THE ROLE OF PHOSPHATIDYLSERINE AS A SCAFFOLD FOR LIPID/PROTEIN COMPLEXES

A Major Qualifying Project Report Submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirements for the
Degree of Bachelor of Science in Biochemistry

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Date: March 2017

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Phosphatidylserine (PS), one of the main components of the inner leaflet of the plasma membrane, plays an important role in various physiological processes including cell signaling. Its interaction with other lipids as well as bivalent cations has shown to lead to domain formation. This project studies how PS interacts with Ca^{2+} as well as phosphatidylinositol 4,5 biphosphate. PS monolayers at the air/water interface were studied in the presence and absence of calcium, where low concentrations of calcium lead to expansion of the monolayer and no domain formation while high concentrations of calcium lead to condensation and stability of the monolayer, as well as domain formation at low pressures. In addition, PS/PI(4,5)P_2 monolayers at the air/water interface were studied in the presence and absence of calcium, where similar behavior was observed.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Arne Gericke, for allowing me to work in his lab from my freshman year at WPI, and for his great advice and support throughout my undergraduate studies, and especially for this project.

I would like to thank all the members of Gericke lab: Anne-Marie for teaching me the basics of lab work, how to use various instruments in the lab, and for always willing to help whenever I had any questions; Katie for teaching me everything I know about lipids and how to work with them as well as for answering any questions I had throughout this project; Brittany for always helping me make sense of my data and fix anything that would stop working.

I would also like to thank Osama and Ashma for always willing to help me in the lab, and for their encouraging talks and positive attitude. Finally, I would like to thank my parents and friends for their support throughout this project. I wouldn't be able to spend so many late hours in the lab without their encouragement.

1.0 INTRODUCTION AND BACKGROUND

1.1 IMPORTANCE OF BIOLOGICAL MEMBRANES

The biological membranes, which are composed both of lipids and proteins, play an important role in many cellular phenomena including cell signaling and flow of nutrients. While proteins are responsible for executing various membrane function, lipids provide the fundamental structure for the membranes as well as the right physicochemical conditions for the proteins to carry out their functions.

1.2 FLUID MOSAIC MODEL

In 1972, the fluid mosaic model of membrane structure was proposed by Jonathan Singer and Garth Nicolson. This model is now adopted as the basic structure for the organization of all biological membranes. As shown by the model, membranes are two-dimensional fluids in which proteins are inserted into lipid bilayers (1,3). In recent years, the fluid mosaic model of membranes was augmented to account for the fact that lipids often show a non-homogeneous lateral distribution (domain formation). These domains have been identified as important platforms for protein functions (13).

1.3 STRUCTURE OF PHOSPHOLIPIDS

The structure of phospholipids plays an important role in the basic function of membranes as barriers between two aqueous compartments (2). The interior of the phospholipid bilayer contains the hydrophobic fatty acid chains, and makes the membrane impermeable to water-soluble molecules. In addition, the double bonds in the fatty acids allow for the presence of kinks in the hydrocarbon chains and make them difficult to pack together, and therefore make the membrane more flexible. The exterior of the phospholipid bilayer contains hydrophilic headgroups. The headgroups can change the hydrogen bonding capabilities of lipids. In addition, while some lipids are neutral at physiological pH, others are negatively charged. Therefore, the differences in charge and hydrogen bonding ability influences interactions between the lipids in the bilayer as well as with other molecules (5).

1.4 LIPID CONTENT OF PLASMA MEMBRANE

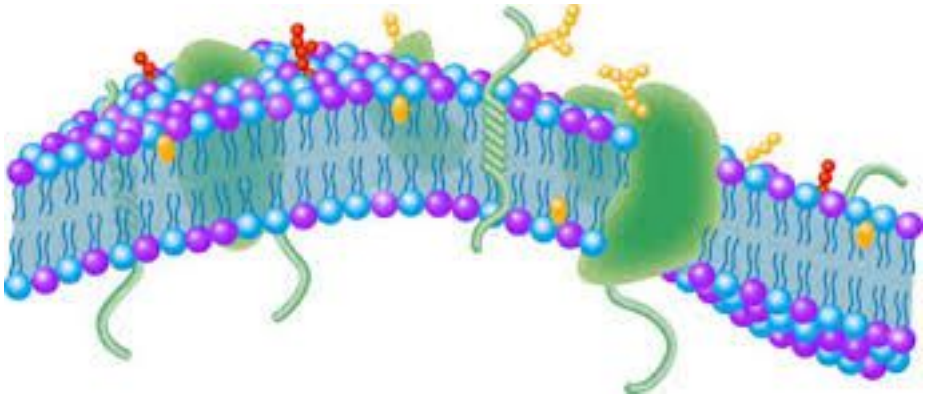

Figure 1: Structure of plasma membrane composed of proteins and lipids. The outer leaflet consists predominantly of phosphatidylcholine, sphingomyelin, and glycolipids, while the inner leaflet is mainly composed of phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol.

The plasma membranes of animal cells are composed mainly of these four phospholipids: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin , which together make up more than half of the lipid composition in most membranes (2). Phosphatidylinositol, which is a minor component of the plasma membrane, plays an important role in cell signaling as well as in the charge on the cytosolic face of the plasma membrane (2). These phospholipids are distributed asymmetrically in the two halves of the membrane bilayer as shown in Figure 1. The outer leaflet of the plasma membrane is composed of phosphatidylcholine and sphingomyelin , while the inner leaflet is mainly composed of phosphatidylethanolamine and phosphatidylserine, and some phosphatidylinositol (2). The head groups of both phosphatidylserine and phosphatidylinositol are negatively charged, so the inner leaflet has an overall negative charge (Figure 2).

1.5 IMPORTANCE OF PHOSPHATIDYLINOSITOL, PHOSPHATIDYLSERINE AND INOSITOL PHOSPHOLIPIDS

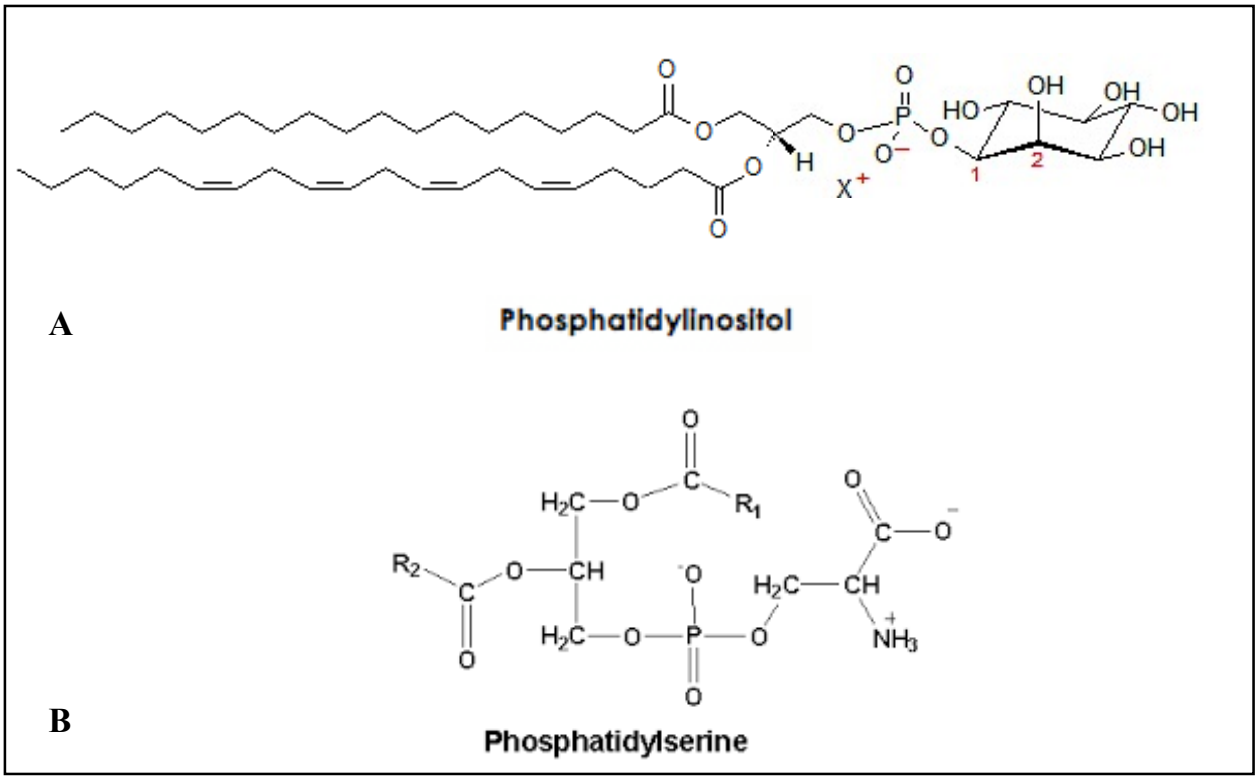

Figure 2: Structure of phosphatidylinositol (A), which makes up 6-10% of the lipids in the inner leaflet of the plasma membrane, and phosphatidylserine (B), which makes up more than 12% of the lipids in the inner leaflet of the plasma membrane.

Phosphatidylinositols (PI) participate in signaling and recognition, while phosphatidylserines (PS), when exposed on the cell surface, act as a signal for phagocytosis and as a propagation signal in blood coagulation (4).

Another important class of lipids that compose the plasma membrane are inositol ring phosphorylated phosphatidylinositols (phosphoinositides), which mediate many interactions through their headgroups, including the control of membrane-cytosol interfaces (6). The headgroups in these lipids can be reversibly phosphorylated to generate seven different species. These lipids identify membranes that are undergoing endocytosis and allow them to recruit proteins from the cytosol that are involved in vesicle trafficking (4). In addition, they play an important role in regulation of membrane traffic, nuclear events and the permeability of membranes (6).

1.6 STRUCTURE AND FUNSTION OF PHOSPHANTIDYLNOSITOL 4,5 BIIPHOSPHATE

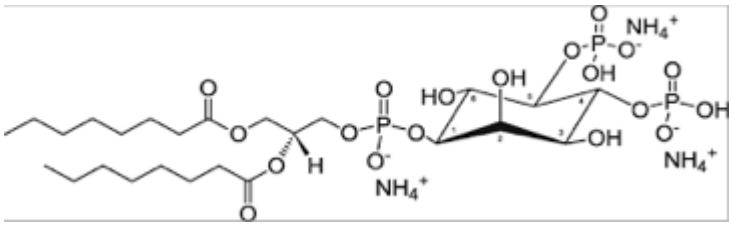

Figure 3 : Structure of Phosphatidylinositol 4,5-bisphosphate, which makes up 1% of inner leaflet in plasma membrane where it plays mainly a signaling role, and 10-15% of nucleus membrane where it mainly plays a structural role.

Phosphatidylinositol 4,5-bisphosphate (PIP_2) (Figure 3) comprises only about 1% of the phospholipids in the cytoplasmic leaflet of the plasma membrane (7). However, it is the source of three second messengers — inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) , diacylglycerol (DAG) and $PI(3,4,5)P_3$, activates many ion channels and enzymes, is involved in both endocytosis and exocytosis, as well as anchors proteins to the membrane through several structured domains (7).

Irvine et al suggest that PIP_2 is able to be involved in so many different functions due to the presence of "multiple independent pools of PIP_2, with different pools governing distinct functions" (8) while Stenmark et al contributes this to the "localized formation and turnover of this lipid" (9). While studies suggest that both of the above are true, it is also claimed that PIP_2 is concentrated in lipid rafts (10), clusters at the plasma membrane that are in a more dense phase.

1.7 PHOSPHATIDYLINOSITOL MONOPHOSPHATE (PIP) DOMAIN FORMATION

One of the theories for the formation of PIP enriched domains is through a hydrogen bond network form (11). It is found that hydrogen bonding plays an important role in the formation of phosphoinositide monophosphate domains in PIP/PC model membrane systems (11). Specifically, it is suggested that the hydroxyl groups of the inositol ring act as hydrogen donors, while the phosphomonoester, phosphodiester and other hydroxyl groups from adjacent molecules act as hydrogen bond acceptors in the bond network (14). However, it was also found that PIP_2 had less of a tendency to form domains (11), which suggests that a more complex PIP model membrane systems needs to be built in order to understand the interactions of PIPs in biological membranes (14).

1.8 LIPID MODEL MEMBRANE SYSTEMS

Given that biological membranes are composed of both lipids and proteins, they are very complex. However, lipid model membrane systems can be used to explore some aspects of these membranes. Organic monolayers at the air–water interface, Langmuir films, are two-dimensional systems that exhibit a very rich phase-transition behavior, and are widely used as potential models for biological membranes (12). In this study, monolayers are used to mimic the inner leaflet of the plasma membrane because the composition of the lipids as well as other molecules can be varied widely. These monolayers are characterized by surface pressure/area isotherms, which can be used to obtain information about lipid-lipid interactions for different lipid compositions and subphase conditions. If a known amount of the lipid or lipid mixture is applied, the isotherm obtained can provide information about lipid packing densities and lipid phases (14).

1.9 LANGMUIR TROUGH

The Langmuir trough contains two movable barriers placed on opposite sides of a trough and a pressure sensor connected to a Wilhelmy plate. The barriers move at a certain speed as controlled by NimaTR516 program, which also collects data points and autogenerates an area/pressure isotherm. In addition, temperature of the trough is regulated with a thermostated water bath. For every experiment, the lipid mixture of interest is dissolved in an organic solvent and is spread on the water or buffer surface in a dropwise manner using a 25μL syringe (14). The organic solvent is then allowed to evaporate, leaving the lipid monolayer of interest on the buffer/water surface. The surface pressure can described by the 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 interphase. “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 ($Å^2$ /molecule) where the area decreases as the surface pressure increases” (14).

1.10 EPIFLUORESCENCE MICROSCOPY

In addition, epifluorescence images of the Langmuir films can be taken to obtain visual information about monolayer morphology. 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 (14). In order to visualize the domains, fluorescently labeled lipids, which partition into ordered or liquid disordered phases, are added to the lipid monolayer of interest. It is important to note that fluorophores can be seen as an impurity in the monolayer, which could provide inaccurate information about the lipid monolayer; however, other techniques have shown similar results (14). For this study, fluorescently labeled lipid (0.1%) that matched the headgroup of the lipid species being investigated were used.

1.11 PREVIOUS STUDY RESULTS

In a previous study, the conditions that lead to formation of phosphoinositide enriched domains in increasingly complex lipid mixtures were explored. The most suitable conditions found will be used in this project to study other lipid mixtures. As in the previous study, lipid systems in the fluid phase were used for lipid monolayers in order to better mimic the actual conditions of biological membranes. However, isotherms are taken at a temperature of 20^{o}C instead of 25^{o}C (previous study) to obtain more stable monolayers. In the previous study, it was found that the interaction of the highly negatively charged head group of PI(4,5)P_2 with calcium leads 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. Under epifluorescence microscopy, domain formation was observed in the presence of calcium. In addition, PI/PI(4,5)P_2mixtures were found to form domains in the presence of calcium and behave as seen in Figure 4, where the presence of Ca^{2+}may be able to bridge the PI molecules leading to a more condensed monolayer and therefore a surface pressure/area isotherm that is shifted to a lower area/molecule.

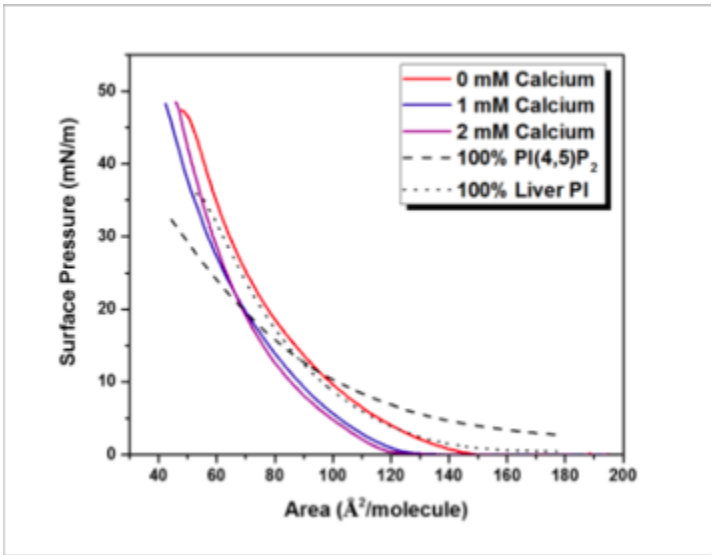

Figure 4: Surface pressure/area isotherms of mixed PI:PI(4,5)P $_2$ monolayers at a 1:1 ratio subphase comparison. Surface pressure/area isotherms of mixed PI:PI(4,5)P $_2$ at a 2:1 ratio in the absence and presence of calcium. Suphase consisted of 10mM Tris, 150mM NaCl, and with either 0.1mM EDTA (0mM CaCl $_2$) (red), 0.01mM CaCl $_2$ (green), or 1mM CaCl $_2$ (blue) at pH 7.4 and T= 25±0.2℃. The surface pressure/area isotherm for 100% PI(4,5)P $_2$ (dashed) and 100% Liver PI (dotted) in the absence of Ca^{2+} are shown for comparison. (14)

1.12 OBJECTIVE OF THIS STUDY

For this project, POPS and PI(4,5)P $_2$ monolayers in the presence and absence of calcium will be studied. We hypothesize that domains will form in the presence of calcium because calcium will increase the interactions between the negatively charged POPS and PI(4,5)P $_2$ head groups. In addition, we will visualize the POPS and PI(4,5)P $_2$ monolayers using epifluorescence microscopy. For these experiments, it is our hypothesis that we will be able to observe domain formation in the presence of calcium. Langmuir monolayers will also be used to understand the lipid-lipid interactions between PS and PI(4,5)P_2 in the presence and absence of calcium.

2.0 MATERIALS AND METHODOLOGY

2.1.BUFFERS

For this project, buffers were prepared in order to create the conditions of a subphase for the Langmuir-Trough Films. 10mM Tris, 150mM NaCl, and 0.1mM EDTA buffer was prepared using Ethylenediamine tetraacetate disodium salt (EDTA) with a formula weight of 372.24 g/mol, and 99.9% purity; Tris Hydroxymethyl Aminomethane Hydrochloride (TRIS) with a formula weight of 157.60g/mol and electrophoresis grade purity; sodium chloride (NaCl), with a formula weight of 58.44 g/mol, and 99.9% purity; and HPLC grade water; all of the above were obtained from Fisher Scientific (FC). Calcium chloride from FC was used to prepare 0.01, 1 and 2 mM Ca2+ buffer. All buffers were adjusted to a pH of 7.4 using pH probe and 5M hydrochloric acid or sodium hydroxide.

2.2.LIPID DISSOLVING

For this project, various lipids were used. Stock lipids from Avanti Polar Lipids were dissolved in a non-polar solvent or mixture of solvents. L-α-Phosphatidylinositol-4,5-bisphosphate (Brain-PI(4,5)P_2) with formula weight 1098.19g/mol and purity >99% was dissolved in 20:9:1 chloroform:methanol:water to a final concentration of 0.4mg/mL. 1-hexadecanoyl-2--(9Z-octadecenoyl)-*sn*-glycero-3-phospho-L-serine (POPS) with a formula weight 783.988g/mol and >99% purity, and 1-hexadecanoyl-2--(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (POPC) with a formula weight 760.076g/mol and >99% purity were dissolved in chloroform to a final concentration of 0.3mg/mL. All dissolved lipids were stored in vials at -20^{oC}.

2.3.PHOSPHATE ASSAY

The exact concentrations of the dissolved stock lipids were determined by running a phosphate assay. First, six test tubes were labeled and the following amounts of phosphorus standard (Table 1) were added.

Table 1: Phosphorus Standard for Phosphorus Determination Method

µmoles of Phosphorus Standard	**µL of Phosphorus Standard**
0	0
0.01625	25
0.0325	50
0.0570	88

0.0815	125
0.114	175

Then, 0.05μmoles of the lipid of interest were placed into the bottom of separate test tubes, and the solvent was removed using a flow of nitrogen. The standards as well as the lipids of interest were run in triplicates in order to produce accurate results. 0.225mL of 8.9N sulfuric acid was added to all tubes, and the tubes were heated in an aluminum block placed on a hot plate in a hood at 215°C for 40 minutes. The tubes were then removed and 75μL of H_2O_2 was added to each tube. Next, the tubes were heated at 215°C for 40 minutes, removed and cooled down to approximately 100°C. Then, 1.95mL of DI water was added, followed by 0.25mL 2.5% Ammonium molybdate (IV) tetrahydrate solution and the tubes were vortexed. Then, 0.25mL 10% Ascorbic Acid solution was added and the tubes were vortexed. Next, the tubes were heated at 100°C for 7 minutes and cooled to room temperature before the absorbance was checked at 820nm by adding 250μL of each sample in micro-titer plate. A standard curve was constructed by using the phosphorous standard concentrations and absorbances. The equation of the line from the standard curve was used to determine the concentration of the lipid of interest.

2.4.SURFACE PRESSURE/AREA ISOTHERMS OF LANGMUIR FILMS

First, a Langmuir trough, described in section 1.9 and shown in Figure 5, and its barriers were cleaned using a mixture of hexane and ethanol, and then HPLC water. The temperature was set to 20^{o}C through the water bath. A buffer solution of 10mM Tris, 150mM NaCl, 0.1mM EDTA, at pH 7.4, made as explained in section 2.1, was added to the trough for subphases without calcium. The barriers were compressed and expanded various times in order to ensure no pressure increase due to impurities. Any impurities that resulted in pressure increase were removed through suction, the barriers were set to the open position and the surface pressure was set to 0. Then, 15-20uL (exact volume calculated depending on lipid concentration) of the desired lipid dissolved in the appropriate non-polar solvent as described in 2.2, and whose concentration was determined as explained in 2.3, was added drop-wise using a 25uL syringe. The solvent was allowed to evaporate for 10-15 minutes and the barriers were compressed at a rate of 5cm^2/minute. A surface pressure/area isotherm was generated through NimaTR516 program and recorded. This was repeated at least three times until the isotherms were reproduced with an area difference of less than 2 Armstrongs.

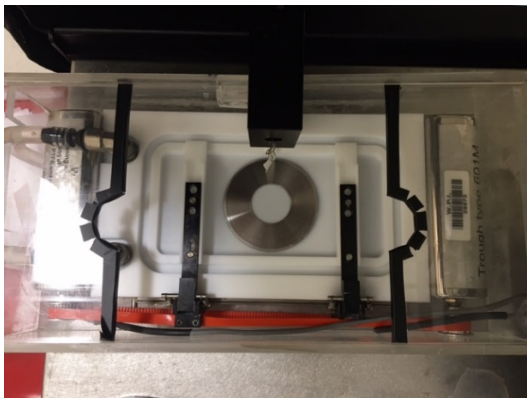

Figure 5: Image of Langmuir-Trough used to take surface pressure/area isotherms

2.5. EPIFLUORESCENCE MICROSCOPY OF LANGMUIR FILMS

Epifluorscence microscopy was used to image the lipid monolayers as well as their morphological changes. The lipid samples were prepared as described in 2.2, and spread as described in 2.4. However, 0.1mol% of fluorescently labeled lipid that matched the headgroup of the lipid species being investigated were used. Given that the fluorophores can be seen as an impurity in the monolayer, which could provide inaccurate information about the lipid monolayer, additional images with fluorescently labeled lipid that had a different headgroup than the lipid species being investigated were taken to determine the presence of impurity or domain formation. The surface pressure/area isotherms were taken as described in 2.4. However, the trough was placed on an Olympus BX51 upright microscope equipped with a Hamamatsu EM-CCD camera (Figure 6). The fluorescent images were taken using a 40X objective and an EM-CCD camera with an exposure of 30 frames per second. Because the trough's pressure sensor was slightly moved when the microscope was being focused, the isotherms recorded at the same time as the fluorescent images were not used, and separate isotherms were taken instead. However, these isotherms were used as a reference for the obtained images. Images were analyzed through ImageJ.

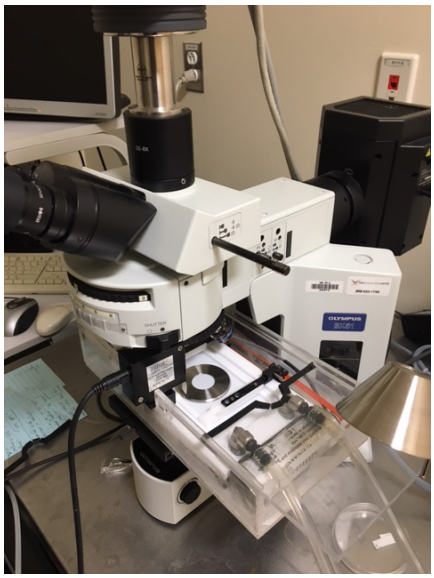

Figure 6: Image of Langmuir-Trough Placed on Olympus BX51 upright microscope equipped with a Hamamatsu EM-CCD camera

3.0 RESULTS AND DISCUSSION

3.1. SURFACE PRESSURE/AREA ISOTHERM AND EPIFLUORESCENCE IMAGES OF PHOSPHOPATIDYLCHOLINE

In order to understand how the isotherm provides information regarding the phase states of the monolayer, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) isotherms were taken (Figure 7), which are known to have all the phase states present. After spreading the lipid, the monolayer is in a gaseous phase state (G), which has a large area/molecule, and low surface pressure. Once the compression takes place, the monolayer enters a liquid-expanded/gaseous (LE/G) coexistence phase. Further compression leads to the monolayer changing from a LE/G phase to a liquid-expanded/liquid-condensed (LE/LC) coexistence phase. Finally, more compression leads to the monolayer entering a solid state (S). The transition to each state correlates to a decrease in the area/molecule and an increase in the surface pressure. The shape of the surface pressure/area isotherm is affected by the different behaviors of the monolayer, which shows changes in the molecular packing of the lipid molecules. The other lipids studied in this project only go through a LE phase. Through the epifluorescence images, the domain formation can be seen at different pressures.

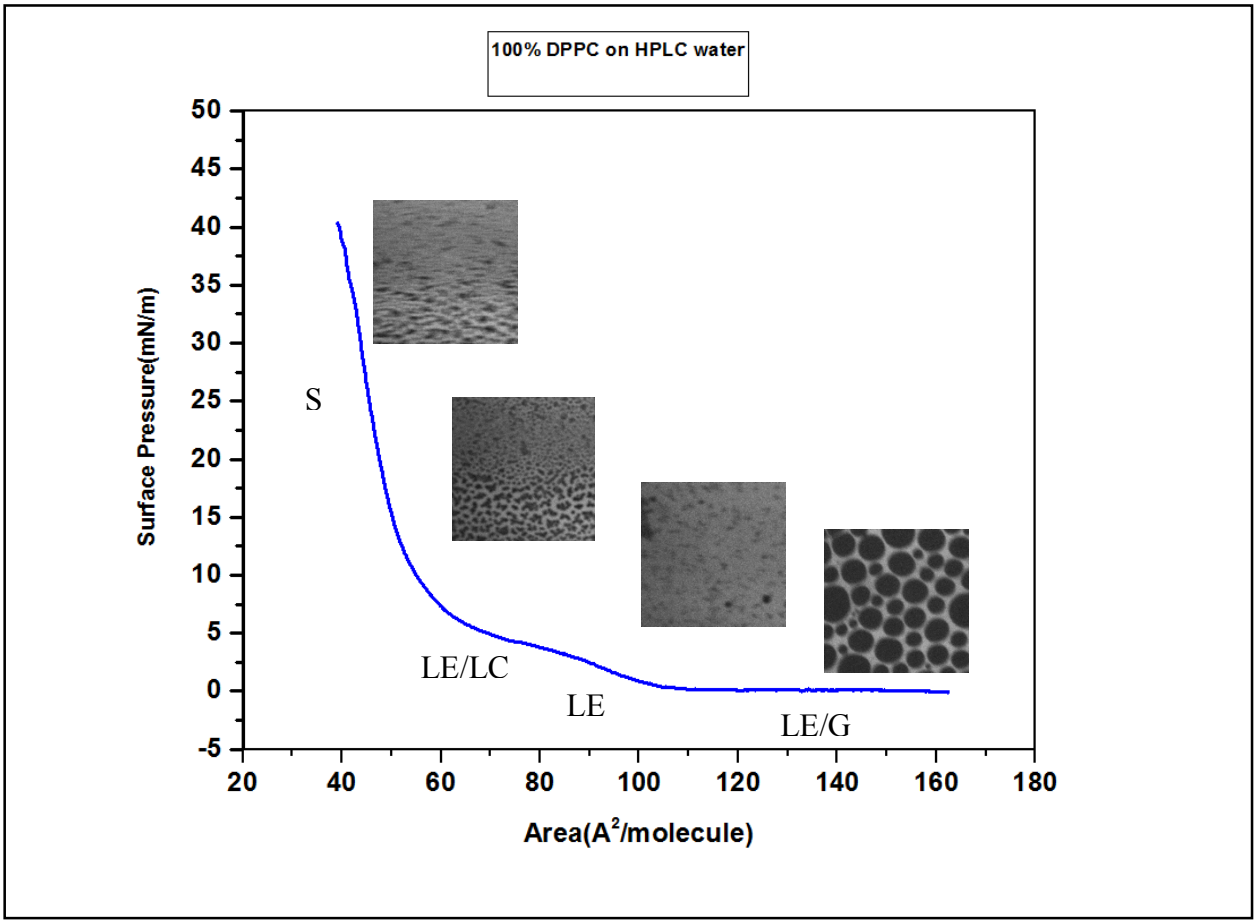

Figure 7: Surface Pressure/Area isotherm and Corresponding Epiflurescence Images of DPPC Monolayer. Suphase consisted of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.5mol% NBD-PC.

3.2. THE EFFECT OF DIFFERENT BARRIER COMPRESSION SPEEDS ON SURFACE PRESSURE/ AREA ISOTHERMS

In order to determine the type of barrier compression that lead to the most accurate isotherm, PI(4,5)P_2 monolayers were compressed continuously and discontinuously at varying times for barrier opening of 2, 4 and 7 seconds. It was expected for the monolayer to have a higher collapse pressure at slow compression rates due to higher film stability.

PI(4,5)P_2 samples were prepared as described in Section 2, and were spread on subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at pH 7.4 and a temperature of 20˚C. Throughout the discontinuous compressions, a lower collapse pressure of the monolayer was noticed (Figure 8), which could be due to the lipid molecules slipping under the barriers.

Therefore, the various isotherms with the different lipids used for this project, were taken through continuous (i.e faster) compressions.

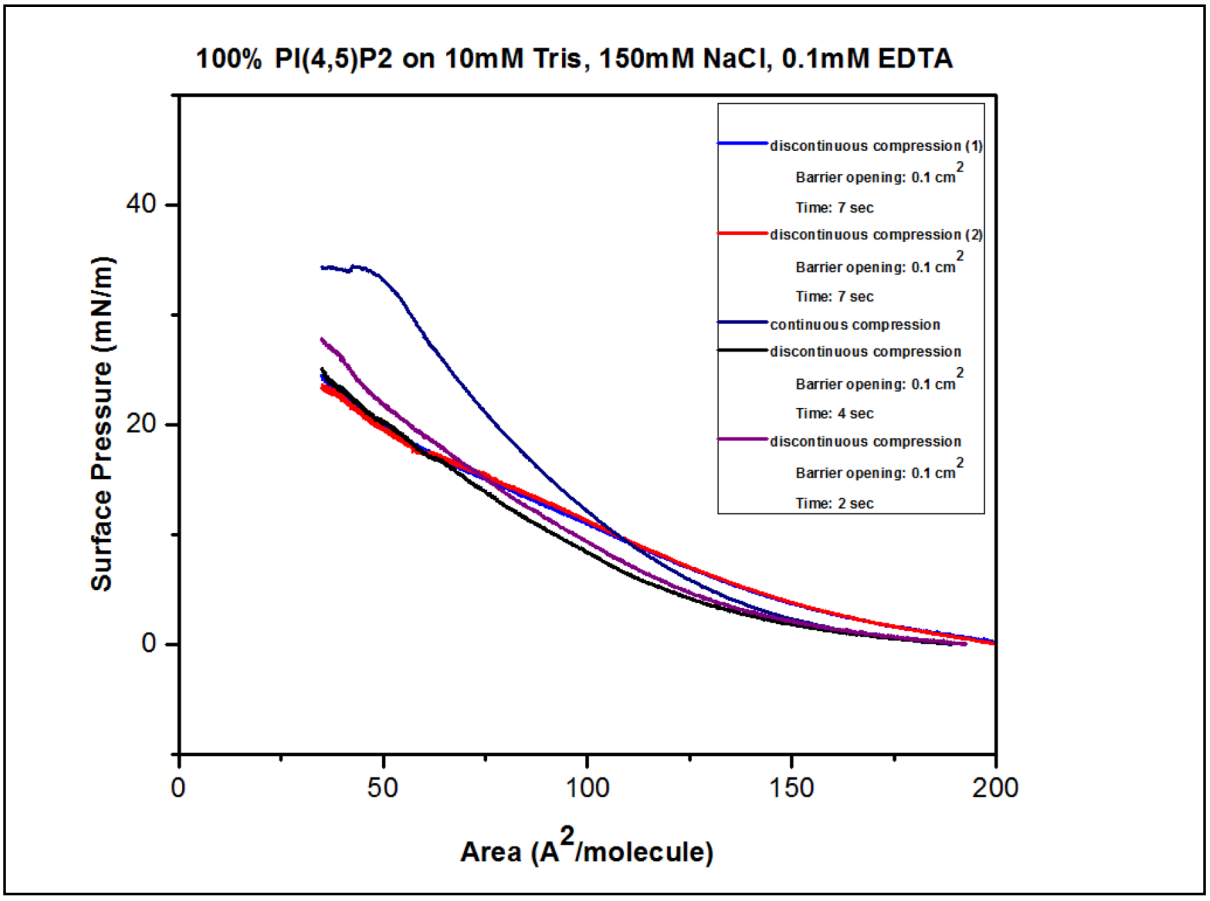

Figure 8: Surface Pressure/Area Isotherms of PI(4,5)P_2 Monolayer obtained Through Continuous and Discontinuous Compressions with Varying Times for Barrier Opening. Monolayers consisting of PI(4,5)P_2, on subphase composition of 0.1mM EDTA, 10mM Tris, 150mM NaCl, pH 7.4 at T=20˚C. Isotherms obtained through continuous compression (blue), and discontinuous compressions with a barrier opening of 0.1cm2 every 7 seconds (red), 4 seconds (black), and 2 seconds (purple).

3.3. THE EFFECT OF CALCIUM ON SURFACE PRESSURE/AREA ISOTHERMS AND MORPHOLOGY OF PHOSPHOPATIDYLSERINE MONOLAYERS

In order to determine the behavior of PS in the plasma membrane, surface pressure/ area isotherms of these lipid molecules were studied and epifluorescence images were obtained at

selected surface pressures. Since PS has a negatively charged headgroup, it is expected that when calcium is added, it will shield and bridge these headgroups of the PS molecules, and therefore lead to the condensation of the monolayer.

PS samples were prepared as described in Section 2, and were spread on a subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA or various concentrations of calcium: 0.01mM Ca^{2+}, 1mM Ca^{2+}, or 2mM Ca^{2+} at pH 7.4 and a temperature of 20˚C. For the epifluorescence images, 0.1mol% labeled PS fluorphore was added to the sample.

Surface pressure/ area isotherms of $PI(4,5)P_2$ on a subphase without calcium show an expanded isotherm (Figure 9). In addition, the monolayer collapses at low surface pressures, which could come as a result of instability of the monolayer due to the unsaturated chains of the lipid, and the lipid slipping under the barriers. In the presence of 0.01mM Ca^{2+}, surprisingly, the monolayer slightly expands, and the isotherm is placed at higher areas/molecule. This could be due to calcium inserting between PS headgroups leading to a competition between the monolayer condensing effect of the Ca^{2+} due to headgroup bridging and monolayer expansion due to insertion of the cation between the headgroup. However, the increase in calcium stabilizes the monolayer leading to a higher collapse pressure. When 1mM Ca^{2+} is added, the monolayer condenses slightly, and the collapse pressure increases which shows higher stability. The 2mM Ca^{2+} results in a slightly more condensed monolayer than the 1mM Ca^{2+}. The data (Figure 9) shows that Ca^{2+} could shield and bridge the negatively charged headgroups of PS, and therefore result in a slightly more condensed and more stable monolayer at the air/water interface. The surface pressure/area isotherm also shows that the subphase containing 2mM Ca^{2+} crosses over with the 1mM Ca^{2+}, which could be due to the increased stability of the PS monolayer in the presence of calcium.

In the absence of Ca^{2+}, under the fluorescence microscopy, PS monolayers showed domain formation at pressure 0mN and no domain formation at any higher pressure throughout the compression of the monolayer (Figure 10), which can be explained with the expanded monolayer and the lack of stability. The domain formation can be explained with the PS headgroups interacting with each other. When 0.01mM calcium is added to the subphase, domains can be seen at low pressures (Figure 11). The addition of 1mM and 2mM calcium in subphase also shows domain formation (Figure 12 and Figure 13). For images at subphases containing any concentration of calcium, at high pressures, the labeled PS seems to form clusters by itself, and

hence the net-like looking domains. Given the tightly packing of lipid molecules at high pressures and high Ca^{2+} concentrations, the labeled PS is excluded from unlabeled PS and therefore no domains are visible. This is also supported by the images in the Appendix.

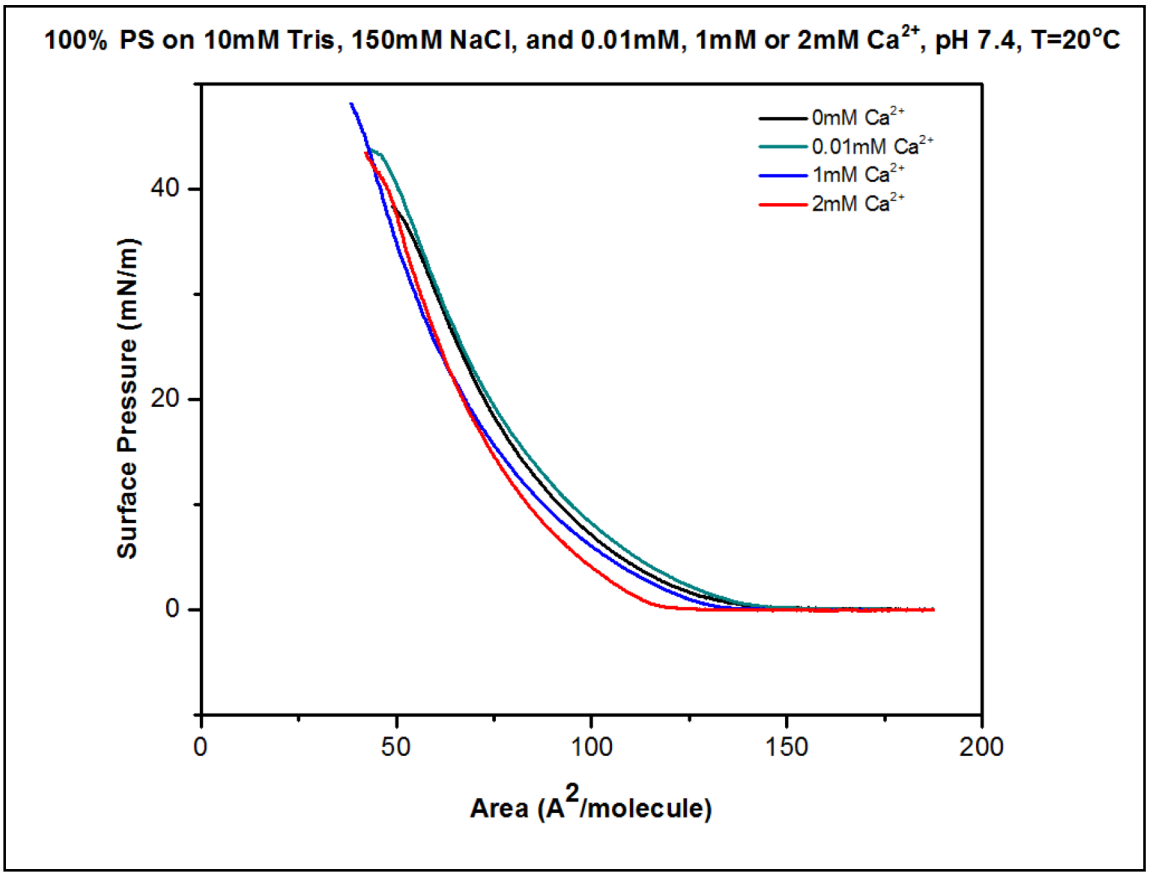

Figure 9: Surface Pressure/Area Isotherms of POPS Monolayer in the Presence and Absence of Calcium. Subphase compositions of 0mM $CaCl_2$ (0.1mM EDTA) (black), 0.01mM Ca^{2+} (green), 1mM Ca^{2+} (blue), or 2mM Ca^{2+} (red), 10mM Tris, 150mM NaCl, pH 7.4 at T=20˚C.

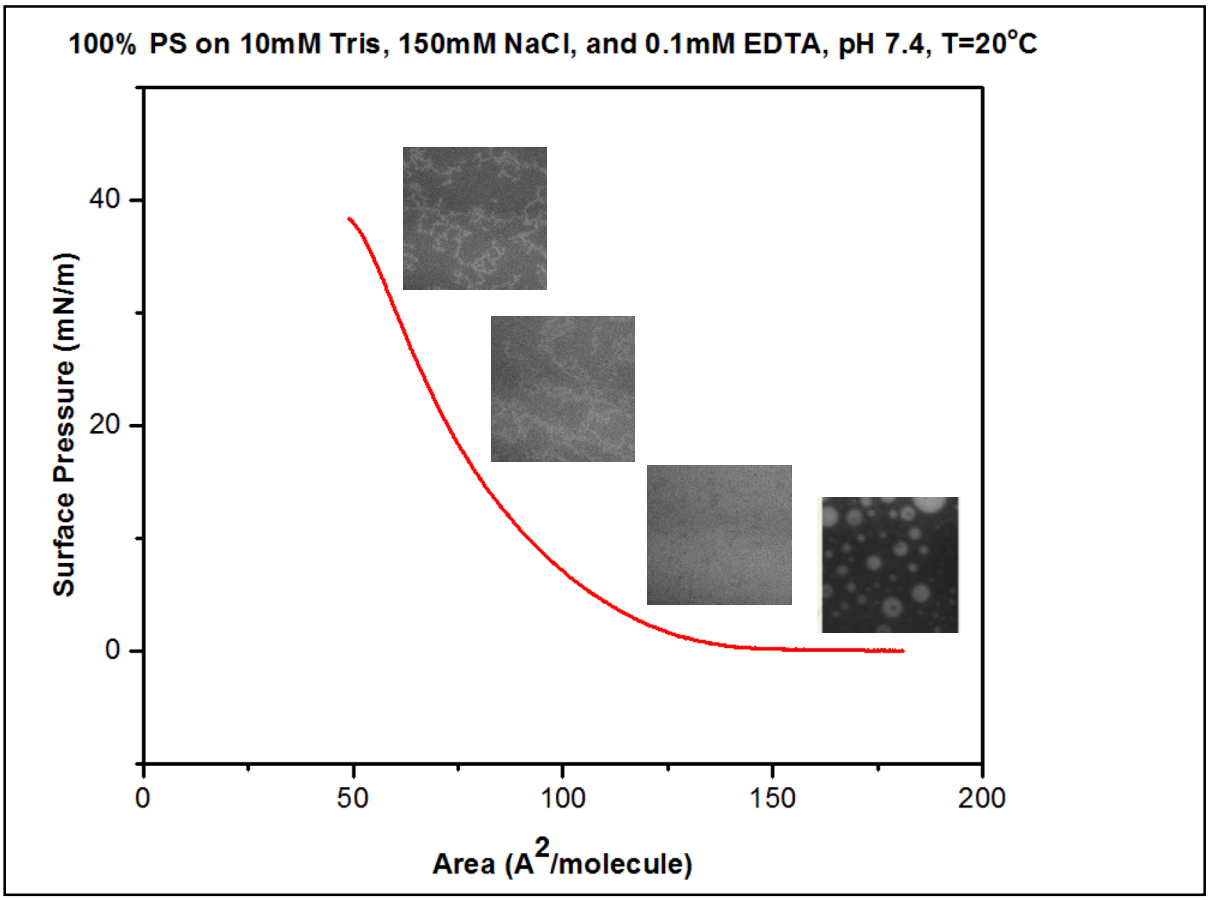

Figure 10: Surface Pressure/Area Isotherm and Corresponding Epiflurescence Images of POPS Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® PS.

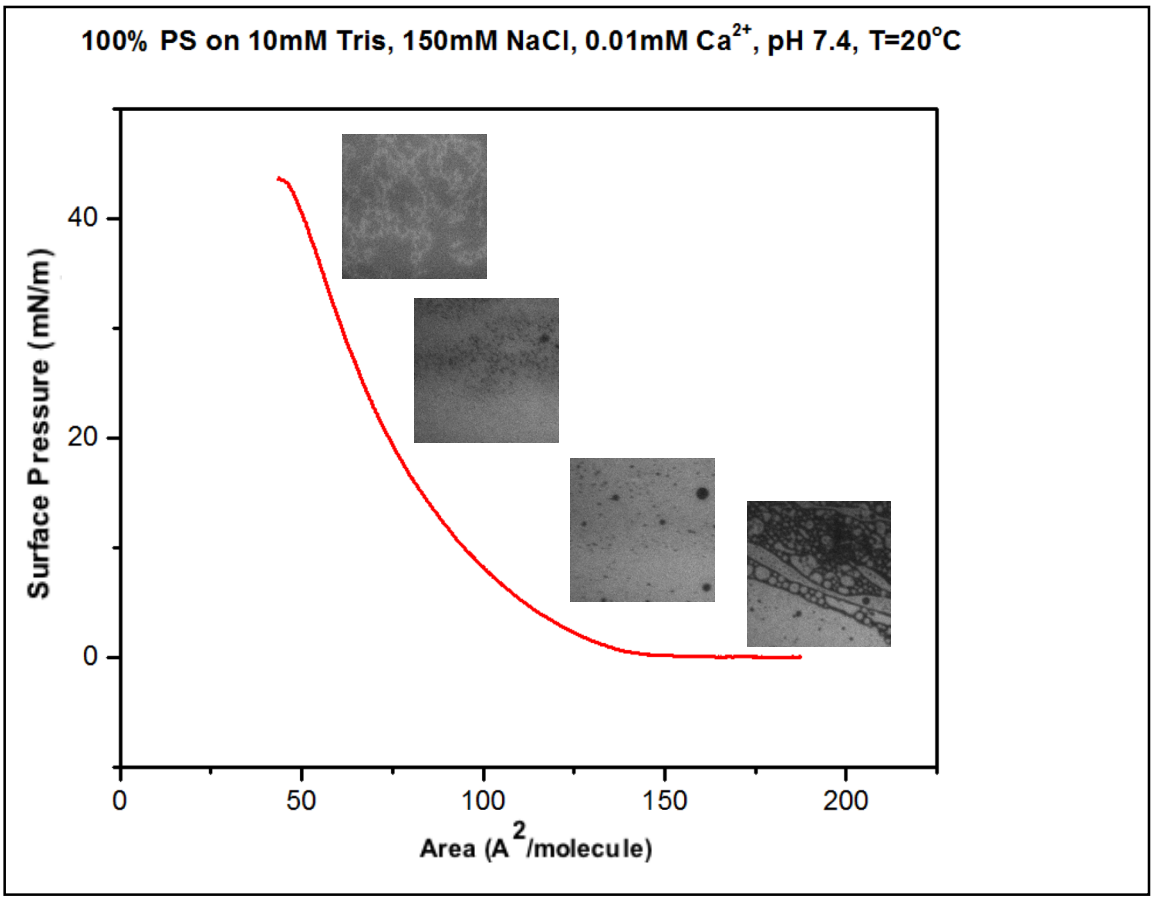

Figure 11: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of POPS Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 0.01mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® PS.

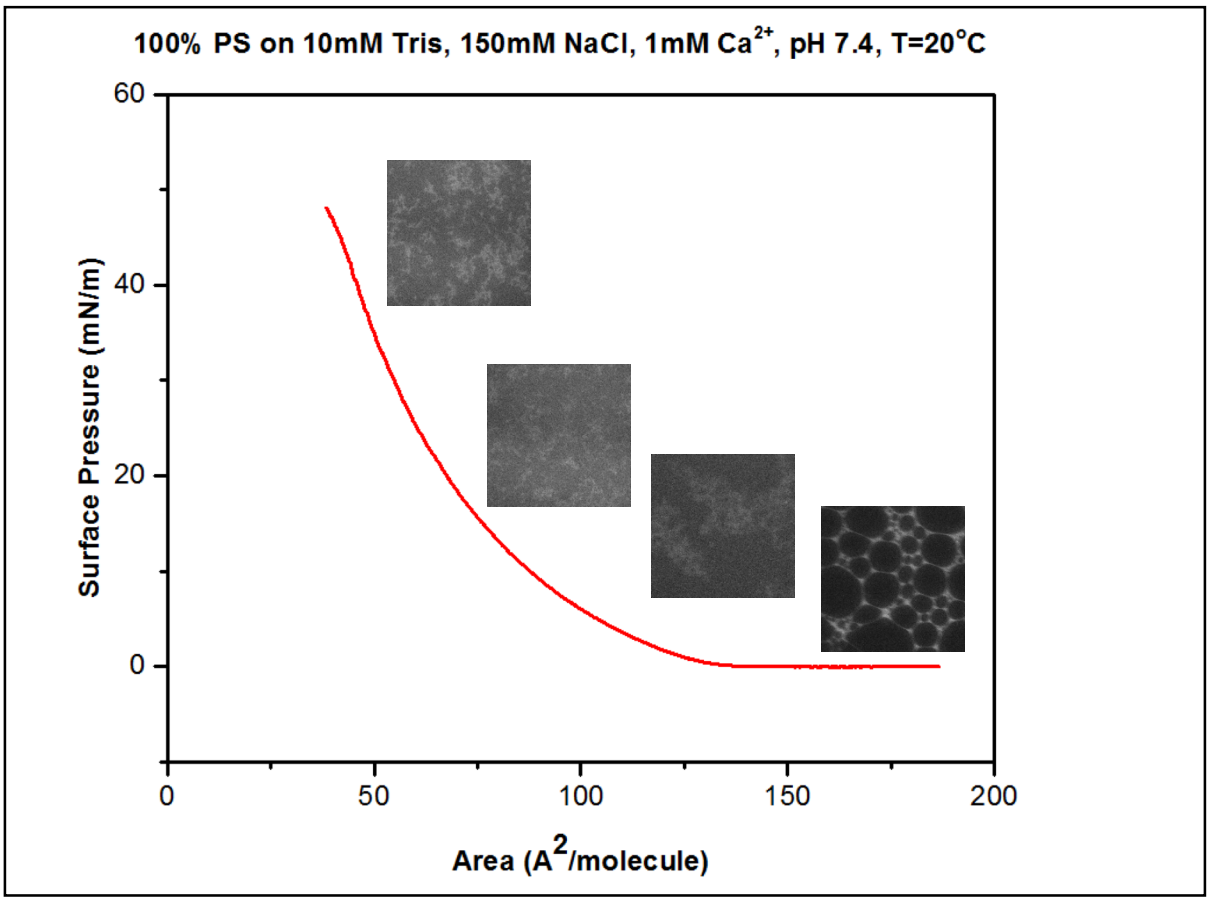

Figure 12: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of POPS Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 1mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® PS.

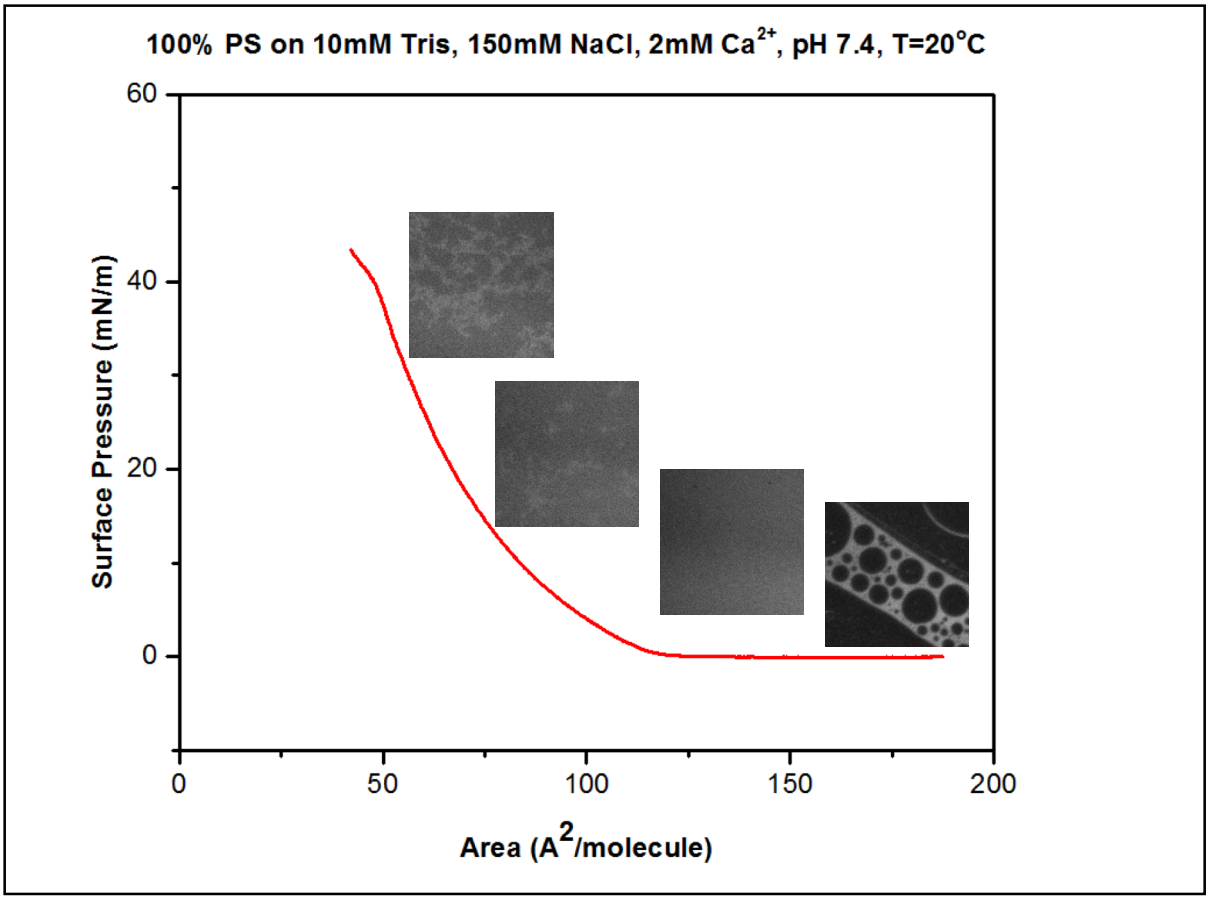

Figure 13: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of POPS Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 2mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® PS.

3.4. THE EFFECT OF CALCIUM ON SURFACE PRESSURE/AREA ISOTHERMS AND MORPHOLOGY OF PHOSPHOPATIDYLINOSITOL 4,5 BISPHOSPHATE MONOLAYERS

In order to determine the behavior of $PI(4,5)P_2$ in the plasma membrane, surface pressure/ area isotherms of these lipid molecules were studied and epifluorescent images were obtained at selected surface pressures. Given that $PI(4,5)P_2$ a has negatively charged headgroup, it is expected that when calcium is added, it will shield and bridge these $PI(4,5)P_2$ headgroups, and therefore lead to the condensation of the monolayer.

$PI(4,5)P_2$ samples were prepared as described in Section 2, and were spread on subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA or various concentrations of

calcium: 0.01mM Ca^{2+}, 1mM Ca^{2+}, or 2mM Ca^{2+} at pH 7.4 and a temperature of 20˚C. For the epifluorescent images,

Surface pressure/ area isotherms of $PI(4,5)P_2$ on a subphase without calcium show an expanded isotherm (Figure 14). In addition, the monolayer collapses at low pressures, which could come as a result of instability of the monolayer due to the unsaturated chains of the lipid, and the lipid slipping under the barriers. In the presence of 0.01mM Ca^{2+}, the monolayer condenses, and the isotherm is placed at lower area/molecule. This increase in calcium also stabilizes the monolayer leading to a higher collapse pressure. When 1mM Ca^{2+} is added, the monolayer condenses even more, and the collapse pressure increases which indicating higher film stability. The addition of 2mM Ca^{2+} results in a slightly more condensed monolayer than what is observed for 1mM Ca^{2+}. The data (Figure 14) shows that Ca^{2+} could shield and bridge the negatively charged headgroups of $PI(4,5)P_2$, and therefore result in a more condensed and more stable monolayer at the air/water interface.

In the absence of Ca^{2+}, under the fluorescence microscope $PI(4,5)P_2$ monolayers showed no domains formation at any pressure throughout the compression of the monolayer (Figure 15), which can be explained with the expanded character of the monolayer. When 0.01mM calcium is added to the subphase, domains can be seen at pressures up to 40mN/m (Figure 16). The addition of 1mM and 2mM calcium in subphase also shows domain formation (Figure 17 and Figure 18). For images at subphases containing any concentration of calcium, at high pressures, the labeled $PI(4,5)P_2$ seems to form clusters by itself, and hence the net-like looking domains. Given the tight packing of lipid molecules at high pressures and high Ca^{2+} concentrations, the labeled $PI(4,5)P_2$ is excluded from unlabeled $PI(4,5)P_2$ and therefore no domains are visible. This is also supported by the images in the Appendix.

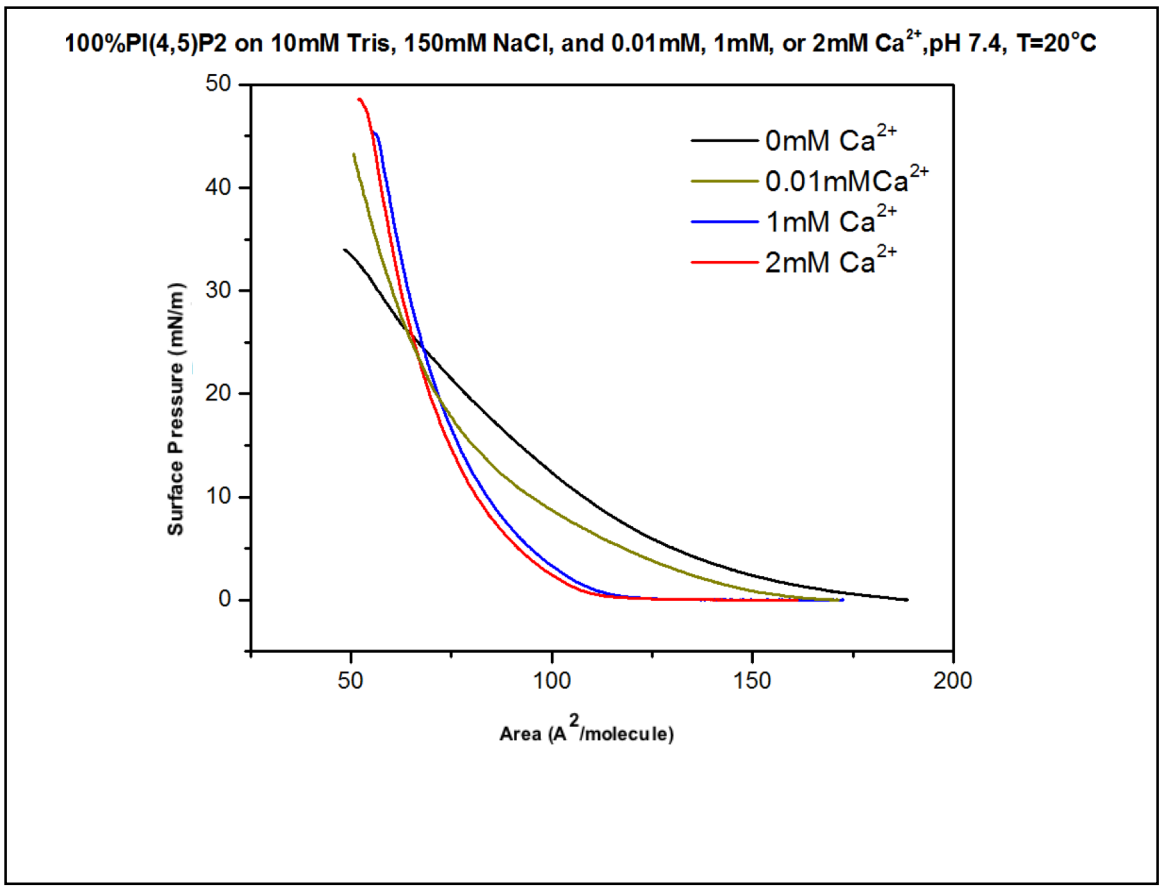

Figure 14: Surface Pressure/Area Isotherms of PI(4,5)P_2 Monolayer in the Presence and Absence of Calcium. Subphase compositions of 0mM $CaCl_2$ (0.1mM EDTA) (black), 0.01mM Ca^{2+} (green), 1mM Ca^{2+} (blue), or 2mM Ca^{2+} (red), 10mM Tris, 150mM NaCl, pH 7.4 at T=20˚C.

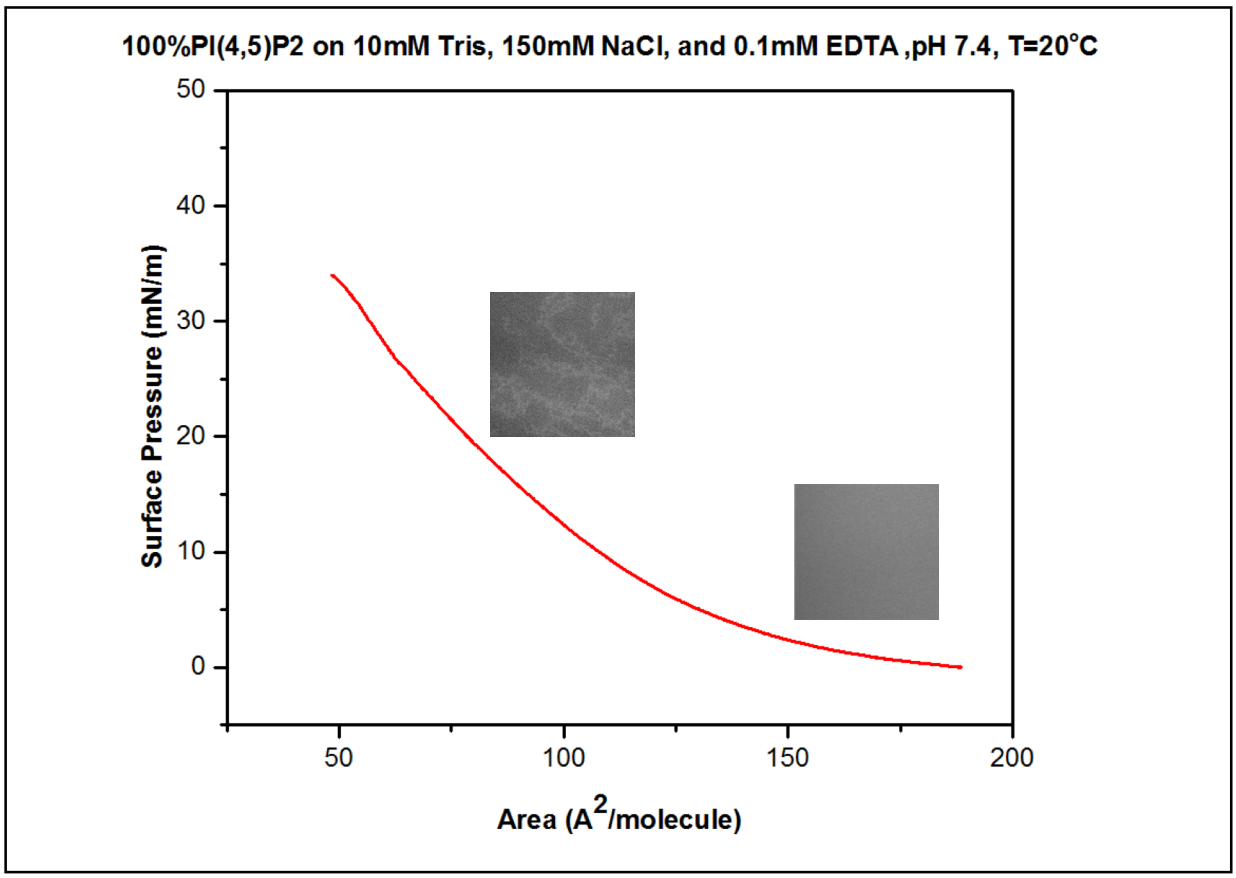

Figure 15: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of $PI(4,5)P_2$ Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® $PI(4,5)P_2$.

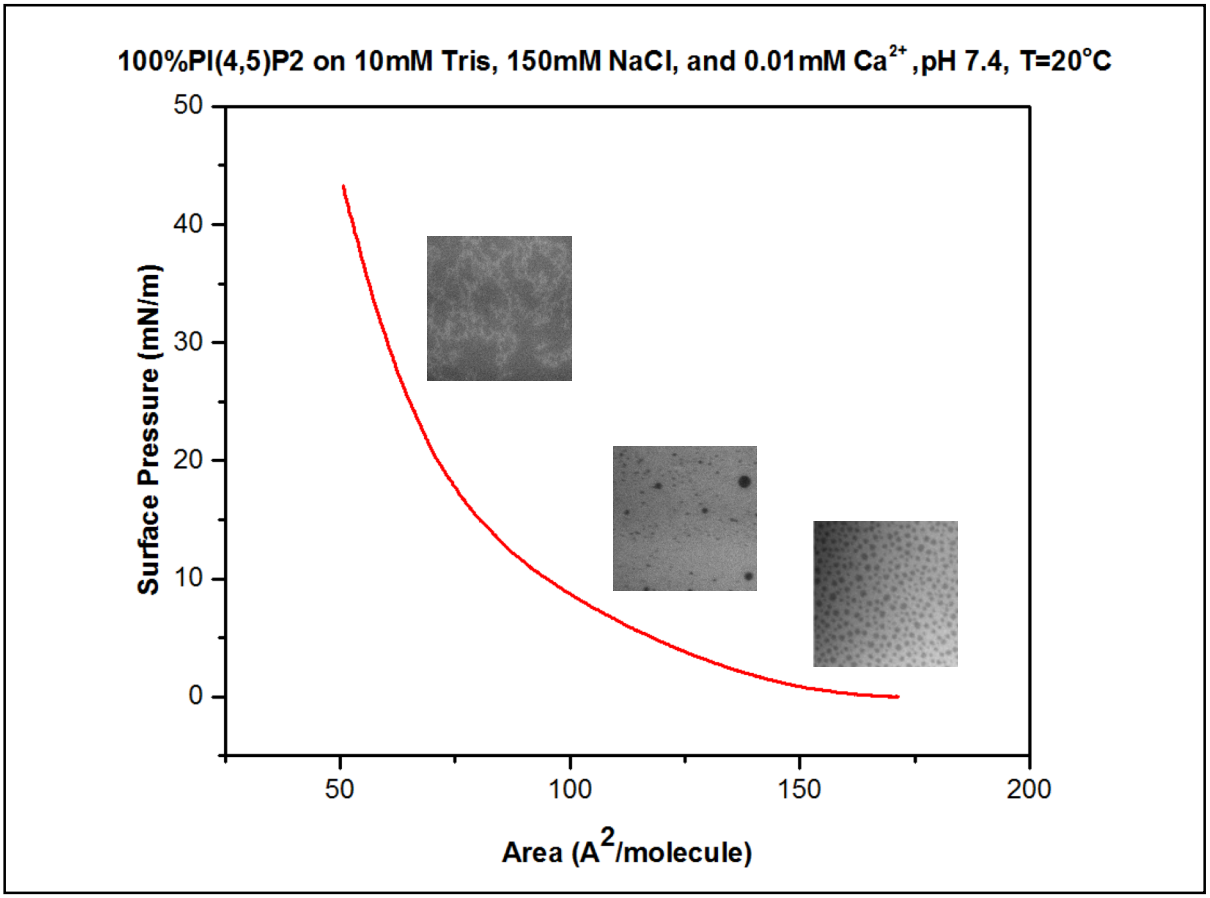

Figure 16: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of $PI(4,5)P_2$ Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 0.01mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® $PI(4,5)P_2$.

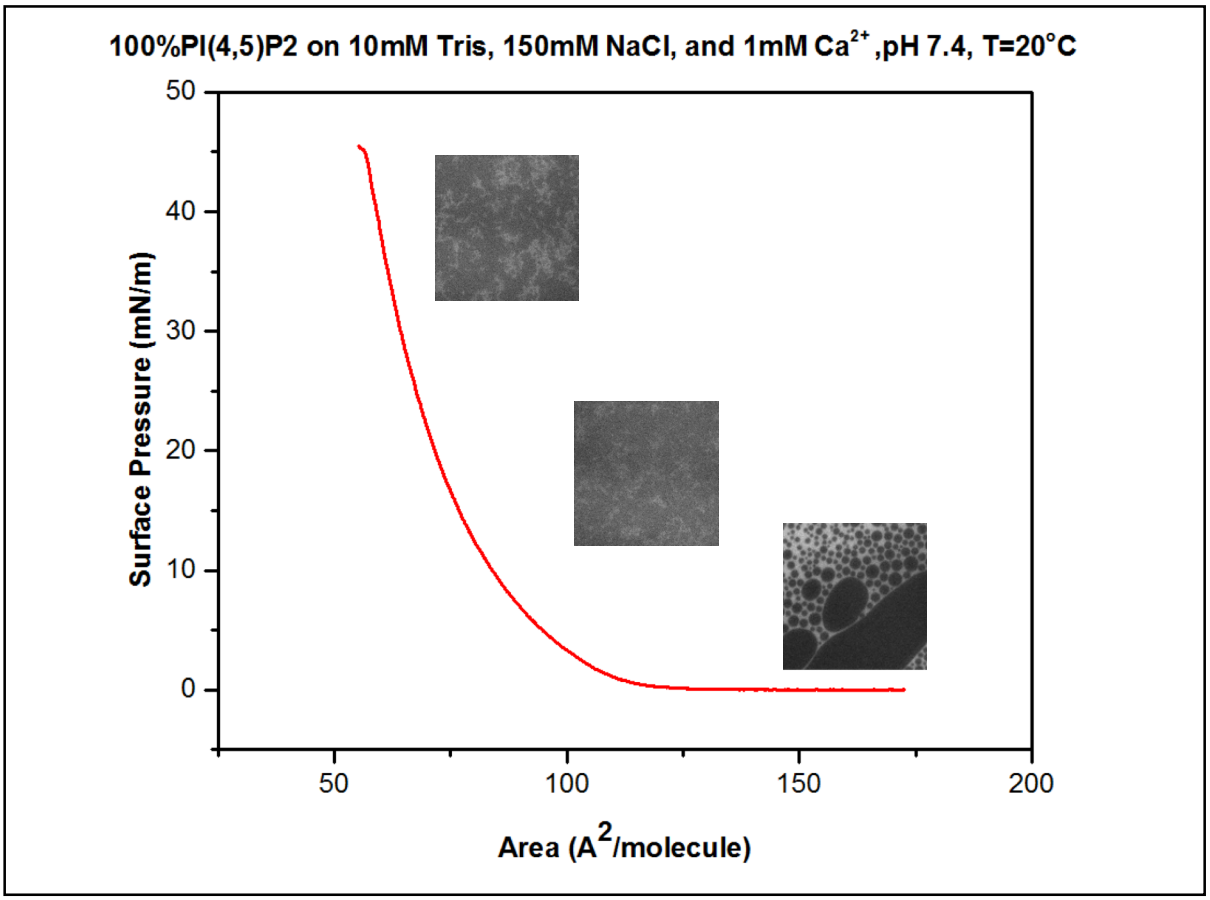

Figure 17: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of $PI(4,5)P_2$ Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 1mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® $PI(4,5)P_2$.

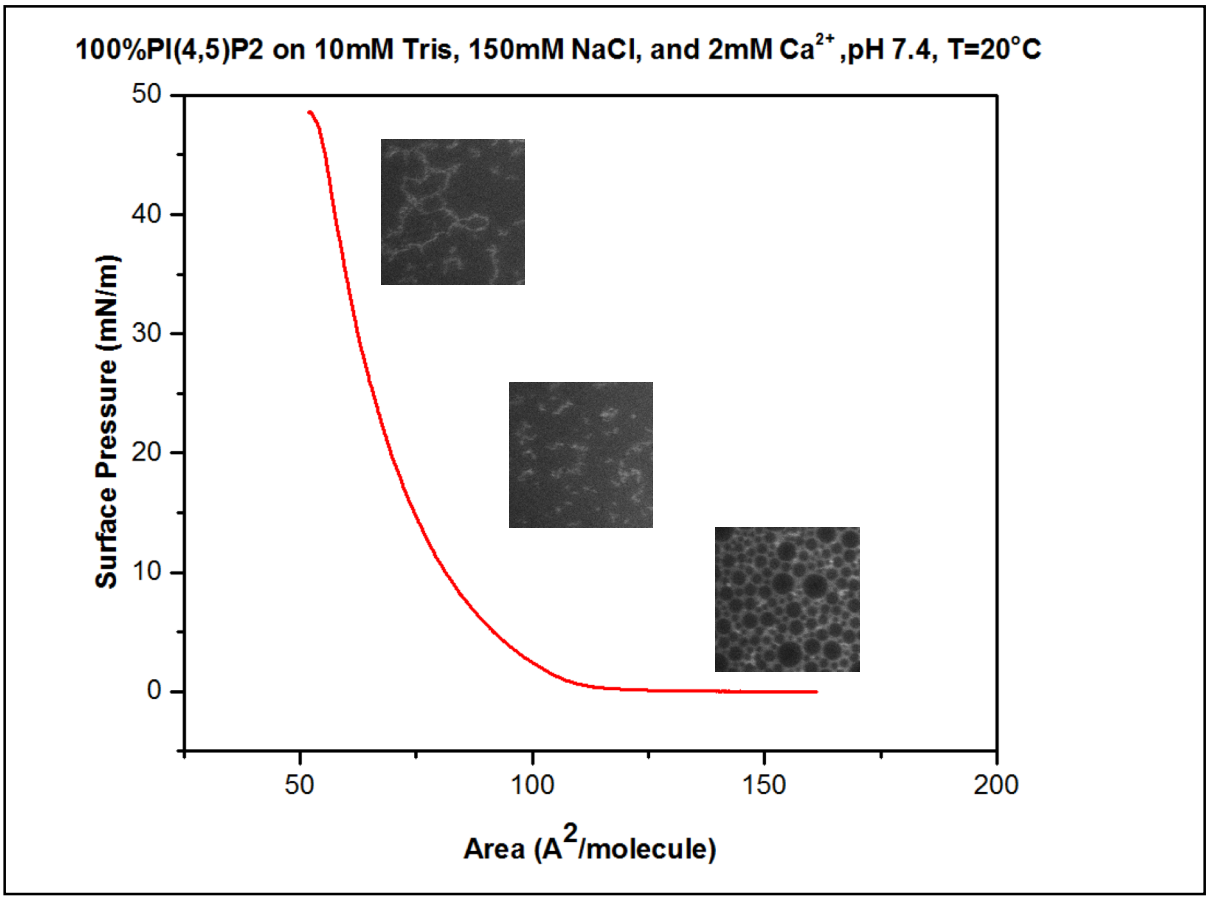

Figure 18: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of $PI(4,5)P_2$ Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 2mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® $PI(4,5)P_2$.

3.5. THE EFFECT OF CALCIUM ON SURFACE PRESSURE/AREA ISOTHERMS OF MIXTURES OF PHOSPHOPATIDYLSERINE PHOSPHOPATIDYLINOSITOL 4,5 BIPHOSPHATE MONOLAYERS

In order to better mimic the lipid content of the plasma membrane and to determine the behavior of PS:$PI(4,5)P_2$ mixtures in the plasma membrane, specifically in 1:1 and 2:1 ratios of PS:$PI(4,5)P_2$, surface pressure/ area isotherms of these lipid molecules were studied and epifluorescence images were obtained at selected surface pressures in presence and absence of calcium subphases. Given the structure of these two lipids, it is expected that some hydrogen

bonding may occur between the two molecules in addition to the shielding and bridging of the negatively charged headgroups from the addition of calcium.

Samples were prepared as described in Section 2, and were spread on subphase consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA or various concentrations of calcium: 0.01mM Ca^{2+}, 1mM Ca^{2+}, or 2mM Ca^{2+} at pH 7.4 and a temperature of 20˚C. For the epifluorescence images, 0.1mol% fluorescently labeled PS or 0.1mol% fluorescently labeled PI(4,5)P_2 was added to the sample.

Surface pressure/ area isotherms of a 1:1 PS:PI(4,5)P_2 mixture on a subphase without calcium show an expanded isotherm (Figure 19). In addition, the monolayer collapses at low pressures, which could come as a result of instability of the monolayer due to the unsaturated chains of the lipids, and the lipids slipping under the barriers. In the presence of 0.01mM Ca^{2+}, surprisingly, the monolayer slightly expands, and the isotherm is placed at higher area/molecule. This could be due to the PS and PI(4,5)P_2 headgroups interacting with each-other in addition to interacting with the calcium, similar to what is observed for our PS monolayer. However, the further increase in calcium stabilizes the monolayer leading to higher collapse pressures. When 1mM Ca^{2+} is added, the monolayer condenses slightly, and the collapse pressure increases which shows higher stability. The addition of 2mM Ca^{2+} results in a slightly more condensed monolayer than the 1mM Ca^{2+}. The data (Figure 19) shows that Ca^{2+} could shield and bridge the negatively charged headgroups of PS and PI(4,5)P_2, and therefore result in a slightly more condensed and more stable monolayer at the air/water interface. The surface pressure/area isotherm also shows that the subphase containing 2mM Ca^{2+} crosses over with the 1mM Ca^{2+}, which could be due to the increased stability of the monolayer in the presence of calcium.

Surface pressure/ area isotherms of 2:1 PS:PI(4,5)P_2 mixture shows similar behavior as the 1:1 PS:PI(4,5)P_2 mixture for non calcium and 0.01mM calcium subphase (Figure 20). However, the surface pressure/ area isotherm of the subphase containing 2mM Ca^{2+} and 1mM Ca^{2+} follow the exact same path with the 2mM Ca^{2+} having a slightly higher collapse pressure. This shows that the lipid mixture at this ratio does not stabilize any further with higher calciumconcentrations, which could be due to the headgroups of the two lipids interacting with each other and not with calcium.

In the absence of Ca^{2+}, under the fluorescence microscopy, 1:1 PS:PI(4,5)P_2 monolayers showed no domain formation at any pressure throughout the compression of the monolayer (Figure

21), which can be explained with the expanded monolayer and the lack of stability. When 0.01mM calcium is added to the subphase, again no domains can be seen (Figure 22), which could be due to the labeled lipid just interacting with itself and not with either of the lipids. The addition of 1mM and 2mM calcium to the subphase results in domain formation (Figure 23 and Figure 24). For images at subphases containing any concentration of calcium, at high pressures, the labeled PS and labeled PI(4,5)P_2 seems to form clusters by itself, and hence the net-like looking domains. Given the tight packing of lipid molecules at high pressures and high Ca^{2+} concentrations, the labeled PS and labeled PI(4,5)P_2, as seen before, is excluded from unlabeled PS and PI(4,5)P_2 and therefore no domains are visible. This is also supported by the images in the Appendix.

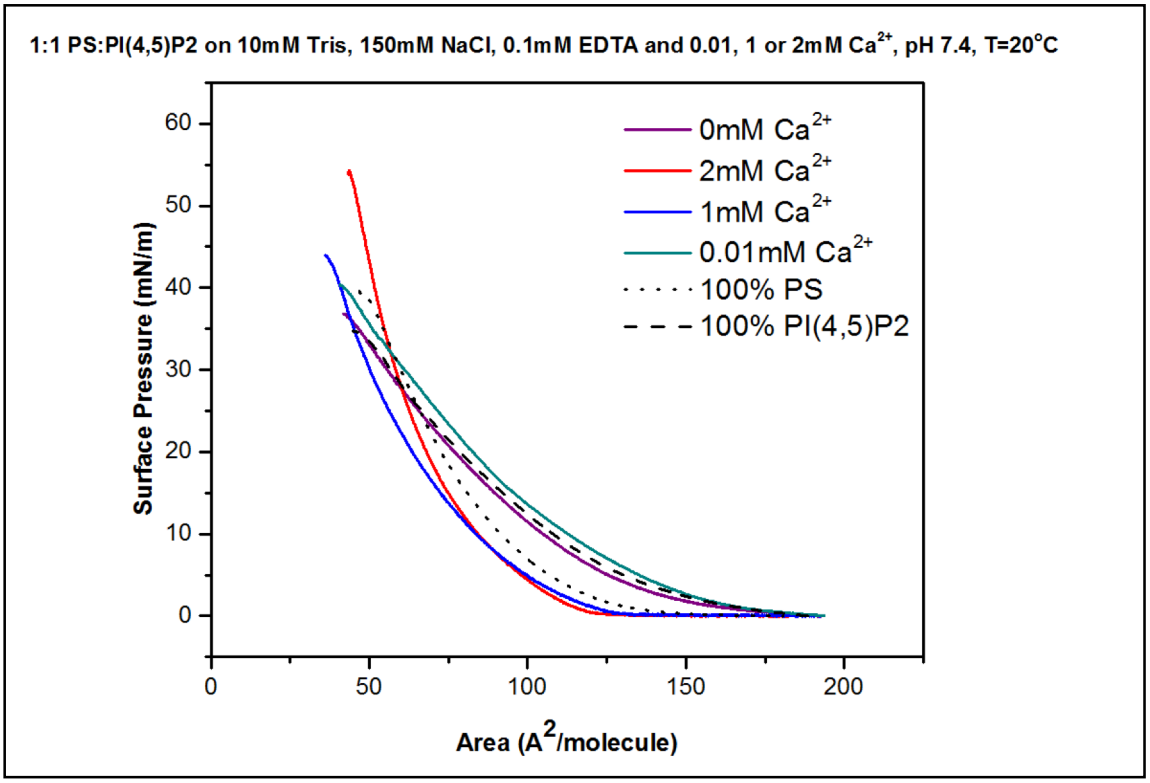

Figure 19: Surface Pressure/Area Isotherms of 1:1 PS:PI(4,5)P_2 Monolayer in the Presence and Absence of Calcium. Subphase compositions of 0mM $CaCl_2$ (0.1mM EDTA) (purple), 0.01mM Ca^{2+} (green), 1mM Ca^{2+} (blue), or 2mM Ca^{2+} (red), 10mM Tris, 150mM NaCl, pH 7.4 at T=20˚C. Isotherms of PS (dots) and PI(4,5)P_2 (dashes) are shown for comparison.

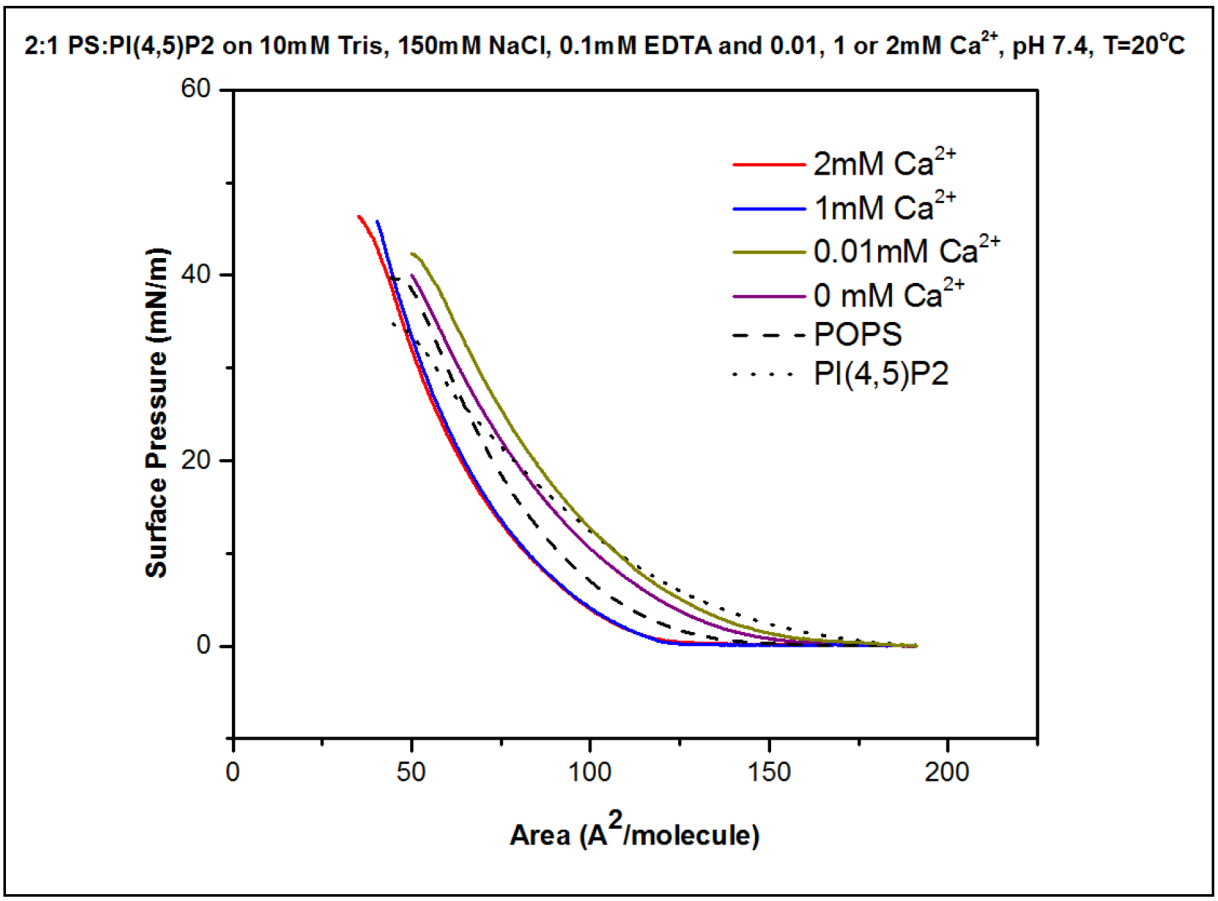

Figure 20: Surface Pressure/Area Isotherms of 2:1 PS:PI(4,5)P_2 Monolayer in the Presence and Absence of Calcium. Subphase compositions of 0mM $CaCl_2$ (0.1mM EDTA) (purple), 0.01mM Ca^{2+} (green), 1mM Ca^{2+} (blue), or 2mM Ca^{2+} (red), 10mM Tris, 150mM NaCl, pH 7.4 at T=20˚C. Isotherms of PS (dots) and PI(4,5)P_2 (dashes) are shown for comparison.

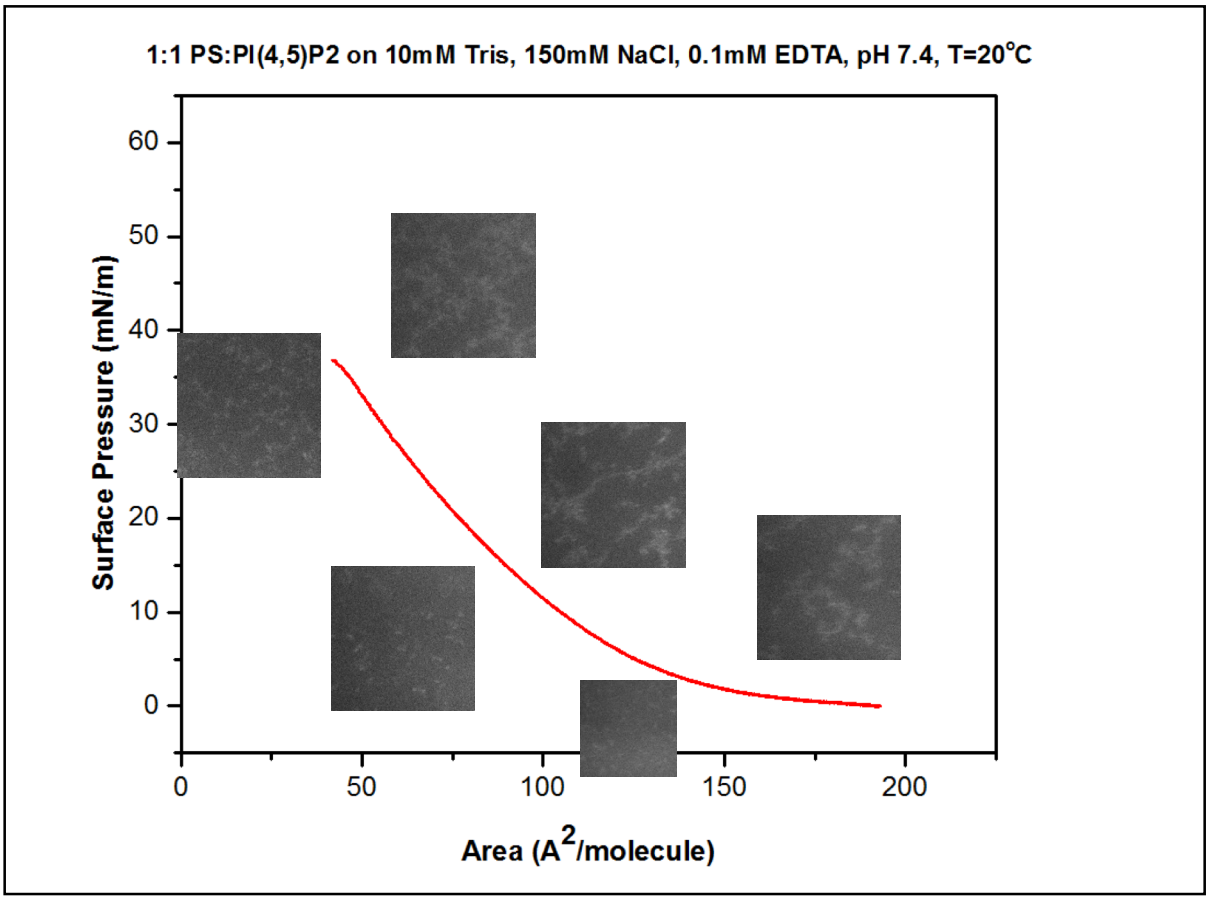

Figure 21: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of 1:1 PS:PI(4,5)P_2 Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® PS (images on left of isotherm) and 0.1mol% Bodipy® PI(4,5)P_2 (images on right of isotherm).

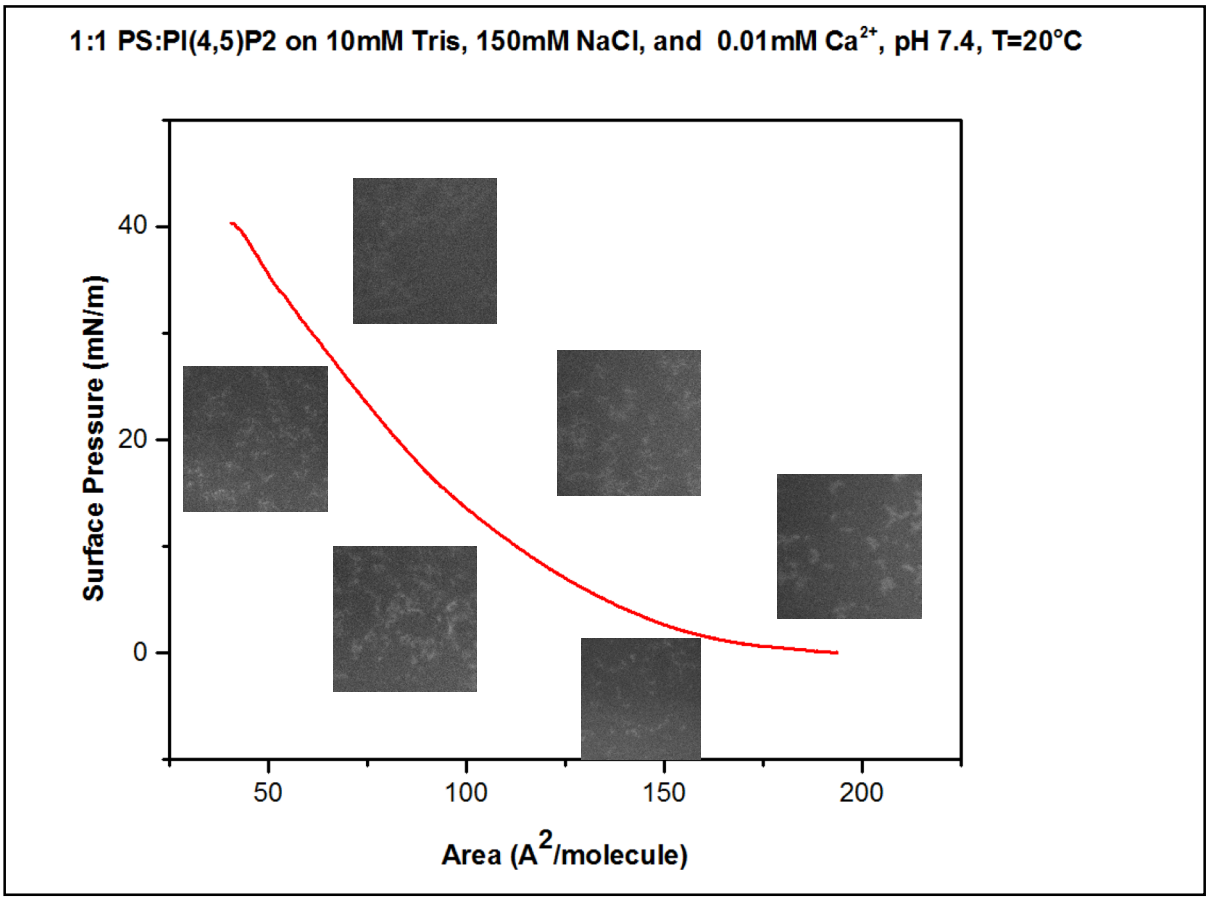

Figure 22: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of 1:1 PS:PI(4,5)P_2 Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 0.01mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® PS (images on left of isotherm) and 0.1mol% Bodipy® PI(4,5)P_2 (images on right of isotherm).

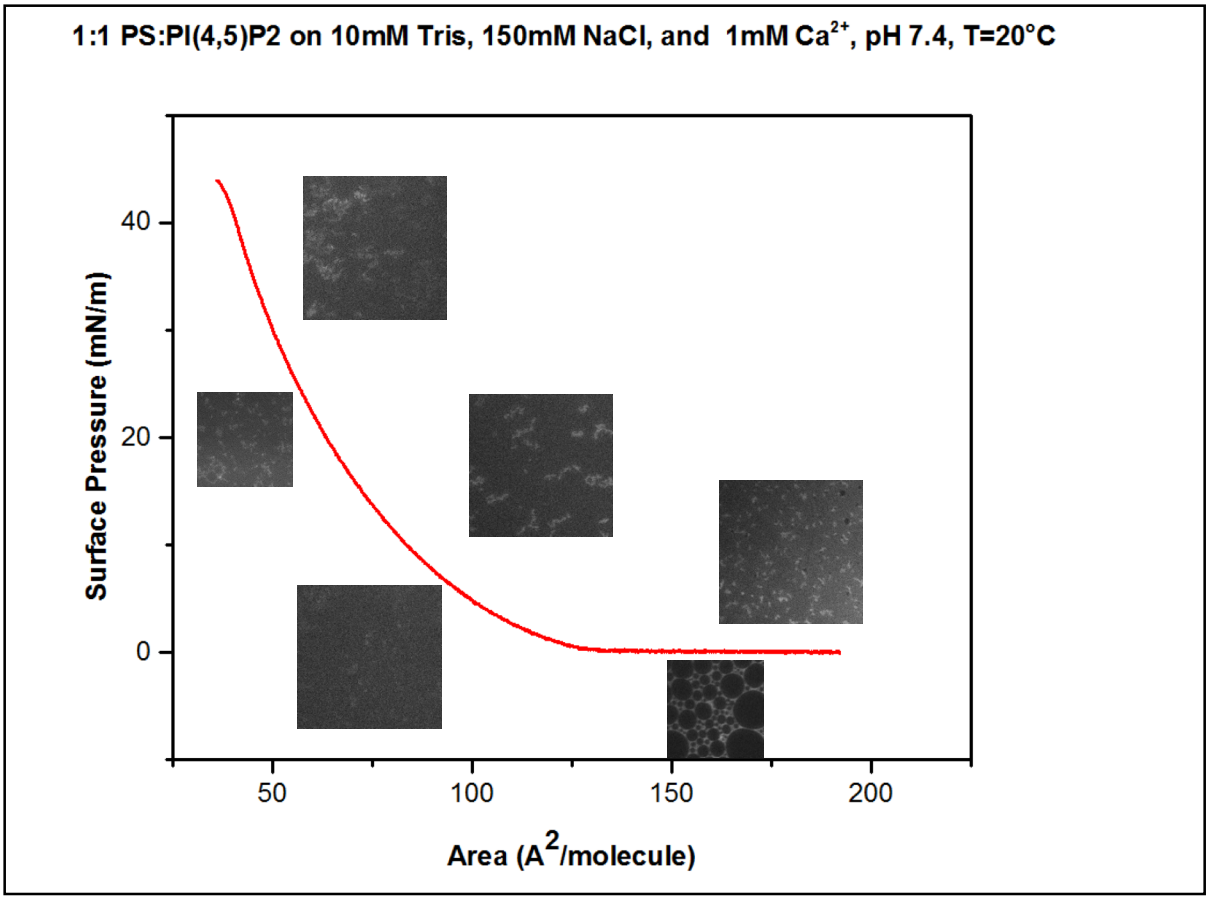

Figure 23: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of 1:1 PS:PI(4,5)P_2 Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 1mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® PS (images on left of isotherm) and 0.1mol% Bodipy® PI(4,5)P_2 (images on right of isotherm).

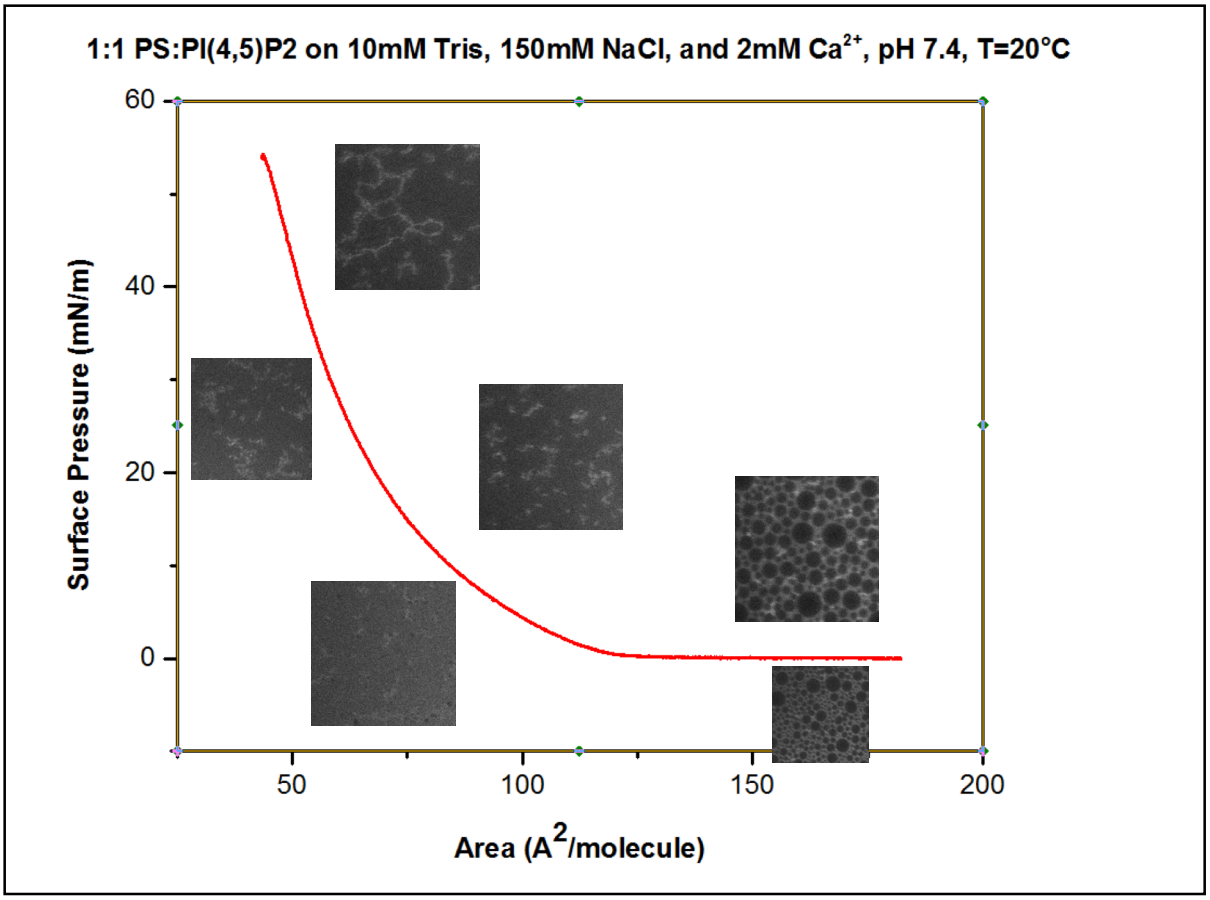

Figure 24: Surface Pressure/Area Isotherm and Corresponding Epifluorescence Images of 1:1 PS:PI(4,5)P_2 Monolayer. Subphase consisted of 10mM Tris, 150mM NaCl, and 2mM Ca^{2+} at pH 7.4 and T= 20℃. Epifluorescence images shown were recorded at various pressures using 0.1mol% Bodipy® PS (images on left of isotherm) and 0.1mol% Bodipy® PI(4,5)P_2 (images on right of isotherm).

4.0 CONCLUSION AND FUTURE STUDIES

In this project, the effect of calcium on PS and PI(4,5)P_2 monolayers was studied at the air/water interphase under conditions mimicking those of the plasma membrane. This study is important for our understanding of the roles of these lipids assume at the inner leaflet of the plasma membrane.

For PI(4,5)P_2, in the presence of any calcium concentration, the bivalent cation appears to stabilize the monolayer and induce the formation of domains at low pressures suggesting that calcium can shield and bridge the interaction between the headgroups. For PS, this is only observed at a high calcium concentration, because low calcium concentrations lead to an expansion of the monolayer and no domain formation, most likely due to the low calcium concentration not allowing for bridging. This can be attributed to a competition between insertion of the cation between the headgroups (resulting in monolayer expansion) and the bridging of the headgroups (resulting in condensation). At low Ca^{2+} concentrations, the bridging of the headgroup is apparently not pronounced enough to overcome the expansion effect due insertion of the cation between the headgroups.

Then, lipid mixtures of 1:1 and 2:1 PS: PI(4,5)P_2 were studied in the presence and absence of calcium. In both mixtures, at low calcium concentrations, the monolayer slightly expanded while at high calcium concentrations, the monolayer condensed, which shows the bridging effect of calcium. Apparently also for these mixed lipid systems, the argument for monolayer condensing and expanding effects holds. In addition, domain formation was only observed at high concentrations. Finally, for the 2:1 mixture, the surface pressure/ area isotherm of the subphases containing 2mM Ca^{2+} and 1mM Ca^{2+} follow the exact same path with the 2mM Ca^{2+} having a slightly higher collapse pressure, which shows that the lipid mixture at this ratio does not stabilize any further with higher calcium concentrations, which could be due to the headgroups of the two lipids interacting with each other and not with calcium.

At the onset of these studies we hypothesized that PS and PI(4,5)P_2 exhibit repulsive interaction in the absence of Ca^{2+}, while the addition of Ca^{2+} leads to PS/PI(4,5)P_2 headgroup bridging. In comparison to 100% PS or PI(4,5)P_2 monolayer, respectively, we found that surface pressure/area isotherm of the 1:1 mixture of the two lipids has a more expanded character than the pure films. This suggests that the interaction between the two lipids is indeed characterized by unfavorable interaction, either due to steric packing problems or electrostatic repulsion. It

appears to be evident that the two types of lipids do not form a hydrogen bond network as it was found for $PI(4,5)P_2$ alone. In the presence of Ca^{2+} the isotherms are clearly condensed relative to the pure monolayers on Ca^{2+} free subphases. This can be explained either through the formation of PS/$PI(4,5)P_2$ bridging by Ca^{2+} or demixing of Ca^{2+} stabilized $PI(4,5)P_2$ and PS phases. The fluorescence images do not support the latter explanation (i.e., no evidence of demixing), suggesting that Ca^{2+} may bridge PS/$PI(4,5)P_2$. However, this needs further investigation.

For future studies, the complexity of the monolayer could be expanded even further. Cholresterol for example, which has been found to stabilize the monolayer even more and leads to domain formation (14), could be added to any of these mixtures.

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APPENDIX

Epifluorescent Images of Lipids and Lipid Mixtures with Different Fluorophores in Subphase Consisting of 10mM Tris, 150mM NaCl, and 0.1mM EDTA at pH 7.4 and T= 20℃ at Pressures of 0, 1-5, >15 and >25mN.