethanolamine. A. The chemical structure for the most abundant chain composition of liver phosphatidylinositol (liver PI) B. The structure of dioleoylphosphatidylethanolamine (DOPE).

Phosphatidylethanolamine (PE) is the second most abundant phospholipid in the plasma membrane and one of the main lipid constituents of the inner leaflet. In comparison to the trimethylammonium headgroup of PC, the ammonium headgroup of PE is much smaller and can engage in hydrogen bond formation with other membrane resident species. (Figure 8B) Because of its small size and its ability to engage in hydrogen bond formation, PE can intercalate between other lipids with larger head groups. (Figure 9) Due to the small size of the PE headgroup relative to the footprint of the acyl chains, it is also able to induce a negative curvature in the plasma membrane, making it essential in roles involving endocytosis and exocytosis. In addition, the headgroup geometry of PE is important for the functional embedding of membrane proteins. (van Meer and de Kroon 2011) The lateral distribution of PE has also been shown to be important for the activation of various membrane proteins such as phospholipase D (PLD), protein kinase C, and calcium pumps. (Navarro, Toivio-Kinnucan et al. 1984, Yeagle and Sen 1986, Bazzi, Youakin et al. 1992, Nakamura, Kiyohara et al. 1996) In addition, it has been suggested that PE plays a pivotal role in cell division. The distribution of PE impacts the function of myosin II, which leads to issues with the proper function of the contractile ring assembly required for cell division. (Kazuo Emoto 1996)

The interaction of divalent cations with phospholipid monolayers has been shown to have a significant impact on their lateral distribution and on the condensation of the monolayer. (Gericke and Huhnerfuss 1994, Bagg, Abramson et al. 1996, Kutscher, Gericke et al. 1996) For lipids with highly negatively charged headgroups, the effect is even more prevalent. (Leventhal, Christian et al. 2009, Slochower, Wang et al. 2014, Wang, Slochower et al. 2014) Though similar in size, calcium seems to be more impactful on the physical state of these monolayers than magnesium, which might be attributable to

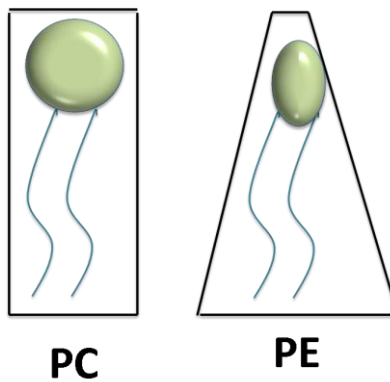


Figure 9: Schematic Representation of Phosphatidylethanolamine Geometry. The geometry of the PE headgroup induces a negative curvature in the plasma membrane.

the differences in the hydration shell between Mg^{2+} and Ca^{2+} (Ca^{2+} has a smaller hydration shell). In the case of calcium, it may cause a bridging and/or screening of the negatively charged phospholipid headgroups leading to a stronger condensation of the monolayer than what is observed for Mg^{2+} . Since the interaction of Mg^{2+} with PIPs has been found to be small, this study focuses on Ca^{2+} .

In previous studies of vesicle fusion, it was found that the presence of Ca^{2+} , triggered an increased tendency for fusion for mixed PE/PI vesicles. (Sundler, Duzgunes et al. 1981) Through the use of NMR, it has also been suggested that PI is able to stabilize PE bilayer systems in the presence of calcium. However, in the absence of PE, the addition of Ca^{2+}

leads to PI vesicle aggregation. (Nayar, Schmid et al. 1982) Taking this data into account, we hypothesize that PE intercalated between PI molecules, leaving the phosphodiester group of PI more accessible for Ca^{2+} .

Cholesterol has also been shown to modulate the lateral organization of plasma membrane lipids. Much attention has been devoted to the formation of liquid-ordered phases (“rafts”) due to the presence of cholesterol in the outer leaflet of the plasma membrane, a mechanism that does not apply in this form for the inner leaflet since the inner leaflet of the plasma membrane is void of lipids that form gel phases at physiological temperatures (the presence of cholesterol transforms a gel phase into a liquid ordered phase). In terms of inner leaflet plasma membrane lipids, our group has shown previously that cholesterol induces domain formation in pure PI and PIP monolayers (Figure 11). (Jiang, Redfern et al. 2014) Jiang et al. furthered their studies by testing cholesterol derivatives, and determined that the hydroxyl group of cholesterol (Figure 10) was essential for this domain formation. Considering the importance of the cholesterol hydroxyl group for the observed interaction, the authors of this study hypothesized that this interaction involves hydrogen bond formation between the cholesterol hydroxyl group and functional groups at the inositol ring. Although preliminary studies by our group have aimed at

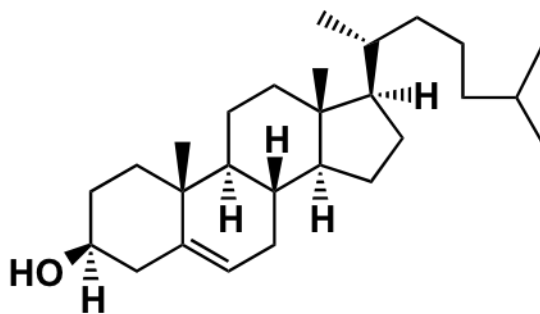


Figure 10: Chemical Structure of Cholesterol.

understanding the impact of cholesterol on phosphoinositide domain formation, the experiments conducted so far utilized rather simplistic models since only binary lipid mixtures were used. The addition of other major inner leaflet membrane components was not considered because these initial experiments aimed at highlighting the interaction of cholesterol with PIPs and not how this interaction is modulated by other chemical species. The experiments outlined in this chapter aim to fill this void.

In our studies we will investigate the effect of calcium and/or cholesterol on increasingly complex, PI containing lipid mixtures that better represent the inner leaflet of the plasma membrane. We will begin with single component lipid monolayers (PI or PE), then move to binary mixtures of lipids (PE:PI). The addition of Ca^{2+} to the monolayer subphase is expected to alter the mutual interactions of PI as well as the interaction with PE by bridging the molecules through the phosphodiester groups. This would lead to a more densely packed monolayer, a more condensed isotherm, and could also impact the monolayer morphology. The impact of Ca^{2+} on PE monolayers is expected to be less significant than for PI. Our last studies in this chapter will focus on the effect of cholesterol in the presence or absence of Ca^{2+} on mixed PE:PI monolayers. The use of Langmuir monolayer systems is especially convenient for studies involving Ca^{2+} since the addition of Ca^{2+} to anionic lipid vesicles almost always results in aggregation, and hence precipitation of the vesicles. Based on previous evidence, we would expect cholesterol and calcium to condense the monolayer synergistically due to interactions through hydrogen bonding and bridging of the headgroups. In Chapter 4 we will compare these results to those for PE: PI(4,5) P_2 mixtures under the same conditions, and in Chapter 5 we will discuss these results in the context of PI:PI(4,5) P_2 mixtures.

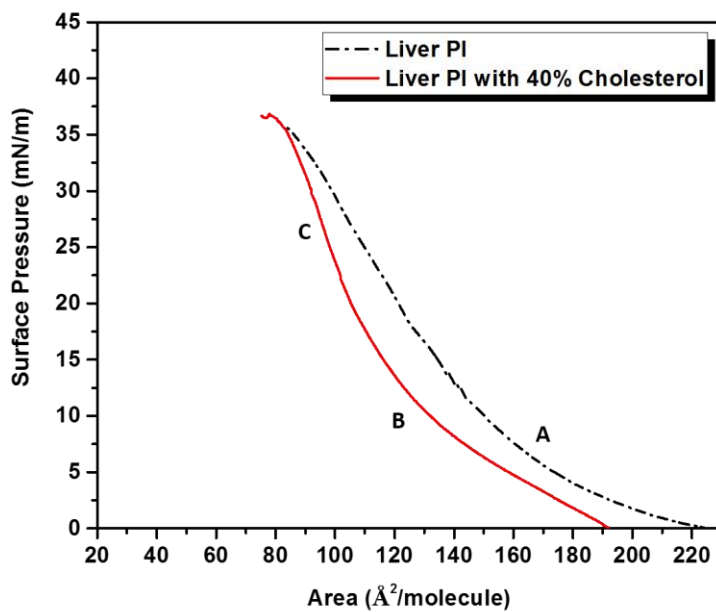
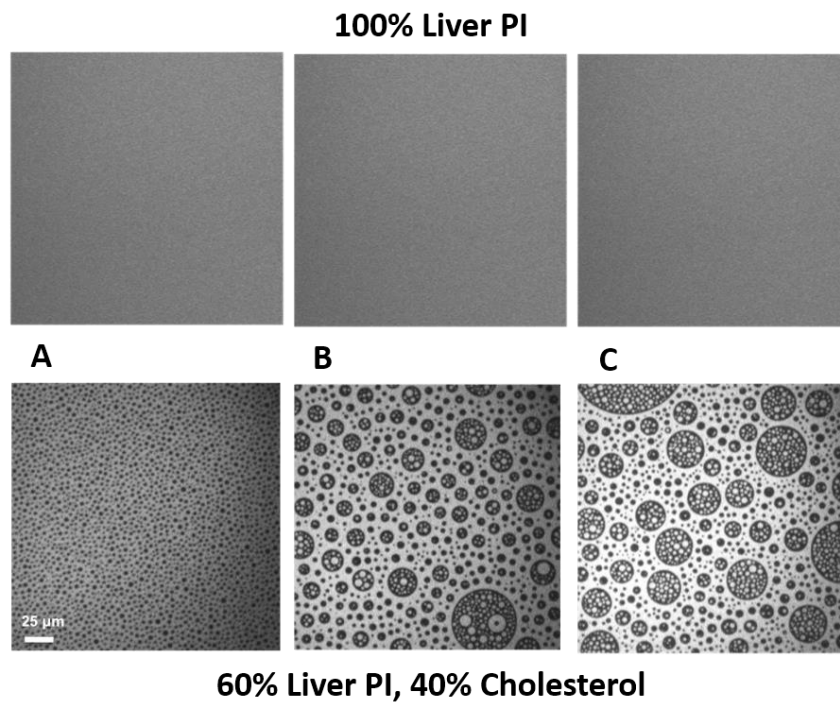


Figure 11: Liver PI Isotherms and Epifluorescence Images in the Absence and Presence of Cholesterol. Monolayers consisting of 100% Liver PI (black) and 60% Liver PI with 40% Cholesterol (red) on subphase composition: 10mM Tris, 150mM NaCl, 0.1mM EDTA, at pH 7.4 and 20°C

3.2 Effect of calcium on the surface pressure/area isotherms of phosphatidylinositol monolayers

In this section, we investigate the effect of varying concentrations of calcium on PI monolayers at the air/water interface. We will investigate Langmuir films at the air/water interface for varying calcium concentrations to obtain surface pressures/area isotherms. The addition of calcium to the subphase will allow us to investigate how the Liver PI monolayer is influenced by the presence of calcium at physiological and non-physiological Ca^{2+} concentrations. The presence of Ca^{2+} may be able to bridge the PI molecules leading to a more condensed monolayer and hence a surface pressure/area isotherm that is shifted to a lower area/molecule. The shape and $\text{\AA}^2/\text{molecule}$ shifts will be reflective of the PI/ Ca^{2+} interactions

The PI samples for these experiments were made as described in Chapter 2. The buffer subphase was composed of 10mM Tris, and 150mM NaCl with varying concentrations of calcium (0.01mM, 1mM, or 2mM) or in the case of 0mM CaCl_2 , 0.1mM EDTA was used. All buffers were at pH 7.4, and the temperature during experiments was maintained at 25 ± 0.2 °C.

Initially, the surface pressure/area isotherm of PI in the absence of calcium is characteristic of an expanded monolayer (Figure 12). This is expected and is a result of the headgroup charge repulsion and the unsaturated nature of the acyl chains. The addition of physiologically relevant concentrations of calcium to the subphase had only a minimal effect on the shape and placement of the π/A -isotherm. A further increase of the calcium concentration (1mM and 2mM) resulted in a condensation of the monolayer and a shift of the surface pressure/area isotherm to smaller areas/molecule. While the addition of the

calcium slightly condensed the monolayer, the π/A -isotherm remained characteristic for a monolayer in an expanded LE phase. The change in the area can be explained with an interaction of the calcium with the phosphate group of PI. While screening of the negative headgroup charge by the bivalent cation leads to a reduction of the charge, and hence a reduction of the repulsive forces (which would lead to a small condensation of the film), it is likely that the observed condensation is predominantly due to bridging of the headgroup by the Ca^{2+} . At all concentrations of calcium, the monolayer remains in a fluid state for all of the calcium concentrations.

3.3 The Effect of calcium on domain formation in Liver PI monolayers investigated by epifluorescence microscopy

In section 3.2, we showed that the impact of Ca^{2+} on the thermodynamic behavior of the PI monolayer was minimal. To investigate if the thermodynamic measurements agreed with the morphology of the monolayer, we utilized epifluorescence microscopy. This allows us to directly visualize any changes in the morphology of the monolayer. The samples were prepared as described in Chapter 2, and the experiments were carried out as previously mentioned.

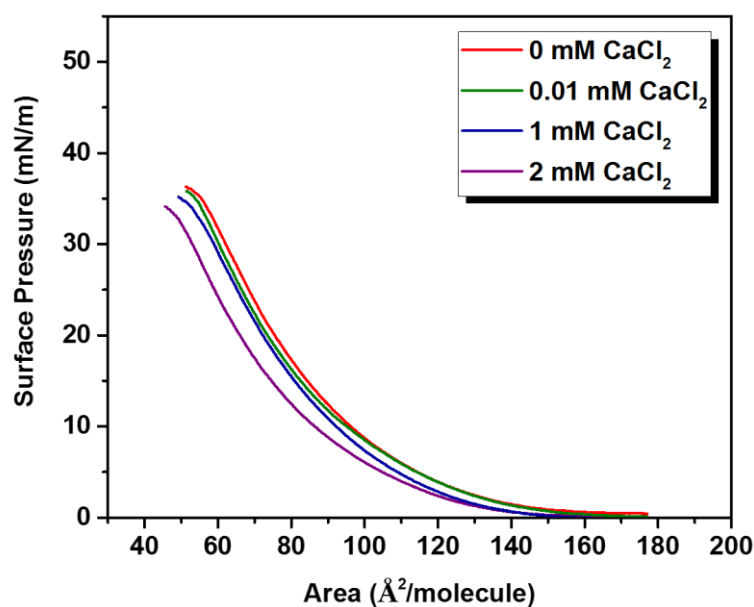
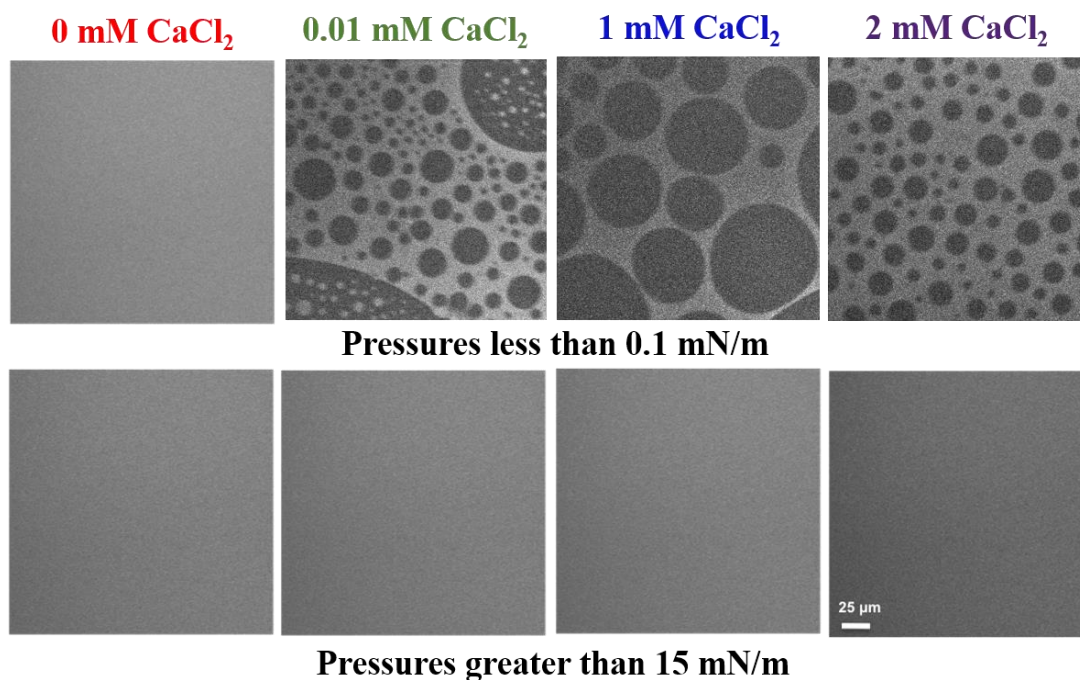


Figure 12: Surface Pressure/Area Isotherms of PI Monolayer in the Presence and Absence of Calcium with Corresponding Epifluorescence Images. Monolayers consisting of PI, on varying subphase composition: 0mM CaCl₂ (0.1mM EDTA) (red), 0.01mM CaCl₂ (green), 1mM CaCl₂ (blue), or 2mM CaCl₂ (purple), 10mM Tris, 150mM NaCl, pH 7.4 at T=25±0.2°C. Epifluorescence images shown were recorded at pressures less than 0.1mN/m and pressures greater than 15mN/m using 0.1% Bodipy® PI.

The epifluorescence images taken of PI on 10mM Tris, 150mM NaCl, 0.1mM EDTA buffer at pH 7.4 showed no domain formation or contrast throughout the compression of the monolayer. (Figure 12) On subphases containing varying concentrations of Ca^{2+} (0.01mM CaCl_2 , 1mM CaCl_2 , 2mM CaCl_2), domains were visible at low pressures. (Figure 12) For all investigated concentrations, condensed regions were similar in both size and shape, but were no longer visible at higher pressures. The round shape of the domains is typical for those domains seen in a LE/G co-existence region. Upon further compression of the monolayer, the gaseous phase disappears and the LE domains coalesce, and a homogenous fluid phase is obtained. In combination, the π/A -isotherm and epifluorescence microscopy measurements show that the addition of Ca^{2+} yields in a slight condensation of the monolayer.

3.4 Effect of calcium on the surface pressure/area isotherms of phosphatidylethanolamine monolayers

In preparation of our experiments aimed at studying mixed PI/PE monolayers, we investigated the effect of Ca^{2+} on PE π/A -isotherms. We will later use this data to interpret the mixed monolayer studies discussed hereafter.

The PE samples prepared for these experiments are outlined in Chapter 2. The buffer subphase was composed of 10mM Tris, 150mM NaCl, with varying concentrations of CaCl_2 . (0.01mM CaCl_2 , 1mM CaCl_2 , 2mM CaCl_2 , and in the case of 0mM CaCl_2 , 0.1 mM EDTA was used instead.) All subphases were buffered to pH 7.4, and the temperature was maintained at $25 \pm 0.2^\circ\text{C}$.

The initial isotherm of PE in the absence of calcium is characteristic of a monolayer

in an expanded, fluid state. Unexpectedly, we observed that at physiologically relevant calcium concentrations of 0.01mM the pressure/area isotherm is shifted to a higher area per molecule than the isotherm in the absence of calcium. (Figure 13) As the calcium concentration is increased, the π/A -isotherm begins to shift back to a lower area/molecule until it returns at 2 mM Ca^{2+} to the area/molecule of PE we observed in the absence of calcium. In all cases, the monolayer is expanded and lacking any distinct phase transitions. At first glance, it is surprising that Ca^{2+} has an effect on a monolayer of a zwitterionic lipid. When interpreting these results, one needs to consider that the presence of Ca^{2+} may impact the protonation state of the PE ammonium headgroup. Apparently, Ca^{2+} inserts between the headgroups at low concentrations, presumably through the interaction with the negatively charged phosphodiester group. We suggest that Ca^{2+} is not able to bridge PE molecules at this concentration. Since the PE headgroup is small, the insertion of Ca^{2+} between the headgroups will lead to an expansion of the monolayer. In addition, it is possible that Ca^{2+} interferes with PE/PE hydrogen bond formation. At higher concentrations, more Ca^{2+} will accumulate in the interfacial region, leading to a reduction of the interfacial pH (because the interface becomes more positively charged and attracts fewer protons), which may lead to a slight increase in protonation of the PE ammonium group. In general, the pH utilized for our experiments is far from the pKa of the PE ammonium group and therefore, this effect is expected to be very minor. Instead, it appears that the Ca^{2+} is able at these concentrations to bridge the headgroups, offsetting in part the steric demand imposed by its presence in the headgroup region.

3.5 Effect of calcium on lateral heterogeneity of PE monolayers at the air/water interface

In the previous section we discussed the effect of calcium on PE surface pressure/area isotherms. Next, we investigated the impact of calcium on the lateral organization of PE monolayers. We would expect that since the isotherms are characteristic of an expanded monolayer and are not indicative of Ca^{2+} condensation effects that we would see no domain formation. It is therefore not surprising that for all calcium concentrations the epifluorescence images lack any macroscopically discernable domain formation, meaning that the monolayer remains in a fluid state. (Figure 13)

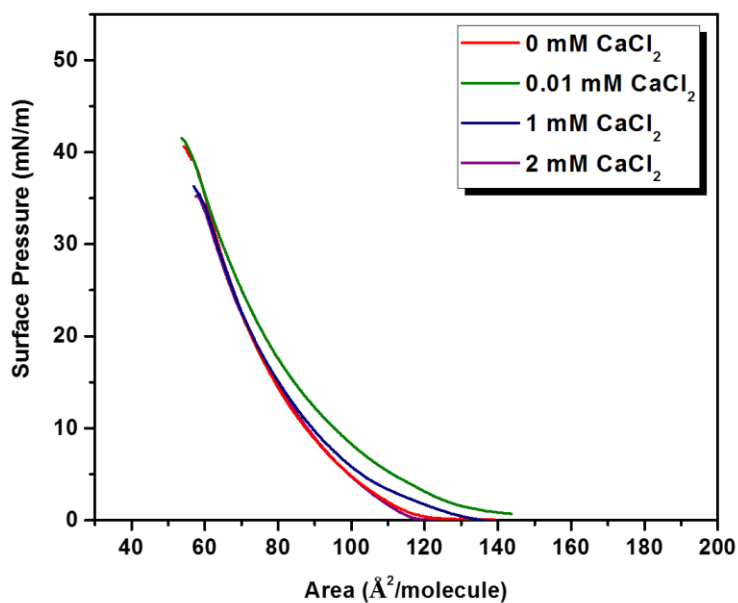
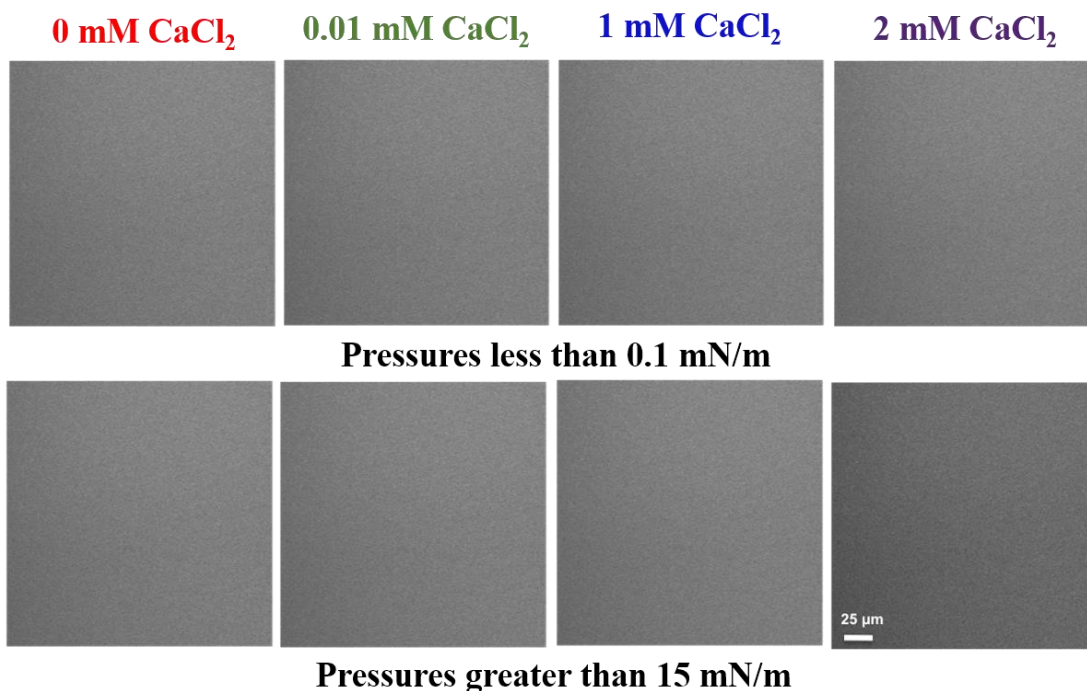


Figure 13: Surface Pressure/Area Isotherm of PE Monolayers in the Presence and Absence of Calcium with Corresponding Epifluorescence. Monolayers consisting of PE, on varying subphase composition: 0mM CaCl₂ (0.1mM EDTA) (red), 0.01mM CaCl₂ (green), 1mM CaCl₂ (blue), or 2mM CaCl₂ (purple), 10mM Tris, 150mM NaCl, pH 7.4 at T=25±0.2°C. Fluorescence images shown were recorded at pressures less than 0.1 mN/m and pressures greater than 15 mN/m using 0.1% NBD-PE.

3.6 Effect of calcium on the surface pressure/area isotherms of mixed phosphatidylethanolamine/phosphatidylinositol monolayers

In this section we investigate how calcium impacts mixed PE/PI monolayers using surface pressure/area isotherm measurements. Using this system we will investigate how calcium modulates PE/PI interactions in monolayers at the air/water interface. Due to the small headgroup geometry, PE may be able to intercalate between the PI headgroups. However, the introduction of calcium to the subphase may impact these interactions. This data will allow us to compare in Chapter 4 how the additional phosphate groups of PI(4,5)P₂, and hence the increased negative charge, influence the interaction with PE.

Samples for these experiments were prepared as described in Chapter 2. The calcium concentration in the subphase was varied (Either 0mM or 2mM). The subphase consisted of 10mM Tris, 150mM NaCl and either 2mM CaCl₂ or 0mM CaCl₂ where 0.1mM EDTA was substituted for the calcium. The subphase was buffered to pH 7.4, and the temperature was maintained at 25±0.2°C.

First, we investigated mixed PE/PI monolayers at the air/water interface at a 1:1 ratio. In the absence of calcium, the mixed monolayer is more expanded than either the isotherm of PE or PI in the presence or absence of calcium. (Figure 14) This suggests that at ratios of 1:1, PE is intercalating between the PI headgroups in a way that the overall space demand of the PE/PI complex is larger than one would obtain for a simple addition of the respective PI and PE footprints. This implies that the two lipids cannot be demixed because in this case the phase behavior would be additive and the π/A -isotherm would be positioned somewhere between those for pure PE and PI monolayer, respectively. At the same time, the data suggest that the interaction between PE and PI is more space consuming than the

PI-PI interaction, which is surprising considering the small size of the PE headgroup. We hypothesize that the PI headgroup changes its orientation upon interaction with PE, which leads to the observed increase in space demand. The addition of 2mM CaCl₂ to the subphase slightly condenses the monolayer (shift of the isotherm to lower areas/molecule); however, the monolayer remains in a more expanded form than the monolayer of PI in the presence of 2mM CaCl₂. This may suggest that PE is able to insert between the PI headgroups, disrupting the interaction of PI with Ca²⁺. At a more physiologically relevant ratio of 4:1 PE:PI, the π/A -isotherm in the absence of calcium is characteristic of a more condensed monolayer than it was found for the 1:1 ratio both with and without CaCl₂. (Figure 15) The difference in positioning of the π/A -isotherm of the 4:1 monolayer when compared to the 1:1 ratio could be a result of stoichiometry. Although our research can neither prove nor disprove that this is the case, the interaction with PI may require more than one PE molecule. The additional PE headgroups could be able to interact with the phosphodiester and/or the hydroxyl groups of the PI headgroup, resulting in a condensation of the monolayer. However, it is also possible that the shift in the isotherm for the 4:1 PE/PI ratio relative to the 1:1 ratio is a result of the reduced space demand of the PE lipid, which is the majority component in the monolayer. The addition of 2mM CaCl₂ to the subphase, has only a slight impact on the condensation of the 4:1 PE:PI monolayer. (Figure 15) Again, this may be because PE is competing with Ca²⁺ for bonding opportunities with the PI headgroup.

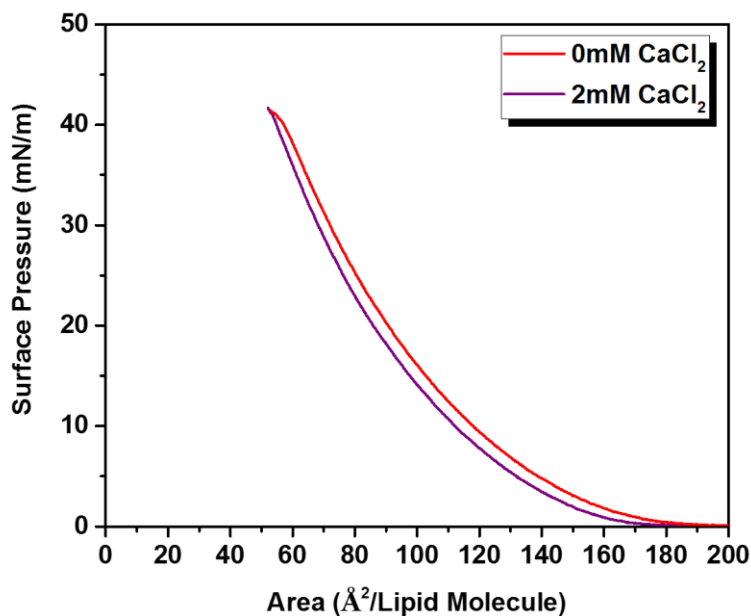
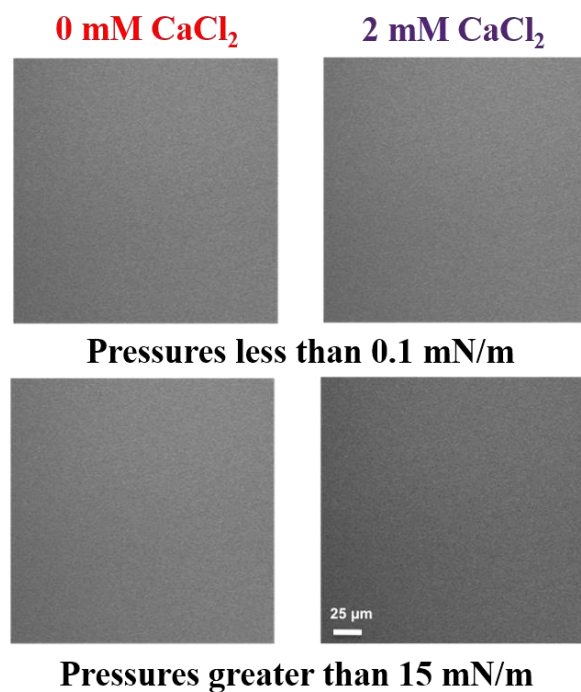


Figure 14: Surface Pressure/Area Isotherms 1:1 PE:PI Monolayers on Varying Subphase Composition with Corresponding Epifluorescence Microscopy Images. Monolayers consisting of 1:1 PE:PI, subphase composition: 2mM CaCl₂ (purple) and 0mM CaCl₂ (0.1mM EDTA) (red), 10mM Tris, 150mM NaCl, pH 7.4 at T=25±0.2°C. Fluorescence images shown were recorded at pressures less than 0.1 mN/m and pressures greater than 15 mN/m using 0.1% NBD-PE.

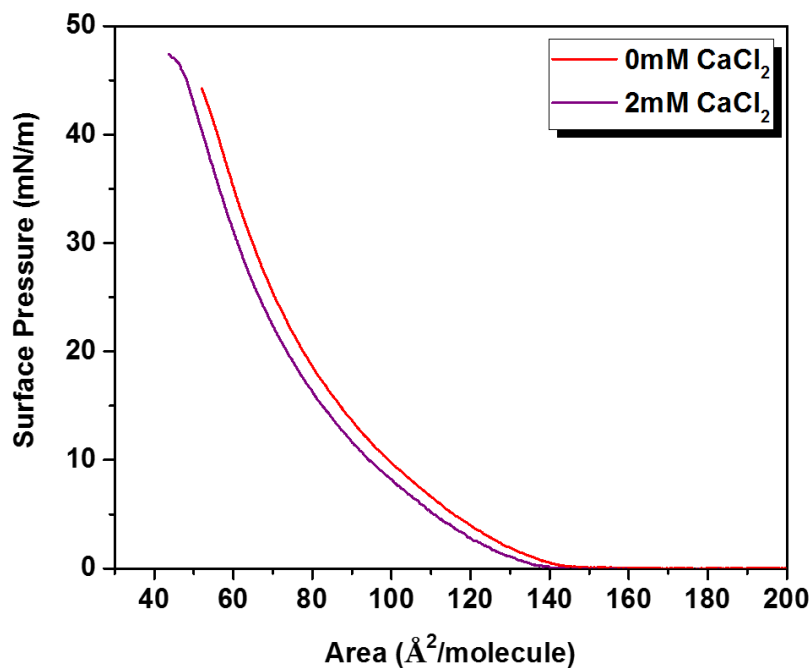
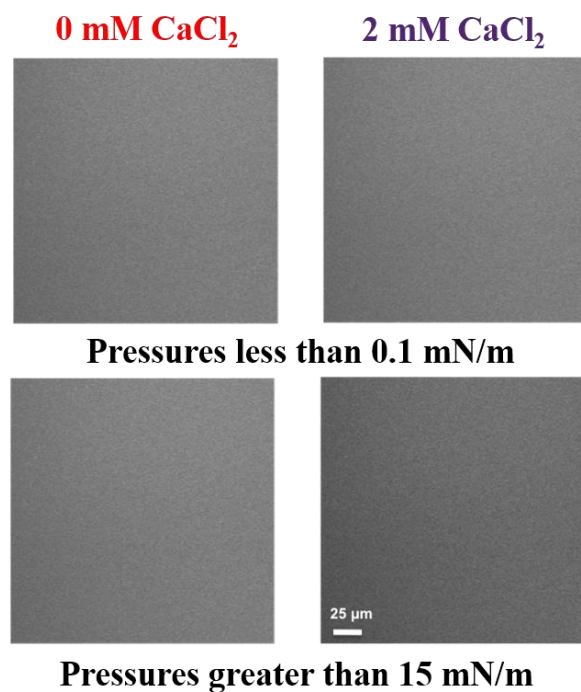


Figure 15: Surface Pressure/Area Isotherms 4:1 PE:PI Monolayers on Varying Subphase Composition with Corresponding Epifluorescence Microscopy Images. Monolayers consisting of 4:1 PE:PI, subphase composition: 2mM CaCl₂ (purple) and 0mM CaCl₂ (0.1mM EDTA) (red), 10mM Tris, 150mM NaCl, pH 7.4 at T=25±0.2°C. Fluorescence images shown were recorded at pressures less than 0.1 mN/m and pressures greater than 15 mN/m using 0.1% NBD-PE.

3.7 Effect of calcium on the morphology of mixed phosphatidylethanolamine/phosphatidylinositol monolayers

Here, we use epifluorescence microscopy to study the changes in PE/PI monolayer morphology due to the presence of calcium. Based on the thermodynamic data obtained in section 3.6, it could be possible that the small headgroup geometry of PE is able to insert between PI.

Experiments in this section were performed as discussed in Chapter 2. All experiments were conducted on the following subphase: 10mM Tris, 150mM NaCl, and either 0mM CaCl₂ (0.1mM EDTA), or 2mM CaCl₂ at pH 7.4 and temperature controlled at 25±0.2°C. Bodipy PI was added to the lipid sample in 0.1mol% to image the monolayer.

Epifluorescence images taken for both the 1:1 and 4:1 ratios of PE:PI lack domain formation either in the presence or absence of calcium, suggesting these monolayers remain in an expanded, fluid state. (Figure 14, Figure 15) This result is not particularly surprising based on the π/A -isotherms obtained in section 3.6, and supports the suggestion that PE is able to insert between the PI headgroups, but that the monolayer expands as a result of this interaction.

3.8 Effect of cholesterol on the surface pressure/area isotherms of mixed phosphatidylethanolamine/phosphatidylinositol monolayers

Here, we investigate the effect of cholesterol on surface pressure/area isotherms of mixed phosphatidylethanolamine/phosphatidylinositol monolayers. Although previous work has focused on how cholesterol impacts phosphatidylinositol individually, studies investigating ternary systems are lacking. Here we aim to determine if the introduction of

cholesterol impacts the physiochemical properties of PE:PI monolayers at the air/water interface.

Experiments in this section are performed as detailed in Chapter 2. Isotherms were recorded on a subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at a pH of 7.4 and temperature controlled at $25\pm 0.2^\circ\text{C}$.

The surface/pressure area isotherm of 4:1 PE:PI with the addition of 40mol% cholesterol is characteristic of a more condensed monolayer (lower areas/molecule) compared to 4:1 PE:PI in the absence of cholesterol. (Figure 16) We know from previous studies that the hydroxyl group of cholesterol is important for hydrogen bond formation with the headgroup of phosphatidylinositol. In addition, we suggest from data obtained in section 3.6 that PE is able to intercalate between PI headgroups. Based on this previous data it could be possible that PE, PI, and cholesterol are all participating in a hydrogen bond network, which results in a condensation of the monolayer. We hypothesize that the addition of cholesterol and PE to the PI monolayer result in a tilting of the inositol ring of PI, allowing both PE and cholesterol to participate in hydrogen bonding via the hydroxyl groups on the inositol headgroup of PI.

3.9 Effect of cholesterol on the morphology of mixed phosphatidylethanolamine/phosphatidylinositol monolayers

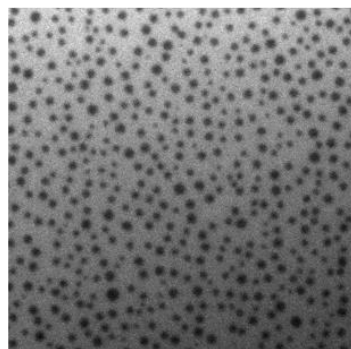
In the previous section, we investigated how cholesterol influences the thermodynamic behavior of monolayers composed of mixtures of 4:1 PE:PI with 40mol% cholesterol. In this section, we focus on how the addition of cholesterol to the monolayer impacts its morphology. As documented previously, domain formation is observed in pure

PI monolayers with the addition of 40mol% cholesterol. In this section we aim to investigate whether or not cholesterol can enhance domain formation in a mixture of PE and PI.

Experiments in this section are carried out as outlined in Chapter 2. Epifluorescence images were recorded at given pressures on a subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at a pH of 7.4 and the temperature was regulated at $25\pm 0.2^{\circ}\text{C}$. In addition, 0.1mol% of the fluorophore Bodipy PI was added to the sample for imaging purposes.

For epifluorescence microscopy images taken, we observe condensation of the monolayer at pressures up to 3mN/m. (Figure 16) From a physiological point of view this suggests that cholesterol is stabilizing the PE/PI domains, which supports our previously mentioned hypothesis stating that cholesterol and PE are jointly participating in a hydrogen bond network via the PI headgroup. For pressures above 5mN/m, the recorded epifluorescence images show no contrast. This may be due to coalescing of the PE/PI/cholesterol domains. This is a fitting hypothesis based on the thermodynamic data obtained in the previous section.

Up to 5 mN/m



Above 5 mN/m

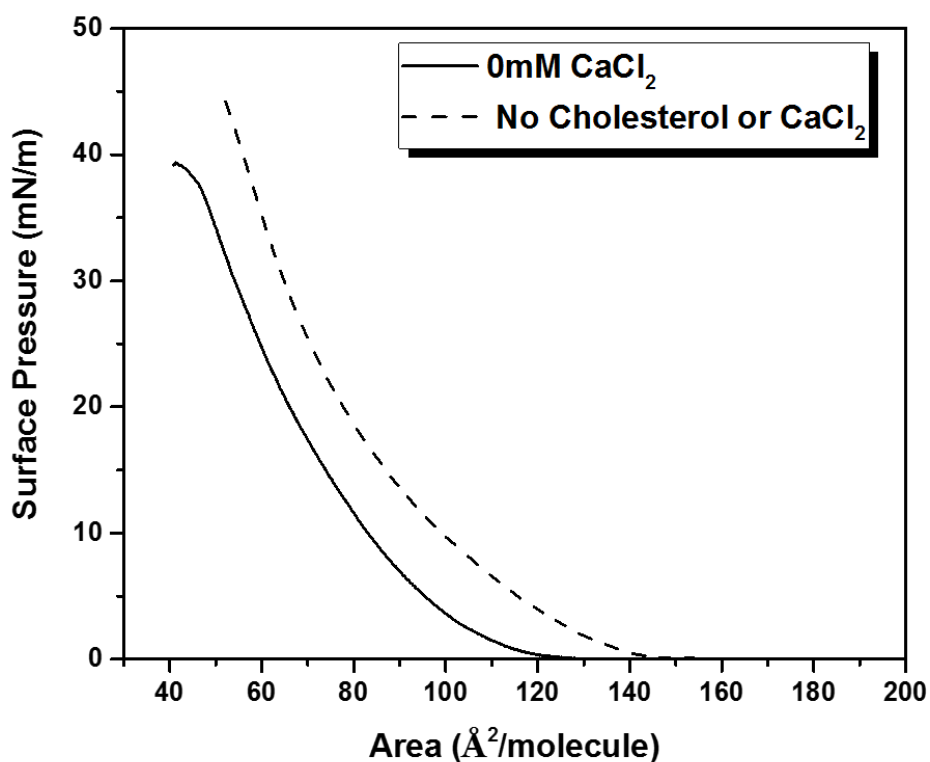
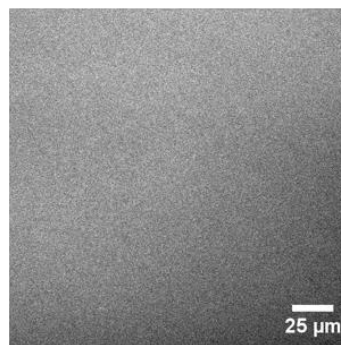


Figure 16: Surface Pressure/Area Isotherms 4:1 PE:PI Monolayers with 40mol% Cholesterol with Corresponding Epifluorescence Microscopy Images. Monolayers consisting of 4:1 PE:PI with 40% cholesterol (black) on a subphase consisting of 10mM Tris, 150mM NaCl, 0.1mM EDTA, pH 7.4 at $T=25\pm 0.2^\circ\text{C}$. Fluorescence images shown are representative of pressures up to 5mN/m and pressures above 5mN/m. Images were taken using 0.1% Bodipy® PI. The surface pressure/area isotherm of 4:1 PE:PI in the absence of cholesterol is shown for comparison. (dashed)

3.10 Effect of cholesterol and calcium on the surface pressure/area isotherms of mixed phosphatidylethanolamine/phosphatidylinositol monolayers

In this section, we focus on the how the presence of varying amounts of calcium in the subphase impact monolayers composed of 4:1 PE:PI with 40mol% cholesterol. Previous studies by our group have shown that the hydroxyl group of cholesterol is important for the participation in the hydrogen bond network formed by phosphoinositides. (Graber, Gericke et al. 2014) In addition, we have discussed in previous sections of this chapter how calcium can impact both PI and PE monolayers as well as how it can influence mixed PE:PI monolayers. The addition of calcium to the PI/PE/cholesterol monolayer will help to determine if calcium is able to bridge the interactions between lipid molecules also in the presence of cholesterol or disrupts the stabilization of the PI domains by cholesterol.

Experiments in this section were carried out as detailed in Chapter 2. Isotherms were recorded on a subphase composed of 10mM Tris, 150mM NaCl, and either 0.01mM CaCl₂ or 2mM CaCl₂ and were maintained at pH 7.4 and 25±0.2°C.

Surface pressure/area isotherms of 4:1 PE:PI with 40mol% cholesterol in the presence of 0.01mM CaCl₂ (Figure 17) show condensation of the monolayer when compared to monolayers composed of 4:1 PE:PI in the absence of calcium and cholesterol, as well as 4:1 PE:PI in the presence of 40mol% cholesterol and the absence of calcium. (Figure 19). In section 3.8, we suggested that the addition of PE and cholesterol to the PI monolayer results in a twisting of the PI inositol ring at the air/water interface. Taking this hypothesis in combination with the 4:1 PE:PI + 40mol% cholesterol in the presence of calcium, we suggest that the small headgroup geometry of the PE headgroup allows it to sit higher in the monolayer, allowing the addition of calcium to bridge interactions between

the phosphodiester groups on the PI headgroups. We see for subphase containing 2mM CaCl₂ (Figure 18) a further condensation of the monolayer than that of the monolayer recorded on subphase containing 0.01mM CaCl₂. This suggests that the condensation effect of calcium and cholesterol is additive in the case of PE/PI monolayer.

3.11 Effect of cholesterol and calcium on the morphology of mixed phosphatidylethanolamine/phosphatidylinositol monolayers

We previously have shown in section 3.9 that the addition of calcium to monolayers composed of 4:1 PE:PI with 40mol% cholesterol had an additive effect with respect to condensation of the monolayer. Here we investigate whether the morphology of the monolayer is consistent with these thermodynamic data. Based on the condensation of the 4:1 PE:PI + 40mol% cholesterol monolayer in the presence of calcium, we would expect to see domain formation.

In this section, experiments were carried out according to the information in Chapter 2. The subphase was composed of 10mM Tris, 150mM NaCl, and either 0.01mM CaCl₂ or 2mM CaCl₂ at pH 7.4 and a temperature of 25±0.2°C. For imaging purposes, 0.1mol% of the fluorophore, Bodipy PI, was added to the lipid sample.

The epifluorescence microscopy images taken in the presence of 0.01mM CaCl₂ (Figure 17) show small linked circular domains and independent larger circular domains that are present at pressures around 0mN/m. As the pressure is increased, the linked circular domains begin to break apart, while the circular domains remain the same. When the pressure reaches 5mN/m the domains begin to blur and are no longer visible. We see similar domain formation in the presence of 2mM CaCl₂ (Figure 18): however, there are

some differences. At low pressures (0mN/m), the chain-like domains are even more apparent. There are some areas of large circular domains interspersed throughout the monolayer at low pressures as well. The chain-like domains begin to break apart at pressures around 3mN/m and continue to break down until they are no longer linked at pressures around 1.3mN/m. Much like the domains in the presence of 0.01mM CaCl₂, the domains in the presence of 2mM CaCl₂ remain visible until 5mN/m when they coalesce and disappear. This data is in agreement with the thermodynamic data detailed in the previous section. The morphologies of these monolayers is unique and lack precedence in the literature. At this point, it can only be concluded that PI/PE/chol monolayers in the presence of Ca²⁺ form a unique complex that leads to an apparently well organized film.

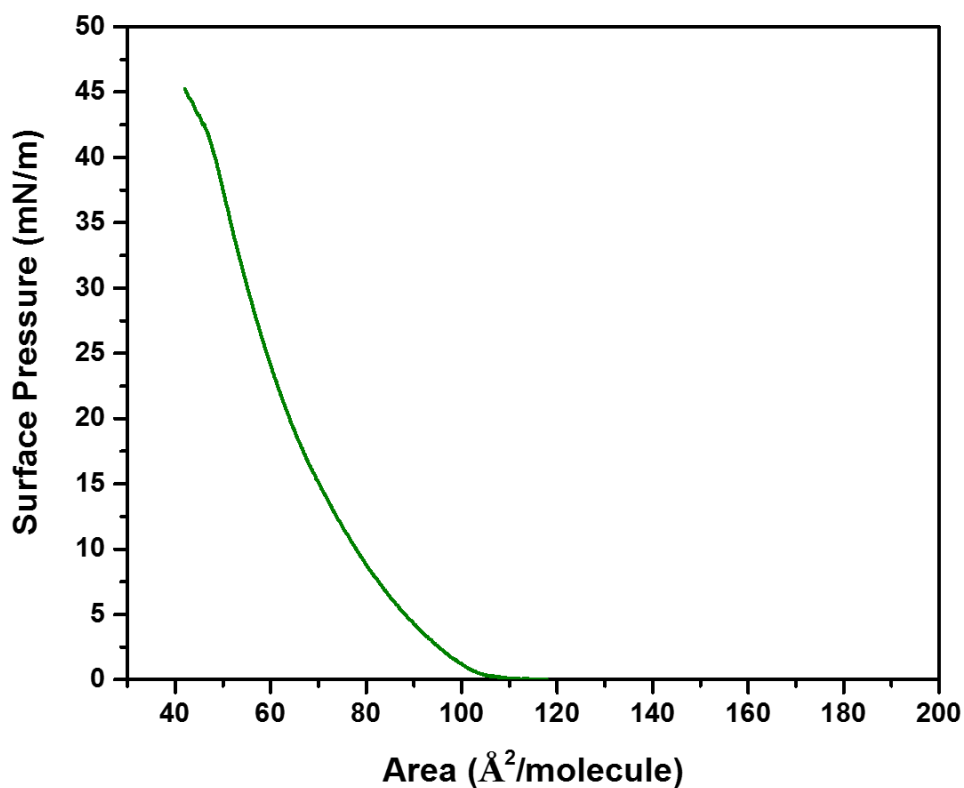
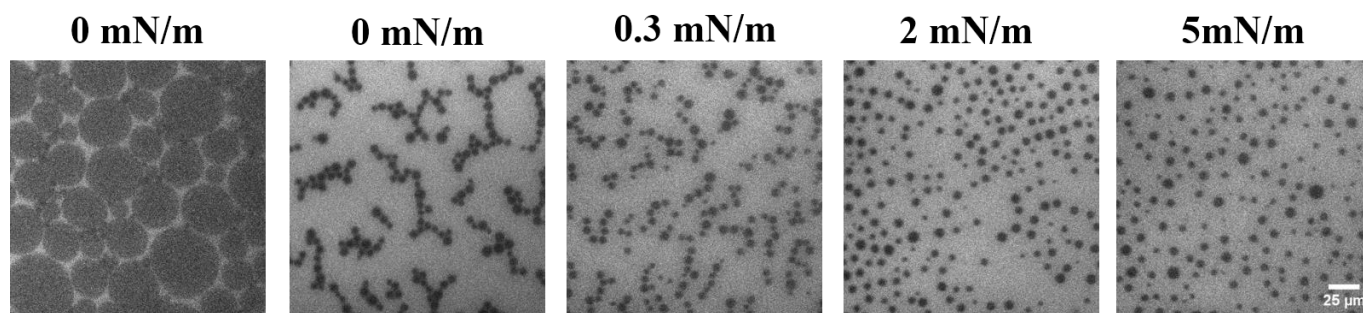


Figure 17: Surface Pressure/Area Isotherms 4:1 PE:PI Monolayers with 40mol% Cholesterol in the Presence of 0.01mM CaCl₂ with Corresponding Epifluorescence Microscopy images. Monolayers consisting of 4:1 PE:PI with 40mol% cholesterol on a subphase consisting of 10mM Tris, 150mM NaCl, 0.01mM CaCl₂, pH 7.4 at T=25±0.2°C. Fluorescence images shown are representative of the corresponding pressures. Images were obtained using 0.1% Bodipy® PI

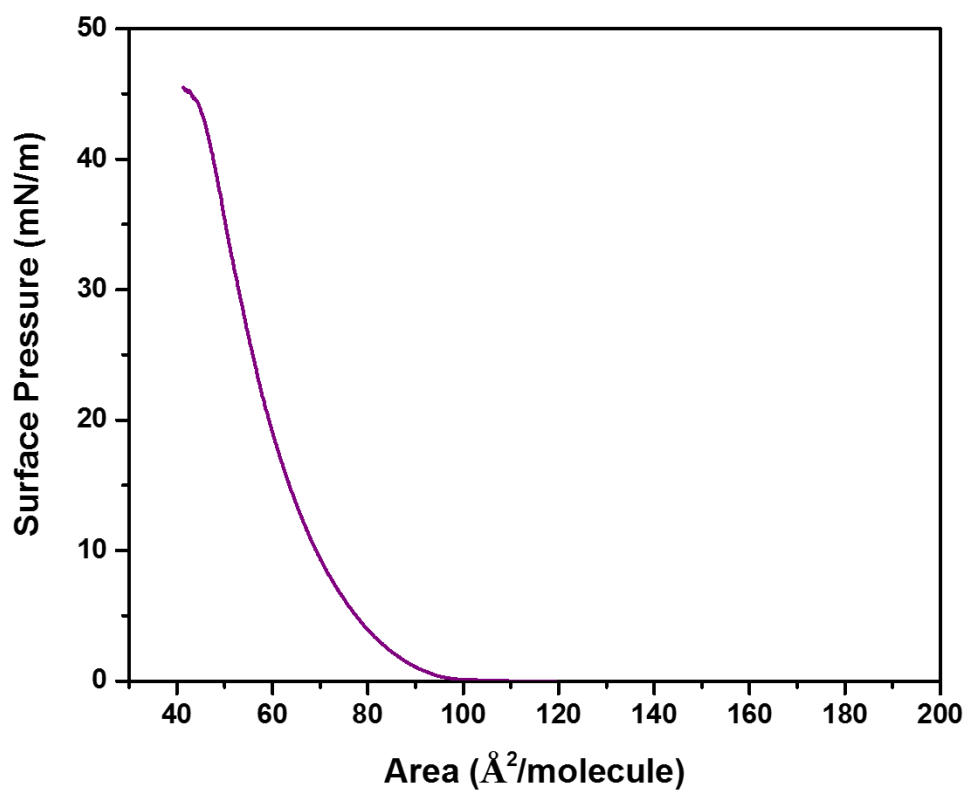
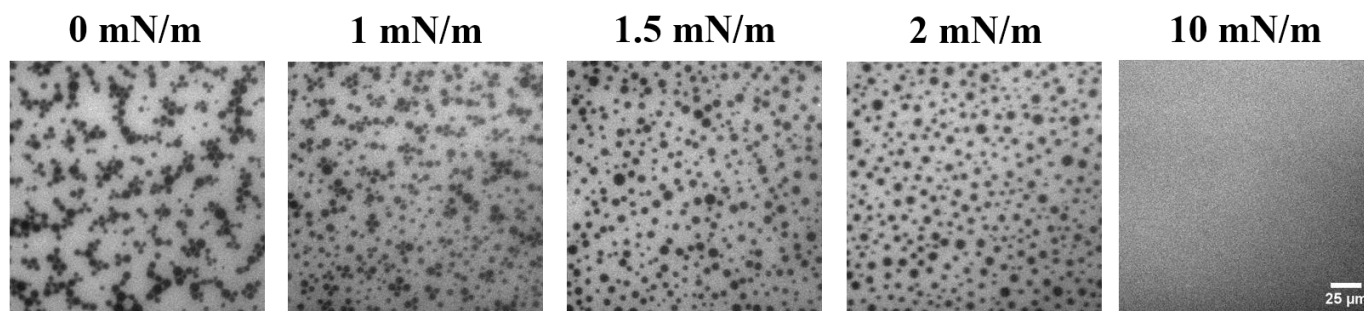


Figure 18: Surface Pressure/Area Isotherms 4:1 PE:PI Monolayer with 40mol% Cholesterol in the Presence of 2mM CaCl₂ with Corresponding Epifluorescence Microscopy Images. Monolayers consisting of 4:1 PE:PI with 40% cholesterol on a subphase consisting of 10mM Tris, 150mM NaCl, 2mM CaCl₂, pH 7.4 at T=25±0.2°C. Fluorescence images shown are representative of the corresponding pressures. Images were obtained using 0.1% Bodipy® PI

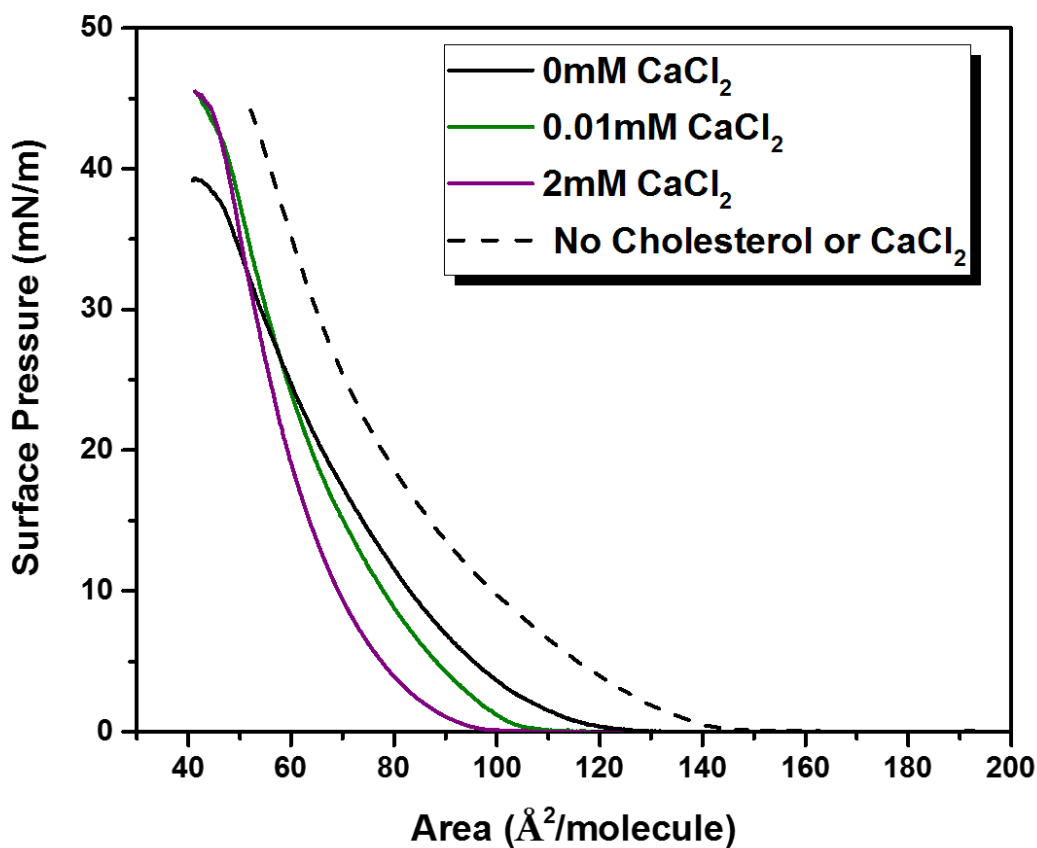


Figure 19: Surface Pressure/Area Isotherms of 4:1 PE:PI with 40mol% Cholesterol with Varying Subphase Composition. Monolayers consisting of 4:1 PE:PI 40% cholesterol, on varying subphase composition: 0mM CaCl₂ (0.1mM EDTA) (black), 0.01mM CaCl₂ (green), or 2mM CaCl₂ (purple), 10mM Tris, 150mM NaCl, pH 7.4 at T=25±0.2°C. The isotherm of 4:1 PE:PI in the absence of cholesterol and calcium (dashed) is added for comparison.

3.12 Chapter discussion and conclusions

In this chapter, we have investigated the influence of plasma membrane modulators (Ca^{2+} and cholesterol) on mixed PE/PI monolayers at the air/water interface. Our main motivation for this was based on previous studies that showed the importance of hydrogen bonding in both phosphatidylinositol-phosphatidylinositol interactions and PI-cholesterol interactions. (Redfern and Gericke 2004, Redfern and Gericke 2005, Kooijman, King et al. 2009, Jiang, Redfern et al. 2014) In addition, the formation of PI rich domains in the inner leaflet of the plasma membrane could serve as platforms for the delivery of additional PI by PITP and the transformation of PI to its phosphorylated derivatives by action of PI kinases. The formation of PI pools may be a first step towards the establishment of PIP pools, which provide for the spatiotemporal control of signaling events.

The results in this chapter show that the incorporation of calcium in the subphase has a slight impact on the condensation of PI monolayers at the air/water interface. Corresponding epifluorescence images show a lack of domain formation in the absence of calcium, and only a slight condensation of the monolayer at low pressures with the addition of calcium. Taken together this data suggests that calcium is able to shield the charge of the PI headgroups and bridge them, however, the effect appears to be minor.

Based upon the charge of the PE headgroup, one would expect that the addition of calcium would have no effect on the monolayer state at the air/water interface. However, we see a change in the area/molecule based on changes in calcium concentration. We suggest that this may be due to calcium inserting between the PE headgroups leading to a more expanded state of the monolayer. In addition, as the calcium concentration is increased, the calcium could begin to bridge the PE headgroups, resulting in an opposing

effect and a slight condensation of the monolayer relative to the low Ca^{2+} concentration case. We also suggest that this is a real effect and not an artifact because all of the Langmuir films are reaching the same collapse pressure. The lack of domain formation in the epifluorescence images at all calcium concentrations supports this conclusion. It should be noted, however, that this hypothesis would require confirmation from other, structurally more sensitive, methods.

The thermodynamic data obtained for mixed PE/PI monolayers at the air water interface suggested calcium does not significantly impact the condensation of the monolayer at ratios of 1:1 and 4:1. Interestingly, the isotherms at a ratio of 4:1 PE:PI were more condensed than those of 1:1 mixtures. This outcome is surprising based on the expanded isotherms of both pure PE and PI monolayers. It could be possible that the higher concentration of PE induces a shift in the inositol ring of PI allowing for a tighter packing of the molecules and a more condensed monolayer (lower areas/molecule).

Previous data suggest that cholesterol plays an important role in the hydrogen bond network of phosphoinositide domain formation. Our results for 4:1 PE:PI with 40% cholesterol suggest that at a low surface pressure and large area/molecule, cholesterol is able to stabilize the PE/PI mixed phases. Because condensation is only observed at low pressures, it may be that as the pressure is increased, the condensed regions of the monolayer are forced together and eventually coalesce.

The addition of varying amounts of calcium to the subphase of mixed PE/PI with 40mol% cholesterol monolayers results in an additive effect on the condensation of the monolayer. We suggest that this is due to PE, PI, and cholesterol participating in a hydrogen bond network, while Ca^{2+} is able to bridge the PI headgroups via the

phosphodiester group. (Figure 19)

CHAPTER 4

MIXED PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE/ PHOSPHATIDYLETHANOLAMINE MONOLAYERS AT THE AIR/WATER INTERFACE

4.1 Introduction

The inositol ring of phosphatidylinositol can be phosphorylated to produce seven naturally occurring phosphoinositides. Of these, PI(4,5)P₂ is the most abundant in the inner leaflet of the plasma membrane. The rich hydrogen bonding capabilities of the PI(4,5)P₂ headgroup (Figure 20) makes it an ideal candidate for participation in a wide array of physiological processes. It has been suggested previously that PI(4,5)P₂ is able to form and participate in a hydrogen bond network via its inositol ring hydroxyl and phosphate groups. (Redfern and Gericke 2004, Redfern and Gericke 2005, Kooijman, King et al. 2009)

Previous research has shown that Ca²⁺ promotes condensation of PI(4,5)P₂/PC mixed monolayers when compared to the Ca²⁺ free case or in the presence of the divalent cation magnesium. (Ellenbroek, Wang et al. 2011) It should be noted that the use of PC limits the physiological relevance of this lipid mixture since PC is a minor plasma membrane inner leaflet lipid component.

The addition of PE instead of PC as a matrix lipid results in a better representation of the inner leaflet of the plasma membrane. In contrast to PC (Figure 27), PE is able to participate in hydrogen bonding through its ammonium headgroup (Figure 8B).

PI(4,5)P₂ has been shown to play an important role in a variety of Ca²⁺ signaling events. Through previous research, the presence of Ca²⁺ is thought to both bridge and shield PI(4,5)P₂, which could create signaling platforms for these signaling events. (Ellenbroek, Wang et al. 2011, Graber, Gericke et al. 2014) For example, PI(4,5)P₂ has been shown to increase the sensitivity of synaptotagmin for calcium, making it an important part of the exocytosis process. (Aoyagi, Sugaya et al. 2005, James, Khodthong et al. 2008, Lam, Tryoen-Toth et al. 2008, van den Bogaart, Meyenberg et al. 2012)

The participation of PI(4,5)P₂ in raft domains remains somewhat controversial. In many cases, the notion that PI(4,5)P₂ is raft resident is rooted in the observation that many PI(4,5)P₂ mediated signaling events respond to changes in cholesterol levels and the authors of these studies suggest that PI(4,5)P₂ is therefore raft resident. (Lingwood and Simons 2010) However, PI(4,5)P₂ does not accumulate in the liquid-ordered phases, which puts PI(4,5)P₂ raft association into question. The observation that cholesterol induces PI(4,5)P₂ domain formation in simple binary model membranes could be an important finding in the context of spatiotemporal regulation of proteins that are recruited to the plasma membrane. Some in vitro studies call into question the existence of protein independent de-mixing of PI(4,5)P₂; however, it should be noted that the lipid

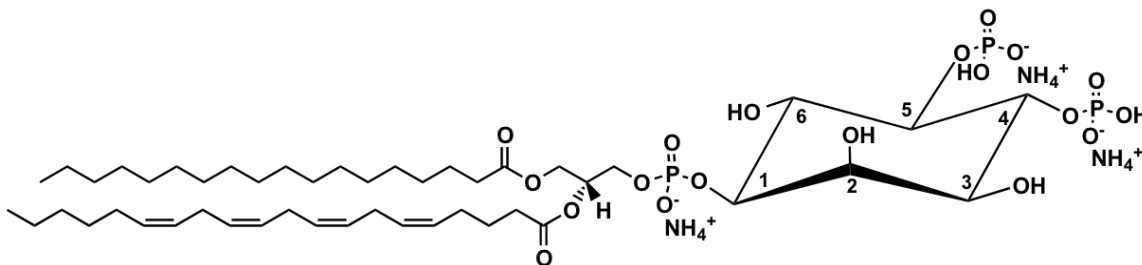


Figure 20: Chemical Structure of Brain PI(4,5)P₂.

compositions used in these studies mimicked the outer leaflet of the plasma membrane rather than the inner leaflet. More recent studies have shown that cholesterol is able to participate in the PI(4,5)P₂ hydrogen bond network via the hydroxyl group. (Jiang, Redfern et al. 2014)

The use of Langmuir monolayers at the air/water interface provides a direct visualization of domain formation at the macroscopic level in addition to providing information regarding the lipid-lipid interactions in the presence of other membrane components. In comparison to previous studies, our study increases the complexity of model membrane systems to investigate how chemical species such as calcium and cholesterol impact PI(4,5)P₂ domain formation in a physiologically more relevant lipid environment, e.g., in the presence of PE. Recent results from NMR experiments have suggested that the interaction of PE with PI(4,5)P₂ leads to an increased ionization of PI(4,5)P₂ due to hydrogen bond formation between phosphomonoester groups of PI(4,5)P₂ with the ammonium group of PE. (Graber, Jiang et al. 2012)

In this chapter, we examine the effect of calcium and/or cholesterol on PI(4,5)P₂ and PE/PI(4,5)P₂ mixtures at the air water interface using Langmuir monolayers and epifluorescence microscopy. First, we study the impact of Ca²⁺ on pure PI(4,5)P₂ monolayers. Comparing these results with the PI data from Chapter 3 we can obtain information on how the increased negativity of the headgroup impacts the isotherm and morphology of the monolayer. In addition, the data from surface pressure/area isotherms and epifluorescence images provide information about how Ca²⁺ impacts PI(4,5)P₂ interactions at the air/water interface. Next, we will increase the complexity of the monolayer by including PE to better mimic the inner leaflet of the plasma membrane.

Several calcium concentrations will be used to determine how mixed PE/PI(4,5)P₂ monolayers are affected by the presence of the divalent cation. Epifluorescence images will be taken at various calcium concentrations to determine the effect of calcium on mixed PE/PI(4,5)P₂ monolayer morphology.

Cholesterol has been shown to have an impact on the condensation of PI(4,5)P₂ surface pressure/ area isotherms as well as the morphology of the monolayer. (Jiang, Redfern et al. 2014) Therefore, we will study whether PE influences the interactions of PI(4,5)P₂ with cholesterol. This data will be compared to the data obtained in Chapter 3 for PE/PI mixtures in the presence of cholesterol. These experiments will not only provide valuable information regarding the influence of PE on PI(4,5)P₂ interactions, but will also give information to what extent the phosphorylation of the PI headgroup affects the interaction with cholesterol and PE. Lastly, mixtures of PE/P(4,5)P₂ and cholesterol will be studied in the presence of calcium. The surface pressure/ area isotherms and epifluorescence images from these experiments will provide information on how calcium influences the interactions at the air/water interface.

4.2 Effect of calcium on the surface pressure/area isotherms of phosphatidylinositol-4,5-bisophosphate monolayers

In this section, we study the impact of varying concentrations of calcium on PI(4,5)P₂ monolayers using surface pressure/area isotherms. The addition of calcium to the subphase will allow us to gain insight into how calcium influences mutual PI(4,5)P₂ interactions. Based on previous data, we would expect Ca²⁺ to shield and bridge the highly negatively charged PI(4,5)P₂ headgroup resulting in condensation of the monolayer.

PI(4,5)P₂ samples for these experiments were prepared as previously described in Chapter 2. The subphase consisted of 10mM Tris, 150mM NaCl, with varying concentrations of calcium (0.01mM CaCl₂, 1mM CaCl₂, or 2mM CaCl₂) or in the case of 0mM CaCl₂, 0.1mM EDTA was substituted. The subphase was maintained at pH 7.4 and a temperature of 25±0.2°C.

Surface pressure/ area isotherms of PI(4,5)P₂ on a subphase lacking calcium show an expanded isotherm typical for a transition from an LE/G state at low surface pressures to an LE state at high pressures. (Figure 21) The π/A -isotherm also shows that in the absence of Ca²⁺, the monolayer collapses at low pressures. Due to the highly unsaturated nature of the chain composition of the PI(4,5)P₂ sample used, it could be possible that the low collapse pressure results from both instability of the monolayer and slippage of the lipid under the barriers. Upon the addition of 0.01mM Ca²⁺, the monolayer condenses, leading to an isotherm placed at significantly lower area/molecule than what is observed for the Ca²⁺ free state. This increase in calcium also stabilizes the monolayer leading to a higher collapse pressure. (Figure 21) Increasing the Ca²⁺ concentration to 1mM results in further condensation of the monolayer. The final calcium concentration of 2mM results in a slightly more condensed monolayer than what was found for the 1mM CaCl₂ data. The π/A -isotherm recorded on a subphase containing 2mM CaCl₂ shows a slight crossover with the 1mM CaCl₂ data. This crossover of the data could be due to an increased stability of the PI(4,5)P₂ monolayer in the presence of calcium. From the data obtained, it is possible that Ca²⁺, is able to shield and bridge the highly negatively charged headgroup, which results in a more condensed monolayer at the air/water interface. In addition, we suggest that the Ca²⁺ also increases the stability of the PI(4,5)P₂ monolayer.

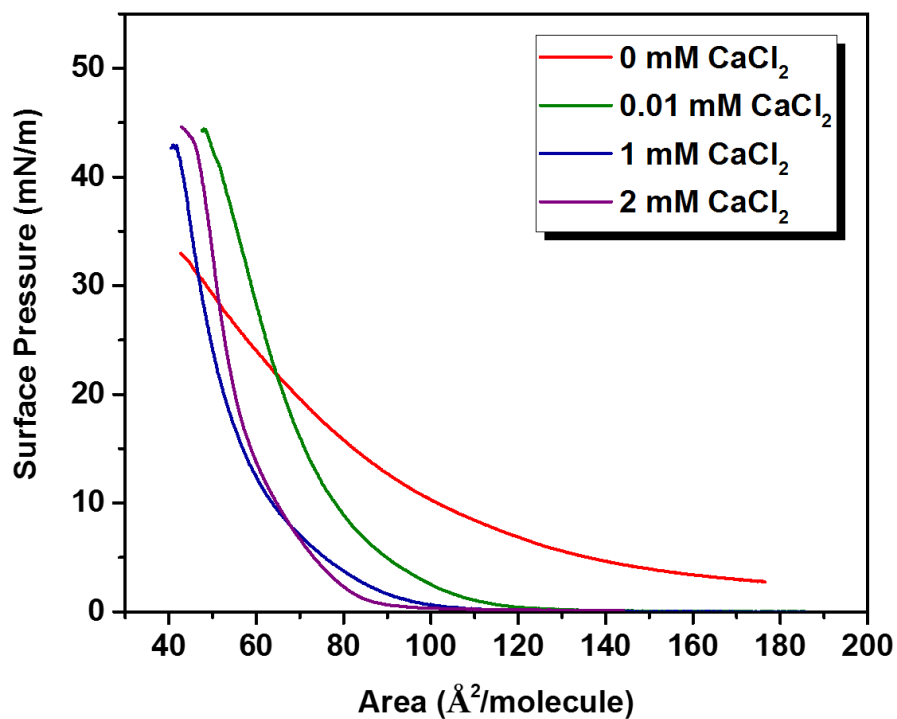


Figure 21: Surface Pressure/Area Isotherms of PI(4,5)P₂ Monolayers in the Absence and Presence of Calcium. Monolayers consisting of PI(4,5)P₂, on varying subphase composition: 0mM CaCl₂ (0.1mM EDTA) (red), 0.01mM CaCl₂ (green), 1mM CaCl₂ (blue), or 2mM CaCl₂ (purple), 10mM Tris, and 150mM NaCl at pH 7.4 and 25±0.2°C.

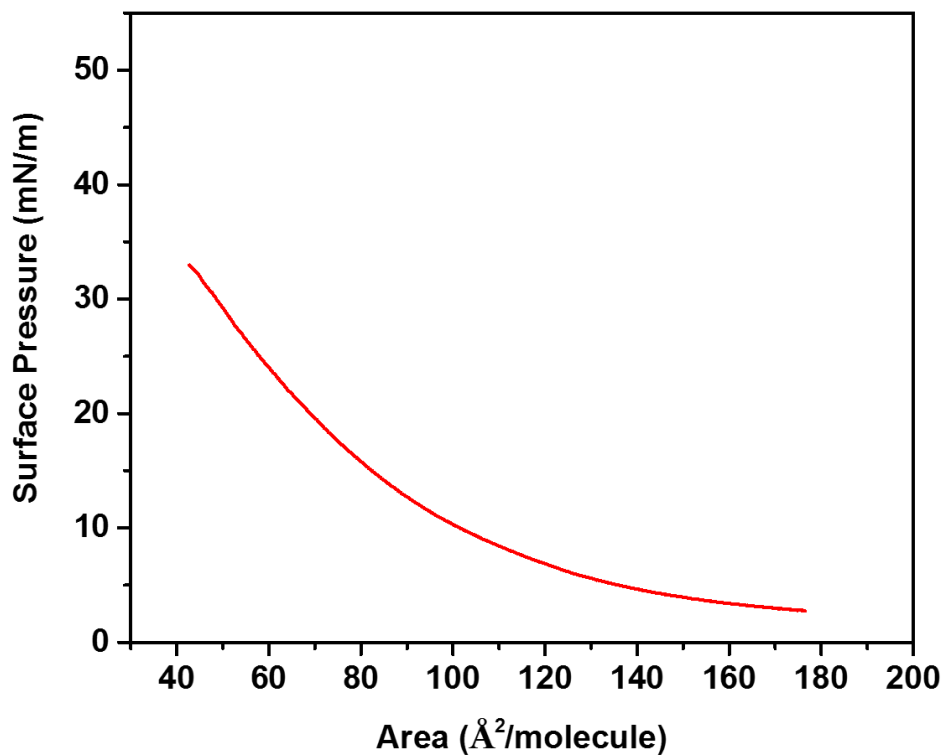
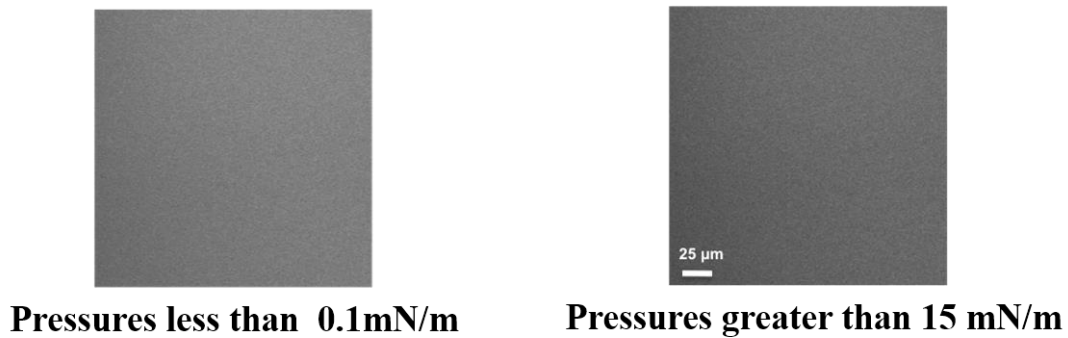


Figure 22: Surface Pressure/Area Isotherms of PI(4,5)P₂ Monolayer in the Absence of Calcium with Corresponding Epifluorescence Microscopy Images. Monolayer consisting of PI(4,5)P₂ on a subphase consisting of 10mM Tris, 150mM NaCl, 0.1mM EDTA, pH 7.4 at T=25±0.2°C. Fluorescence images shown are representative of pressures lower than 0.1mN/m and pressures above 15mN/m. Images were obtained using 0.1% Top Fluor™ PI(4,5)P₂

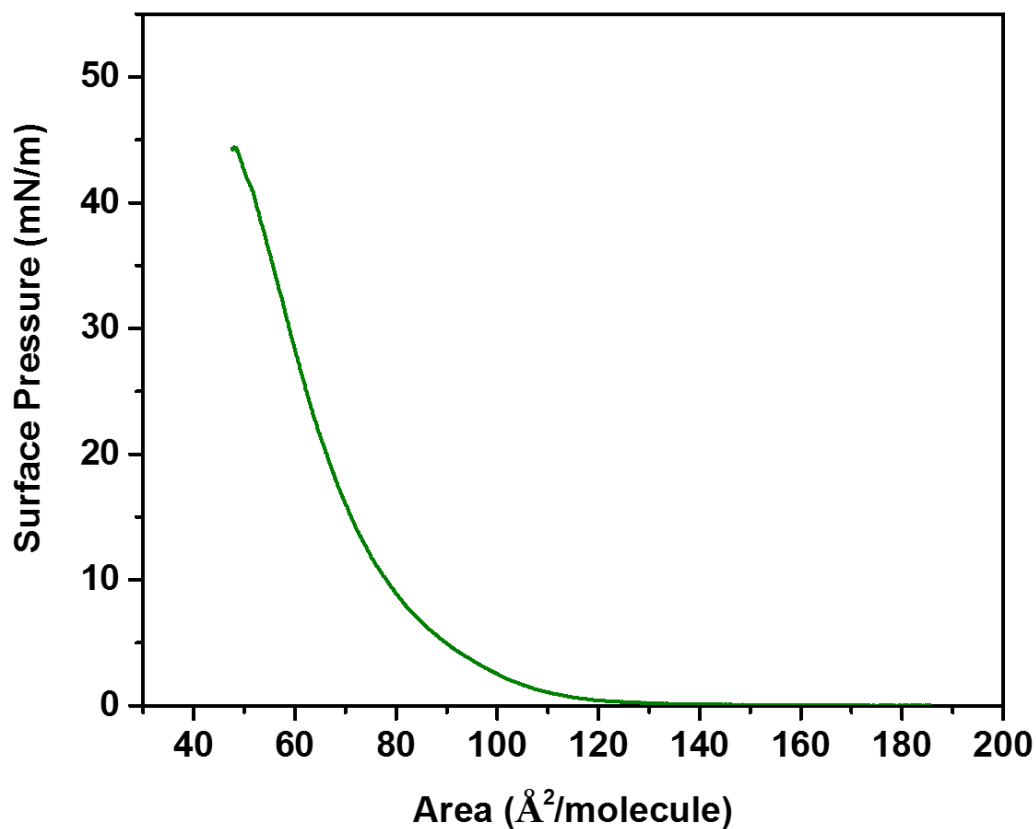
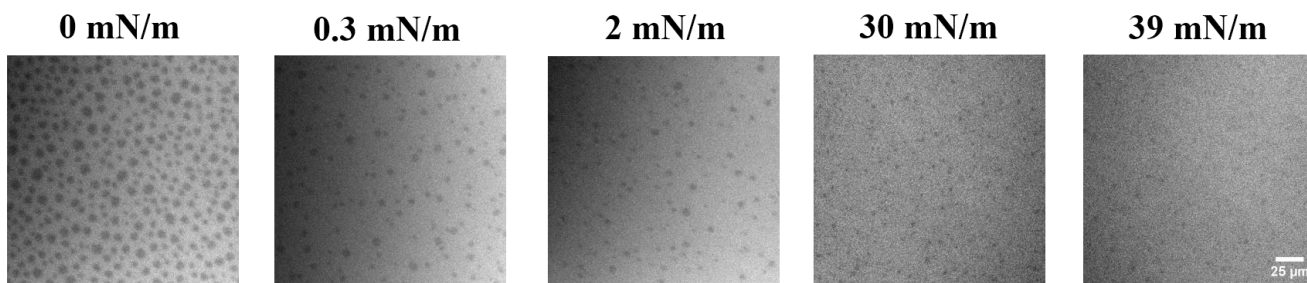


Figure 23: Surface Pressure/Area Isotherm of PI(4,5)P₂ Monolayer in the Presence of 0.01mM CaCl₂ with Corresponding Epifluorescence Microscopy Images.

Monolayer consisting of PI(4,5)P₂ on a subphase consisting of 10mM Tris, 150mM NaCl, 0.01mM CaCl₂, pH 7.4 at T=25±0.2°C. Fluorescence images shown are representative of corresponding pressures. Images were obtained using 0.1% Top Fluor™ PI(4,5)P₂

4.3 Effect of calcium on the morphology of phosphatidylinositol-4,5-bisophosphate monolayers

In section 4.2 we discussed the impact of calcium on surface pressure/area isotherms of PI(4,5)P₂ monolayers at the air/water interface. In this section, we investigate how the addition of calcium impacts the morphology of PI(4,5)P₂ monolayers using epifluorescence microscopy. Based on the significant condensation of the PI(4,5)P₂ monolayer in the presence of Ca²⁺, we would expect to see changes in the morphology of the monolayer when Ca²⁺ is present.

The samples for these experiments were prepared according to the procedure outlined in Chapter 2, and the experiments were carried out as previously described. For these experiments, the subphase consisted of 10mM Tris, 150mM NaCl, and either 0.01mM CaCl₂, 1mM CaCl₂, or 2mM CaCl₂. For experiments where the subphase lacks calcium, 0.1mM EDTA was used. All of the buffers were at pH 7.4 and a temperature of 25±0.2°C was maintained during the experiments. Top Fluor® PI(4,5)P₂ (0.1mol%) was added to the lipid sample to image the monolayer.

PI(4,5)P₂ monolayers in the absence of Ca²⁺ showed no macroscopically discernible domains at all pressures throughout the compression of the monolayer. (Figure 22) Due to the highly negatively charged headgroup of PI(4,5)P₂, and the lack of stability and highly expanded nature of the monolayer in the previous section, this is not a surprising result. Upon the addition of 0.01mM calcium to the subphase, epifluorescence images revealed macroscopically discernible domains at pressures up to 39mN/m. (Figure 23). Similarly, clustering was observed at low pressures for both 1mM and 2mM calcium containing

subphases; however, at higher pressures, the Top Fluor® PI(4,5)P₂ fluorophore seems to form clusters by itself. (Figure 24, Figure 25) Based upon the isotherms obtained in the presence of Ca²⁺ it appears that the lipid molecules become tightly packed. Apparently at high Ca²⁺ concentrations, this results in an exclusion of the labeled PI(4,5)P₂ from the phase enriched in the unlabeled PI(4,5)P₂. Presumably this occurs because the fluorophore label imposes too much of a steric demand to pack efficiently with the unlabeled PI(4,5)P₂. The strength of the PI(4,5)P₂/Ca²⁺ interaction manifests itself in the fact that the labeled PI(4,5)P₂ forms highly condensed, very bright small domains at 2 mM Ca²⁺. It is worth noting that the shape of the π/A -isotherm shows features that are typical of a condensed monolayer.

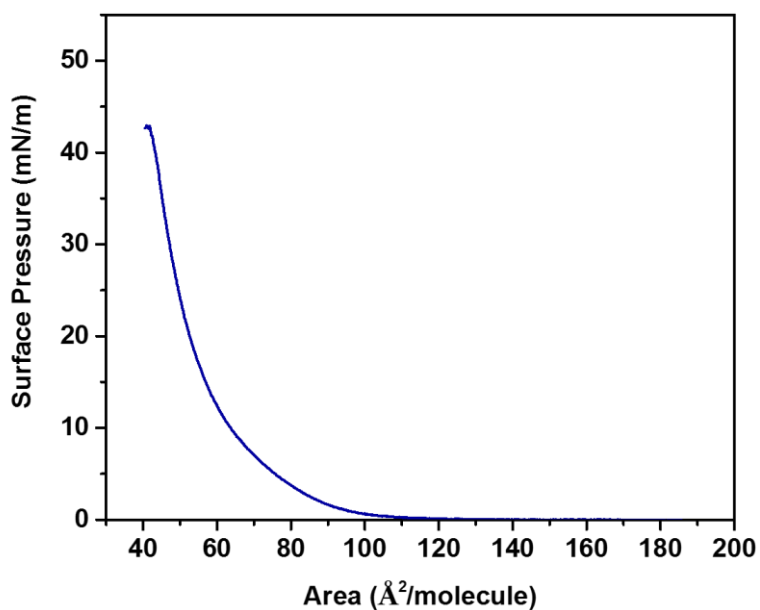
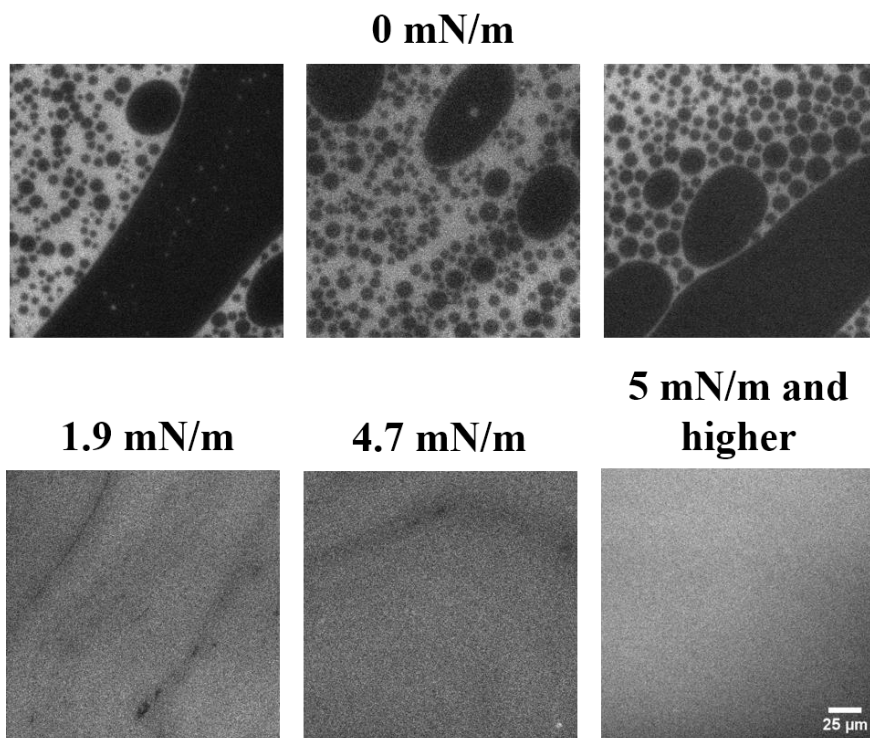


Figure 24: Surface Pressure/Area Isotherm of PI(4,5)P₂ Monolayer in the Presence of 1mM CaCl₂ with Corresponding Epifluorescence Microscopy Images. Monolayer consisting of PI(4,5)P₂ on a subphase consisting of 10mM Tris, 150mM NaCl, 1mM CaCl₂, pH 7.4 at T=25±0.2°C. Fluorescence images shown are representative of corresponding pressures. Images were obtained using 0.1% Top Fluor® PI(4,5)P₂

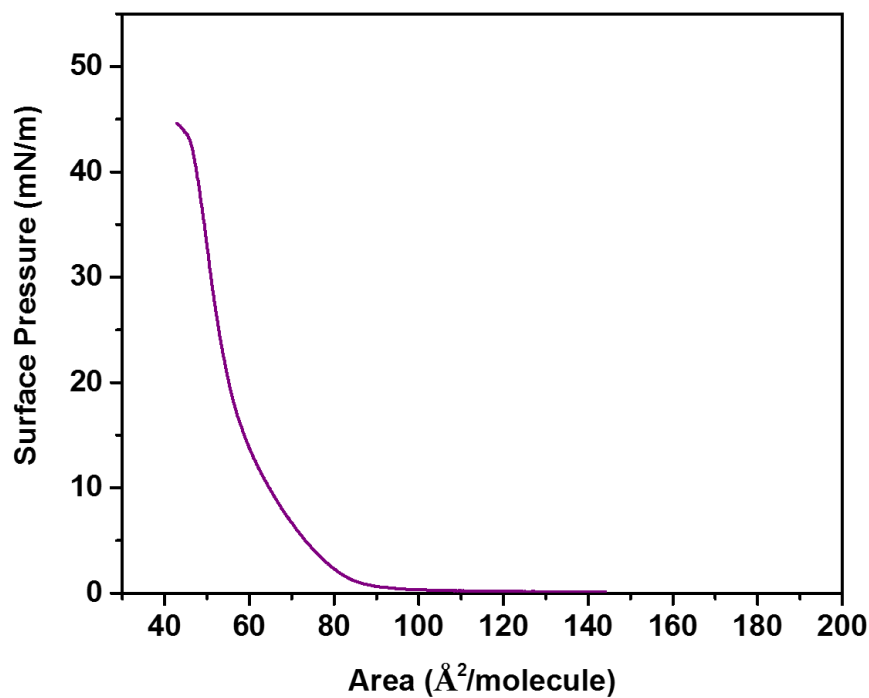
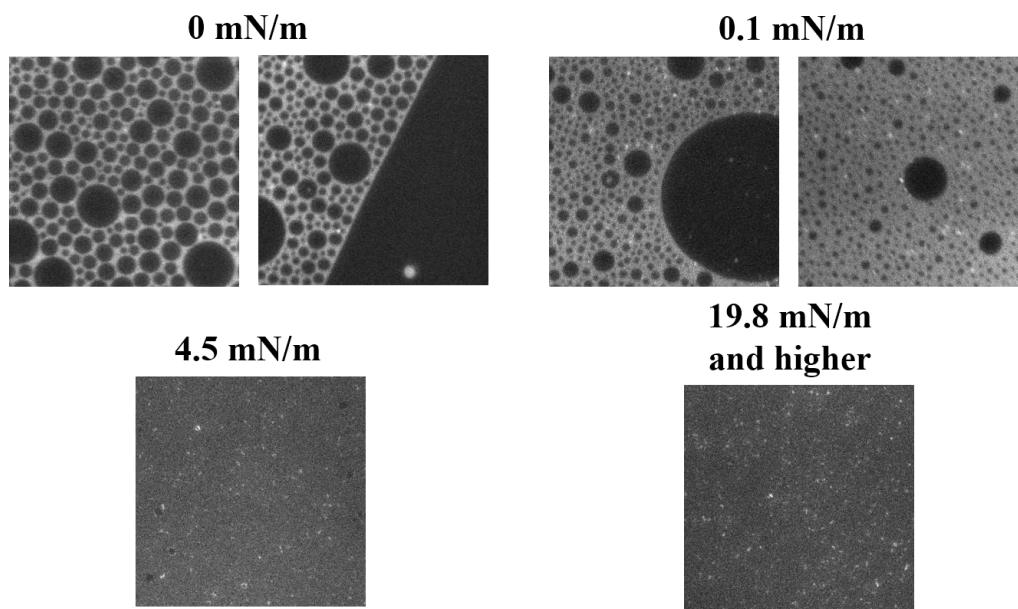


Figure 25: Surface Pressure/Area Isotherm of PI(4,5)P₂ Monolayer in the Presence of 2mM CaCl₂ with Corresponding Epifluorescence Microscopy Images. Monolayer consisting of PI(4,5)P₂ on a subphase consisting of 10mM Tris, 150mM NaCl, 2mM CaCl₂, pH 7.4 at T=25±0.2°C. Fluorescence images shown are representative of corresponding pressures. Images were obtained using 0.1% Top Fluor® PI(4,5)P₂

4.4 Effect of calcium on the surface pressure/area isotherms of mixed phosphatidylethanolamine/phosphatidylinositol-4,5-bisphosphate monolayers

To better mimic the behavior of the inner leaflet of the plasma membrane we used next mixtures of PE and PI(4,5)P₂. In this section we will investigate 1:1 and 4:1 ratios of PE:PI(4,5)P₂ to determine how PE influences PI(4,5)P₂ phase behavior. In addition we will add varying concentrations of calcium to the subphase to investigate how this effects mixed PE/PI(4,5)P₂ monolayers. The small headgroup geometry and hydrogen bonding capability of the ammonium headgroup of PE, may allow for the intercalation of PE between PI(4,5)P₂ headgroups and participation in hydrogen bonding.

All samples for this section were prepared according to the procedure discussed in Chapter 2 and the experiments were carried out as previously mentioned. The subphase for these experiments contained 10mM Tris, 150mMNaCl, and either 0.01mM CaCl₂, 1mM CaCl₂, or 2mM CaCl₂. In cases where the subphase lacked calcium, 0.1mM EDTA was used. The subphase was at pH 7.4 and a temperature of 25±0.2°C.

We initially started with a 1:1 ratio of PE:PI(4,5)P₂. (Figure 26) Surprisingly, when using a subphase lacking calcium, the isotherm is even more expanded than that of either PE (Figure 13) or PI(4,5)P₂ (Figure 22) in the absence of calcium. A possible explanation for this behavior is that due to intercalation of PE between the PI(4,5)P₂ headgroups, a change in the PI(4,5)P₂ headgroup orientation occurs like it was postulated in chapter 3 PI in the presence of PE (see Chapter 3). The addition of physiologically relevant concentrations of calcium (0.01mM) results in a significant condensation of the monolayer. (Figure 26) Based on the data from PI(4,5)P₂ monolayers in the presence of calcium, the condensation of 1:1 PE:PI(4,5)P₂ in the presence of 0.01mM Ca²⁺ could be

two fold, the Ca^{2+} could shield the negative charge of the PI(4,5)P₂ headgroups while the PE is able to intercalate between PI(4,5)P₂ molecules. Increasing the calcium concentration in the subphase to either 1mM or 2mM Ca^{2+} only slightly condenses the monolayer further. (Figure 26) Considering the data obtained previously, this outcome is justifiable. The shift of the isotherm to lower areas/molecule (more condensed monolayer) is only slightly altered by an increase in Ca^{2+} because the PE molecules are placed between the PI(4,5)P₂ headgroups, giving it a fixed area to which it can be condensed.

We then moved to a more physiological representation of the inner leaflet of the plasma membrane by using a 4:1 ratio of PE:PI(4,5)P₂. (Figure 27) When PE is at a higher concentration in the monolayer, the monolayer is more condensed than that of 1:1 PE:PI(4,5)P₂ when the subphase is lacking calcium. This suggests that the stoichiometry of the PE:PI(4,5)P₂ interaction may play a role in the condensation of the monolayer. This further supports our hypothesis that the headgroup of PE is able to insert between the PI(4,5)P₂ headgroups.

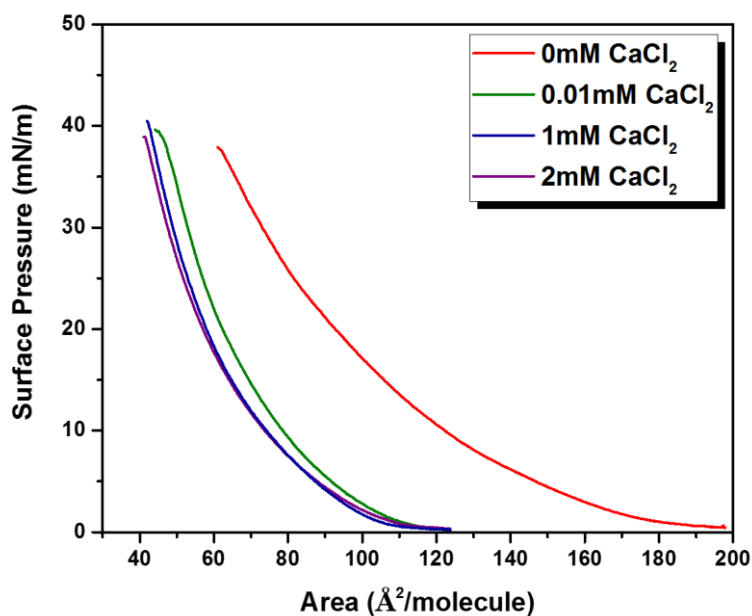
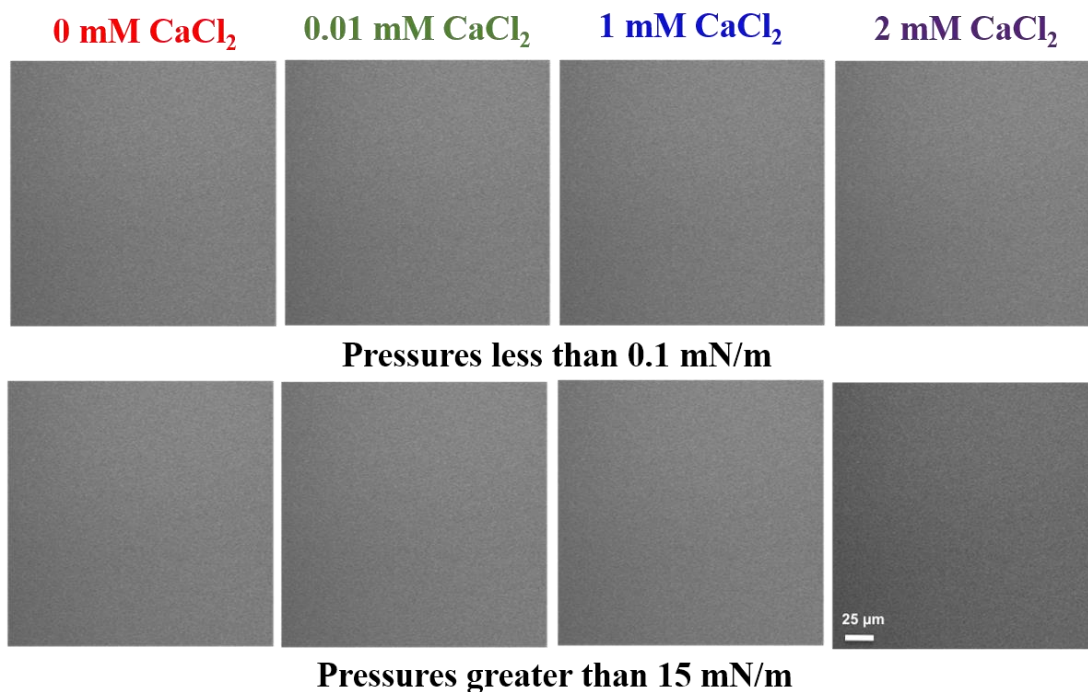


Figure 26: Surface Pressure/Area Isotherms of 1:1 PE:PI(4,5)P₂ with Corresponding Epifluorescence Microscopy Images. Monolayers consisting of 1:1 PE:PI(4,5)P₂, on varying subphase composition: 0mM CaCl₂ (0.1mM EDTA) (red), 0.01mM CaCl₂ (green), 1mM CaCl₂ (blue), or 2mM CaCl₂ (purple), 10mM Tris, and 150mM NaCl at pH 7.4 and 25±0.2°C. 0.1mol% Top Fluor™ PI(4,5)P₂ was added to lipid mixtures for imaging.

4.5 Effect of calcium on the morphology of mixed phosphatidylethanolamine/phosphatidylinositol-4,5-bisphosphate monolayers

In the previous section we discussed the influence of calcium on the thermodynamic behavior of mixed PE/PI(4,5)P₂ monolayers. Here we discuss how the lateral organization of the monolayer is impacted by the addition of calcium concentrations.

Preparation of samples and experimental procedure for these experiments are outlined in Chapter 2. The buffers used for these experiments were composed of 10mM Tris, 150mM NaCl, and either 0.01mM CaCl₂, 1mM CaCl₂, or 2mM CaCl₂. All buffers were pH 7.4 and at a temperature of 25±0.2°C. Top Fluor™ PI(4,5)P₂ was incorporated (0.1mol%) in the lipid sample for imaging purposes.

For epifluorescence images taken of mixed PE:PI(4,5)P₂ monolayers at ratios of 4:1 and 1:1, no domain formation or condensation was observed for any of the calcium concentrations used. (Figure 26, Figure 27) Although the π/A -isotherms indicated a significant condensation of the monolayer for 1:1 PE/PI(4,5)P₂ ratio, the monolayer remains quite expanded and the fluorophore appears to be distributed evenly, and essential all of the components of the monolayer are forming one phase (they are not demixing). It is possible that because PE is inserting between the PI(4,5)P₂ headgroups that incorporation of a fluorophore yields no domain formation in the monolayer.

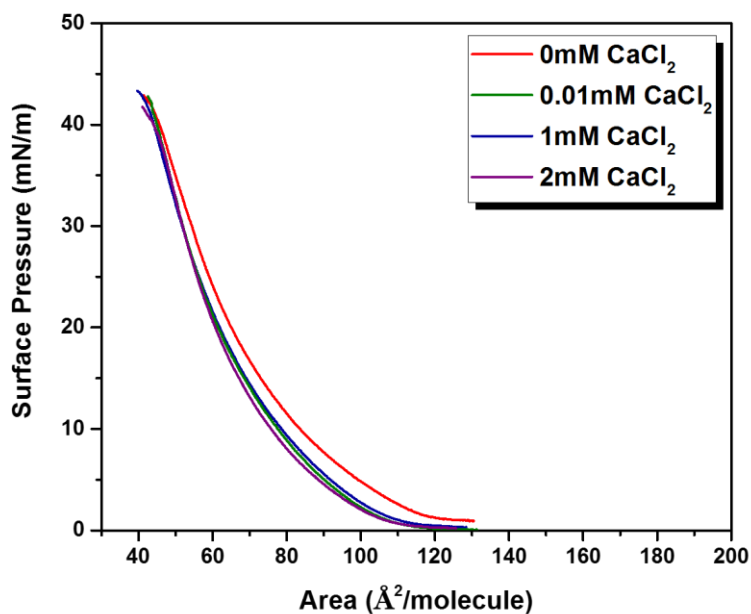
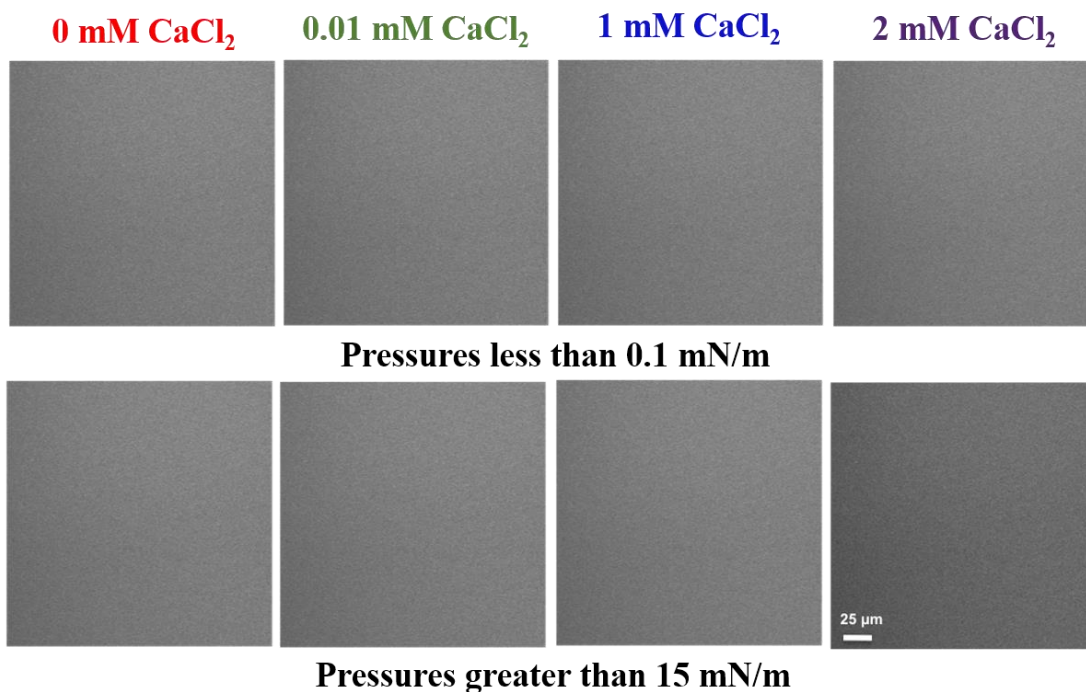


Figure 27: Surface Pressure/Area Isotherms of 4:1 PE:PI(4,5)P₂ with Corresponding Epifluorescence Microscopy Images. Monolayers consisting of 4:1 PE:PI(4,5)P₂, on varying subphase composition: 0mM CaCl₂ (0.1mM EDTA) (red), 0.01mM CaCl₂ (green), 1mM CaCl₂ (blue), or 2mM CaCl₂ (purple), 10mM Tris, and 150mM NaCl at pH 7.4 and 25±0.2°C. 0.1mol% Top Fluor™ PI(4,5)P₂ was added to lipid mixtures for imaging.

4.6 Effect of calcium on the surface pressure/area isotherms of mixed phosphatidylcholine/ phosphatidylinositol-4,5-bisphosphate monolayers

In the previous sections we investigated how mixed PE/PI(4,5)P₂ monolayers were influenced by the addition of Ca²⁺ to the subphase. Although the main motivation for our studies is to use lipid mixtures that mimic the inner leaflet of the plasma membrane, it is beneficial to investigate the interaction of PI(4,5)P₂ with PC to elucidate how the hydrogen bonding capabilities and small headgroup geometry of PE differ from the larger headgroup of PC (Figure 28), which is unable to participate in hydrogen bonding. Based on our previous experiments, we would expect to see less condensation (shifting to lower areas/molecule) due to the larger trimethylammonium headgroup of PC.

Experiments carried out in this section were performed according to the details discussed in Chapter 2. Surface pressure/area isotherms were recorded on subphases consisting of 10mM Tris, 150mM NaCl, and either 0mM CaCl₂ (0.1mM EDTA), or 2mM CaCl₂, and were maintained at a pH of 7.4 and a temperature of 25±0.2°C.

We first measured surface pressure/area isotherms for monolayers composed of PC:PI(4,5)P₂ at a 1:1 ratio. (Figure 29) This initial isotherm is shifted to larger areas/molecule than that of isotherms recorded for either PC or PI(4,5)P₂. This is characteristic of a monolayer that is less condensed (lower molecular density). The

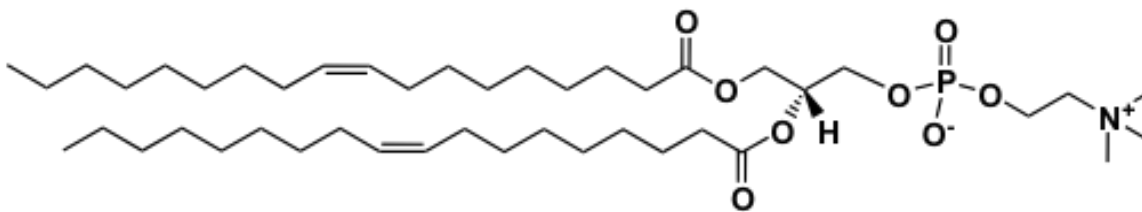


Figure 28: Chemical Structure of DOPC

addition of 2mM CaCl₂ to the subphase results in a surface/pressure isotherm that is similar to that of the 1:1 PC:PI(4,5)P₂ in the absence of calcium, i.e., the condensation of the monolayer is marginal. The most significant difference in the isotherms is that the isotherm recorded on the subphase containing 2mM CaCl₂ is more stable, and hence has a higher surface pressure at the collapse of the monolayer than that of the isotherm in the absence of Ca²⁺. We then used a 4:1 ratio of PC:PI(4,5)P₂. (Figure 30) The results were similar to those seen in the 1:1 PC:PI(4,5)P₂ in both the absence and presence of Ca²⁺. For 4:1 PC:PI(4,5)P₂ samples in the absence of calcium shows an isotherm characteristic of an expanded monolayer, while the addition of 2mM CaCl₂ shows an increased in the stability of the monolayer based on the collapse pressure of the monolayer. Taken together, this data suggests that the inability of the PC headgroup to contribute to hydrogen bonding, along with the larger headgroup geometry makes it unable to condense the monolayer. This data strengthens the argument for PE insertion between PI(4,5)P₂ headgroups due to small headgroup geometry and hydrogen bonding capabilities.

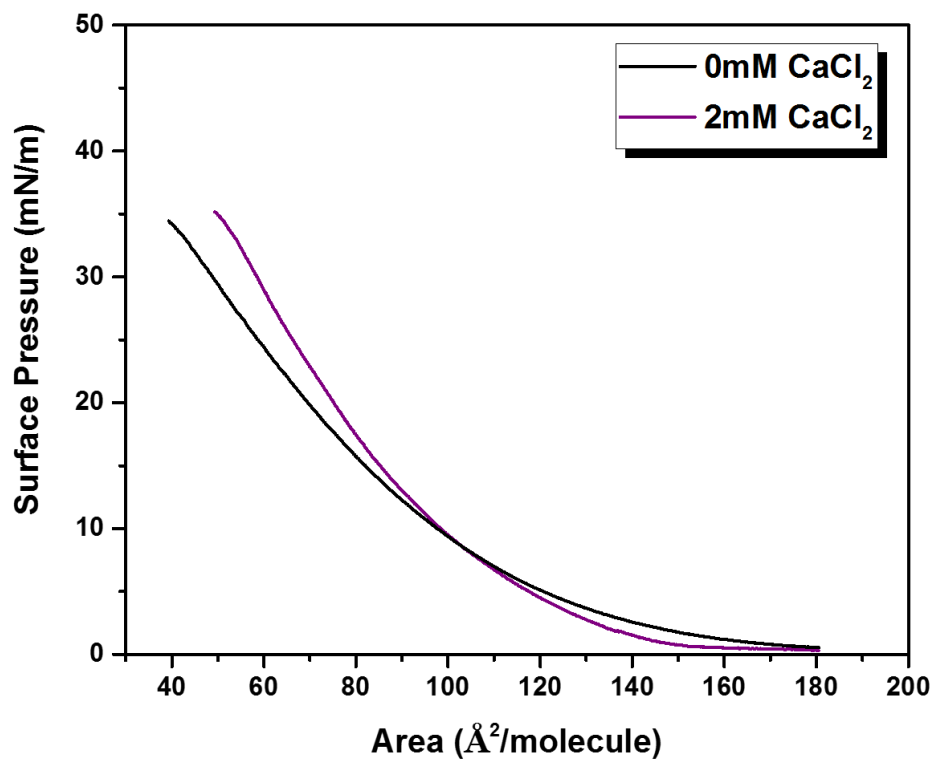


Figure 29: Surface pressure/area isotherms of monolayers consisting of 1:1 PC:PI(4,5)P₂ in the presence and absence of calcium. Monolayers consisting of 1:1 PC:PI(4,5)P₂, on varying subphase composition: 0mM CaCl₂ (0.1mM EDTA) (black) or 2mM CaCl₂ (purple), 10mM Tris, and 150mM NaCl at pH 7.4 and 25±0.2°C.

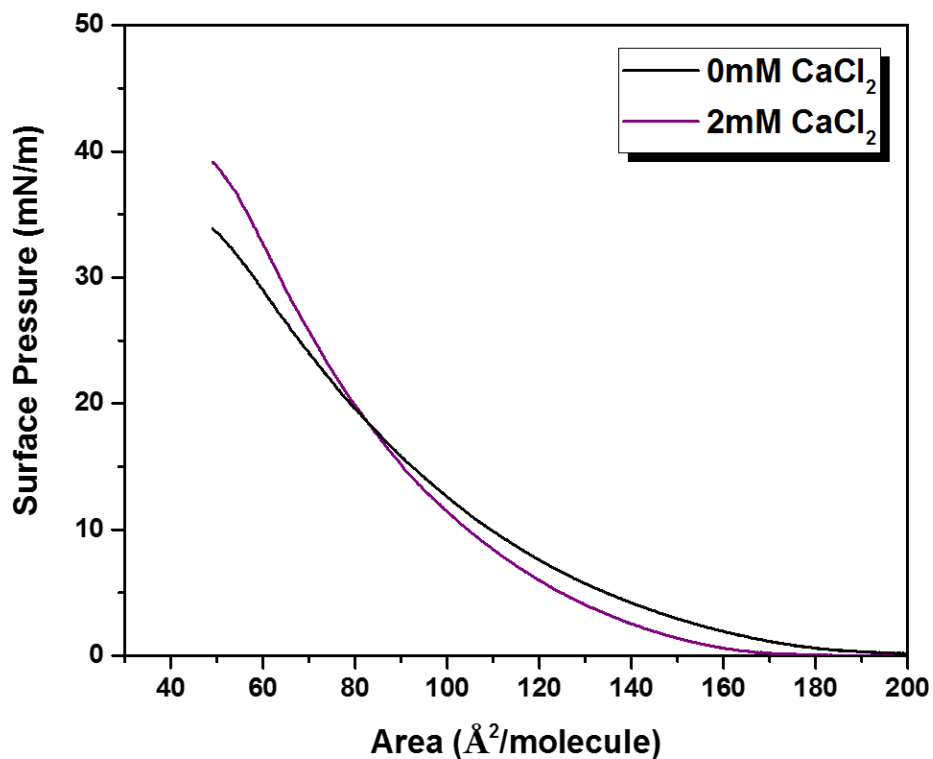


Figure 30: Surface pressure/area isotherms of monolayers consisting of 4:1 PC:PI(4,5)P₂ in the presence and absence of calcium. Monolayers consisting of 4:1 PC:PI(4,5)P₂, on varying subphase composition: 0mM CaCl₂ (0.1mM EDTA) (black) or 2mM CaCl₂ (purple), 10mM Tris, and 150mM NaCl at pH 7.4 and 25±0.2°C.

4.7 Effect of Cholesterol on Mixed PE/PI(4,5)P₂ surface pressure/area isotherms.

In this section, we investigate the influence of cholesterol on the phase behavior of mixed PE/PI(4,5)P₂ monolayers at air/water interface. Previous work from our group has shown that the hydroxyl group of cholesterol is integral for participation in the hydrogen bond network formed between phosphoinositides. The hydrogen bonding capabilities of PE may be able to disrupt the cholesterol-PI(4,5)P₂ hydrogen bonding; however it could also further condense the monolayer by participating in the hydrogen bonding network.

Experiments carried out in this section were performed as detailed in Chapter 2. Experiments were carried out on a subphase composed of 10mM Tris, 150mM NaCl, 0.1mM EDTA, and were maintained at a pH of 7.4 and a temperature of 25±0.2°C.

At a ratio of 4:1 PE:PI(4,5)P₂ with 40mol% cholesterol, the π/A -isotherm is characteristic of a more condensed monolayer when compared to 4:1 PE:PI(4,5)P₂ in the absence of cholesterol (Figure 31). The shift to lower areas/molecule is significant, suggesting that PE/PI(4,5)P₂/chol form a ternary complex via hydrogen bonding.

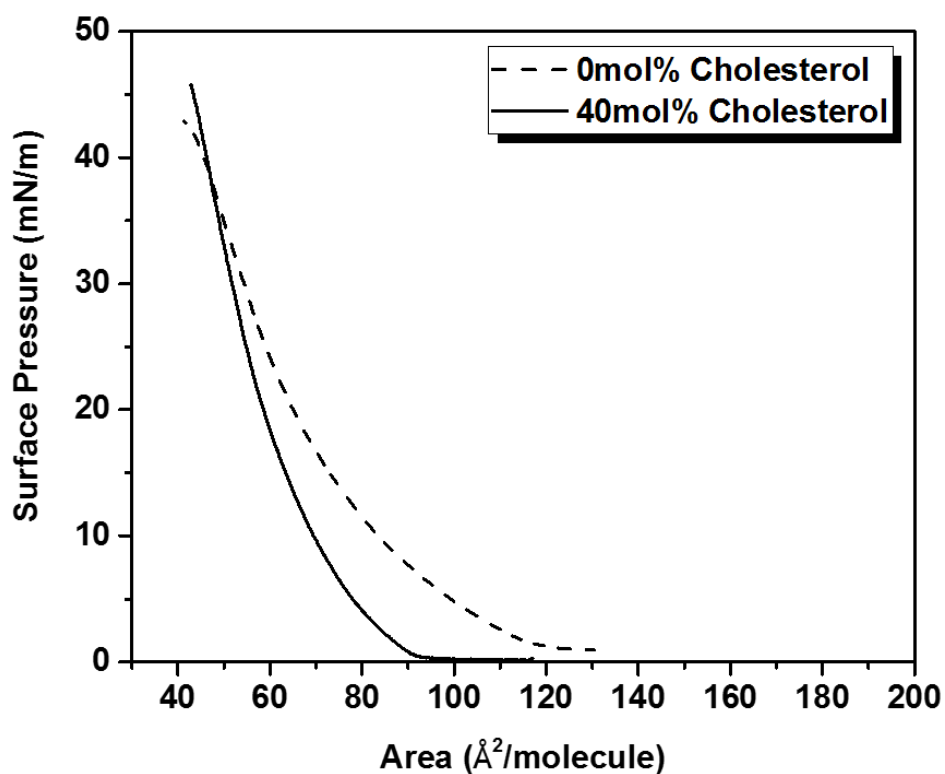
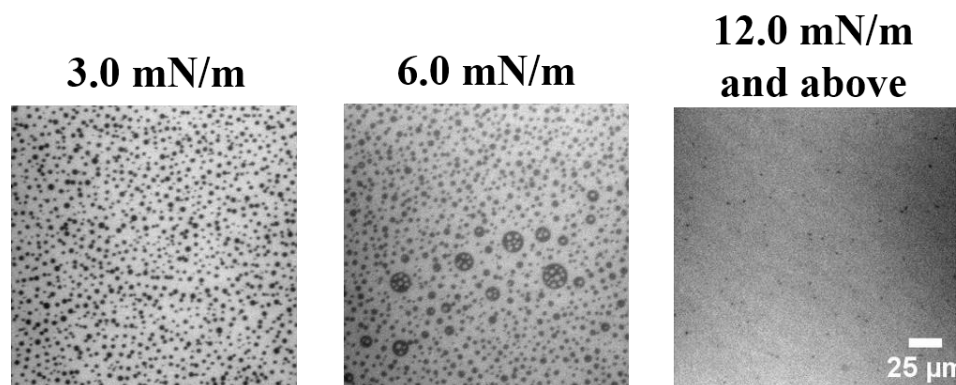


Figure 31: Surface pressure/area isotherm of 4:1 PE:PI(4,5)P₂ with an additional 40mol% cholesterol. Monolayers consisting of 4:1 PE:PI(4,5)P₂ with an additional 40% cholesterol on subphase consisting of 10mM Tris, 150mM NaCl, 0.1mM EDTA at pH 7.4 and maintained at T=25±0.2°C. For epifluorescence images, 0.1mol% Top Fluor™ PI(4,5)P₂ was incorporated in the lipid sample. The isotherm of 4:PI(4,5)P₂ in the absence of cholesterol is shown for comparison. (dashed)

4.8 Effect of Cholesterol on Mixed PE/PI(4,5)P₂ monolayer morphology.

In the previous section, we have described how cholesterol influences the phase behavior of mixed PE:PI(4,5)P₂ monolayers at a 4:1 ratio. In this section, we investigate how the addition of cholesterol to the monolayer influence the lateral homogeneity of the monolayer. From our thermodynamic data, we see a condensation of the 4:1 PE:PI(4,5)P₂ monolayer with the addition of 40mol% cholesterol. Based on this information we would expect to see domain formation in the corresponding epifluorescence images.

Experiments in this section were performed as detailed in Chapter 2. Epifluorescence images of the monolayer were taken on a subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA. The buffer subphase was maintained at a pH of 7.4 and a temperature of 25±0.2°C. For these studies 0.1mol% Top Fluor™ PI(4,5)P₂ was incorporated in the lipid sample for imaging purposes.

The epifluorescence images taken for samples consisting of 4:1 PE:PI(4,5)P₂ with an additional 40mol% cholesterol show small, circular domain formation at low pressures. As the pressure increases, the domains become smaller and are less condensed. Although small, the domains persist throughout the isotherm. (Figure 31) Taken together with the thermodynamic data, this suggests that cholesterol is able to condense and stabilize mixed PE:PI(4,5)P₂ monolayers.

4.9 Impact of Cholesterol and Calcium on Mixed PE/PI(4,5)P₂ surface pressure/area isotherms

In the previous section, we have shown that both cholesterol and calcium can modulate PE/PI(4,5)P₂ monolayer phase behavior. Here we investigate how the combination of cholesterol and calcium influence mixed PE/PI(4,5)P₂ monolayers at the air/water interface. These experiments are designed to determine if cholesterol and calcium have a synergistic or competing effect on PE/PI(4,5)P₂ interaction. The addition of Ca²⁺ may disrupt the hydrogen bond network formed between cholesterol, PE, and PI(4,5)P₂. In Chapter 3, we saw that the effect of the addition of cholesterol and calcium was additive in terms of the condensation of a PE/PI monolayer. Through our experiments in this chapter, we will be able to determine if the more negatively charged headgroup of PI(4,5)P₂ will behave similarly as the less charged PI headgroup.

Experiments performed in this section were completed as detailed in Chapter 2. All isotherms we recorded on a subphase consisting of 10mM Tris, 150mM NaCl, and either 0mM CaCl₂ (0.1mM EDTA), 0.01mM CaCl₂, or 2mM CaCl₂. Buffer subphases were maintained at a pH of 7.4 and a temperature of 25±0.2°C.

The addition of 0.01mM CaCl₂ to the subphase results in a 4:1 PE:PI(4,5)P₂ with 40mol% cholesterol monolayer (Figure 32) that is more expanded (higher areas/molecule) than 4:1 PE:PI(4,5)P₂ with 40mol% cholesterol in the absence of calcium. Equally, when the Ca²⁺ concentration in the subphase is increased to 2mM, the 4:1 PE:PI(4,5)P₂ with 40mol% cholesterol monolayer is more expanded when compared to the same monolayer in the absence of Ca²⁺. (Figure 32) When comparing the surface pressure/area isotherms of 4:1 PE:PI(4,5)P₂ with 40mol% cholesterol in the presence of

0.01mM CaCl₂ and 2mM CaCl₂, it is found that they are quite similar, suggesting that the increase in Ca²⁺ from 0.01mM to 2mM has little effect on the expansion of the monolayer. Apparently, the incorporation of Ca²⁺ in the PE/PI(4,5)P₂/cholesterol complex leads to an arrangement of the lipid headgroup that is more space consuming. This will be discussed further below.

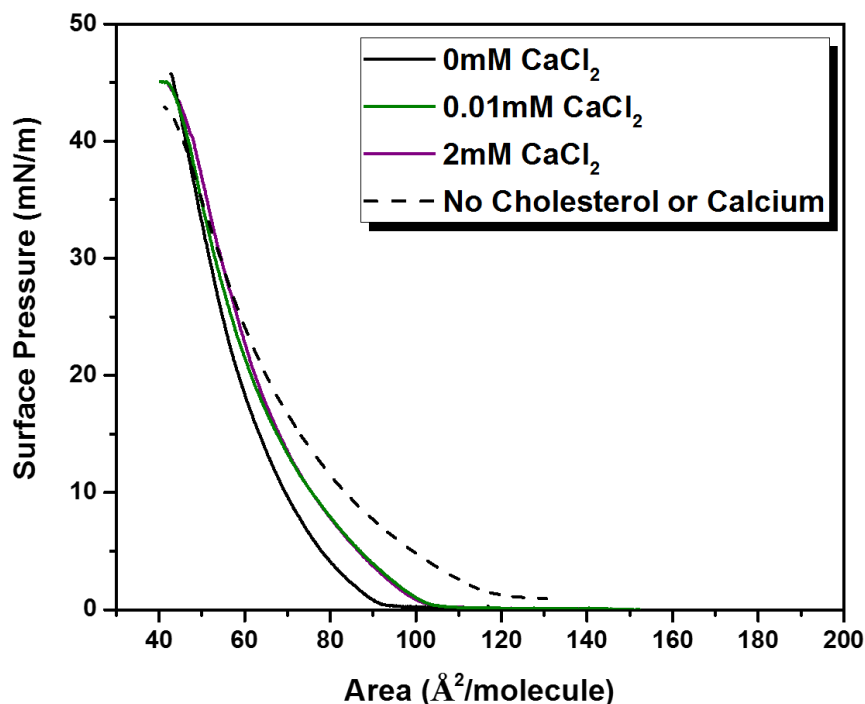


Figure 32 Surface Pressure/Area Isotherms of 4:1 PE:PI(4,5)P₂ monolayers with 40mol% Cholesterol on the Absence and Presence of Calcium. Surface pressure/area isotherms composed of 4:1 PE:PI(4,5)P₂ with 40mol% cholesterol on subphase consisting of 0mM CaCl₂ (0.1mM EDTA) (black), 0.01mM CaCl₂ (green), or 2mM CaCl₂ (purple), 10mM Tris, and 150mM NaCl at pH 7.4 and T=25±0.2°C.

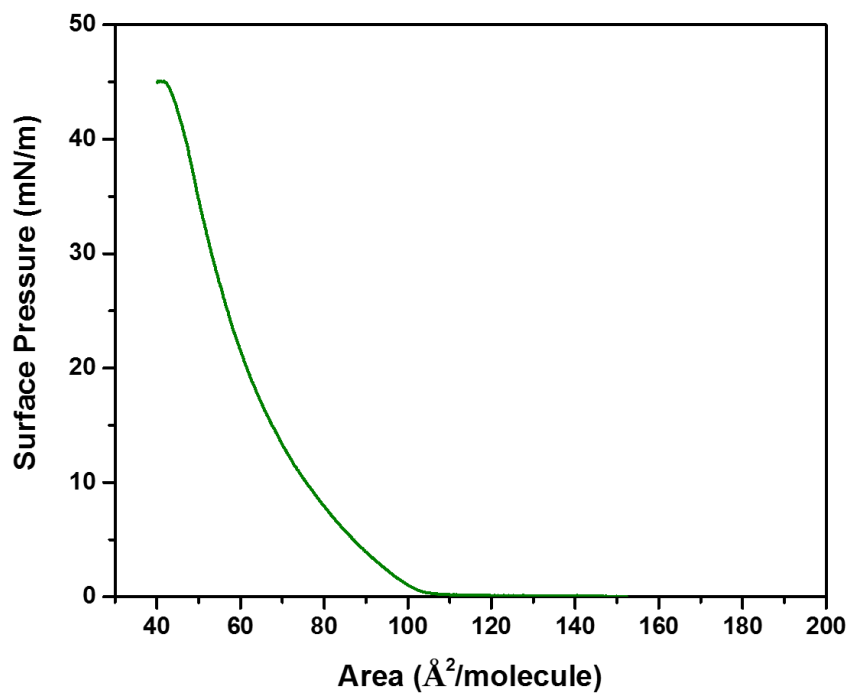
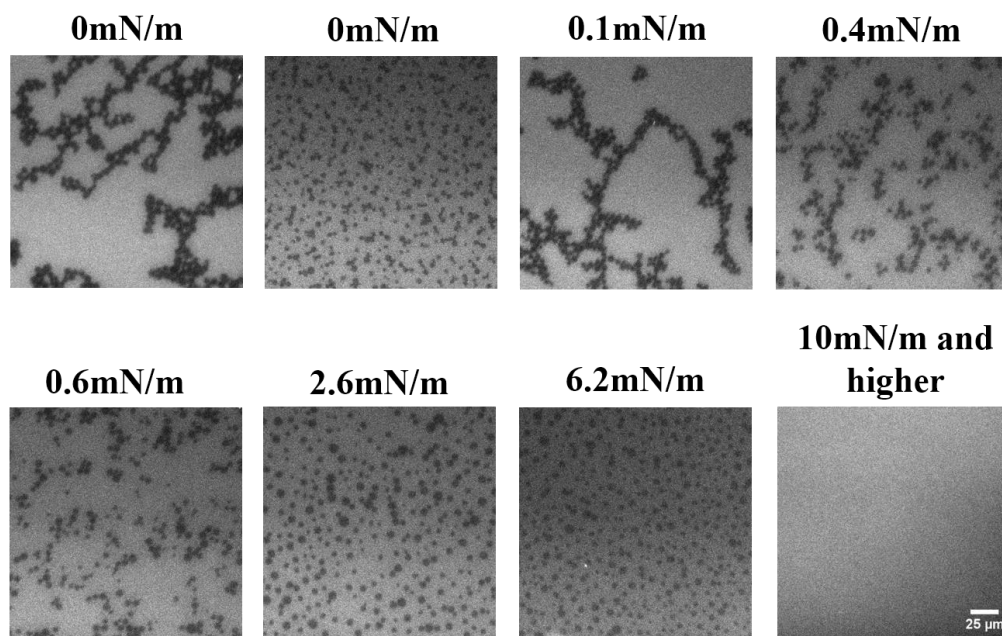


Figure 33: Surface pressure/area isotherm of 4:1 PE:PI(4,5)P₂ with an additional 40mol% cholesterol in the presence of 0.01mM CaCl₂. Monolayer consisting of 4:1 PE:PI(4,5)P₂ with an additional 40% cholesterol on subphase consisting of 10mM Tris, 150mM NaCl, and 0.01mM CaCl₂ at pH 7.4 and maintained at T=25±0.2°C. For epifluorescence images, 0.1mol% Top Fluor™ PI(4,5)P₂ was incorporated in the lipid sample.

4.10 Influence of Cholesterol and Calcium on Mixed PE/PI(4,5)P₂ monolayer morphology

In the previous section, we have investigated how the addition of cholesterol and calcium influences the phase behavior of mixed PE:PI(4,5)P₂ monolayers at the air/water interface. In this section we focus on how the addition of the membrane modulators, calcium and cholesterol, impact the morphology of PE:PI(4,5)P₂ monolayers.

Experiments in this section were conducted according to the procedure in Chapter 2. All isotherms were performed on a subphase composed of 10mM Tris, 150mM NaCl, and either 0.01mM CaCl₂ or 2mM CaCl₂ at pH 7.4 and a temperature of 25±0.2°C. Top Fluor™ PI(4,5)P₂ was added (0.1mol%) to the lipid sample for imaging.

Epifluorescence images recorded on a subphase with 0.01mM CaCl₂ (Figure 33), display at 0mN/m both dark circular areas and circular areas that are linked. As the pressure increases, the chained, circular areas begin to break apart into individual circular areas. The circular areas remain present until pressures of 10mN/m when they blur and coalesce. At Ca²⁺ concentrations of 2mM (Figure 34), chained, circular areas are visible at 0mN/m. As the pressure is increased, regions of a mixture of linked circular dark areas and individual circular areas are present. Further compression of the barriers (increase in pressure) leads to a separation of the linked circular areas, which remain visible until 10mN/m when they blur and disappear. The question arises whether the dark regions are domains from which the fluorophore is being excluded or areas where no film material is present. The image in Figure 34 for 0.1mN/m suggests that the latter is the case, since typical features for an LE/G phase (large “bubbles”) are in co-existence with the small pearl chain like structures, suggesting that also these structures are areas of low or no

monolayer coverage. Why do these areas look distinctively different from those that are typically found for a LE/G transition phase, and why do these structures persist up to quite high surface pressures? This can only be explained by assuming that lateral mobility of the lipid molecules is quite limited, which prevents the lipid molecules from filling the “holes” upon moderate compression. We hypothesize that the PE/PI(4,5)P₂/cholesterol/Ca²⁺ complex results in a headgroup network that strongly reduces the mobility of the film forming molecules.

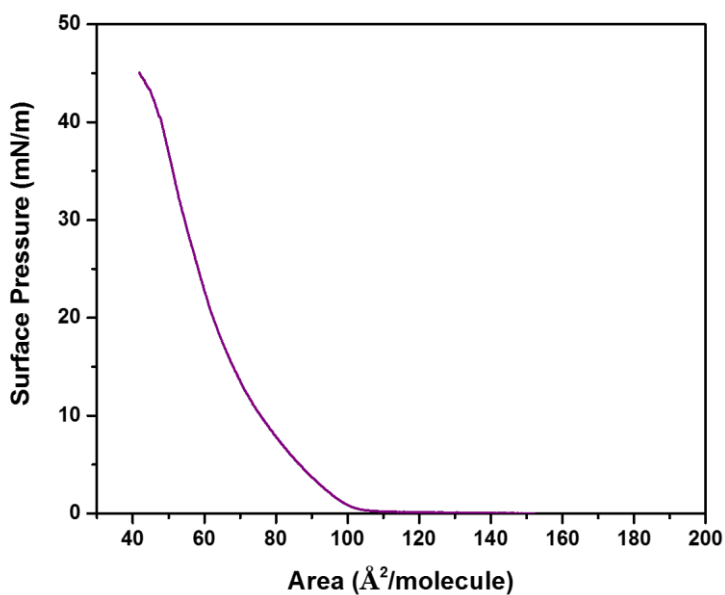
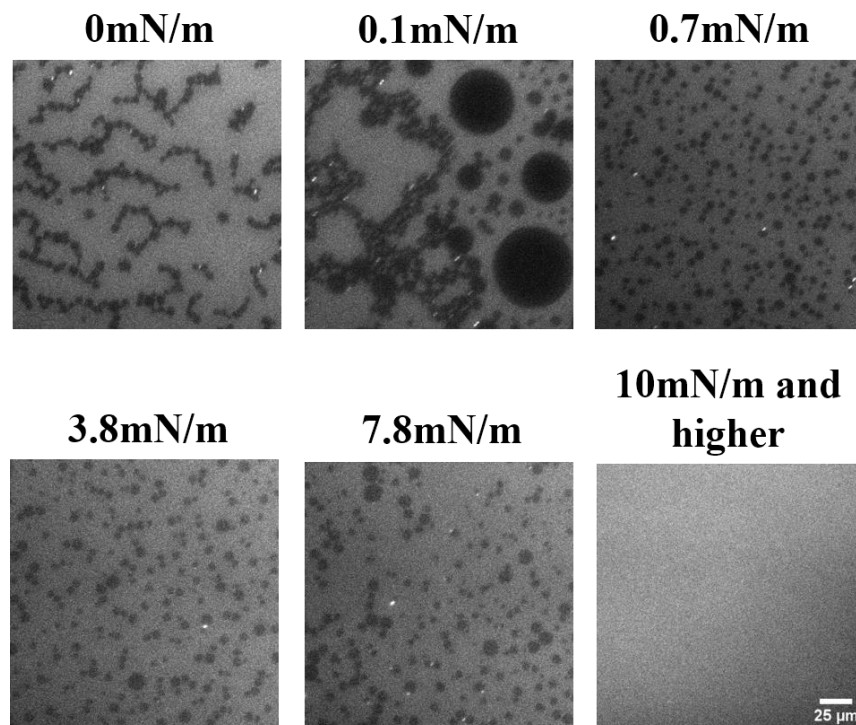


Figure 34: Surface pressure/area isotherm of 4:1 PE:PI(4,5)P₂ with an additional 40mol% cholesterol in the presence of 2mM CaCl₂. Monolayer consisting of 4:1 PE:PI(4,5)P₂ with an additional 40% cholesterol on subphase consisting of 10mM Tris, 150mM NaCl, and 2mM CaCl₂ at pH 7.4 and maintained at T=25±0.2°C. For epifluorescence images, 0.1mol% Top Fluor™ PI(4,5)P₂ was incorporated in the lipid sample

4.11 Conclusion

PI(4,5)P₂ monolayers in the absence of calcium lead to a surface pressure/area isotherm characteristic of an expanded monolayer. When compared to the data obtained for PI in the absence of calcium (Chapter 3), the isotherms are more expanded and have a lower collapse pressure. The expanded monolayer behavior is most likely a result of the repulsive forces from the highly negatively charged head group of PI(4,5)P₂ and the unsaturated nature of the *sn*-2 arachidonoyl acyl chain. The negatively charged headgroups prevent the lipid from packing tightly, leading to a less dense monolayer. In addition, the low collapse pressure of the monolayer suggests that the monolayer is not very stable, supporting the notion that the lipids cannot pack tightly. The instability of the monolayer might also be, at least in part, attributable to the lipid slipping underneath the barriers of the Langmuir trough. The epifluorescence images for this particular experiment show a completely gray image, meaning there is no condensation of the monolayer. This data is in agreement with the thermodynamic data obtained from the surface pressure/area isotherms.

The addition of calcium to the subphase results in condensation of the PI(4,5)P₂ monolayer even at low Ca²⁺ concentrations. We suggest that this is due to the ability of Ca²⁺ to shield and bridge the negatively charged headgroup of PI(4,5)P₂. We also observe that the addition of calcium leads to a stabilization of the monolayer, as evidenced by the higher surface pressure at the collapse of the monolayer. When isotherms of PI(4,5)P₂ in the presence of calcium are compared to those with PI in the presence of calcium, the isotherms for PI(4,5)P₂ are shifted to lower areas/molecule. Once again, this could be due to the additional phosphate groups and higher negative

charge of PI(4,5)P₂ leading to a more effective bridging of the PI(4,5)P₂ headgroups. The epifluorescence images show that at low calcium concentrations (0.01mM CaCl₂), there is some condensation at low pressures (less than 0.1mN/m). The further addition of calcium to the subphase also shows condensation at low pressures: however, for 2mM CaCl₂, the fluorophore is excluded from the monolayer, probably due to high steric demand of the fluorophore. We suggest that the condensed regions of the monolayer are eventually pushed together to form a very rigid monolayer at high concentrations of calcium. When taken together with the data obtained from the surface/pressure area isotherms, this supports our hypothesis that the calcium is stabilizing the monolayer as the calcium concentration is increased.

We next investigated how the addition of PE would impact monolayers of PI(4,5)P₂ in the absence and presence of calcium. The isotherm of 1:1 PE:PI(4,5)P₂ in the absence of calcium is expanded. Surprisingly, the 1:1 isotherm is even more expanded than that of either PI(4,5)P₂ or PE in the absence of calcium. This may be due to the fact that the interaction with PE leads to a change in the orientation of the PI(4,5)P₂ headgroup. In addition, this monolayer is more expanded than that of the 1:1 PE:PI data obtained in Chapter 3, possible due to the increased larger steric demand of a tilted PI(4,5)P₂ headgroup vs. a PI headgroup. The 1:1 PE:PI(4,5)P₂ epifluorescence images in the absence of calcium reveal no condensation and look much like those of PI(4,5)P₂ and DOPE in the absence of calcium. This data is in agreement with the data obtained for the isotherms. When calcium was added to the subphase the condensation of the monolayer as inferred by the surface pressure/ area isotherms for 1:1 ratios of PE:PI(4,5)P₂ was drastic. Even at concentrations of 0.01mM CaCl₂, the isotherm is shifted to a much lower

area/molecule. It was surprising that while the condensing effect of calcium was so strong, there were no macroscopically visible domains in the corresponding epifluorescence images. Based on previous work, we know that mixtures of PI(4,5)P₂ and PC form domains in the presence of calcium. We also know that PE should be able to form hydrogen bonds where PC cannot: therefore, we suggest that we may not be seeing domain formation/condensation because PE, PI(4,5)P₂ and calcium are all interacting with one another forming one homogeneous monolayer. In addition, the condensation of 1:1 PE:PI(4,5)P₂ isotherms is impacted more than those of 1:1 PE:PI. It could be that the highly negative charge of PI(4,5)P₂ plays an important role in the interaction with PE. Furthermore we suggest that calcium is able to bridge the interaction of PE and PI(4,5)P₂, which allows the PE to incorporate itself in between the headgroups of PI(4,5)P₂.

When the ratio of PE:PI(4,5)P₂ was tested at a more physiologically relevant level (4:1), the initial isotherm was more condensed than that of the 1:1 ratio. This is not surprising since the monolayer is expected to exhibit behavior more similar to PE than to PI(4,5)P₂ due to the stoichiometry of PE:PI(4,5)P₂. When compared to the surface/pressure area isotherms of 4:1 PE:PI, the monolayer of 4:1 PE:PI(4,5)P₂ is more condensed. It is possible that the additional PE is able to interact with PI(4,5)P₂ to further condense the monolayer or the orientation of the PI(4,5)P₂ headgroup in the presence of PE is less space consuming than the respective orientation of the PE headgroup. The addition of calcium to the subphase has only a minor impact on the condensation of the monolayer. This could be because PI(4,5)P₂ is already stabilized by the higher concentration of PE and the addition of Ca²⁺ does not lead to further condensation. It is particularly interesting that the

addition of calcium in cases of both 1:1 and 4:1 are very similar. The epifluorescence images in the case of 4:1 PI(4,5)P₂ reveal no condensation of the monolayer. This is further support of the theory that PE, PI(4,5)P₂, and calcium are all interacting with one another.

The addition of cholesterol and calcium to mixed PE/PI(4,5)P₂ monolayer resulted in a surprising outcome. When compared to 4:1 PE:PI(4,5)P₂ monolayers with 40mol% cholesterol, the addition of calcium shifted the isotherm to larger areas/molecule, which is characteristic of a more expanded monolayer. This is an interesting finding since the effect of condensation of similar monolayers with PI was additive. In the case of PI(4,5)P₂, we suggest that PE/PI(4,5)P₂/cholesterol/Ca²⁺ are forming a singular complex, which results in strong hydrogen bonding between the headgroups.

CHAPTER 5

MIXED PHOSPHATIDYLINOSITOL/PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE MONOLAYERS AT THE AIR/WATER INTERFACE

5.1 Introduction

The role of phosphoinositides in physiological events is vast, and the ability of phosphoinositides to form domains may have significant implications for the spatiotemporal regulation of cell membrane signaling pathways. Among all phosphoinositide mediated signaling pathways, the PI3K pathway is arguably one of the most important. We have reported previously, that PTEN is able to bind phosphatidylserine and PI(4,5)P₂ synergistically: however, based on ³¹P NMR data, PS and PI(4,5)P₂ do not interact and unpublished results suggest that they don't co-localize in ternary mixtures of PC/PS/PI(4,5)P₂. (Graber, Jiang et al. 2012) Even though the conditions in biological membranes are significantly more complex than in these model systems, this raises the question whether PI(4,5)P₂ and PS are co-localized cell membranes. The current model for PTEN function is that the protein binds non-specific, electrostatic via its C2 domain to negatively charged membrane patches and if PI(4,5)P₂ is present in these membrane patches, it locks the protein down by binding to its N-terminal domain. Unpublished data from our group suggest that PI, like PS, is able to provide PTEN with an appropriate electrostatic environment for the initial binding and the question arises whether for optimal function, PTEN binds to PI rather than PS rich membrane platforms.

We have shown in previous chapters how plasma membrane modulators such as cholesterol and calcium can cause changes in the morphology of mixed PE/PI and PE/PI(4,5)P₂ monolayers at the air/water interface. While PE is a major lipid constituent of the inner leaflet of the plasma membrane, its role in cell signaling events is minimal when compared to phosphoinositides. Previously, ³¹P NMR spectroscopy and giant unilamellar vesicle fluorescence microscopy studies have shown that PI is able to induce PI(4,5)P₂ domain formation. (Graber, Jiang et al. 2012) While these studies were done using PC as a matrix lipid, our studies aim to determine the influence of cholesterol and calcium on mixed PI/PI(4,5)P₂ model membranes.

The notion that PI is able to promote phosphoinositide domain formation has potentially far reaching consequences for our understanding of the spatiotemporal control of phosphoinositide signaling events. While PI is present in quantities of 6-10%, PI(4,5)P₂ is only present in quantities of about 1% in the inner leaflet of the plasma membrane. A PI/PI(4,5)P₂ platform (domain) would be ideal for phosphoinositide binding proteins to interact with because the high concentration of PI would provide the protein in question with the negative electrostatic environment that supports non-specific electrostatic interactions, while the co-localized PI(4,5)P₂ binds with high specificity the respective protein. In addition to providing an excellent platform for the protein to interact with, remodeling of this platform as a result of particular physiological cues would provide an additional temporal control of the spatially resolved protein activity. To better understand the factors that lead to a stabilization or break-up of these PI/PI(4,5)P₂ platforms, we will investigate the effect of cholesterol and/or Ca²⁺ on PI/PI(4,5)P₂ monolayer phase behavior and morphology. In other words, our monolayer

experiments are designed to explore the properties of a PI/PI(4,5)P₂ rich domain and to identify the factors that may stabilize such membrane platforms.

5.2 Effect of calcium on the surface pressure/area isotherms of mixed phosphatidylinositol/phosphatidylinositol-4,5-bisphosphate monolayers.

In this section, we investigate the influence of calcium on the phase behavior of mixed PI/PI(4,5)P₂ monolayers using surface pressure/area isotherm measurements.

Given previous studies, we would expect that PI and PI(4,5)P₂ would be able to participate in hydrogen bonding between the hydroxyl groups of PI and the phosphomonoester groups of PI(4,5)P₂. Furthermore, we hypothesize that the addition of calcium may be able to shield the highly negatively charged headgroup of PI(4,5)P₂ and potential bridge the PI/PI(4,5)P₂ phosphodiester and or PI phosphodiester and PI(4,5)P₂ phosphomonoester groups, which should lead to further condensation of the monolayer.

Experiments in this section were carried out according to the details described in Chapter 2. Surface pressure/area isotherms were recorded on a subphase composed of 10mM Tris, 150mM NaCl, and either 0mM CaCl₂ (0.1mM EDTA), 1mM CaCl₂, or 2mM CaCl₂, and were maintained at pH 7.4 and a temperature of 25±0.2°C.

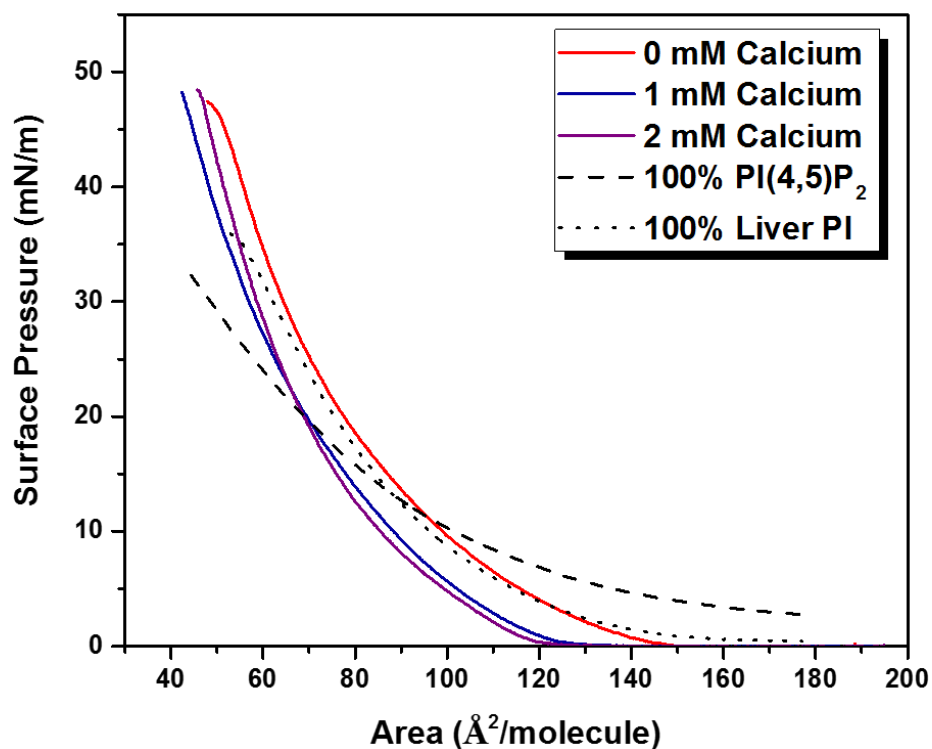


Figure 35: Surface pressure/area isotherms of mixed PI:PI(4,5)P₂ monolayers at a 1:1 ratio subphase comparison. Surface pressure/area isotherms of mixed PI:PI(4,5)P₂ at a 1:1 ratio in the absence and presence of calcium. Subphase consisted of 10mM Tris, 150mM NaCl, and with either 0.1mM EDTA (0mM CaCl₂) (red), 1mM CaCl₂ (blue), or 2mM CaCl₂ (purple) at pH 7.4 and T= 25±0.2°C. The surface pressure/area isotherm for 100% PI(4,5)P₂ (dashed) and 100% Liver PI (dotted) in the absence of Ca²⁺ are shown for comparison.

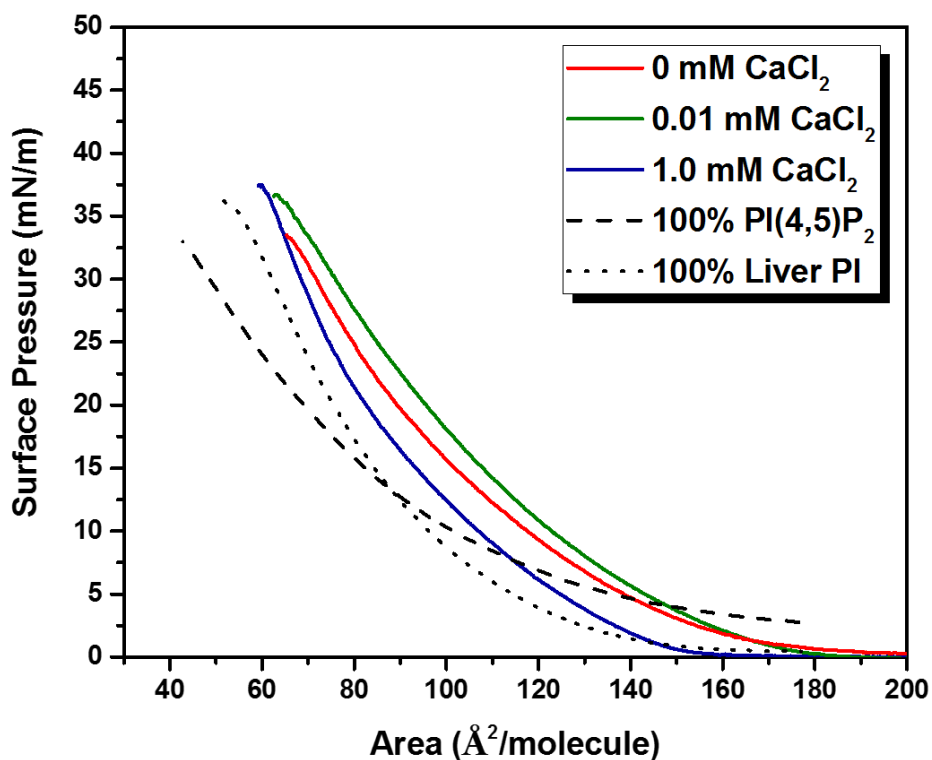
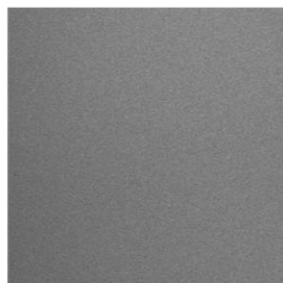


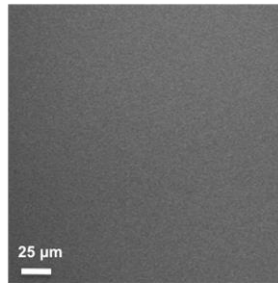
Figure 36: Surface pressure/area isotherms of mixed PI:PI(4,5)P₂ monolayers at a 2:1 ratio subphase comparison. Surface pressure/area isotherms of mixed PI:PI(4,5)P₂ at a 2:1 ratio in the absence and presence of calcium. Subphase consisted of 10mM Tris, 150mM NaCl, and with either 0.1mM EDTA (0mM CaCl₂) (red), 0.01mM CaCl₂ (green), or 1mM CaCl₂ (blue) at pH 7.4 and T= 25±0.2°C. The surface pressure/area isotherm for 100% PI(4,5)P₂ (dashed) and 100% Liver PI (dotted) in the absence of Ca²⁺ are shown for comparison.

We first investigated PI/PI(4,5)P₂ monolayers at the air/water interface at a 1:1 ratio using surface pressure/area isotherms. In the absence of Ca²⁺, the monolayer is less expanded than that of PI(4,5)P₂ in the absence of Ca²⁺. (Figure 35) Although the π/A -isotherm for 1:1 PI:PI(4,5)P₂ monolayer is similar to that of PI in the absence of Ca²⁺, the slope is steeper and the pressure at which the monolayer collapses is higher, which suggests a stabilization of the monolayer. The fact that the combination of PI/PI(4,5)P₂ leads to a more stable phase than what is observed for PI alone, highlights the effectiveness of the hydrogen bond network that is formed between PI and PI(4,5)P₂. This observation is in perfect agreement with the GUV PC/PI/PI(4,5)P₂ microscopy experiments carried out by Jiang et. al. that showed PI/PI(4,5)P₂ demixing from PC. (Jiang, Redfern et al. 2014) The addition of 1mM CaCl₂ to the subphase (Figure 35), results in a condensation of the monolayer (shift to lower areas/molecule). Further increasing the Ca²⁺ concentration to 2mM does not lead to a significant stronger condensation than what is observed in the presence of 1mM CaCl₂; however, the slight crossover of the isotherms suggests that the monolayer is in the presence of 2mM CaCl₂ even more stabilized (Figure 35). Taken together, we suggest that PI is able to stabilize PI(4,5)P₂ in the absence of Ca²⁺ via the hydroxyl groups of PI and the phosphomonoester of PI(4,5)P₂. This could in part be due to the smearing out of the charges along the interface. In addition, we suggest that the addition of Ca²⁺ leads to a shielding of the negative charges and a bridging of the PI/PI(4,5)P₂ headgroups. To explore the potential of other PI/PI(4,5)P₂ domain stoichiometries, we investigated the properties of a monolayer with a 2:1 PI:PI(4,5)P₂ ratio, which is closer to the global PI/PI(4,5)P₂ concentration ratio found in biological membranes. The Langmuir film is found to be

more expanded in both the presence and absence of calcium than what was found for the 1:1 ratio. In the absence of Ca^{2+} , the 2:1 PI:PI(4,5) P_2 monolayer is expanded (Figure 36), resulting in a shift to higher areas/molecule. When comparing this result to the data for PI in the absence of Ca^{2+} (Figure 35), we observe an isotherm that is characteristic of a more expanded monolayer. For PI(4,5) P_2 monolayers in the absence of Ca^{2+} (Figure 35), we observe an isotherm that is representative of a more condensed monolayer than that of 2:1 PI:PI(4,5) P_2 in the absence of Ca^{2+} . This suggests that the presence of PI is able to minimally stabilize the PI(4,5) P_2 monolayer. The addition of 0.01mM CaCl_2 , results in a slight expansion of the monolayer (Figure 36); however, this may be due to a stabilization of the monolayer. (e.g., less of the lipid sample is able to slip under the barriers, prevention of premature collapse) Further increasing the concentration of Ca^{2+} to 2mM in the subphase results in an isotherm that is characteristic of a more condensed monolayer (Figure 36) than the 2:1 PI:PI(4,5) P_2 lipid sample in the absence of Ca^{2+} or in the presence of 0.01mM Ca^{2+} . Interestingly, even in the presence of 1mM Ca^{2+} , the 2:1 PI:PI(4,5) P_2 remains more expanded than the 1:1 PI:PI(4,5) P_2 in the absence of Ca^{2+} . Overall, the comparison of the 1:1 and 2:1 PI/PI(4,5) P_2 data suggest that the best stoichiometry for the PI/PI(4,5) P_2 interaction is 1:1. Following this argument, we hypothesize that in cells, PI/PI(4,5) P_2 domains would show such a ratio. However, in the cellular context several other chemical species may alter this ratio in one direction or another.



Pressures less than 0.1mN/m



Pressures greater than 15 mN/m

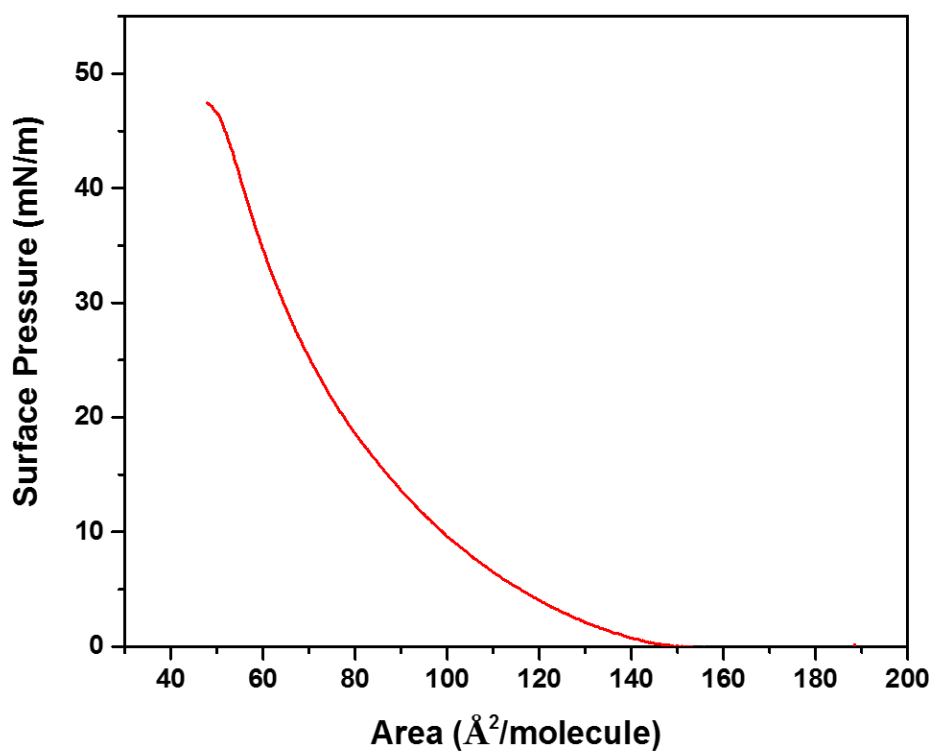


Figure 37: Surface pressure/area isotherm of 1:1 PI:PI(4,5)P₂ in the absence of calcium. Monolayer consisting of 1:1 PE:PI(4,5)P₂ on subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at pH 7.4 and maintained at T=25±0.2°C. Images were taken using 0.1mol% Top Fluor™ PI(4,5)P₂.

5.3 Effect of calcium on the morphology of mixed phosphatidylinositol/phosphatidylinositol-4,5-bisphosphate

In the previous section, we investigated how Ca^{2+} influenced mixed PI/PI(4,5) P_2 Langmuir monolayers using surface pressure/area isotherms. Here, we investigate how Ca^{2+} influences the morphology of mixed PI/PI(4,5) P_2 monolayers. From data in the previous section, we would expect to see some domain formation in the presence of Ca^{2+} .

Experiments in this section were carried out according to the details described in Chapter 2. Surface pressure/area isotherms were recorded on a subphase composed of 10mM Tris, 150mM NaCl, and either 0mM CaCl_2 (0.1mM EDTA), 1mM CaCl_2 , or 2mM CaCl_2 , and were maintained at pH 7.4 and a temperature of $25 \pm 0.2^\circ\text{C}$. Top Fluor PI(4,5) P_2 or Bodipy PI were added (0.1mol%) were added to the lipid samples for imaging purposes.

For mixed PI/PI(4,5) P_2 monolayers at a ratio of 1:1, we see no macroscopically discernible domain formation in the absence of Ca^{2+} (Figure 37); however, the addition of 1mM Ca^{2+} to the subphase results in domain formation at low pressures (pressures at or below 0.1mN/m) (Figure 38). The fluorescence images show round features (“bubbles”) that are typical for an LE/G transition region with a monolayer that shows some tendency for condensation. Upon compression of the monolayer, these circular features become significantly smaller in size as the monolayer reaches a pressure of 0.1mN/m before disappearing at pressures above 0.1mN/m. Interestingly, increasing the Ca^{2+} concentration to 2mM results in the formation of small domains even at high surface pressures (Figure 39), where the fluorophore is excluded from the domain (domains are small and dark). One can only speculate about the exact nature and composition of these

domains. Overall, this data suggests that the addition of Ca^{2+} stabilizes the 1:1 PI:PI(4,5)P₂ monolayers.

For 2:1 PI:PI(4,5)P₂ monolayers, domain formation is absent in the absence of calcium and in the presence of 0.01mM Ca^{2+} (Figure 40, Figure 41). The addition of 1mM CaCl_2 to the subphase results in a condensation of the monolayer and the formation of a monolayer phases characterized by circular, monolayer free “bubbles” at pressures up to 0.1mN/m. (Figure 42) As the pressure is increased, these monolayer free areas disappear and a continuous monolayer is formed. However, the fluorescently labeled lipid is excluded from the remainder of the PI/PI(4,5)P₂ monolayer, leading to bright patches throughout the monolayer. We hypothesize that the additional PI leads to an overall change in the interaction with PI(4,5)P₂, which manifests itself as a lack of domain formation in the absence of Ca^{2+} and in the presence of 0.01mM Ca^{2+} . Increasing the Ca^{2+} concentration to 1mM helps to shield the negative charge of the PI and PI(4,5)P₂, apparently leading to a more effective interaction between the two lipid species and hence some domain formation. (i.e., condensation of the monolayer).

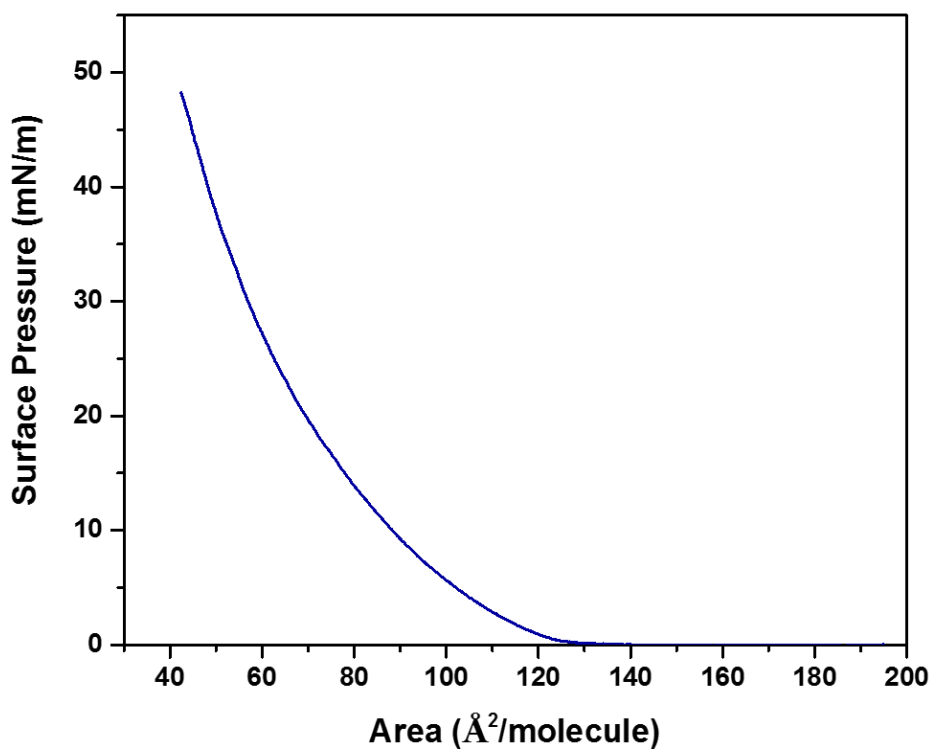
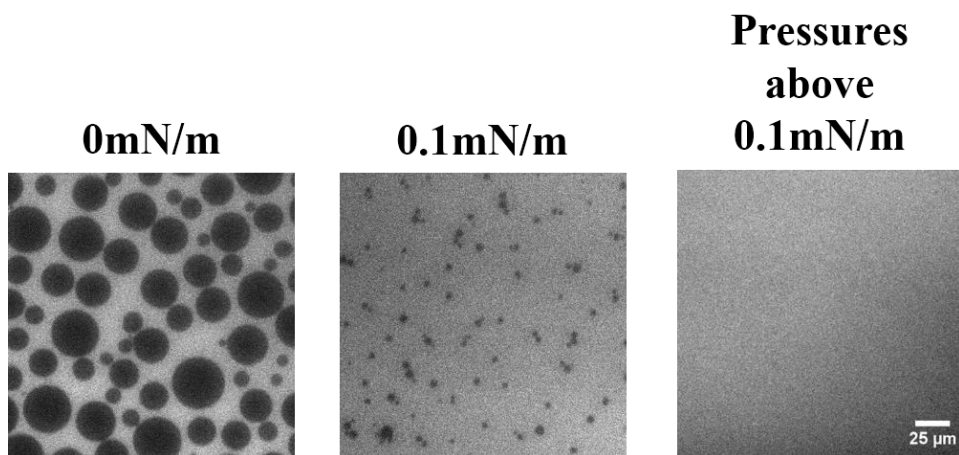


Figure 38: Surface pressure/area isotherm of 1:1 PI:PI(4,5)P₂ in the presence of 1mM calcium. Monolayer consisting of 1:1 PE:PI(4,5)P₂ on subphase consisting of 10mM Tris, 150mM NaCl, and 1mM CaCl₂ at pH 7.4 and maintained at T=25±0.2°C. Images were taken using 0.1mol% Top Fluor™ PI(4,5)P₂.

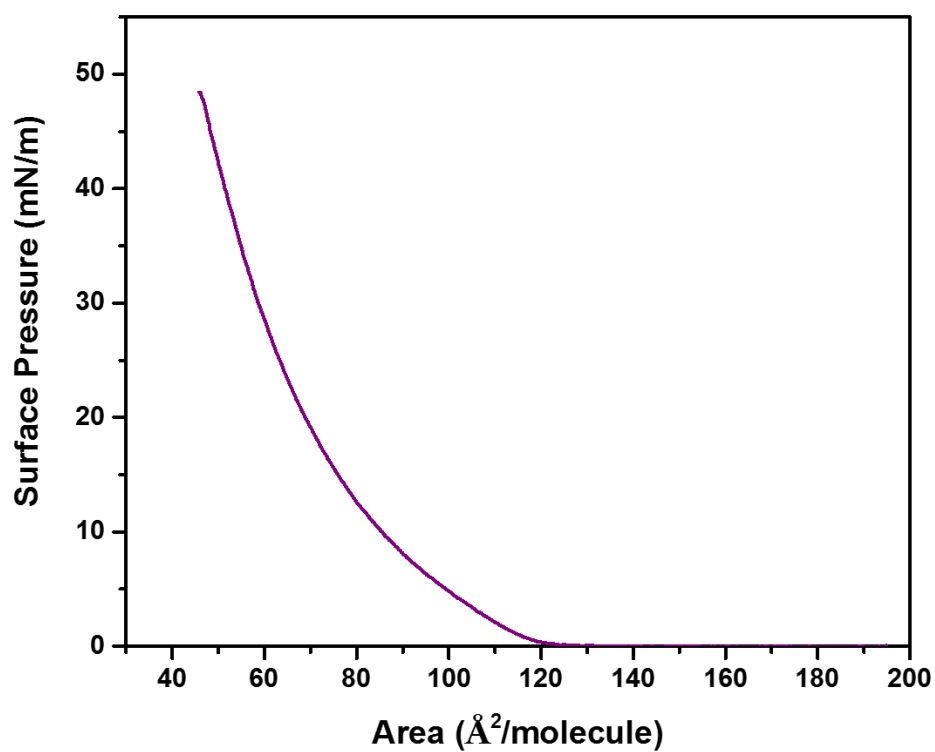
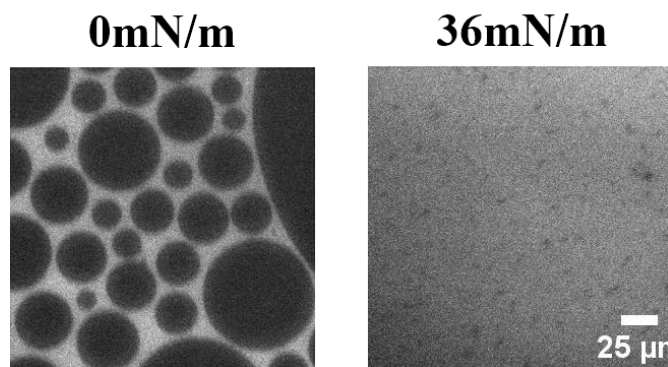
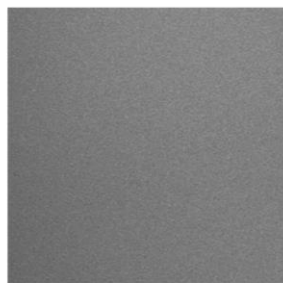
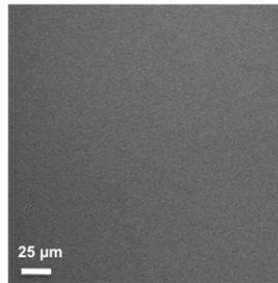


Figure 39: Surface pressure/area isotherm of 1:1 PI:PI(4,5)P₂ in the presence of 2mM calcium. Monolayer consisting of 1:1 PE:PI(4,5)P₂ on subphase consisting of 10mM Tris, 150mM NaCl, and 2mM CaCl₂ at pH 7.4 and maintained at T=25±0.2°C. Images were taken using 0.1mol% Top Fluor™ PI(4,5)P₂.



Pressures less than 0.1mN/m



Pressures greater than 15 mN/m

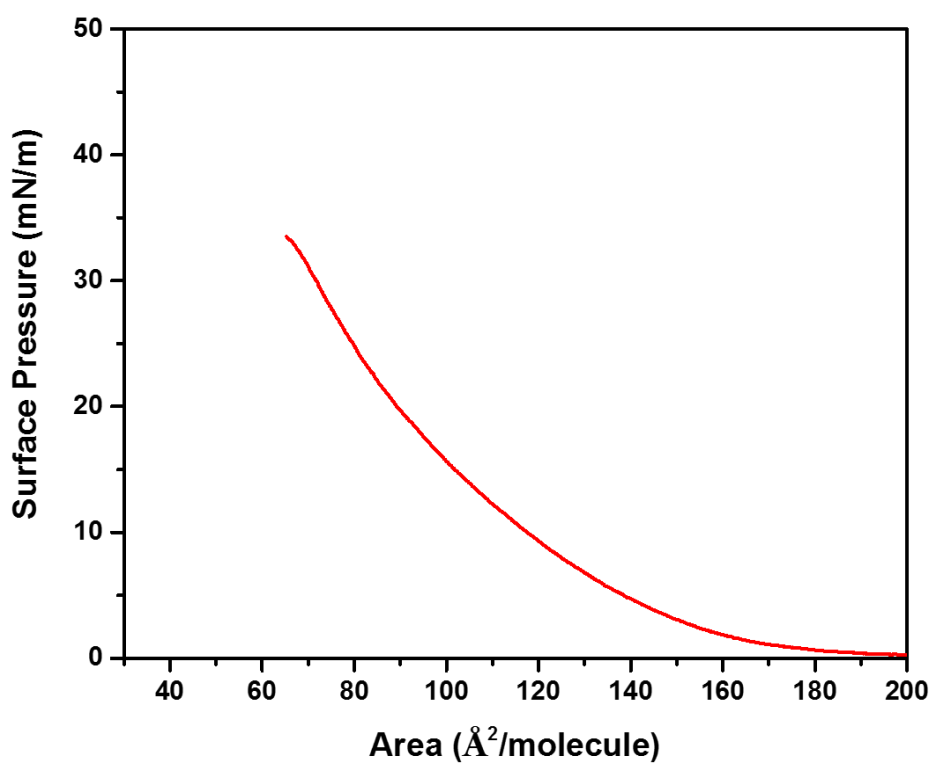
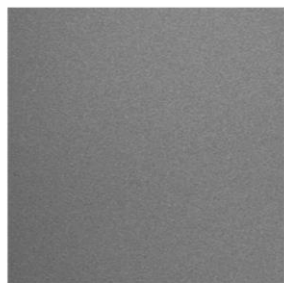
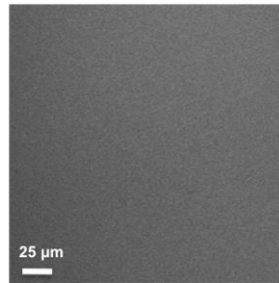


Figure 40: Surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ in the absence of calcium. Monolayer consisting of 2:1 PE:PI(4,5)P₂ on subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at pH 7.4 and maintained at T=25±0.2°C. Images were taken using 0.1mol% Top Fluor™ PI(4,5)P₂.



Pressures less than 0.1mN/m



Pressures greater than 15 mN/m

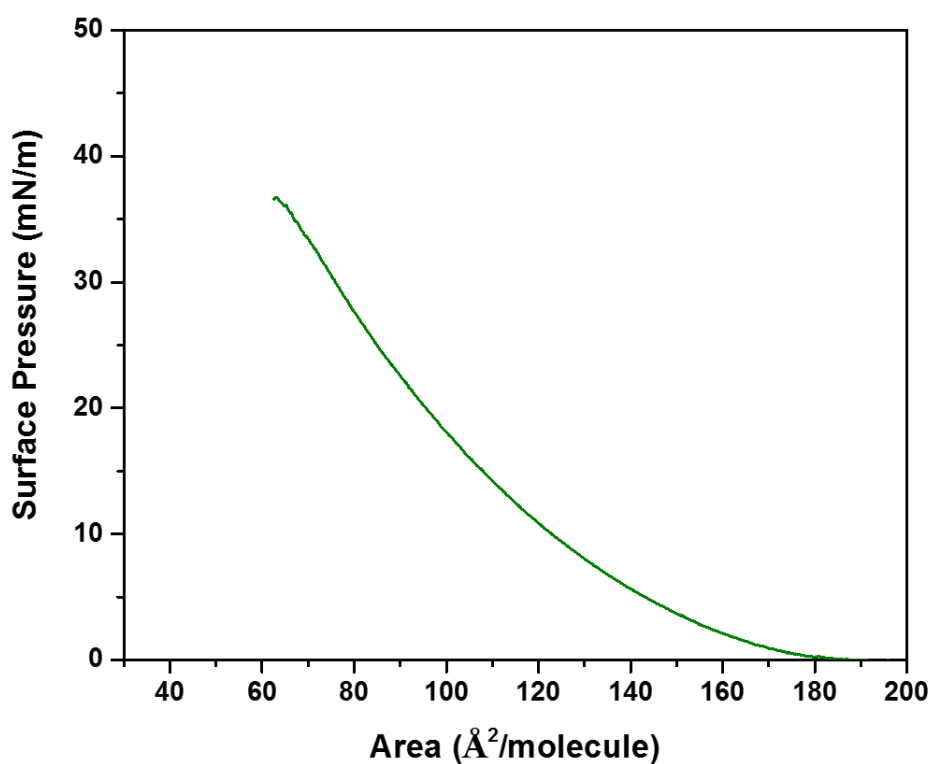


Figure 41: Surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ in the presence of 2mM calcium. Monolayer consisting of 2:1 PE:PI(4,5)P₂ on subphase consisting of 10mM Tris, 150mM NaCl, and 0.01mM CaCl₂ at pH 7.4 and maintained at T=25±0.2°C. Images were taken using 0.1mol% Top Fluor™ PI(4,5)P₂.

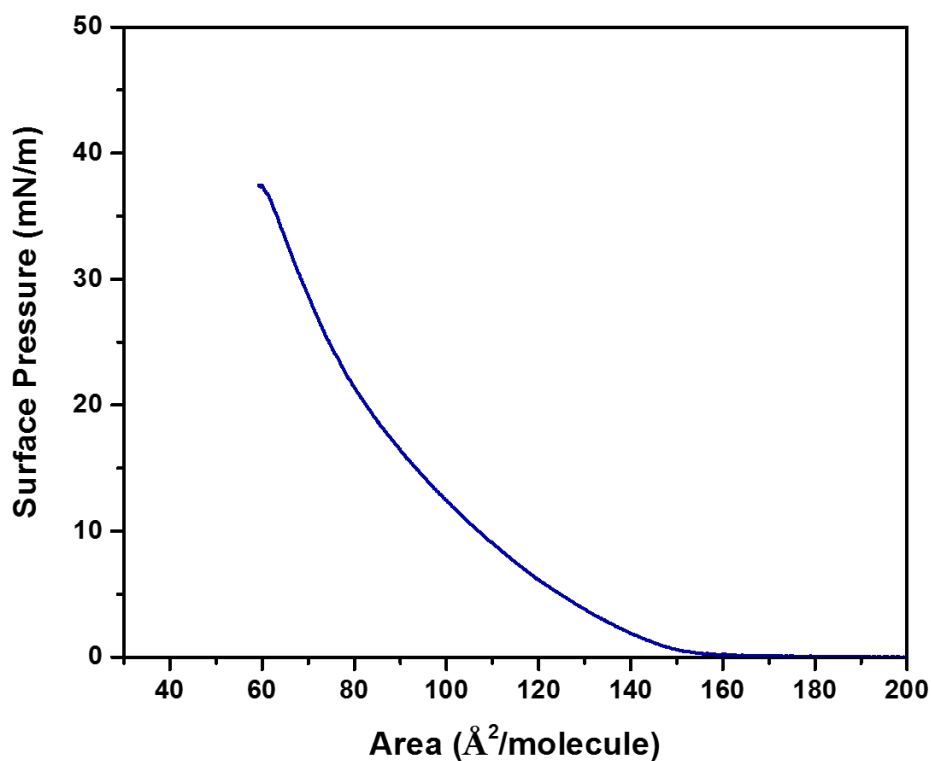
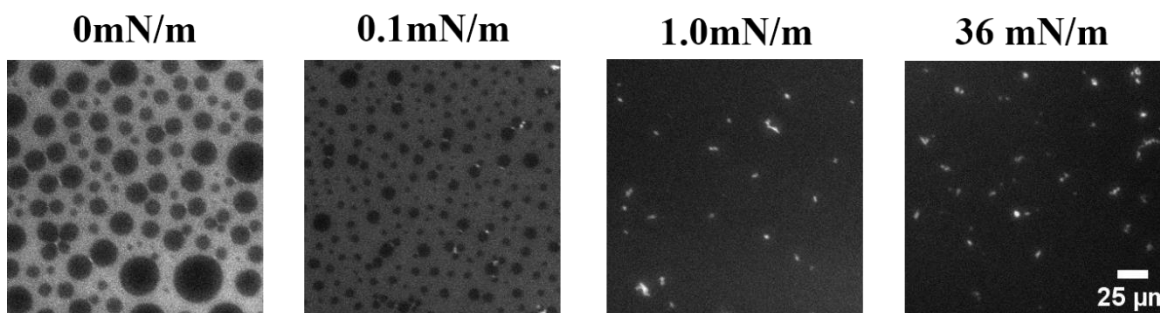


Figure 42: Surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ in the presence of 2mM calcium. Monolayer consisting of 2:1 PE:PI(4,5)P₂ on subphase consisting of 10mM Tris, 150mM NaCl, and 1mM CaCl₂ at pH 7.4 and maintained at T=25±0.2°C. Images were taken using 0.1mol% Top Fluor™ PI(4,5)P₂.

5.4 Influence of cholesterol on the surface pressure/area isotherms of mixed phosphatidylinositol/phosphatidylinositol-4,5-bisphosphate monolayers.

In this section we investigate the effect of cholesterol on mixed PI/PI(4,5)P₂ model membrane systems. Based on previous results from our lab, we would expect that cholesterol, possibly through its hydroxyl group and the hydroxyl groups at the 2 and/or 6 positions of the inositol ring of PI and/or PI(4,5)P₂, would be able to participate in the hydrogen bond network formed between the headgroups of PI and PI(4,5)P₂.

The experiments carried out in this section were performed as detailed in Chapter 2. All isotherms were recorded on a subphase composed of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at a pH of 7.4 and maintained at 25±0.2°C.

The surface pressure/area isotherm for 2:1 PI:PI(4,5)P₂ with 40% cholesterol is characteristic of a significantly more condensed monolayer than that of 2:1 PI/PI(4,5)P₂. (Figure 43) The addition of cholesterol also leads to a higher collapse pressure of the monolayer, and the shape of the isotherm is much steeper. Based on these data, we suggest that cholesterol plays a significant role in the stabilization of 2:1 PI:PI(4,5)P₂ monolayers at the air/water interface. We hypothesize that the hydroxyl groups of cholesterol are able to stabilize the interactions between the PI and PI(4,5)P₂ headgroups by participating in a hydrogen bond network through the hydroxyl groups at the 2 and 6 positions of the inositol rings.

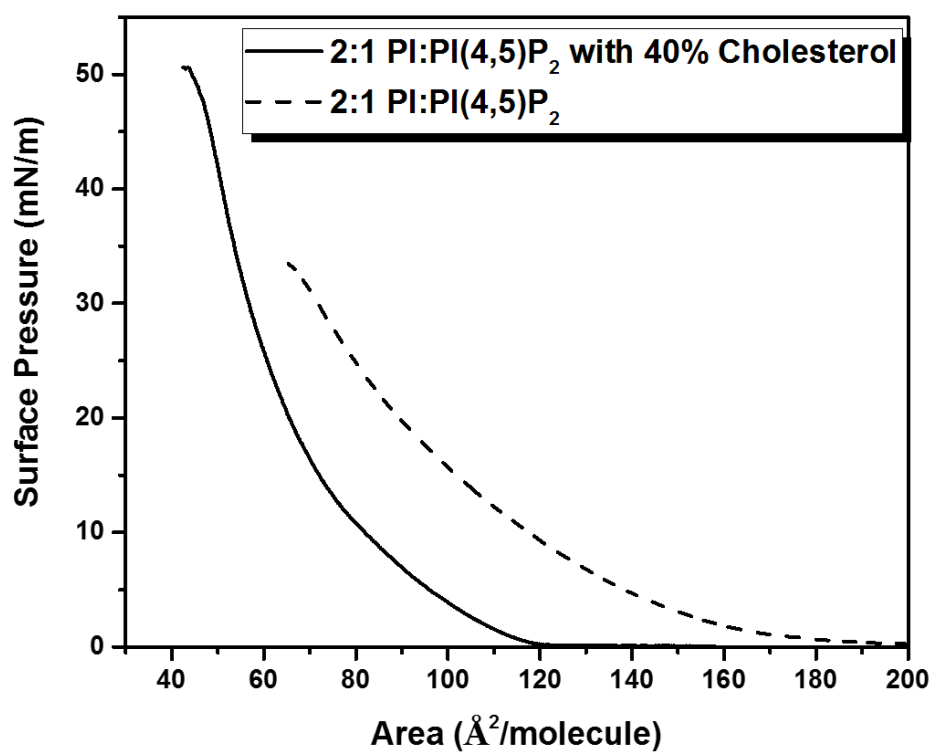


Figure 43: Surface pressure/area isotherms of 2:1 PI:PI(4,5)P₂ in the absence and presence of cholesterol. Surface pressure/area isotherms of 2:1 PI:PI(4,5)P₂ in the absence (dashed) and presence (black) of 40mol% cholesterol. Subphase consists of 10mM Tris, 150mM NaCl and 0.1mM EDTA at pH 7.4 and a temperature of 25±0.2°C.

5.5 Effect of cholesterol on the morphology of mixed phosphatidylinositol/phosphatidylinositol-4,5-bisphosphate monolayers.

Here, we determine the effect of cholesterol on the morphology of mixed PI/PI(4,5)P₂ monolayers using epifluorescence microscopy. Based on the surface pressure/area isotherms, which are characteristic of a more condensed monolayer, we would expect to see domain formation even at higher pressures.

The epifluorescence microscopy experiments carried out in this section were performed as mentioned in Chapter 2. The subphase consisted of 10mM Tris, 150mM NaCl, and 2mM CaCl₂. The buffer was maintained at a pH of 7.4 and a temperature of 25±0.2°C. Either Bodipy PI or Top Fluor™ PI(4,5)P₂ were incorporated in the lipid sample (0.1mol%) for imaging purposes.

Monolayers composed of a 2:1 ratio of PI:PI(4,5)P₂ with 40% cholesterol were initially imaged with incorporation of 0.1mol% Top Fluor™ PI(4,5)P₂. At low pressures, the images show the formation of small, circular domains. (Figure 44) As the pressure is increased to 20mN/m, the domains remain essentially the same. While, we were able to image the 2:1 PI:PI(4,5)P₂ with 40mol% cholesterol monolayer using Top Fluor™ PI(4,5)P₂, we also tried using Bodipy PI to see if we could improve the contrast. For these monolayers, we see similar small, circular domains; however, the domains are no longer visible at pressures above 10mN/m. (Figure 44) We suggest based on this data that cholesterol is able to stabilize the PI/PI(4,5)P₂ interactions via interactions of the cholesterol hydroxyl group with the hydrogen bond network formed by PI and PI(4,5)P₂. This is an important finding since the 2:1 PI/PI(4,5)P₂ monolayer (Figure 36) was less condensed and stable than the 1:1 PI/PI(4,5)P₂ (Figure 35). From a physiological point of

view a domain with a 1:1 lipid ratio is less favorable than a domain with a higher ratio (e.g., 2:1) because the low PI(4,5)P₂ concentration in the PM would still limit the size and abundance of PI(4,5)P₂ containing platforms. The fact that cholesterol stabilizes domains with a PI/PI(4,5)P₂ ratio that more closely aligns with the global PM ratio, is therefore an important finding.

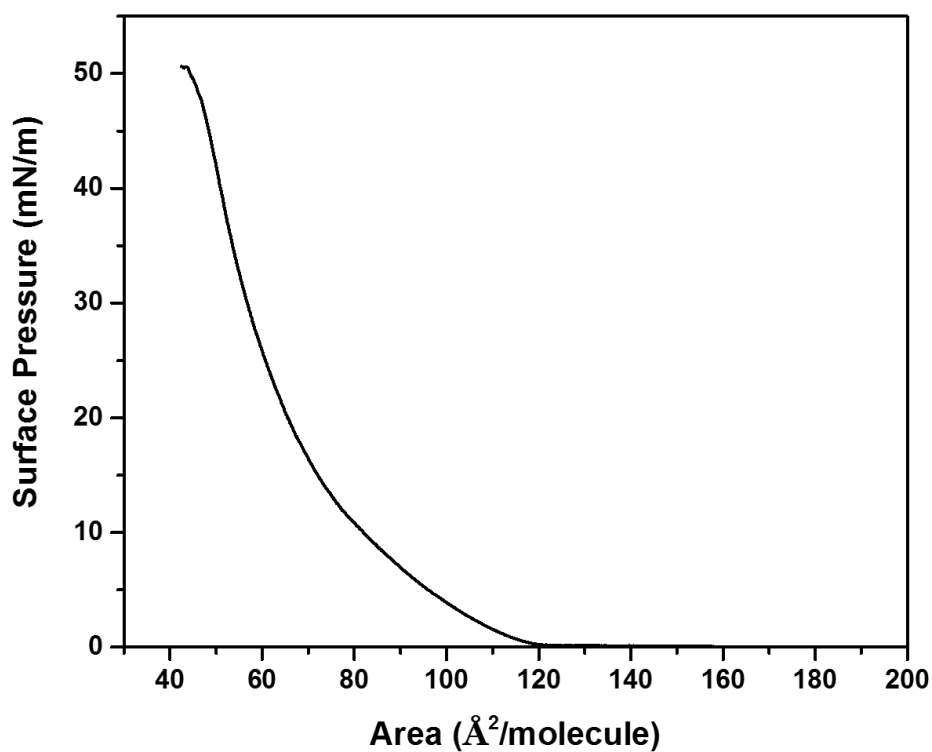
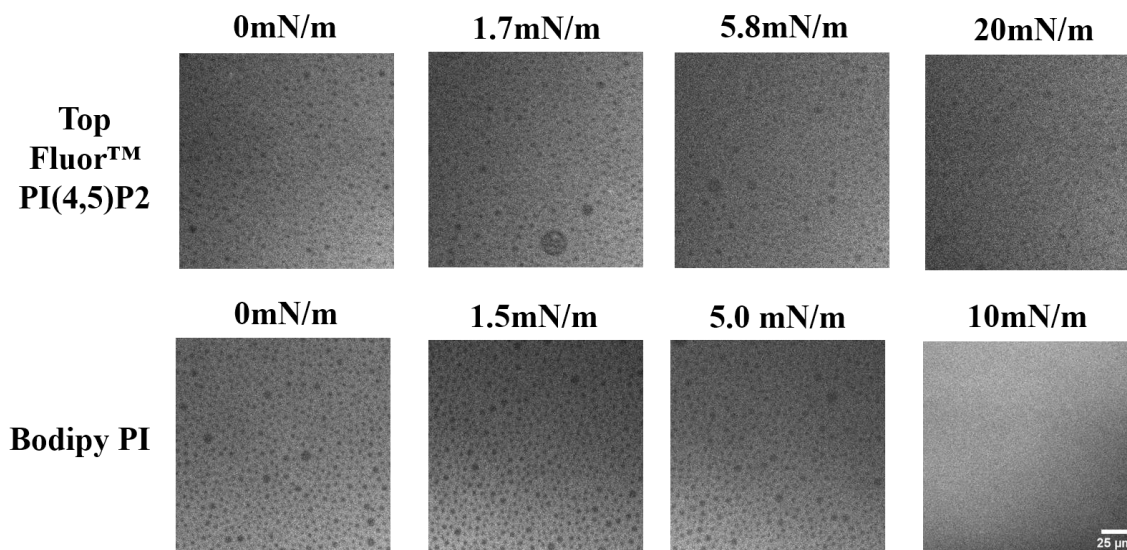


Figure 44: Surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ with 40% cholesterol with corresponding epifluorescence images. Surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ with 40mol% cholesterol. Subphase consists of 10mM Tris, 150mM NaCl and 0.1mM EDTA at pH 7.4 and a temperature of 25±0.2°C. Epifluorescence images were taken by incorporating 0.1mol% or either Top Fluor™ PI(4,5)P₂ or Bodipy PI as indicated.

5.6 Effect of cholesterol and calcium on the surface pressure/area isotherms of phosphatidylinositol/phosphatidylinositol-4,5-bisphosphate monolayers.

We have previously investigated the influence of calcium and cholesterol separately on mixed PI:PI(4,5)P₂ monolayers. In this section, we aim to elucidate the effect of calcium and cholesterol in combination on PI:PI(4,5)P₂ monolayers. Previously, we have shown that independently, calcium and cholesterol result in the condensation of mixed PI:PI(4,5)P₂ monolayers. We would expect that the addition of these plasma membrane modulators may cause an additive effect on the condensation of the monolayer; however, it could also be possible based on the PI/PI(4,5)P₂/cholesterol data that the interactions are stronger and will compete with PI/PI(4,5)P₂/Ca²⁺ leading to no significant change in the condensation.

Experiments in this section were performed as outlined in Chapter 2. All monolayers were recorded on a subphase consisting of 10mM Tris, 150mM NaCl, and 2mM CaCl₂ and were maintained at pH 7.4 and a temperature of 25±0.2°C.

The surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ in the presence of 2mM CaCl₂, is characteristic of a monolayer that is more condensed than the monolayer of 2:1 PI/PI(4,5)P₂ in the absence of cholesterol or Ca²⁺. (Figure 45) When the surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ with 40% cholesterol is compared to the isotherm of 2:1 PI:PI(4,5)P₂ with 40% cholesterol in the presence of 2mM CaCl₂, there is virtually no change, suggesting that the monolayer does not condense further in the presence of Ca²⁺. Based upon these data it is unclear whether Ca²⁺ participates in the PI/PI(4,5)P₂/cholesterol complex. The packing of the lipids might be already to close for

an additional condensation to happen. On the other hand, it is obvious that Ca^{2+} does not interfere negatively with the formation of this complex.

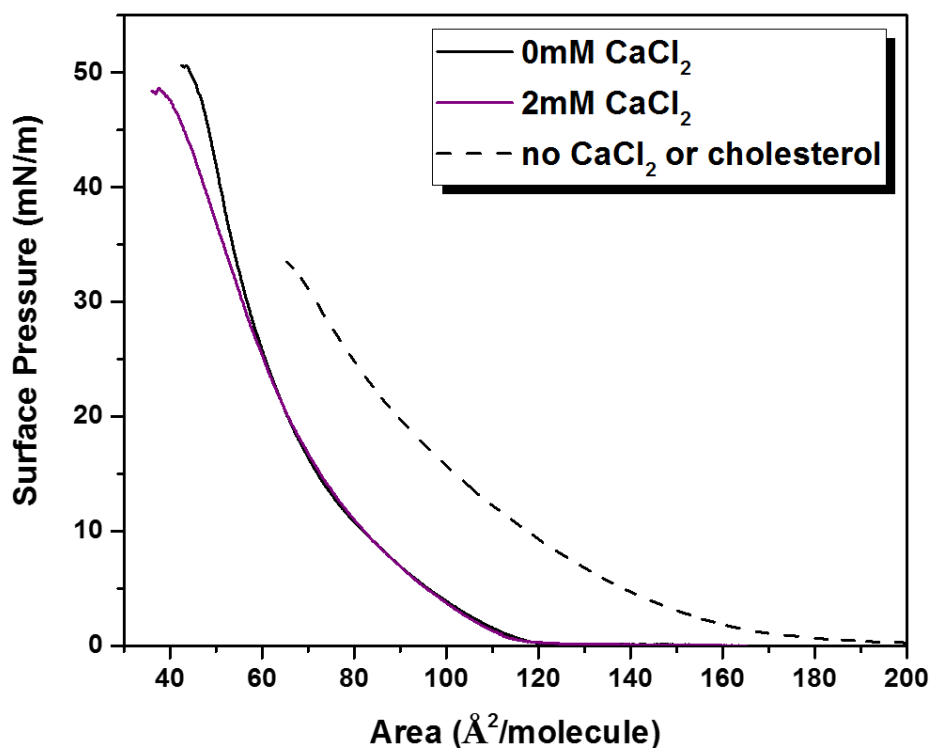


Figure 45: Surface pressure/area isotherms of 2:1 PI:PI(4,5)P₂ with 40% cholesterol in the absence and presence of calcium. Surface pressure/area isotherms of 2:1 PI:PI(4,5)P₂ with 40mol% cholesterol in the absence and presence of calcium. Subphase consists of 10mM Tris, 150mM NaCl and 0.1mM EDTA (0mM CaCl_2) (black) or 2mM CaCl_2 (purple) at pH 7.4 and a temperature of $25\pm 0.2^\circ\text{C}$. The isotherm representative of 2:1 PI:PI(4,5)P₂ in the absence of both cholesterol and calcium is shown for comparison (dashed line)

5.7 Influence of cholesterol and calcium on the morphology of mixed phosphatidylinositol/phosphatidylinositol-4,5-bisphosphate monolayers.

In this section, we study the influence of cholesterol and calcium on the morphology of mixed PI/PI(4,5)P₂ monolayers using epifluorescence microscopy. From the data obtained previously, we would expect to see domain formation similar to that of 2:1 PI/PI(4,5)P₂ with 40mol% cholesterol.

Imaging experiments in this section were carried out as described in Chapter 2. The subphase was composed of 10mM Tris, 150mM NaCl, and 2mM CaCl₂. The pH was maintained at 7.4 and the temperature was held constant at 25±0.2C°. Top Fluor™ PI(4,5)P₂ was added (0.1mol%) to the lipid mixture for imaging purposes.

Monolayers composed of 2:1 PI:PI(4,5)P₂ with 40% cholesterol were imaged on a subphase containing 2mM CaCl₂. At low pressures, images show small, circular domains similar to those previously seen in lipid mixtures of 2:1 PI:PI(4,5)P₂ with cholesterol in the absence of Ca²⁺. (Figure 46) The small, circular domains remained present to pressures reaching ~20mN/m. At pressures above ~20mN/m, the fluorophore begins to be excluded, making it hard to image the monolayer. This data seems to be in agreement with our thermodynamic data from the previous section. The images are similar to those of 2:1 PI:PI(4,5)P₂ with 40% cholesterol in the absence of Ca²⁺, suggesting that the addition of Ca²⁺ has little effect on the monolayer.

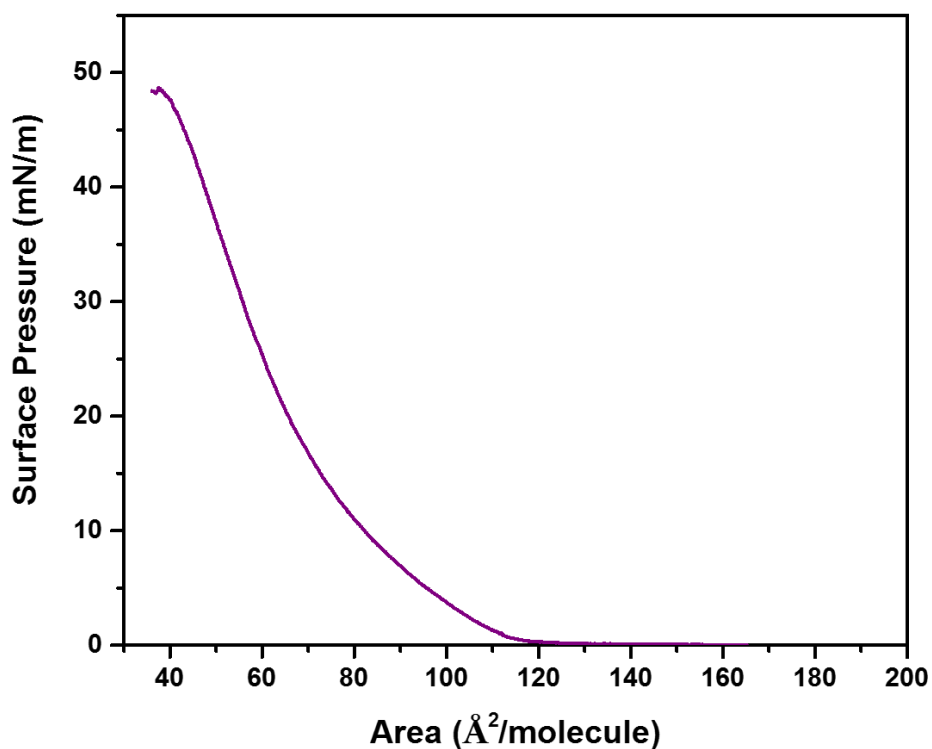
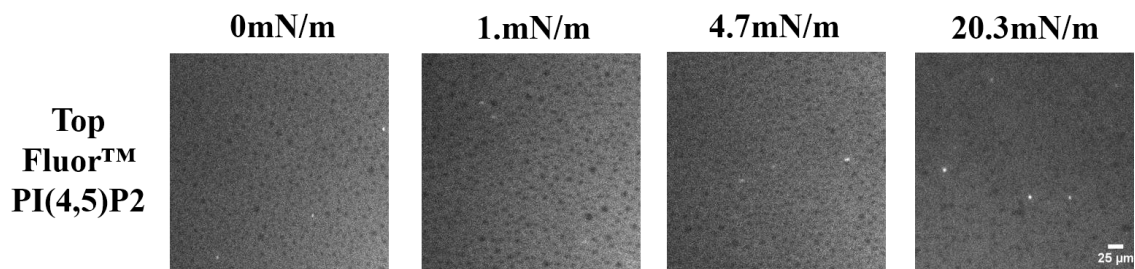


Figure 46: Surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ with 40% cholesterol in the presence of calcium with corresponding epifluorescence images. Surface pressure/area isotherm of 2:1 PI:PI(4,5)P₂ with 40mol% cholesterol. Subphase consists of 10mM Tris, 150mM NaCl and 2mM CaCl₂ at pH 7.4 and a temperature of 25±0.2°C. Epifluorescence images were taken by incorporating 0.1mol% Top Fluor™ PI(4,5)P₂.

5.8 Conclusion

In this chapter, we studied the influence of plasma membrane modulators such as calcium and cholesterol on mixed PI/PI(4,5)P₂ monolayers at the air/water interface. The results from previous studies indicated that PI stabilizes PI(4,5)P₂ domains in mixed PC/PI/PI(4,5)P₂ vesicles were the major driving force behind this investigation. (Graber, Jiang et al. 2012)

The data in this chapter show that at 1:1 ratios of PI:PI(4,5)P₂ the monolayer is more condensed than those of 2:1 PI:PI(4,5)P₂ in the absence of calcium or cholesterol. Upon the addition of calcium to the subphase, the monolayer is further condensed in both cases. This suggests that calcium is able to shield the headgroups (i.e., smear out the charge). These data are in agreement with Graber (2012) and support the notion that at 1:1 ratios of PI:PI(4,5)P₂, hydrogen bonding is able to occur most efficiently between the PI and PI(4,5)P₂ headgroups via the hydroxyl groups of PI and the phosphomonoester group of PI(4,5)P₂. At 2:1 ratios of PI:PI(4,5)P₂ we see a shift to higher areas/molecule, which is characteristic of a more expanded monolayer. This might be due to the fact that for a 2:1 ratio of PI/PI(4,5)P₂ complex is formed that is more space consuming than the 1:1 complex. Alternatively, a demixing might occur where PI only and PI/PI(4,5)P₂ domains are formed. Overall, these data suggest that a 1:1 PI/PI(4,5)P₂ ratio is favored for optimal interaction.

The addition of 40mol% cholesterol to 2:1 PI/PI(4,5)P₂ resulted in a significant condensation of the monolayer. This is not surprising based on the condensation effect of

cholesterol on PI and PI(4,5)P₂ individually. (Jiang, Redfern et al. 2014) We hypothesize that the structural details of the PI/PI(4,5)P₂ complex are fundamentally different from those found for the PI/PI(4,5)P₂/Chol complex. From a physiological point of view this is potentially an extraordinary important finding. First, it opens up the possibility of the formation of larger domains where PI is providing an appropriate electrostatic environment for proteins to bind electrostatically, while PI(4,5)P₂ is present in sufficient quantities to bind with high specificity the protein in question.

CHAPTER 6

CONCLUSION

Many physiological processes involving phosphoinositides have been either shown or postulated to be associated with the formation of phosphoinositide enriched membrane platforms. While the exact nature and composition of these platforms has not been discerned, this study aimed to identify some of the parameters from a lipidic point of view that stabilize or de-stabilize such phosphoinositide-enriched domains. Studying phosphoinositide domain formation is particularly challenging because the domains will be in a fluid state and the main driver for the domain formation will be interactions involving the phosphoinositide headgroup. Monolayers at the air/water interface are exquisitely well suited to meet this challenge because the monolayer composition can be varied widely and the interaction with bulk phase resident species like Ca^{2+} can be studied comfortably. On one hand, Langmuir films allow for the investigation of lipid demixing, i.e., domain formation in mixed systems can be studied. Most importantly, it is possible to study the properties of domains likely to occur in membranes, by using a monolayer composition that is reflective of what is found in these domains.

In this study, we initially investigated the effect of Ca^{2+} on PE, PI, and PI(4,5)P₂ monolayers individually at the air/water interface. Our results for PI and PI(4,5)P₂ monolayers in the presence of Ca^{2+} were not all that surprising based on previous studies, and the ability of Ca^{2+} to both shield and bridge the interaction of the phosphoinositide inositol headgroups supported our hypothesis. The influence of Ca^{2+} on PE monolayers was far more surprising. At low (0.01mM) Ca^{2+} concentrations, it appears as though

Ca^{2+} is able to insert between the PE headgroups and as the Ca^{2+} concentration is increased, Ca^{2+} is apparently able to bridge the PE headgroups, resulting in a condensation of the monolayer and a shift to lower areas/molecule.

We then increased the complexity of our lipid mixtures by studying PE/PI and PE/PI(4,5) P_2 in presence and absence of calcium. PE is found in the inner leaflet of the plasma membrane in high concentrations and considering its hydrogen bonding capability, it is highly likely that PE interacts with phosphoinositides. Despite of the physiological importance of this potential interaction, only little is known about PE/phosphoinositide interaction. For PE/PI monolayers we observe for 1:1 ratios, a slight condensation of the monolayer as inferred from the surface pressure/area isotherm. We suggest that this is due to the ability of PE to insert between the PI headgroups. In contrast, the 4:1 PE:PI monolayer is significantly more condensed than that of the 1:1 ratio. We hypothesize that the condensation of the 4:1 PE:PI monolayer could be due to a difference in stoichiometry. The addition of Ca^{2+} to both the 1:1 and 4:1 ratios of PE:PI result in only a slight further condensation, suggesting that Ca^{2+} has a minimal effect on the condensation of the monolayers. Overall, these data suggest that PE and PI may co-localize in biological membranes, however, PE appears to be not a major driver for PI domain formation.

For lipid mixtures of 1:1 PE:PI(4,5) P_2 in the absence of Ca^{2+} , we see a significant shift to higher areas/molecule (more expanded monolayer). We hypothesize that this is due to a change in the orientation of the PI(4,5) P_2 inositol headgroup that results in a more space consuming arrangement of the PI(4,5) P_2 molecules. In the presence of even relatively Ca^{2+} low concentrations, the monolayer is significantly more condensed than

that of 1:1 PE:PI(4,5)P₂ in the absence of Ca²⁺. This suggests that Ca²⁺ is able to both bridge and shield the head group interactions. Based upon the analysis of the fluorescence images, it appears that PE remains mixed with PI(4,5)P₂ even in the presence of Ca²⁺. For 4:1 lipid mixtures in the absence of Ca²⁺, we see a more condensed monolayer than for 1:1 PE:PI(4,5)P₂ in the absence of Ca²⁺. This suggests that the additional PE is able to interact with PI(4,5)P₂ to further condense the monolayer. The addition of Ca²⁺ to the 4:1 PE:PI(4,5)P₂ results only in a slightly more condensed monolayer. We hypothesize that the lack of further condensation is because PE is already able to stabilize the PI(4,5)P₂ headgroups. Overall, our studies aimed at delineating the interaction between PE and PI(4,5)P₂ indicated a stronger interaction than what was found for PI. However, in comparison to other phosphoinositide domain formation promoting species is the effect of PE on PI or PI(4,5)P₂ domain formation less pronounced.

Our study further extended the investigation of mixed PE/PI and PE/PI(4,5)P₂ monolayers to include both cholesterol and calcium. While for both PE/PI and PE/PI(4,5)P₂ mixtures in a 4:1 ratio we observed a condensation of the monolayer in the presence of cholesterol, we found distinct differences between the PI and PI(4,5)P₂ containing mixtures. For 4:1 PE:PI with 40mol% cholesterol, we see an additive effect of cholesterol and Ca²⁺ on the condensation of the monolayer, suggesting that PE, PI, and cholesterol are participating in a hydrogen bond network, while the presence of calcium is able to bridge the PI inositol headgroups, which leads to an increased stabilization of this phase. For 4:1 PE:PI(4,5)P₂ with 40mol% cholesterol, we initially see a condensation of the monolayer (shift to lower areas/molecule); however, the addition Ca²⁺ results in a shift to higher areas/molecule when compared to the Ca²⁺ free state. We

suggest that this is due to a singular PE/PI(4,5)P₂/cholesterol/Ca²⁺ complex. While a monolayer expansion normally suggests a more fluid phase state, in this case, the monolayer is quite stiff. Apparently, the PE/PI(4,5)P₂/cholesterol/Ca²⁺ is more space consuming than the Ca²⁺ free complex .

Lastly, we studied how both Ca²⁺ and cholesterol influence PI/PI(4,5)P₂ monolayers. For 1:1 mixtures in the absence of calcium and cholesterol we observe a more condensed monolayer than we found for 2:1 mixtures under the same conditions. The addition of Ca²⁺ leads to a condensation of the monolayer in both the 1:1 and the 2:1 ratios. This suggests that the presence of Ca²⁺ is able to shield and bridge the headgroups. From these experiments it appears that a 1:1 complex is more stable and preferred. The addition of cholesterol to 2:1 PI:PI(4,5)P₂ monolayers results in a significant condensation of the monolayer, suggesting that cholesterol participates in the hydrogen bond network via the 2 and/or 6 position of the inositol rings. The fact that cholesterol stabilizes PI/PI(4,5)P₂ complexes at stoichiometric ratios larger than 1 is potentially of major physiological importance because it provides a roadmap towards significantly large and robust lipid platforms for proteins to interact with.

Overall, our study confirms that cholesterol is likely to be a major modulator of phosphoinositide domain formation in biological membranes. This would explain why many phosphoinositide mediated signaling events show a strong cholesterol dependence. Highly speculative, but tempting to propose is that such domains would represent the inner leaflet raft domains. PE does not inhibit PIP domain formation, but it is also not a major PIP domain promoter. In most cases, we found that Ca²⁺ further stabilizes phosphoinositide domains.

6.1 Future Directions

While our studies have focused on monolayers at the air/water interface, it may be of interest to see if our data holds true in a bilayer model membrane system such as giant unilamellar vesicles (GUVs). This will prove to be difficult experiments, especially in the presence of Ca^{2+} . Although there are newer methods for the formation of GUVs in the presence of divalent cations, the yield of usable domains is low and the GUVs are prone to rupture due to osmotic swelling.

Obviously, it might be also of interest to expand the complexity of the monolayer even further. Obvious questions are the impact of PE on the PI/PI(4,5) P_2 /chol system. Equally, it might be of interest to add PS to any of the lipid mixtures studies in this thesis. As the complexity of the monolayer is increased, it might be also useful to study the interaction of Mg^{2+} with these more complex films even though Mg^{2+} did not show a strong interaction with pure PIP monolayer.

The evidence for domain formation in the inner leaflet of the plasma membrane could have far reaching implications for many physiological processes. Our lab is particularly interested in the binding properties of PTEN, hence it may be of interest to study PTEN binding in those lipid mixtures studied here, in particular those forming domains.

Furthermore, it may be of interest to study how the presence of PTEN influences the quantity of and shape of domains formed and/or where PTEN is located in terms of the monolayer. Using the data obtained in our studies, the monolayers of interest could be used in two ways. A PTEN tagged with the appropriate fluorophore could be injected

under the surface of the monolayer of interest. Epifluorescence microscopy could then be used to image the cluster and/or location of PTEN at the monolayer. On the other hand, unlabeled PTEN could be injected under the surface of the monolayer and the clustering of the lipids could be imaged in the same way the monolayer are images in this study.

A major but very challenging goal is the confirmation of these experiments in a cellular context. A major obstacle is that currently no PI specific probe molecule exists, i.e., it currently impossible to image cellular PI pools. The development of such a probe molecule would be a major step forward.

APPENDIX

ABBREVIATION LIST

PC: Phosphatidylcholine

PE: Phosphatidylethanolamine

PS: Phosphatidylserine

PI: Phosphatidylinositol

PA: Phosphatidic Acid

PIPs: Phosphoinositides

PI(3)P: Phosphatidylinositol-3-Monophosphate

PI(4)P: Phosphatidylinositol-4-Monophosphate

PI(5)P: Phosphatidylinositol-5-Monophosphate

PI(4,5)P₂: Phosphatidylinositol-4,5-Bisphosphate

PI(3,4)P₂: Phosphatidylinositol-3,4-Bisphosphate

PI(3,5)P₂: Phosphatidylinositol-3,5-Bisphosphate

PI(3,4,5)P₃: Phosphatidylinositol-3,4,5-Trisphosphate

PH: Pleckstrin Homology

BAR: Bin-Amphiphysin-Rvs

FYVE: Fab1, YOTB, Vac1p, EEA1

PLC: Phospholipase C

IP₃: Inositol 1,4,5 Trisphosphate

DAG: Diacylglycerol

IP₃R: Inositol 1,4,5 Trisphosphate Receptor

NMR: Nuclear Magnetic Resonance Spectroscopy

PM: Plasma Membrane

L_o: Liquid Ordered

STED: Stimulated Emission Depletion

FCS: Fluorescence Correlation Spectroscopy

PTEN: Phosphatase and Tensin Homologue Deleted on Chromosome 10

PDZ: Post Synaptic Density protein (PSD95), Drosophila Disc Large Tumor Suppressor (Dlg1), Zonula Occludens-1 protein (zo-1)

PTP: Protein Tyrosine Phosphatases

PI3K: Phosphatidylinositol 3-Kinase

P-Loop: Phosphate Binding Loop

DUSPs: Dual Specificity Protein Phosphatases

PDK1: Phosphoinositide Dependent Kinase-1

BAM: Brewster Angle Microscopy

LE: Liquid Expanded

G: Gaseous

LC: Liquid Condensed

S: Solid

PI4K: Phosphatidylinositol 4-Kinase

PI5K: Phosphatidylinositol 5-Kinase

PITP: Phosphatidylinositol Transfer Proteins

GPI: Glycophosphatidylinositol

PLD: Phospholipase D

Table A1: List of Chemicals

Chemical Name	Abbreviation	Chemical Formula	Company	Formula Weight	Purity
L- α -phosphatidylinositol	Liver PI	C ₄₇ H ₈₂ Na O ₁₃ P	Avanti Polar Lipids, Inc. (Alabaster, AL)	902.133	>99%
1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine	DOPC	C ₄₄ H ₈₄ NO ₈ P	Avanti Polar Lipids, Inc. (Alabaster, AL)	786.113	>99%
1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	DOPE	C ₄₁ H ₇₈ NO ₈ P	Avanti Polar Lipids, Inc. (Alabaster, AL)	744.034	>99%
L- α -Phosphatidylinositol-4,5-bisphosphate	Brain-PI(4,5)P ₂	C ₄₇ H ₉₄ N ₃ O ₁₉ P ₃	Avanti Polar Lipids, Inc. (Alabaster, AL)	1098.19	100%
Cholesterol		C ₂₇ H ₄₆ O	Avanti Polar Lipids, Inc. (Alabaster, AL)	386.66	99%
Bodipy FL Phosphatidylinositol	Bodipy PI	C ₄₃ H ₇₂ BF ₂ N ₄ O ₁₄ P	Echelon Biosciences, Salt Lake City, UT	948.83	
1-oleoyl-2-{6-[4-(dipyrrrometheneboron difluoride)butanoyl]amino)hexanoyl- <i>sn</i> -glycero-3-phosphoinositol-4,5-bisphosphate (ammonium salt)	Top Fluor PI(4,5)P ₂	C ₅₀ H ₉₂ N ₆ O ₂₀ P ₃ F ₂	Avanti Polar Lipids, Inc. (Alabaster, AL)	1239.023	>99%
Ethylenediamine tetraacetate disodium salt	EDTA	C ₁₀ H ₁₄ O ₈ Na ₂ N ₂ 2H ₂ O	Fisher Scientific (Fairlawn, NJ)	372.24	99.9%

Chemical Name	Abbreviation	Chemical Formula	Company	Formula Weight	Purity
Tris Hydroxymethyl Aminomethane Hydrochloride	TRIS	$\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3\text{HCl}$	Fisher Scientific (Fairlawn, NJ)	157.60	Electrophoresis grade
Calcium Chloride		CaCl_2	Fisher Scientific (Fairlawn, NJ)	110.99	Biotech Grade
Hexanes		C_6H_{14}	Fisher Scientific (Fairlawn, NJ)	86.18	HPLC Grade
Ethyl Alcohol	Ethanol	$\text{C}_2\text{H}_6\text{O}$	Acros Organics (Morris Plains, NJ)	46.06	99.5+%
Methanol		CH_3OH	Fisher Scientific (Fairlawn, NJ)	32.04	99.9%
Cholesterol		$\text{C}_{27}\text{H}_{46}\text{O}$	Avanti Polar Lipids, Inc. (Alabaster, AL)	386.66	99%
Sodium Chloride		NaCl	Fisher Scientific (Fairlawn, NJ)	58.44	99.9%
Hydrochloric Acid		HCl	Fisher Scientific (Fairlawn, NJ)		
Sodium Hydroxide		NaOH	Fisher Scientific (Fairlawn, NJ)		
Chloroform		CHCl_3	Fisher Scientific (Fairlawn, NJ)	119.38	99.9%
Water		H_2O	Fisher Scientific (Fairlawn, NJ)	18	HPLC grade

A2: Total phosphorous determination method

μmoles of Phosphorous Standard	μL of Phosphorus Standard
0	0
0.01625	25
0.0325	50
0.0570	88
0.0815	125
0.114	175

Table A2: Total volumes and μmoles of phosphorous standard required for phosphate determination

A total of six test tubes were labeled, and the calculated volume of phosphorous standard was added to create a standard curve. The μmoles of phosphorous standard added are shown in table X in triplicate. Then, 0.05μmoles of the stock lipid of interest were added to the bottom of three separate tubes and dried down using compressed nitrogen. Next, 0.225mL of 8.9N sulfuric acid was added to each tube. The tubes were then placed in a custom built aluminum block and heated at 215°C for 40 minutes. Following the heating, the tubes were removed from the block and allowed to cool. Then, 75μL of 30% hydrogen peroxide was added to each tube, and the tubes were placed back in the aluminum block at 215°C. After 40 minutes, the tubes were removed and the aluminum block was allowed to cool to 100°C. Finally, 1.95mL of distilled water, 0.25mL of 2.5% Ammonium molybdate (IV) tetrahydrate solution, and 0.25mL of 10% ascorbic acid solution were added to all of the tubes, making sure to vortex between each addition. The tubes were placed in the heating block once again, and were heated to 100°C for 7 minutes. The test tubes were then removed from the heating block and were allowed to cool in a dark area. Once the tubes had cooled to room temperature, the samples were analyzed in a microplate

reader at an absorbance of 820 nm.

Data Analysis

Using the data collected from the microplate reader, a standard curve can be constructed, and the equation of the line can be obtained. The equation was used to determine the μ moles of phosphorous present in each lipid sample. From this information, the concentration of each lipid sample can be determined based on the number of phosphates present in each lipid structure. This process was carried out in triplicate for each lipid stock solution to ensure an accurate molar concentration.

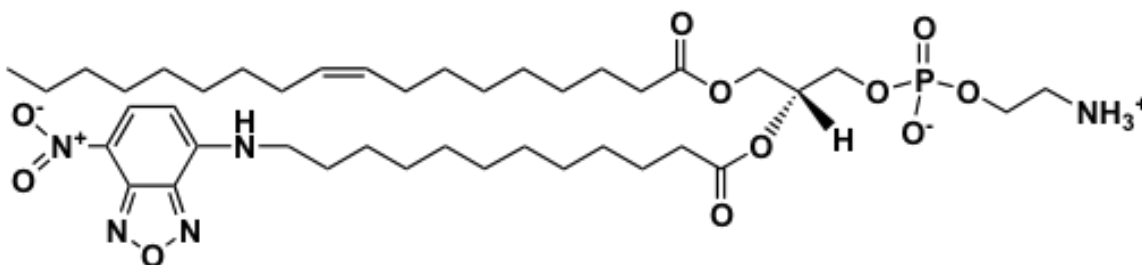


Figure A1: Chemical Structure of NBD-PE.

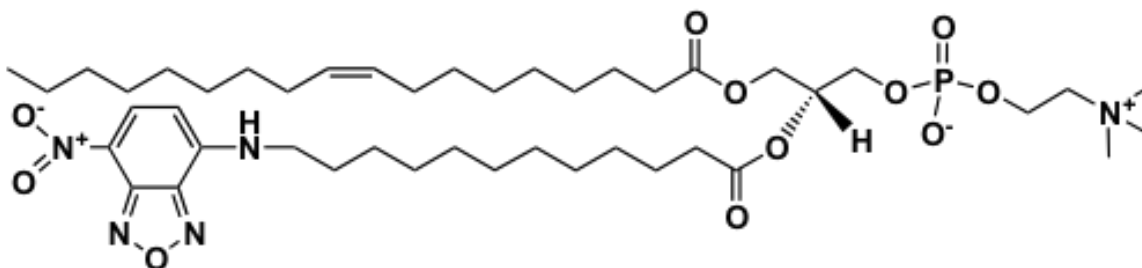


Figure A2: Chemical Structure of NBD-PC.

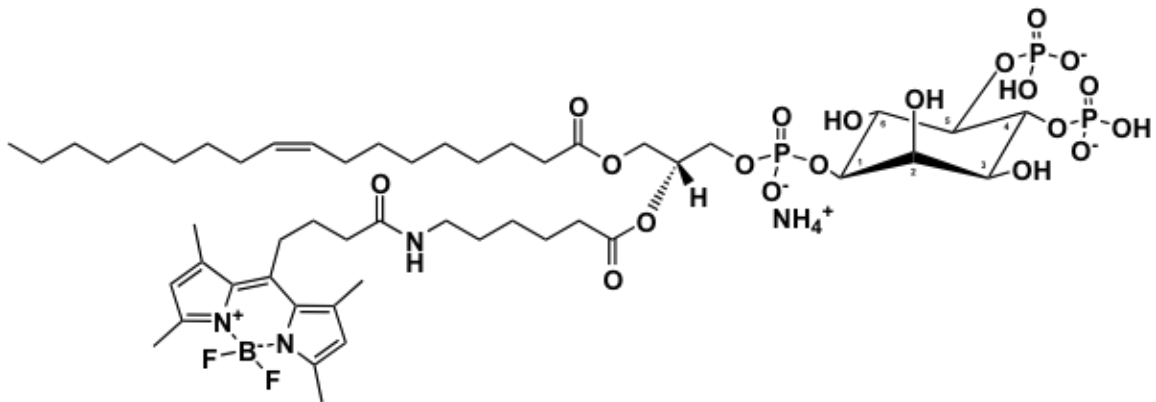


Figure A3: Chemical Structure of Top Fluor PI(4,5)P₂.

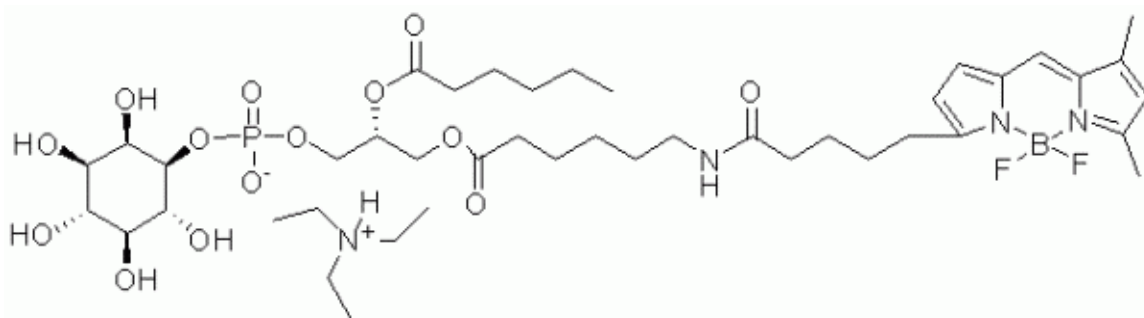


Figure A4: Chemical Structure of Bodipy FL PI.

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