Multiscale Simulations of Phosphatidylinositol Bisphosphate: Understanding Its Biological Role Through Physical Chemistry

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Multiscale Simulations of Phosphatidylinositol Bisphosphate: Understanding Its Biological Role Through Physical Chemistry

Abstract
Proper functionality of biological membranes depends on the regulation of lipid composition and localization. Spatial localization of molecules within the lipid bilayer depends on both steric effects due to their acyl chains and attractive or repulsive interactions between lipid head groups, such as those mediated by the electrostatic charge of the lipid. Most eukaryotic lipids are zwitterionic or have a charge of -1 at physiological pH, but some lipids such as phosphatidylinositol bisphosphate (PtdInsP2) bear a net charge of -4. The ability of these highly charged lipids to interact with monovalent and divalent cations affects their spatial organization and temporal distribution on the cytoplasmic side of membranes. In turn, these lipids act as important effectors of apoptosis, inflammation, motility, and proliferation through their interactions with proteins at the membrane interface and transmembrane ion channels. We hypothesize that in some settings, the isomers of PtdInsP2 - PtdIns(3,5)P2 and PtdIns(4,5)P2 - act by changing the physical-chemical properties of the membrane rather than by specific biochemical binding to proteins. We also predict that PtdIns(3,5)P2 and PtdIns(4,5)P2 alter the mechanical properties in distinct ways due to the larger size and altered charge distribution in the head group of PtdIns(3,5)P2. We used multiscale computational techniques, ranging from quantum-level electronic structure calculations to all-atom molecular dynamics simulations of bilayers to characterize the biological role of PtdInsP2. Our results demonstrate that the different roles of PtdIns(4,5)P2 and PtdIns(3,5)P2 in vivo are not simply determined by their localization, but also due to intrinsic factors different between them such as molecular size, propensity to bind cellular divalent cations, and the partial dehydration of those ions which may affect the ability of PtdIns(3,5)P2 and PtdIns(4,5)P2 to form phosphoinositide-rich clusters in vitro and in vivo.

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MULTISCALE SIMULATIONS OF PHOSPHATIDYLINOSITOL BISPHOSPHATE:
UNDERSTANDING ITS BIOLOGICAL ROLE THROUGH PHYSICAL CHEMISTRY

David R. Slochower

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I am grateful to many individuals for their support and encouragement during my time as a graduate student. My advisor, Paul Janmey, provided the perfect balance between instruction and freedom. He allowed me to adopt a project and drive it in a totally new direction. Although unfamiliar with the methodology, he was always willing to discuss any detail and minute concern with me. As my work grew beyond Paul’s expertise, Ravi Radhakrishnan became like a second advisor, and mentor, to me. Ravi helped me implement the latest computational techniques and provided me with the resources to run countless simulations.

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Proper functionality of biological membranes depends on the regulation of lipid composition and localization. Spatial localization of molecules within the lipid bilayer depends on both steric effects due to their acyl chains and attractive or repulsive interactions between lipid head groups, such as those mediated by the electrostatic charge of the lipid. Most eukaryotic lipids are zwitterionic or have a charge of -1 at physiological pH, but some lipids such as phosphatidylinositol bisphosphate (PtdInsP_2) bear a net charge of -4. The ability of these highly charged lipids to interact with monovalent and divalent cations affects their spatial organization and temporal distribution on the cytoplasmic side of membranes. In turn, these lipids act as important effectors of apoptosis, inflammation, motility, and proliferation through their interactions with proteins at the membrane interface and transmembrane ion channels. We hypothesize that in some settings, the isomers of PtdInsP_2 – PtdIns(3,5)_2 and PtdIns(4,5)_2 – act by changing the physical-chemical properties of the membrane rather than by specific biochemical binding to proteins. We also predict that PtdIns(3,5)_2 and PtdIns(4,5)_2 alter the mechanical properties in distinct ways due to the larger size and altered charge distribution in the head group of PtdIns(3,5)_2. We used multiscale computational techniques, ranging from quantum-level electronic structure calculations to all-atom molecular dynamics simulations of bilayers to characterize the biological role of PtdInsP_2. Our results demonstrate that the different roles of PtdIns(4,5)_2 and PtdIns(3,5)_2 in vivo are not simply determined by their localization, but also due to intrinsic factors different between them such as molecular size, propensity to bind cellular divalent cations, and the partial dehydration of those ions which may affect the ability of PtdIns(3,5)_2 and PtdIns(4,5)_2 to form phosphoinositide-rich clusters in vitro and in vivo.
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In 1772, Benjamin Franklin and John Pringle noted that adding just a teaspoon of olive oil, which contains a blend of saturated and unsaturated fatty acids, to the water surface could calm waves in a pond. They observed the olive oil spread out until a quarter of the pond was covered and became “as smooth as a looking glass” (Franklin et al., 1774). In the 1890s, Lord Rayleigh, who was working to repeat Franklin’s experiment and perform calculations to determine the thickness of an oil layer on water, received a letter from Agnes Pockels. While living as caretaker for her parents and washing their dishes, Pockels carefully documented the way soap is able to form bubbles in water and devised a way to measure surface tension through “increasing or diminishing the surface of a liquid in any proportion, by which its purity may be altered at pleasure” (Rayleigh, 1891).

Shortly after that in 1902, Overton discovered that the plasma membrane has a “fatty nature”, after the observation that lipid soluble compounds are able to enter cells quicker than substances that are not soluble in lipids (Benson, 1964). A group of molecules isolated from human brain cells and characterized by their solubility in ether but not in ethyl alcohol, were separated into two fractions in 1946 and assigned the names phosphatidylserine and phosphatidylethanolamine (Folch, 1945). A small amount of a third constituent, containing the six member sugar ring inositol, was also found and called diphosphoinositide (Folch, 1949). By the 1960s, the structure of diphosphoinositide and the ratio of other phosphorylated inositol derivatives (polyphosphoinositides; PPIs) were determined,
and the phosphorylated inositol rings were shown to be attached to glycerol and fatty acid chains (Dawson and Dittmer, 1961; Dittmer and Dawson, 1961). Unlike other phospholipids isolated from animal cells, PPIs were more unstable; they rapidly degraded sitting on laboratory benches and their stability depended on the buffers used during extraction (Akhtar and Abdel-Latif, 1978; Grove et al., 1981; Best et al., 1982). PPIs extracted from guinea pig brain with solvents that contained potassium were colored and left an insoluble residue that remained after drying whereas the calcium salt fractions did not (Michell et al., 1970).

PPIs are among the most highly charged molecules in the cell, carrying a net charge that can range from -3 to perhaps as much as -5 for one such molecule, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) at biologically relevant pH, and the physical chemistry of PPIs has been of interest since their discovery. The negative charge of PPIs originates by the deprotonation of phosphomonoester and phosphodiester groups on the lipid that have dissociation constants in a physiological range. This makes PPIs very sensitive to cationic counterions present in biological contexts, such as the monovalent ions Na⁺ or K⁺ and the divalent ions Ca²⁺, Mg²⁺, and Zn²⁺.

Even the enzymes that act on PPIs, themselves carrying clusters of charged amino acids necessary to coordinate the negatively charged phosphates on PPIs, are very sensitive to counterions. The enzymes that remove a phosphate group from inositol 1,4-bisphosphate and inositol 4-phosphate are sensitive to the monovalent cation of lithium, Li⁺. It is hypothesized that Li⁺ reduces the supply of inositol, a key substrate for downstream cell signaling events, which is responsible for the ability of Li⁺ to treat manic-depressive episodes and stabilize mood when administered medically (Berridge and Irvine, 1984).

In addition to the unique physical chemistry of PPIs, the vital biological role of one PPI in particular – PtdIns(4,5)P₂ the major PPI in mammalian cells that accounts for approximately 1% of the total phospholipid content – began to become clear through a series of elegant experiments in the 1980s and 1990s. The binding or detection of PPIs by proteins has been implicated in myriad cell processes: attachment of the cytoskeleton to the membrane, cell adhesion, cell motility and
proliferation, exocytosis, endocytosis, membrane fusion, membrane trafficking, regulation of ion channels, and both activation and inhibition of enzymes (reviewed in (Janmey et al., 1987; Janmey and Lind, 1987; Janmey and Stossel, 1987; Katso et al., 2001; McLaughlin et al., 2002; Di Paolo and De Camilli, 2006)). PPIs are found in multiple membrane compartments throughout the cell, with specific isoforms more or less populous in specific membranes; although the distribution changes upon stimulation, in general PtdIns(4,5)P2 is enriched in the plasma membrane, PtdIns(4)P in the Golgi complex; PtdIns(3) in early endosomes; PtdIns(3,5)P2 in late endosomes, and PtdIns in the endoplasmic reticulum (Di Paolo and De Camilli, 2006; Hilgemann, 2007). The relative levels of these isoforms varies from 0.0001% of the total phospholipid content in a cell for PtdIns(3,5)P2 to perhaps 1-1.5% of the total phospholipid content in the cell for PtdIns (Lemmon, 2008); 1% of the total phospholipid in the cell suggests a concentration of roughly 10 µM (McLaughlin et al., 2002). Although a measurement of the PtdIns(4,5)P2 mass in extracts from 10^9 platelets yielded the equivalent of ~150 µM PtdIns(4,5)P2 per cell. Defects in signaling pathways dependent on PPI recognition have been implicated in melanoma, breast, lung, and ovarian cancers as well as, Charcot-Marie-Tooth disease, amyotrophic lateral sclerosis, and primary lateral sclerosis among others (Faiderbe et al. 1992; Lin et al., 1999; Katso et al., 2001; Engelman, 2007; Miled et al., 2007; Bunney and Katan, 2010; Sun et al., 2010).

1.1 The many roles of PtdInsP2 in the cell

Cells devote up to 5% of their genome to synthesis of the lipids that are the primary ingredients of biological membranes (van Meer et al., 2008). When aggregated in droplets, lipids serve as energy reservoirs (often in the form of triacylglycerol or TAG) that can be catabolized to generate energy for the cell in the form of ATP. These droplets are also stores of fatty acids and sterols that can be used to generate other membrane components such as cholesterol. Degradation of lipids in the membrane by specialized proteins serves as a starting point for signaling cascades; when a lipid
is cleaved in half, information extends across the membrane via the diffusion of hydrophobic lipid fragments and propagates into the cytosol via the diffusion of the polar lipid fragment. Modification of phospholipids that alter their charge, such as by phosphorylation of the head group, can initiate or prevent a cellular signaling cascade, and in one case, the so-called “futile cycle” of repeated phosphorylation and dephosphorylation of phosphoinositides may consume up to 7% of a cell’s energy in the form of ATP turnover \cite{Verhoeven1987}. It is very interesting that the synthesis of many charged phospholipids, in at least some prokaryotes and all eukaryotes, involves the cleavage of CDP to CMP giving rise to the phosphodiester linkage between the head group and acyl chains, using cytosine as the nucleobase instead of adenosine \cite{Cronan2003}.

![Phospholipids](image)

Figure 1.1: Common phospholipids in the mammalian plasma membrane. In this thesis, I abbreviate phosphatidylcholine as PtdCho, phosphatidylethanolamine as PtdEtn, phosphatidylserine as PtdSer, phosphatidylglycerol as PtdGly, and phosphatidic acid as PtdA.

Hydrolytic cleavage of the head group of PtdIns(4,5)P$_2$ on the inner leaflet of the plasma membrane by phospholipase C (PLC), under hormonal and neurotransmitter control in addition to being regulated by subcellular processes, via activation of cell surface receptors, releases two so-called second messengers: D-myo-inositol 1,4,5-trisphosphate (InsP$_3$; the phosphate on the 1 position originates from the phosphodiester linkage between the head group of PtdIns(4,5)P$_2$ and the glycerol moiety; D-myo indicates the chair conformation of the ring such that the second hydroxyl group is axial and the others are equatorial) and diacyl glycerol (DAG). Hydrophobic DAG remains in
the plasma membrane to diffuse among phospholipids and is an activator of protein kinase C (PKC) whereas soluble InsP₃ diffuses into the cytosol, binds to the InsP₃ receptor on the endoplasmic reticulum, and releases internal stores of Ca²⁺ (Berridge and Irvine, 1989). PPIs are only found in eukaryotic cells, although *E. coli* have a signaling cascade involving DAG and another anionic lipid, PtdGly, analogous to the signaling of PPIs in eukaryotic cells (Cronan, 2003). Some bacteria contain phosphatidylinositol without any phosphorylation on the head group (such as *Mycoplasma*, where it is essential for survival) as is the case of phosphatidylinositol in yeast, as well (Gardocki et al., 2005).

![Figure 1.2: The structure of PtdIns(4,5)P₂ drawn in various representations. a. The two-dimensional chemical structure of PtdIns(4,5)P₂ marking the physiological negative charges in red. b. A three-dimensional stick drawing (hydrogen: white, carbon: green, oxygen: red, phosphorus: gold). c. A space-filling model of panel b. d. The solvent accessible surface area of panels b and c. e. A space filling model highlighting the two common acyl chains (R₁ and R₂ in panel a) illustrating the straight, saturated stearate and bent, unsaturated, arachidonate.](image)

Among phospholipids, the role of PtdInsP₂ isoforms as regulatory molecules is unrivaled. The cellular roles of PtdInsP₂ isomers are not limited to passive substrates for kinases and phosphatases that reside in the interfacial region of the plasma membrane. Rather, hundreds of cytoplasmic proteins have been shown to bind PtdIns(4,5)P₂ in vitro and numerous cellular processes are controlled the level and location of PtdInsP₂ isoforms in the cell (Gamper and Shapiro, 2007). Many kinds of proteins bind to the plasma membrane but only some of these specifically interact with
At least ten different classes of proteins interact with PtdInsP₂ in the plasma membrane (Lemmon, 2007, 2008; Moravcevic et al., 2010). One consequence of localizing proteins to the membrane interface is to increase the apparent concentration of potential reactants. These reactants can either be lipids within the membrane or other proteins bound to it. For example, by confining a protein such as phospholipase C to diffuse along a two-dimensional surface instead of throughout the entire cell, the apparent concentration of its substrate PtdIns(4,5)P₂ relative to the protein is nearly 1000 fold higher than if the protein were unconstrained (McLaughlin et al., 2002). Similar effects can also alter the reaction kinetics of PPI-binding protein kinases and their ligands when both bind to the membrane.

C1 domains, identified in the peripheral membrane associated kinase PKC, bind to DAG. By analyzing the sequence of plekstrin, another substrate of PKC, Plekstrin Homology (PH) domains were identified as a region of approximately 100 amino acids found in at least 300 other globular proteins in humans (Lemmon and Ferguson, 2000). Certain PH domains, such as the N-terminal region of phospholipase C1 (PLC), are potent PPI binders with specificity for PtdIns(4,5)P₂ and InsP₃, although most PH domains show weak specificity for PPIs and only 10% bind strongly with specificity and sensitivity to PPIs (McLaughlin et al., 2002). Many PPI binding domains are evolutionarily conserved across eukaryotes, from humans to yeast (Lemmon, 2008). When the PLCδ domain binds to PtdIns(4,5)P₂ it anchors the protein to the membrane (McLaughlin et al., 2002). In fact, InsP₃ can displace PtdIns(4,5)P₂ from the PLCδ binding pocket, with the effect that cleavage of PtdIns(4,5)P₂ into DAG and InsP₃ leads to dissociation of PLC from the plasma membrane. Other domains, reviewed in (Lemmon, 2008), bind to PPI isoforms with varying levels of sensitivity and specificity. Some temporal and spatial specificity in binding arises through the necessity of a second message, such as Ca²⁺, to be present as a cofactor, perhaps helping to neutralize the negative charge of the inner leaflet of the plasma membrane (McLaughlin et al., 2002; Lemmon, 2008).

The unstructured basic region of another PKC substrate, the myristoylated alanine-rich C
kinase substrate (MARCKS) protein, containing 13 basic residues (the basic region is usually 25 total residues: 13 basic and 5 hydrophobic), binds to PtdIns$_{P_2}$ containing membranes with high affinity, but does not discriminate between PtdIns(3,5)$_{P_2}$ and PtdIns(4,5)$_{P_2}$ and its attraction is probably mediated by nonspecific electrostatic effects (McLaughlin et al., 2002). MARCKS has been found concentrated in the ruffles of fibroblasts and has been implicated in exocytosis, membrane transport, cell secretion, and many other activities that take place at the plasma membrane interface (Blackshear, 1993). As MARCKS is unstructured and highly positively charged, it is believed to bind as many as three PtdIns$_{P_2}$ molecules at a time; other small positively charged peptides, such as poly-lysine, with charges less than +7 do not bind selectively to PtdIns$_{P_2}$-containing membranes because their linear charge density is not compatible with the coordination of multiple PtdIns$_{P_2}$ molecules at the same time (McLaughlin et al., 2002).

Yet another class of peptides that bind to PtdIns(4,5)$_{P_2}$ is epsin N-terminal homology (ENTH) domains. Unlike MARCKS, a well-defined PtdIns(4,5)$_{P_2}$ binding pocket has been identified in ENTH domains and upon binding to PtdIns(4,5)$_{P_2}$ ENTH domains insert an $\alpha$-helix in contact with PtdIns(4,5)$_{P_2}$ into the membrane. This insertion, coupled with binding to PtdIns(4,5)$_{P_2}$ causes a deformation in the membrane and may contribute the generation of curvature at the sites of endocytosis. Coarse-grained simulations have shown the oligomerization of ENTH domains on a membrane bilayer alters the curvature of the membrane, leading to a tubular geometry (Lai et al., 2012).

### 1.2 Lipid heterogeneity in cells

Early characterization of membrane fractions isolated from cells revealed a population of detergent insoluble glycolipid (also called detergent resistant membranes) (Simons and Ikonen, 1997; Simons and Toomre, 2000; Simons and Vaz, 2004). These findings formed the basis of the “lipid raft” hypothesis which posits that the plasma membrane is heterogeneous and some membrane
components exist segregated in a separate phase (or domain).

Recent studies from 2011 show the formation of nanoscale PtdIns(4,5)P₂ clusters during critical PtdIns(4,5)P₂-dependent vesicle recruitment to the plasma membrane and PtdIns(4,5)P₂-dependent exocytosis [van den Bogaart et al., 2011; Honigmann et al., 2013]. Clustering of the SNAP receptor protein syntaxin-1A on the plasma membrane [Sieber et al., 2007], which enables neuronal exocytosis, requires formation of lipid domains. Stimulated emission depletion (STED) microscopy revealed that PtdIns(4,5)P₂ in the plasma membrane of a PC12 cell forms nanoclusters with a relatively narrow size distribution centered at 73 nm in diameter and a peak surface coverage of 80% PtdIns(4,5)P₂ in these clusters. Similar size distributions of PtdIns(4,5)P₂ nanoclusters in a plasma membrane have also been reported using the same cell line but different imaging techniques (Wang et al., 2012a).

The fact that only the inner leaflet of eukaryotic cells usually contains the acidic phospholipids under physiological conditions results in a significant negative surface charge density. Mobile counterions in the cytoplasm are drawn to the membrane and interact with the negative lipids, sometimes forming a bond, reducing the overall electrostatic potential [McLaughlin, 1989]. Non-specific, electrostatically driven adsorption of counterions to the charged lipid surface (forming the Stern layer) in some cases in combination with specific binding of multicationic solutes to unique anionic phospholipid species, can lead to significant changes in membrane structure including changes in membrane curvature and surface patterning. The concentration of Ca²⁺ in the Stern layer near the membrane can be more than ten times the bulk concentration in the cytosol [McLaughlin et al., 1981], and there is likely competition between Ca²⁺ and other physiological divalent cations, such as Mg²⁺ [Wang et al., 2012b]. The interplay among ions may result in competition for specific binding to anionic phospholipids that carry a valence of more than -2 or simple electrostatic attraction, usually to anionic phospholipids with a valence of -1. However, important differences remain between the effects of Ca²⁺ and Mg²⁺ and the concentration of counterions near a highly charged anionic membrane depends on both the valence and hydrated diameter of the cation (Shapovalov and
The clusters of PtdIns(4,5)P₂ that are found in cells closely resemble the structures formed by PtdIns(4,5)P₂ in vitro after the addition of Ca²⁺ to mixed lipid monolayers (van den Bogaart et al., 2011). An outstanding question is whether PtdIns(4,5)P₂ clusters play an important role in the cell, our approach is to understand what conditions promote cluster formation and how the behavior of PPIs in clusters is different from free single molecules. It has been suggested that the lateral organization of PPIs is responsible for catalyzing specific downstream biochemical effects. However, the causality of cluster formation in the cell is still unclear; it is not known whether effector domains recognize and bind to regions of increased concentration of PPIs or if effector domains sequester and enhance the local concentration of PPIs. In vitro experimental evidence accumulated over the past five years has elucidated the lateral organization and cluster-forming properties of PtdIns(4,5)P₂ in the presence of physiological counterions and proteins such as gelsolin (Wang, 2013).

1.3 The characterization of PtdInsP₂ in vitro

PPIs are not only among the most highly charged molecules in the cell, they have an extremely large charge density; the phosphomonoester groups on the inositol ring of PtdIns(4,5)P₂, each carrying a charge between -1 and -2, are separated by just a couple of Ångstroms. The pH-dependent charge of PtdInsP₂ can be determined at least four ways: by measuring the electrophoretic mobility of vesicles containing PtdInsP₂ (Ohki, Müller et al. 2010), from pH-dependent shifts in ³¹P-NMR peaks of multilamellar stacks containing PtdInsP₂ (Kooijman et al., 2009), from a theoretical model of the electrostatic contribution to the surface pressure of monolayers containing PtdInsP₂ (Levental et al., 2008b), or from an ab initio electronic structure calculation (Slochower et al., 2013). Although these methods were generally performed in different buffering conditions, taken together, these data indicate the charge of PtdIns(4,5)P₂ is likely in the range of -3 to -4 at physiological pH and in the presence of 100 mM monovalent counterions. Moreover, the charge per PtdInsP₂ phosphate group
can be calculated using site-resolved pKa values obtained either from \(^{31}\)P-NMR experiments or from the theoretical modeling.

One striking result of the theoretical modeling of Levental, et al. concerning the electrostatic contribution to the surface pressure of monolayers containing PtdIns(4,5)\(P_2\) is that the area per phospholipid increases when the ionic strength of the fluid in contact with the monolayer is increased from 10 mM to 250 mM upon addition of monovalent salt [Levental et al., 2008a]. The protonation state of PtdIns(4,5)\(P_2\) determined using quantum mechanics/molecular mechanics simulations appears to follow the same trend as phosphatidic acid, whose phosphate group becomes doubly protonated at physiological pH in the presence of divalent or trivalent ions, particularly Ca\(^{2+}\) due to electrostatic correlations [Wang et al., 2012a; Slochower et al., 2013].

In the absence of divalent cations, it has been proposed that the lateral distribution of PtdIns\(P_2\) is a balance of electrostatic repulsion and hydrogen bond networking among adjacent PtdIns\(P_2\) head groups (at the exclusion of water molecules) [Levental et al., 2008a]. The hydrogen bond network between serves to reduce the average area per molecule by counteracting the electrostatic repulsion between highly charged phosphomonoester groups.

![Figure 1.3: A model of how ion binding to monolayers containing PtdIns(4,5)\(P_2\) might lead to clustering for some ions but not others.](image-url)
1.4 Early simulations of PtdInsP$_2$

Theoretical modeling of membrane systems and molecular dynamics (MD) simulations are able to provide a multiscale view of the physical chemistry of PPIs that is inaccessible to almost all experimental modalities. The properties that we know are essential for the biologic function of PPIs – the net charge of PPIs, their interaction with divalent cations, hydrogen bonding with water and other lipids – can be interrogated across several orders of magnitude in time and space using a combination of quantum-level and all-atom MD simulations as well as predictions from analytical modeling. This thesis benefits from the unique ability to directly compare the computational results with experiments done by other members of our group on precisely matched systems. First, it is necessary to discuss the theoretical and computational underpinnings of our results, beginning with a discussion of charged membranes and progressing to the results of early MD simulations of PtdInsP$_2$, using a variety of methods, now considered outdated, to model PtdInsP$_2$.

The average charge density of the inner leaflet of a membrane bilayer composed of 20% negatively charged monovalent lipids is about 1 electronic charge per 300 Å$^2$ to 1000 Å$^2$ (a single phospholipid may occupy anywhere from 35 Å$^2$ to 100 Å$^2$) [McLaughlin, 1977]. In clusters of PtdInsP$_2$ molecules, the charge density could be an order of magnitude higher. The negative surface charge density produces a negative electrostatic potential that extends immediately adjacent to the membrane, into the cytosol (Figure 1.4). Mobile counterions in the cytosol are attracted to the negative electrostatic potential and reduce the surface charge density to approximately -30 to -60 mV (relative to bulk, where the electrostatic potential is fixed to be zero) [McLaughlin, 1977], given that the counterions are monovalent and have an average concentration of 100 mM. The concentration of counterions at the surface of the membrane may be an order of magnitude greater than in the bulk phase (similarly, the concentration of coions may be an order of magnitude lower than bulk), and therefore, the local pH at the membrane interface may be 1 pH unit lower than in bulk [McLaughlin, 1977; Honig et al., 1986]. The distribution of ions away from the membrane can
be predicted based on a number of theories. One popular theory, the Debye-Hückel expression for the electrostatic potential as a function of the distance from the membrane surface, falls off proportional to the exponential Debye length (which takes into account the permittivity, valence, and concentration of the counterions in the bulk phase); this is essentially a version of Coulomb’s law which includes an additional screening term due to the ionic strength of the aqueous phase. Regardless of which approximation is included, all of the theories must effectively balance the attraction due to electrostatics and randomly oriented diffusion, which will smear out the counterion cloud.

Figure 1.4: The negative charge density in a membrane creates an electrostatic potential that extends perpendicular to the membrane. If the negative charge is uniformly distributed, the equipotential lines above the membrane surface will be relatively flat. If the negative charge is not distributed evenly, the equipotential lines will follow the regions of negative charge close to the membrane, but will be generally flat by ∼6 Å for a membrane containing $-1 \ e/68 \, \text{Å}^2$, packed hexagonally. Figure adapted from [Peitzsch et al., 1995], Figure 5A, copyright Elsevier with permission.

The first MD simulations of PtdIns$P_2$ in 2003 investigated how a small number of PtdIns$P_2$ molecules added to one leaflet of an equilibrated bilayer of DMPtdCho altered the structure of this membrane and explored the mechanism by which a PtdIns$P_2$-binding domain of the protein gelsolin might interact with phospholipid membranes [Liepina et al., 2003]. After 3 ns of simulation, a small cluster of 4 PtdIns(4,5)$P_2$ molecules (carrying a charge of -5 per PtdIns(4,5)$P_2$) caused fluctuations in the thickness of the membrane, and the phosphate groups on the inositol ring of PtdIns(4,5)$P_2$ became fully exposed to the solvent environment. The addition of the gelsolin-derived peptide to the
membrane system was able to disrupt the bilayer through the formation of hydrophobic interactions between nonpolar side chains of the gelsolin peptide and the hydrocarbon tails of PtdIns(4,5)P₂. It is unclear whether proteins that localize to the plasma membrane interface in cells, but do not penetrate the membrane by inserting structural elements (such as amphipathic or transmembrane helices), are able to form close contacts with the hydrocarbon tails of phospholipids. However, some proteins have been shown to bind to membrane phospholipids only in either a liquid ordered or disordered state, implying that the fluidity of the hydrophobic hydrocarbon acyl chains is an important factor that can regulate protein binding.

These MD simulation parameters (Liepinš et al., 2003) were modified to use united atom carbons for the acyl chains of PtdIns(4,5)P₂ and a hydrogen was attached to the phosphate group on the 5-position of the inositol ring, bringing the total charge of PtdIns(4,5)P₂ equal to -4 instead of -5 (Lorenz et al., 2008). As discussed above, setting the total charge of PtdIns(4,5)P₂ to be -4 agrees with chemical shift data from NMR experiments on PtdIns(4,5)P₂-containing vesicles (Kooijman et al., 2009), which are able to report the pKa of each phosphate group separately, and a theoretical model of the relationship between the pKa of membrane phospholipids and their net charge (Levental et al., 2008b).

Lorenz, et al. (2008) performed simulations of a 13-mer of the positively charged amino acid lysine in the presence of monolayers containing 38 phosphatidylcholine (PtdCho) lipids, 8 phosphatidylserine (PtdSer) lipids, and 3 PtdIns(4,5)P₂ molecules. In a 20 ns simulation, the peptide sequesters 3 PtdIns(4,5)P₂ molecules stabilized by long-lived hydrogen bonds. In a separate system without PtdIns(4,5)P₂, the authors note that sequestration does not occur with PtdSer, which carries a negative charge of -1, due to the short lifetime of the interactions that form between the lipids and the peptide. This result is consistent with experiments involving the tail of a myosin-I isoform (Myo1c), which contains a string of positively charged (basic) residues, and binds weakly to physiological PtdSer concentrations (< 40% of an LUV membrane) yet binds tightly to LUVs containing only 2% PtdIns(4,5)P₂ (Hokanson and Ostap, 2006). Further, kinase associated-1 (KA1) domains, which
posses the same Pleckstrin Homology (PH) domain as Myo1c, bind to immobilized membranes containing 3% PtdIns(4,5)P₂ as well as they bind to membranes containing 20% PtdSer and almost three times as well as that to membranes containing 10% PtdIns(4,5)P₂ (Moravcevic et al., 2010).

Without proper MD parameters, the physiological relevance of simulations is impossible to interpret. The simulations mentioned above either used ad hoc parameters for PtdIns(4,5)P₂ or mixed parameters for PtdIns(4,5)P₂ with poor models of other phospholipids. In 2009, parameters for sugars, including inositol, were developed for the CHARMM all-atom pairwise additive force field (Hatcher et al., 2009). After adopting previously established values for bond and angle constants, dihedral parameters were fit to the free energy surface of quantum mechanical (QM) calculations. The bond, angle, dihedral, and non-bonded parameters were then optimized by matching condensed phase MD simulations to densities and diffusion coefficients from experimental solutions (Klauda et al., 2005). Just as important, the torsion parameters for the acyl chains were revised twice (C27r and then C36) so that the current CHARMM lipid force field, C36, is in excellent agreement with the self-diffusivity and viscosity of heptane (Klauda et al., 2010; Pastor and MacKerell, 2011). The suitability of these parameters for simulating bilayer systems has also been tested extensively for PtdCho with saturated and polyunsaturated chains, matching area per molecule, deuterium order parameters, and X-ray form factors (Klauda et al., 2012).

The work in this thesis begins by performing electronic structure calculations on the quantum level to assess the validity of MD parameters and then adopts the CHARMM C36 force field for all-atom molecular dynamics simulations.

1.5 Other biopolymer polyelectrolyte results

Lipid bilayers are not the only cellular structures that create surfaces of large charge densities that organize the distribution of counterions and coions. I have also looked at linear charged polyelectrolytes which share some behaviors of PtdInsP₂ but will not be discussed further in this
thesis. Other charged biopolymers in the cell are able to react to divalent cations in both general and specific mechanisms. In particular, highly charged filaments, such as DNA and F-actin, form organized structures in the presence of counterions that are not explained by simple electrostatic neutralization. Cytoskeletal polymers are strong ligands for a variety of metal ions, polyamines, and cationic peptides, and the binding of these species can produce a variety of specific structures. Indeed polylsine, spermine, lanthanide ions, and histones are among the most potent actin bundling agents identified (Tang and Janmey [1996]), and it is notable that these polycations are also strong condensing agents for DNA (Bloomfield [1997]).

All cytoskeletal polymers: actin, tubulin, and intermediate filament proteins, are strong polyelectrolytes. Depending slightly on the species, isoform, and type of tightly bound nucleotide, actin monomers carry a nominal charge of -13, tubulin dimers a charge of -53, and a representative intermediate filament protein like vimentin, a charge of -19 (Janmey et al. [2014]). These features do not alone provide any special electrostatic properties, but what makes cytoskeletal proteins different from other cytoplasmic elements is their assembly into linear polymers, and these negatively charged filaments have electrostatic properties fundamentally different from those of individual subunits as recognized decades ago by Oosawa (Oosawa [1971]). Two other biological polymers that have charge densities similar to those of cytoskeletal polymers are the filamentous virus Pf1, which has a length of 2 µm, and a diameter and charge density that are very similar to those of F-actin (Crowther [1980], Zimmermann et al. [1986]) and hyaluronan (HA), the long semi-flexible anionic polysaccharide that forms the pericellular matrix as the well as the transparent gel in the vitreous body of the eye (Haxaire, Braccini et al. 2000), which differs from the other biopolymers considered here by its much smaller persistence length (4 to 8 nm) (see Figure 1.5 for a comparison between cytoskeletal polymers).

Effects of divalent cations on rheology of cytoskeletal polymer networks have generally been attributed to the binding of these ions to specific sites on the natively folded surface of the proteins within the polymers rather than to the same type of generic counterion-dependent effects that govern
the bundling transition. There is evidence for preferential binding of divalent cations to sites formed at the junctions between actin subunits in a filament (Kang et al., 2012) that can promote actin polymerization, and for structures protruding from the surface of neurofilaments (Gou et al., 1998) or vimentin (Lin et al., 2010) that might bind preferentially to divalent cations and therefore act as a specific crosslinker between filaments. However, the generic effect of counterion condensation on the filaments is difficult to differentiate, especially when the concentration of divalent cations required to increase the elastic moduli of cytoskeletal networks is orders of magnitude greater than the critical crosslink density needed for gelation, and close to that where structures like raft phases and other crosslinked structures are predicted from theory for cytoskeletal polymers. The free energy of a divalent ion binding to a site on two highly charged filaments, forming a crosslink, is approximately -1.5 k_BT (Janmey et al., 2014).

There are functional consequences to such interactions with implications for drug design and delivery. The effect of polycations on anionic polyelectrolytes in extracellular fluids is not limited to the formation of large filament bundles that increase fluid viscosity. The sequestration of polycationic solutes into these bundles also inhibits their functions, potentially contributing to the failure of antimicrobial agents to function at sites of chronic infection. Both F-actin and DNA inhibit the ability of LL37, beta defensin, lysozyme, and other cationic antimicrobial agents to kill bacteria (Weiner et al., 2003; Bucki et al., 2007; Jones et al., 2013; Lewenza, 2013), presumably because the anionic polyelectrolytes compete with the negatively charged bacteria for binding the cationic antimicrobials. This effect is not limited to native components of the innate immune system, but extends to antibiotic drugs such as tobramycin which is also polycationic. An X-ray scattering study of tobramycin bound within DNA bundles (Purdy Drew et al., 2009) reveals how this cationic drug is sequestered within DNA bundles and therefore cannot access its target bacteria.

Negatively charged intracellular polymers and anionic extracellular polyelectrolytes alike interact with cations to form complex and unexpected structural elements that enhance or reduce their functioning. We use these observations to set the stage for investigating the detailed bonding,
Figure 1.5: Some common cellular polyelectrolytes. Left: Electrostatic potential plotted on an inflated van der Waals surface of the polyelectrolytes. The electrostatic potential maps were computed using the PDB2PQR package (Dolinsky et al. 2007) to assign charges to the atoms and the APBS package (Baker et al. 2001) to solve the Poisson-Boltzmann equation numerically on a ride. Red is negative and blue is positive; colors are not normalized between molecules. Sketches on the right show persistent lengths for long polymers of the corresponding polyelectrolytes on the left can be taken to represent the configuration of a 2 µm polymer of the polyelectrolytes.
bridging, and dehydration of divalent cations with PtdInsP₂, at a level beyond classical electrostatics.
1.6 Objectives of thesis and approach

The objective of this thesis is to gain an atomic-level understanding of the interaction between PPIs and counterions that may be present in a cellular context and to investigate the lateral organization of PPIs in a bilayer setting. In order to achieve these results, we embarked on multiscale modeling of these molecules using computational techniques such as quantum level ab initio electronic structure calculations and classical all-atom molecular dynamics (MD) simulations ranging from a single molecule to bilayer systems containing 800 lipids and cholesterol. These bilayer simulations incorporate a mixture of lipid compositions, membrane leaflet symmetry or asymmetry, presence of absence of cholesterol, monovalent or divalent counterions, charge states for PtdIns(4,5)P₂ and the specific isomer of PtdIns(3,5)P₂ or PtdIns(4,5)P₂.

1.7 Specific aims and hypothesis of work

The hypothesis of this work is that understanding the physical chemistry of PtdIns(3,5)P₂ and PtdIns(4,5)P₂ will lead to insights about the biological role of these phospholipids and shed light on how PPIs induce different cellular responses at different times and different locations in the cell. Specifically, this thesis is aimed at answering the following questions.

**Chapter 2** What is the size and shape of PtdIns(4,5)P₂ and PtdIns(3,5)P₂ and what is their net charge at physiological pH?

**Chapter 3** How sensitively and specifically do PtdIns(4,5)P₂ and PtdIns(3,5)P₂ bind to abundant intracellular counterions? How does binding to counterions affect the structure of these isomers?

**Chapter 4** How do PtdIns(4,5)P₂ and PtdIns(3,5)P₂ affect the structure and dynamics of membrane bilayer systems? Does the introduction of counterions increase the propensity for PtdIns(4,5)P₂ and PtdIns(3,5)P₂ to form clusters in membranes?
Portions of this chapter were published in (Slochower et al., 2014) and (Wang et al., 2014), copyright 2014, with permission from Elsevier; (Janmey et al., 2014), Reproduced by permission of The Royal Society of Chemistry, 2014.
Single molecule simulations of PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$

Experimental studies of the phase behaviors of PtdInsP$_2$ have revealed several interactions among these charged lipids that might be relevant to how their biological effects are regulated. These interactions include the unexpected attractive interaction between adjacent PtdInsP$_2$ molecules, Ca$^{2+}$-induced heterogeneity in monolayers containing PtdInsP$_2$, and cholesterol-dependent phase separation. Therefore, understanding the interaction of PtdInsP$_2$ with other PtdInsP$_2$ residues, water, and counterions on the atomic level emerged as an important goal to help define mechanisms by which the biological effect of PtdInsP$_2$ on the proteins it controls can be modulated (Levental et al., 2008a,b; Ellenbroek et al., 2011; Wang et al., 2012b). In silico calculations and molecular dynamics simulations that allow the inspection and manipulation of atomic coordinates are the ideal tool for this task; hardware advances have allowed simulations to reach hundreds of nanoseconds for even the most complex systems and concomitantly, force fields for biomolecules have improved tremendously over the last 30 years with particular attention given to lipids in the past five years.

At the outset of this project, there were a limited number of molecular dynamics (MD) parameters available for PtdInsP$_2$. The accuracy of MD simulations relies on the underlying accuracy of the force fields by which forces on every atom in the system are calculated and the dynamics of the system are
propagated by moving atoms proportional to the force on them. Typically, empirical force fields (here this means force fields designed to reproduce select macroscopic experimental observations) contain energy terms for bonded (bond stretching, angle bending, dihedral and improper dihedral terms) and nonbonded (Coulomb for electrostatics and Lennard-Jones interactions for van der Waals repulsion) interactions ([Leach] 2001). When new molecules are introduced to an established force field, the parameters for bond stretching and angle bending can usually be adopted by analogy by assigning existing atom types to atoms in the target molecule. This also takes care of the Lennard-Jones interactions which are based on the atom types. However, in order to reproduce proper electrostatic behavior, determining the partial atomic charges of atoms is necessary. This cannot be done by analogy because the specific, three-dimensional spatial distribution of charges sets up an electric field that, in turn, acts on the partial atomic charges. Therefore, the process of assigning partial atomic charges is usually undertaken iteratively, in conjunction with a geometry optimization. Along with the geometry optimization and charge determination, dihedral angles sometimes also need to be refined for a specific molecule if the sequence of four atom types does not occur in other molecules.

As far back as 1970 in the work of Arieh Warshel, the idea of using force fields that attempt to take into account the polarizability of atoms and molecules, where a dipole or higher order multipole is distributed across a molecule or even an atomic center, was proposed ([Antila and Salonen] 2013). These methods have seen increased usage in recent years, but have not been widely accepted, partially due to their computational cost. Another reason for their limited usage is that although there is a plethora of polarizable models for water and ions, there are significantly fewer polarizable force field models that include biomolecules, limiting their usage in simulations of large physiological systems.

It should be noted that one area where polarizable models see considerable success is matching the solvation shell around ions; whereas classical force fields are good at reproducing solvation free energies, historically they have had issues reproducing the experimental number of waters in the first
solvation shell around ions, particularly divalent ions (Saxena and Sept, 2013). On close approach (i.e. where two atoms have a minimum in their Lennard-Jones potential), polarization effects may alter the interaction energy of molecules by 10-20% (Antila and Salonen, 2013). In our work, we have not used any polarizable force fields and instead, where polarization effects may be important, opted to use hybrid quantum mechanics / molecular mechanics (QM/MM) simulations.

In this chapter, I describe electronic structure calculations to determine the proper geometry and partial charge distribution for PtdIns(3,5)P_2 and PtdIns(4,5)P_2 with at a net charge of -5 and -4, with a proton on either the 4-phosphate or 5-phosphate. Further investigation was carried out using QM/MM simulations in the presence of pure water or with added monovalent and divalent cations, including sodium, potassium, calcium, and magnesium.

2.1 Initial simulations of PtdIns(4,5)P_2 with force field parameters from Lupyan, et al.

Lupyan, et al., published force field parameters for PtdInsP_2 with patches for phosphate groups (patches provide rules for how to modify a base molecule with small chemical groups at specific attachment sites, e.g. phosphorylation or protonation) in 2010 based on the CHARMM C27 force field (Lupyan et al., 2010). The patches allow a modular approach, allowing the base PtdIns to be merged with any combination of phosphate groups at the 3, 4, or 5 position of the inositol ring, all carrying a net charge of -2, identically distributed across the atoms. These parameters used a combination of quantum-level methods to fit the partial atomic charges of the inositol ring to the calculated electrostatic potential and parameterized the inositol phosphate group patches so the net charge of PtdIns(4,5)P_2 would be -5, eliminating the choice of whether or how to protonate the phosphate groups. However, their choice of how to fit the electrostatic potential (using the RESP package (Case et al., 2005; Weiner and Kollman, 1981)) is not entirely consistent with the latest CHARMM force fields and is now considered obsolete (personal communication, Jérôme Hénin
and Benoît Roux). The torsional parameters associated with the connection between the PtdIns head group and the glycerol part of the lipid were tuned based on the results of mapping a potential energy surface computed by scanning the key dihedral angles while keeping all other atoms in the head group fixed (i.e., a rigid potential energy surface scan). Another issue with these parameters is due to the use of a sizable portion of the lipid, instead of a minimal fragment for the potential energy scan, it is possible that an unfavorable dihedral angle measured in this way could be caused by a steric clash in portions of the molecule not adjacent to the dihedral under study. Thus, the potential energy surface for the dihedral angle would become conflated with other energy terms in the force field and this has arguably led to unusually high energy barriers for the phosphodiester dihedral angle, effectively locking the molecule into specific conformations.

As PtdIns(4,5)$P_2$ comprises >99% of the doubly phosphorylated PPIs in cells and has the most physical chemical characterization, we tested the parameters of Lupyan et al. on small patches of PtdIns(4,5)$P_2$ (McLaughlin et al., 2002). Understanding the arrangement and dynamics of PtdIns(4,5)$P_2$ molecules in small clusters, to complement experimental data from Langmuir troughs where excess surface pressure induces tighter packing of the lipids, was a goal of our early work. I approached this task by performing simulations of PtdIns(4,5)$P_2$ using the parameters of Lupyan, et al. (provided at that time via personal communication) using the NAMD molecular dynamics package (Phillips et al., 2005). A small grid of 25 PtdIns(4,5)$P_2$ molecules were seeded in the (x,y) plane and simulated for up to 80 ns in the NVT ensemble where N is number of particles, V is the system volume, and T is the system temperature. Either NVT or NPgT simulations have been used for monolayer and membrane simulations; in the former, area per molecule is controlled via system volume and in the latter the surface tension (g) and pressure (P) are controlled (further discussion of bilayer simulation ensembles will occur in 4). The molecular system can expand and contract in the z direction, however, at the expense of (x,y) area, because there are no resistive

\[1\text{Cubic periodic boundary conditions, real space electrostatics cut off at 12 Å and smooth switching distance of 10 Å, Particle Mesh Ewald (PME) grid spacing of 1 Å, non-bonded interaction pair list distance of 13.5 Å, MD time step of 2.0 fs, and temperature control by Langevin dynamics at 310 K with a damping coefficient 5 ps}^{-1}.\]
forces in the vacuum.

Charge neutrality was ensured using mobile monovalent or divalent cations and monovalent anions (MgCl$_2$, CaCl$_2$, or NaCl) to match experimental conditions, and the electrostatics were handled via the Particle Mesh Ewald (PME) formalism which splits the calculation of the Coulombic force into a part that is evaluated in real space and a part that is evaluated in Fourier space along a smoothed grid. Outside the “cut off distance,” the charges of the atoms, centered at the nuclei, are mapped onto the grid points. Choosing a small enough grid spacing is an important consideration when dealing with highly charged systems and certainly when the distance between charges is less than the Bjerrum length, the characteristic length below which electrostatic interactions are stronger than thermal energy and is about 7 Å in water. Two unit charges separated by 10 Å in vacuum contribute 33.2 kcal/mol to the potential energy of a system whereas screening by a material with a dielectric of 80 (e.g. water) reduces that to 0.42 kcal/mol. In a membrane system the relative dielectric constant may be around 2-4 and therefore, electrostatic forces are quite long-ranged and must be accounted for accurately.

These simulations resulted in two clear abnormalities that deviated from physiological expectations. First, the clusters developed a strong curvature, with a radius of curvature on the order of a few molecule lengths (<5 nm) (Figure 2.1, top) After several attempts with different simulation parameters and combination of MD restraints (e.g., gently enabling electrostatic forces after a minimization without electrostatics), I was unable to find a stable monolayer configuration at a charge per phosphate group of -2 (charge per PtdIns(4,5)P$_2$ of -5). Second, the simulations showed strong and irreversible binding of Ca$^{2+}$ and Mg$^{2+}$ ions to the negative charge sites on the PtdIns(4,5)P$_2$ molecules (Figure 2.1b). This had the effect of gluing together adjacent PtdIns(4,5)P$_2$ molecules leading to aggregation. Although we expect strong binding of Ca$^{2+}$ to PtdIns(4,5)P$_2$, the rapid and irreversible sticking of every Ca$^{2+}$ to every phosphate group was not consistent with our chemical intuition and the experimentally determined affinity of divalent cations for PtdInsP$_2$.

Identical simulations were re-run with the charge per inositol phosphate group reduced from -2 to
Figure 2.1: On the left, snapshots showing the head group of PtdIns(4,5)P_2 in simulations where the charge per phosphate group was -2 (green) or -1 (cyan). In both cases, the acyl chains are pointing down but omitted for clarity. On the right, Ca^{2+} (bright green) binding to phosphate oxygens (red spheres that are adjacent to gold spheres) from a simulation where the charge per inositol phosphate was -2.

-1 in steps of 0.1 (reducing the charge per PtdIns(4,5)P_2 from -5 to -4), and the anomalous effects were similarly reduced. Although this ad hoc adjustment was not rigorous, it was a perspicacious way to illustrate just how sensitive the system (lipids, counterions, and water) was to the charge per PtdIns(4,5)P_2 molecule. In molecular simulations of this kind, the charge per molecule is determined by positing a fractional elementary charge on the center of mass of each atom such that the sum of each partial atomic charge is the net charge of the molecule. We set out to determine the partial atomic charge distribution and geometry using electronic structure calculations consistent with established methods for the CHARMM force fields.
2.2 Electronic structure calculations on PtdIns(3,5)P₂ and PtdIns(4,5)P₂

The properties of PPIs are largely controlled by the position and amount of phosphorylation (1, 2, or 3 phosphate groups) on the inositol ring. Several proteins bind with high affinity to a specific PtdInsP₂ isomer but not others, suggesting precise molecular targets. Investigations of PtdIns(4,5)P₂-containing monolayers at varying pH have demonstrated that the degree of ionization of the head group alters the phase behavior and transition temperature of such monolayers (Blume and Eibl, 1979). Quantum-level electronic structure calculations were used to determine the optimized molecular geometry of PtdIns(3,5)P₂ and PtdIns(4,5)P₂ in different charge states. Additionally, the electronic structure calculations reveal information about the stability, shape, and orientation of the isomers.

Geometry optimizations and vibrational calculations were performed primarily using the Gaussian 09 program (Frisch et al., 2009) with Hartree-Fock (HF) 6-31+G(d) model chemistry, which includes diffuse and polarizable functions, unless otherwise noted. In Hartree-Fock theory, the multi-electron wavefunction is expressed in terms of N single electron wavefunctions for N number of electrons (with no explicit correlation between electrons and thus no correlation energy in canonical HF theory). The basis set, 6-31+G(d), is a collection of functions that are used to represent the atomic orbitals in the system. Basis sets with more functions, e.g. 6-31+G(d,f), are needed to keep track of the greater number of electrons in larger (usually nonorganic) atoms but come with a large computational penalty, as computational time usually scales with \( N^4 \) for \( N \) electrons. By default, calculations are carried out in vacuum, and a polarized continuum model (PCM) can be implemented to mimic the effects of arbitrary solvents, whereby the energy of the solute and solvent system is calculated by making the solvent reaction field self-consistent (the so-called self-consistent reaction field; SCRF) with the solute electrostatic potential. Details of this approach can be found in (Improt et al., 2006).
We also attempted a geometry optimization using second-order Møller-Plesset perturbation theory MP2/6-31G(d) and MP2/6-31G, to include electron-correlation effects in HF, but were unable to achieve convergence.

For the described electronic structure calculations, I truncated the stearate and arachidonate acyl chains after two carbons to improve the probability of self-consistent field convergence and decrease the computational resources (56 atoms, 300 electrons). The choice and location of truncation, along with the level of theory, are consistent with quantum mechanical calculations performed on other biomolecules parameterized in classical force fields (Pastor and MacKerell 2011; MacKerell 2004). Partial atomic charges were calculated using CHarges from ELectrostatic Potentials using a Grid based method (CHELPG) (Szabo and Ostlund 1996; Breneman and Wiberg 1990). The vibrational eigenvalues and eigenvectors were computed at HF/6-31G model chemistry and after applying suitable scaling factors (Szabo and Ostlund 1996).

As discussed in section 1.3, the most likely experimental value for the physiological charge of PtdIns(3,5)P_2 and PtdIns(4,5)P_2 is -4 (Kooijman et al. 2009; Levental et al. 2008b) with PtdIns(3,5)P_2 being slightly more negative at perhaps -4.25. We set out to determine (a) the position of the proton, (b) the charge distribution across atoms and the total charge of isomers, and (c) the optimal geometry adopted by the isomers. Following the simulations in section 2.1, PtdIns(4,5)P_2 was selected for the first round of calculations in the presence of a proton initially placed between the 4-phosphate and the 5-phosphate of the inositol ring. The proton found a stable position covalently bound to the 5-phosphate oxygen. Preliminary calculations at a lower level of theory showed that the proton is 6 kcal/mol more stable bound to an oxygen on the 5-phosphate compared with the 4-phosphate. The hydrogen-oxygen bond distance is 0.96 Å. In vacuum, there are several intramolecular hydrogen bonds that span the inositol ring, which is in the chair conformation.

Using a polarized continuum model, the free energy of having a proton exclusively on the 4-phosphate, exclusively on the 5-phosphate, or shared between the two was evaluated. The most favorable configuration had the proton on the 5-phosphate also forming a hydrogen bond with the 4-
phosphate. The proton may also be predominantly on the 5-phosphate and forming a weak hydrogen bond with the 4-phosphate ester oxygen (the oxygen connecting the phosphate group to the inositol ring), at a cost of +15.4 kcal/mol. The strength of hydrogen bonds vary, depending in part on the charge of the acceptor atom, but are generally on the order of 10 kcal/mol \cite{kollman1972, espinosa1998}. The 4-phosphate ester oxygen is less negative than the phosphate oxygens and this hydrogen bond is not as energetically favorable. However, the total energy difference between structures (after geometry optimization) is greater than the energy difference between hydrogen bonding to either the phosphate or the ester, and other subtle structural rearrangements can account for this (Figure \ref{fig:2.2}). One such structural difference is the formation and breakage of other hydrogen bonds, for example, involving the other hydroxyl groups on the inositol ring. If the proton is localized solely to the 4-phosphate, the energy is +22.0 kcal/mol compared to having a proton shared between the two phosphate groups.

![Proton on the 5-phosphate, Proton shared between 4 and 5, Proton on the 4-phosphate](image)

Figure 2.2: The relative stabilities of protonated PtdIns(4,5)$P_2$ computed with Gaussian 09 at the HF/3-21G level of theory with implicit water solvent using a polarized continuum model.

It is also favorable for PtdIns(3,5)$P_2$ to have a proton on the 5-phosphate group. Due to the spread of the phosphate groups, in this case the optimal geometry allows the proton on the 5-phosphate group to hydrogen bond with the 4-hydroxyl and the proton on the 4-hydroxyl to hydrogen bond with a 3-phosphate oxygen (see Figure \ref{fig:2.3}). This is shown in the starting configuration for umbrella sampling discussed in section \ref{sec:2.3} where the stability of this position was tested.
Along with the geometry optimization, we measured the potential energy surface while scanning the dihedral angle connecting the head group to the acyl chains (the phosphodiester dihedral angle) in increments of $10^\circ$. In a bilayer setting, the orientation of the head group determines the accessibility of the lipid residue to its binding partners. Accessibility, in turn, determines when proteins in the cell manipulate PPI levels and generate second messengers responsible for responding to extracellular events. Crystal structures have revealed that the PLC$\delta$ PH domain forms a stereo-specific hydrogen bonding network with the entire PtdIns(4,5)$P_2$ head group in an orientation that appears to be perpendicular to the membrane bilayer (Lemmon [2008]). The structure of inositol 1,4,5-triphosphate bound to the ENTH domain, which is found in the protein epsin and required for clathrin-mediated endocytosis, further highlights coordination of multiple arginine and lysine residues to the 4- and 5-phosphate groups (Ford et al. [2002]). Neutron diffraction studies have suggested the orientation of the PtdIns(4,5)$P_2$ head group is perpendicular to the membrane plane (Bradshaw et al. [1996, 1997]).

Figure 2.3: The optimized geometry of PtdIns(4,5)$P_2$ with and without a proton on the 5-phosphate group and PtdIns(3,5)$P_2$ with a proton on the 5-phosphate group, optimized in vacuum.

A simulation study in 2009 used modified CHARMM C27r parameters for PtdIns(4,5)$P_2$ in the NPAT (number, pressure, surface area of the lipids [seeded at 68Å$^2$ per lipid], temperature) ensemble with neutralizing Na$^+$ counterions. In these simulations, the PtdIns(4,5)$P_2$ head group is tilted at an average angle of $\sim 40^\circ$ with respect to a phosphatidylcholine bilayer (PtdCho) (Li et al. [2009], for
comparison the phosphorus-nitrogen vector in PtdCho was tilted $17^\circ$ relative to the bilayer normal.

That paper also described the twist angle of the inositol ring, fluctuating around $0^\circ$ and $\pm35^\circ$.

**Figure 2.4**: The relationship between the head-tail angle and the dihedral angle in PtdIns(4,5)P$_2$ and the free energy of adjusting the dihedral angle measured in a relaxed potential energy scan.

We define the angle between the inositol ring and the acyl chains (referred to as the “head-tail angle”), as the angle between the vector connecting the fourth carbon in the inositol ring to the phosphorus in the phosphodiester and the vector connecting the phosphorus in the phosphodiester to the first carbon in the glycerol moiety (see Figure 2.4 bottom). As the $C-O-P-O$ dihedral angle was scanned, and the geometry allowed to find a new minimum energy configuration, the head-tail angle stayed between 80-110$^\circ$. in both vacuum and the polarized continuum solvent. The most favorable phosphodiester angle was between approximately 120-240$^\circ$ where the head-tail
angle was between 90-95° in vacuum and 105-115° in the polarized continuum solvent. These data suggest that in that in the absence of other factors, the PtdIns(4,5)P$_2$ head group would lie parallel to the membrane bilayer [2,4]. We were unable to obtain a relaxed structure with the head-tail angle near 0° or 180°, where the head group would be completely perpendicular to the bilayer. These results are confirmed in bilayer simulations, discussed in chapter [4]. These curves qualitatively agree with the CHARMM energy functional for this dihedral angle which is a modified cosine wave.

2.3 Hybrid QM/MM simulations

To investigate the behavior of PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$ with a more realistic solvent environment while retaining the ability to treat atoms of interest with quantum-level detail, we turned to hybrid QM/MM simulations [Warshel and Levitt, 1976]. In the QM/MM approach, the system is broken down into two regions which are treated through separate equations of motion. The effective Hamiltonian ($H_{\text{effective}}$) is the sum of terms representing the QM (quantum mechanical) region, the MM (molecular mechanical) region, and the interaction between the regions,

$$H_{\text{effective}} = H_{\text{QM}} + H_{\text{MM}} + H_{\text{QM/MM}},$$

and the energy of the system is given by

$$E = \phi^T H_{\text{effective}} \phi = \phi^T H_{\text{QM}} \phi + \phi^T H_{\text{QM/MM}} \phi + E_{\text{MM}},$$

where $\phi$ is the wavefunction describing the QM atoms, and $\phi^T$ is its transpose. Minimization of $\phi^T H_{\text{effective}} \phi$ is the expectation value for the ground state characterized by the vector $\phi$.

This multiscale description of force fields provides a route to extend the electronic structure methods to the nanometer scale to enabling the study of reactive events in biological systems. A single PtdInsP$_2$ molecule was placed, using the geometry optimized in vacuum, in a water sphere...
containing approximately 10,000 TIP3 water molecules (15 Å buffer on all sides). Na\(^+\), Ca\(^{2+}\), or Mg\(^{2+}\) were added to neutralize the -4 charge of the singly protonated PtdIns\(P_2\). Water molecules greater than 8 Å away from the PtdIns\(P_2\) were fixed in position. The system was split into two quantum regions (see Figure 2.5): QM1, which contains only phospholipid atoms, and QM2, which contains interacting waters and ions. Two single-link atoms connect QM1 to the classical region (Das et al., 2002). The first link atom was placed between the third and fourth carbon of the inositol ring for PtdIns(4,5)\(P_2\) and between the second and third carbon of the inositol ring for PtdIns(3,5)\(P_2\). The second link atom was placed between the fifth and sixth carbon of the inositol ring for both isomers. The choice and sensitivity to location of the link atoms on ring structures has been investigated in our earlier studies and was found to not impact the optimized geometry significantly.

QM2 typically contains the five or six closest interacting waters and a divalent ion (if present). In one case, QM2 was expanded to include nine waters to cover all hydrogen bonds between the phosphomonoester groups and water molecules. We performed these calculations using a custom-compiled combination of GAMESS-UK (Guest et al., 2005) and CHARMM c36b2 (Brooks et al., 2009). Both QM regions were treated using density functional theory (DFT) using the hybrid functional B3LYP/6-31G. In DFT, the total energy is calculated from the electronic density distribution instead of the Slater determinant of the N-electron wavefunction (Leach, 2001).

In this step, the electrostatic coupling between the QM and the MM sub-regions is accounted for: i.e., the charges in the MM region are allowed to polarize the electronic wave functions in the QM region. The forces in the quantum region are calculated using DFT on-the-fly assuming that the system moves on the Born-Oppenheimer surface (Senn and Thiel, 2007). That is, we assume a clear timescale of separation between the electronic and nuclear degrees of freedom and the electronic degrees of freedom are in their ground state around the instantaneous configurations of the nuclei. The forces on the classical region are calculated using a classical force field. In addition, a mixed Hamiltonian (\(H_{QMM}\)) accounts for the interaction of the classical and the quantum regions. Bonded terms and electrostatic terms between the atoms of the QM region and those of the classical
region are typically included.

![Figure 2.5: A schematic of the QM/MM simulations. Left: On the lipid, link atoms connect the classical and quantum portions, shown as blue spheres on PtdIns(4,5)P₂ for illustration. There are also water molecules in another quantum region shown as a red surface rendering to demonstrate their electron density. Right: The lipid and QM waters are embedded in a water sphere containing classical water molecules.](image)

We note that prior studies have investigated proton free energy landscapes using similar functionals [Venkatramani and Radhakrishnan, 2010]. These studies have discussed the merits and limitations of these methods, such as accurate electrostatics, neglect of quantum effects, etc. Some of the simulations were repeated at a higher level of theory using either B3LYP/6-31G(d) or PBE/6-31G(d), including a higher basis set and a different formulation for combining the DFT and HF electron-electron exchange and electron-electron correlation energies, to verify that we captured all salient hydrogen bonds and the correct orientation of the water molecules. The classical PtdInsP₂ atoms and mobile ions were treated using the CHARMM C36 all-hydrogen lipid forcefield parameterized for lipids and the CHARMM-consistent water model TIP3P [Klauda et al., 2010].
The C36 forcefield is significantly more accurate in reproducing experimental quantities for lipids than the previous iteration forcefield (C27r), particularly in matching the surface tension of bilayers simulated in the NPT ensemble [Pastor and MacKerell 2011; Klauda et al. 2012]. The parameters for the inositol ring and the phosphate groups have been derived from Hatcher, et al. (2009) and Mallajosyula, et al. (2012). These combined parameters are obtained from the C36 version and differ from those of Li, et al. (2009) who used the C27r forcefield, as discussed by Pastor and MacKerell (2011). Our parameters are also different from those used by Lupyan et al. (2010) who parameterized PtdIns(4,5)P$_2$ at a net charge of -5 and explicitly included monovalent salts in all simulations.

In the QM/MM simulations, we performed the usual energy minimization (5000 steepest descent followed by 5000 steps of adopted basis Newton-Raphson) and constant temperature equilibration using Langevin dynamics at 300 K using a piston frequency of 10 ps$^{-1}$ before regular integration procedures in operation for pure MM systems using a standard 1 fs time step of integration. The QM/MM simulations were run for a total of 5 or 10 ps.

We also ran preliminary studies using Car-Parinello molecular dynamics (CPMD), an implementation of DFT that uses plane waves and pseudopotentials to describe the wavefunction of the system. Electrons undergo fictitious dynamics that relies on a fictitious electron mass to approximate dynamics on the Born-Oppenheimer surface (i.e., there is no energy transfer from the electrons to the nuclei). These simulations, completed by Peter Huwe, also characterized the hydrogen bond network for PtdIns(4,5)P$_2$ at a charge of -5. We were able to subjectively identify water molecules in both QM/MM and the CPMD simulations forming the same hydrogen bonds with PtdIns(4,5)P$_2$.

The output of the CPMD simulations were used as the initial coordinates of another geometry optimization of PtdIns(4,5)P$_2$ including three explicit waters. We were able to optimize this system first at HF/3-21G and then again at HF/6-31+G(d). The geometry with and without water molecules had excellent agreement. At this point, it would be possible to derive new parameters for classical force fields. The partial atomic charges on PtdIns(4,5)P$_2$ would be recalculated in the presence of
water, along with the vibrational modes, and an iterative procedure would be undertaken to match
the eigenvalues and eigenvectors estimated using the electronic structure calculations to a normal
mode analysis using the classical force field. One promising approach is to do this automatically
using a merit function and genetic algorithm (Liu, 2010).

**Area of PtdIns(4,5)P₂ at charge -4 and -5**

It would be beneficial to have an analog of the area per molecule of PtdIns(4,5)P₂ and PtdIns(3,5)P₂
that was measured by Levental (2008). In MD simulations of bilayer systems, the average area
per molecule is usually reported by dividing the vectors of the periodic boundary by the number of
molecules in that dimension. This method is not applicable for a single molecule simulation; here
the reported average molecular area was computed by squaring the maximum distance between
oxygens on different phosphomonoester groups at each time step during the simulation. That is,
the area per molecule is a lower bound for the area of a square that would fit the head group of
PtdIns(4,5)P₂ or PtdIns(3,5)P₂.

In the presence of neutralizing Na⁺ ions kept at least 5 Å away from PtdIns(4,5)P₂ (i.e., Na⁺ is
not in the QM region and not bound) with a charge of -4 and a proton on the 5-phosphate, the
average area of the head group calculated from our QM/MM simulations is found to be 66.8 ± 2.2 Å².
However, with a charge of -5 and without the proton, the area is reduced to 63.4 ± 3.2 Å² (see
Figure 2.6, left). Both histograms follow a Gaussian distribution with relatively small standard
deviation, so the difference in areas is statistically significant. Figure 2.3 shows that at charge -5, the
phosphate groups of PtdIns(4,5)P₂ appear to be fully spread, whereas at charge -4, the phosphate
groups hydrogen bond with each other. In the QM/MM simulations, hydrogen bonding between the
phosphate group of PtdIns(4,5)P₂ at charge -5 and an explicit QM water molecule draws the two
phosphate groups together. Figure 2.7 shows that although PtdIns(4,5)P₂ at charge -4 and charge
-5 maintain an average of 3.6 hydrogen bonds with QM water molecules, the water molecules come
much closer to the PtdIns(4,5)P₂ atoms at charge -5.

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There is experimental evidence that the area per molecule of PtdIns(4,5)P$_2$ in monolayers measured by a Langmuir trough at a surface pressure of 30 mN/m (approximating physiological conditions) depends on pH, and thus, charge of PtdIns(4,5)P$_2$. At a pH of 1.8 (where PtdIns(4,5)P$_2$ carries less negative charge) the area per PtdIns(4,5)P$_2$ is approximately 60 Å$^2$ compared to 73.1 ± 3.0 Å$^2$ at pH 7.4 (where PtdIns(4,5)P$_2$ carries more negative charge) (Levental, 2008). This effect also depends on salt content, as the areas increase in the presence of 250 mM NaCl yet the expansion does not occur for PtdIns or other anionic lipids, like PtdSer (Levental, 2008).

**Area of PtdIns(4,5)P$_2$ compared to PtdIns(3,5)P$_2$**

Similarly, we measured the average area per molecule of PtdIns(3,5)P$_2$ at charge -4 under the same conditions. The average area molecule of PtdIns(3,5)P$_2$ is significantly larger than PtdIns(4,5)P$_2$ as the two phosphate groups on the inositol ring are not vicinal and are spread much further apart. The average area per molecule for PtdIns(3,5)P$_2$ is 95 ± 4 Å$^2$, making it significantly larger than other common phospholipids in the cell.

In the absence of 250 mM NaCl (Levental, 2008) did not find the area of PtdIns(3,5)P$_2$ to be greater than PtdIns(4,5)P$_2$ but the addition of 250 mM NaCl caused an expansion in the area per
molecule of 22 Å² for PtdIns(3,5)P₂ and an expansion of only 11 Å² for PtdIns(4,5)P₂. These data suggest the presence of salt may disrupt the structure of water-mediated hydrogen bonds between adjacent molecules and that PtdIns(3,5)P₂ is more sensitive to this than PtdIns(4,5)P₂. These simulations are consistent with this hypothesis as we find that the increased spread of the phosphate groups in PtdIns(3,5)P₂ result in more interactions with water molecules and more waters localized between the phosphate groups (see Figure 2.8).
Figure 2.7: Top: the distance of QM waters to PtdIns(4,5)P$_2$ at charge -4 and -5. Five QM waters come within 2 Å of the nearest PtdIns(4,5)P$_2$ atom when the charge is -5 but only only two QM waters at a time come within 2 Å of PtdIns(4,5)P$_2$ with the addition of a proton to the 5-phosphate group. Middle: The number of hydrogen bonds that PtdIns(4,5)P$_2$ makes with QM water molecules is constant in both charge states. Bottom: starting structures for QM/MM calculations. The colored atoms are treated quantum mechanically, the grey atoms are treated classically, and surrounding classical waters are not shown.
Figure 2.8: Top: the distance of QM waters to PtdIns(3,5)P$_2$ at charge -4. The QM waters do not localize quite as close to PtdIns(3,5)P$_2$ as they do for PtdIns(4,5)P$_2$, yet PtdIns(3,5)P$_2$ maintains nearly 6 hydrogen bonds with water molecules compared to 3.6 for PtdIns(4,5)P$_2$ (middle). Bottom: On average, four QM waters come between the two phosphate groups of PtdIns(3,5)P$_2$. 

4 waters on average between phosphates
Stability of protonation of PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\)

We applied the technique of umbrella sampling (US) to delineate the free energy of proton binding and relative stability of the proton-bound and unbound states for PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\). This technique, using hybrid QM/MM simulations with DFT, is an orthogonal approach to the purely quantum-level HF geometry optimization that ended with a proton on the 5-phosphate for both isomers (Figure 2.3). In umbrella sampling, the simulation is biased to a region of interest via the application of a biasing potential that is added to the potential energy of the system; this is a non-Boltzmann weighted simulation. The biasing potential is typically harmonic along a reaction coordinate chosen before the simulation. Several windows (that is, separate simulations that can be run in parallel) are constructed for increasing (or decreasing) values of the reaction coordinate, and a simulation is run for each window with the harmonic potential centered around the target distance. The value of the reaction coordinate is recorded for each window; if the reaction coordinate is a bond length, then this will be the distance between the atoms on either end of the bond. The distribution of the reaction coordinate in any given window must overlap with the distribution from adjacent windows, giving rise to the namesake “umbrella” sampling.

The 5-phosphate oxygen-hydrogen bond vector was used as the \textit{a priori} reaction coordinate for US on PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\). Overlapping windows were chosen to span the distance from the equilibrium bond length of 0.962 Å to 3.5 Å at which point we assume the covalent bond between oxygen and hydrogen is broken. A biasing potential of either 50 kcal/mol/Å\(^2\) with 0.2-0.4 Å windows or 100 kcal/mol/Å\(^2\) with 0.1 Å windows was applied for 1 ps of simulation in each window using a 1 fs time step of integration.

For the free energy calculation, the probability distribution \(P(\xi_i)\), is calculated by dividing the maximum range of the order parameter \(\xi_i\) (oxygen-hydrogen bond distances) into several windows. We processed the US data to calculate the multidimensional potential of mean force (PMF) using the weighted histogram analysis method (WHAM) \cite{Roux1995}. The number of windows for US/WHAM
ranged from 20-40 and the tolerance for the iterative convergence of the PMF was set at 10^{-7}. A histogram is made for each window \(i\) from which the potential mean force \(\lambda_i(\xi_i)\) is calculated according to,

\[
\lambda_i(\xi_i) = -k_B T \ln [P(\xi_i)] + \text{constant}.
\]

In our first attempt to ascertain the free energy of having a proton on the 5-phosphate group of PtdIns(4,5)\(P_2\) compared with not having a proton bound to the molecule at all (i.e., the free energy difference between charge state -5 and -4), we used the 5-phosphate oxygen-hydrogen (O–H) bond distance as the \textit{a priori} reaction coordinate (Figure 2.9d, blue arrow). As the O–H bond vector was increased, the proton moved from the 5-phosphate to the ester oxygen connecting the 4-phosphate group to the inositol ring (Figure 2.9). In the final umbrella sampling window, the proton is 0.98 Å from the 4-phosphate ester oxygen and 3.70 Å from the 5-phosphate oxygen. This internal proton transfer is entirely uphill and energetically unfavorable (Figure 2.9) by approximately 22 kcal/mol.

To disfavor binding to the 4-phosphate group as well, we used two-dimensional US to force the proton to dissociate from PtdIns(4,5)\(P_2\) onto a nearby water molecule in the QM region. The second reaction coordinate was a harmonic restraint between the proton and the 4-phosphate ester oxygen that had the same magnitude and increased at the same rate as the restraint between the proton and the 5-phosphate oxygen. The proton dissociated from PtdIns(4,5)\(P_2\) and two short-lived hydronium ions (\(\sim 1\) ps) were formed during this process via the Grotthuss mechanism (Agmon, 1995). The two-dimensional energy landscape was converted to a single degree of freedom by integrating along the path through the minimum energy position for each coordinate pair (Figure 2.10).

As before, the starting structure had a proton on the 5-phosphate group (Figure 2.10 image at 1.8 Å). After a brief increase in free energy, as the O–H bond was stretched, the proton was transferred to a 4-phosphate oxygen, not the ester linkage. Both reaction coordinate constraints, between 1 and 2 Å at this point in phase space, were obeyed here. As expected, this conformation is higher in energy than protonation of the 5-phosphate (Figure 2.10 image at 2.8 Å). As the constraints
increase, complete deprotonation occurs (Figure 2.10 blue sphere in the image at 5.8 Å) and a water in the QM region forms a hydrogen bond with the 4-phosphate group, partially replacing the lost proton. Complete deprotonation of PtdIns(4,5)P₂ is unfavorable by nearly 40 kcal/mol and this suggests that PtdIns(4,5)P₂ in vivo has a proton on the 5-phosphate group and the net charge of the phospholipid is -4 at pH 7.

![Figure 2.9: Umbrella sampling of the proton on the 5-phosphate of PtdIns(3,5)P₂ or PtdIns(4,5)P₂. The shading is the difference in computed free energy using WHAM when analyzing only the first half of each window and using the entire trajectory.](image)

One-dimensional US was used to determine the free energy of deprotonating the 5-phosphate of PtdIns(3,5)P₂. The same 5-phosphate O–H bond vector was used as the a priori reaction coordinate (Figure 2.9a, blue arrow). This resulted in a double proton transfer event. First, the proton on the 5-phosphate group was transferred to the 4-hydroxyl group (Figure 2.9b, grey sphere) and the proton on the 4-phosphate group was transferred to the 3-phosphate. So the final umbrella sampling window corresponds to deprotonation of the 5-phosphate and protonation of the 3-phosphate, remaining at
a charge of -4. Although disfavorable by approximately 10 kcal/mol, this energy landscape is more rugged and the wells are not as deep or narrow. It is likely there significantly more movement along this landscape, and conformations sampled by PtdIns(3,5)P₂ in the cell, compared with the single, deep well for PtdIns(4,5)P₂.

Another noteworthy feature to come out of these simulations was the finding of a more stable configuration than the initial window seeded from the purely quantum geometry optimization. When the O–H bond vector reached approximately 1.5 Å allowing both the 5-phosphate proton and the 4-hydroxyl proton to be shared between two equidistant oxygen atoms, the energy was more stable than the initial configuration by about 6 kcal/mol. Intramolecular hydrogen bonding arises as an important factor in the stability of PtdIns(3,5)P₂, which supports the observation that the area of PtdIns(3,5)P₂ is affected nearly twice as much as PtdIns(4,5)P₂ by the addition of 250 mM NaCl (Levental, 2008).

Figure 2.10: Complete deprotonation of PtdIns(4,5)P₂ using two-dimensional umbrella sampling.

Areas and head-tail angle of PtdIns(4,5)P₂ and PtdIns(3,5)P₂ in the presence of monovalent and divalent cations

It is clear that the physical chemistry of PPIs – their size, shape, orientation, charge distribution, aggregation, phase separation, and other factors – is hugely affected by their ionic environment.
Divalent ions have an outsized effect; they disrupt the hydrogen bond network of water and they can bind tightly to PtdIns(4,5)P₂ and PtdIns(3,5)P₂. This binding, of course, cannot be explained by simple electrostatics as it is abundantly clear that Ca²⁺ and Mg²⁺, both deficient in two electrons in their outermost s electron orbital, do not induce the same effects on monolayers and membranes containing PPIs. Unfortunately, most exiting experimental modalities are not able to describe the interaction between a single PPI and Ca²⁺ and Mg²⁺. Here, we report how divalent ions alter the size, orientation, charge distribution, and protonation state of PtdIns(4,5)P₂ and PtdIns(3,5)P₂.

On the purely quantum-level, we can revisit the geometry optimizations performed in vacuum and with a polarized continuum solvent in section 2.2. A separate calculation with Gaussian 09 can be used to plot the electron density (from the SCF density) and map the electrostatic potential onto the electron density surface, giving an impression of the charge distribution in a continuous manner across the molecule. The charge density of PtdIns(3,5)P₂ (truncated after two carbons of the acyl chains) in vacuum can be seen in Figure 2.11A. The overall negative charge density of the molecule (charge -4) is distributed nearly evenly across the molecule, despite the presence of a proton on the 5-phosphate. Figure 2.11B shows dramatic charge redistribution in the presence of a Ca²⁺ placed nearly in between the two phosphate groups. There are expected charge transfer effects; the positive charge on the Ca²⁺ is reduced and the negative charge on both phosphate groups is reduced. The introduction of the polarized continuum model is seen in Figure 2.11C. Here the Ca²⁺ has moved slightly towards the unprotonated 3-phosphate, nearly neutralizing that region yet more negative charge is localized to the 5-phosphate compared with the optimization in vacuum. The binding of Ca²⁺ does not simply reduce the overall charge of PPIs by 2, but rather, it affects the charge distribution over a large region of the molecule. Binding of divalent ions thus has the potential to alter the hydrogen bond forming ability of PPIs, the electrostatic repulsion between adjacent PPIs, and their structural orientation.

In the presence of Ca²⁺, QM waters localize to PtdIns(4,5)P₂ as they do in the absence of Ca²⁺ when the charge on PtdIns(4,5)P₂ is -5 (compare Figure 2.12a with the top of Figure 2.7). The
Figure 2.11: The electrostatic potential of PtdIns(3,5)\(P_2\) mapped onto the electron density surface. 
A: PtdIns(3,5)\(P_2\) in vacuum. B: PtdIns(3,5)\(P_2\) in vacuum with Ca\(^{2+}\). C: PtdIns(3,5)\(P_2\) in a polarized continuum solvent (water) with Ca\(^{2+}\).

The presence of Mg\(^{2+}\) (Figure 2.12b) looks more similar to the QM water distances when the charge on PtdIns(4,5)\(P_2\) is -4. Despite the proximity of the waters, the number of hydrogen bonds made between PtdIns(4,5)\(P_2\) and the waters is slightly below 3 in the presence of Ca\(^{2+}\) and slightly below 2 in the presence of Mg\(^{2+}\), compared with 3.6 for both charge states without the divalent ions. Mg\(^{2+}\) in particular seems to inhibit waters from coming close to PtdIns(4,5)\(P_2\) and interacting with PtdIns(4,5)\(P_2\).

We also investigated the free energy for the phosphate groups on the inositol ring of PtdIns(4,5)\(P_2\) to spread apart, increasing the effective molecular size of the molecule, in the presence of Ca\(^{2+}\), Mg\(^{2+}\), and K\(^{+}\) using one-dimensional US. In these simulations, the ions were not placed between the two phosphate groups, but on the side of the 4-phosphate where other classical simulations showed was a stable binding position. If Ca\(^{2+}\) is localized to the 4-phosphate group, then the phosphate groups prefer to be separated by 5.1 Å. When the Ca\(^{2+}\) is replaced by Mg\(^{2+}\), the phosphate groups prefer to be closer, at around 4.4 Å and do not experience a large energy barrier for spreading (Figure 2.13). K\(^{+}\) does not bind tightly to either of the phosphate groups and represents the intrinsic energy landscape for spreading the phosphate groups of PtdIns(4,5)\(P_2\) while not bound to an ion.

Additionally, we used QM/MM simulations to track the head-tail angle and area per molecule of
PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\) in the presence of Ca\(^{2+}\), Mg\(^{2+}\), and Na\(^+\) using the same methods as Figure 2.6 which was calculated in the presence of non-interacting Na\(^+\). In the presence of Na\(^+\), constrained to be far away from the lipid residue, PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\) have a head-tail angle of between 100\(^\circ\) to 110\(^\circ\). The addition of Ca\(^{2+}\) increases this angle for both isomers, leading to a more erect, extended, and solvent-accessible head group. In the case of PtdIns(4,5)\(P_2\) the head-tail angle becomes nearly 130\(^\circ\), and the head-tail angle of PtdIns(3,5)\(P_2\) becomes closer to 110\(^\circ\). However, the addition of Mg\(^{2+}\) tends to lower this angle just above the level with Na\(^+\) for PtdIns(4,5)\(P_2\) whereas the addition of Mg\(^{2+}\) to PtdIns(3,5)\(P_2\) further increases this angle, past what is seen after the addition of Ca\(^{2+}\).

At the same time, the area per molecule was measured using the spread between phosphate groups as an analog to what might be reported experimentally. The addition of Mg\(^{2+}\) (unconstrained in the water sphere) lowered the average area per molecule of PtdIns(4,5)\(P_2\) to 58.5 ± 2.1 Å\(^2\) and the addition of Ca\(^{2+}\) (also unconstrained in the water sphere), lowered the average area per molecule even further to 50.3 ± 1.0 Å\(^2\). These area estimates are expected to match experimental values from a Langmuir trough system only up to a constant, because in the simulations there is no external surface pressure acting on the PtdIns(4,5)\(P_2\) molecule and there is only one PtdIns(4,5)\(P_2\) molecule, eliminating the effect of electrostatic repulsion or hydrogen bonding between neighboring molecules. However, these data confirm the quantitative trend that binding of Mg\(^{2+}\) slightly lowers the size of PtdIns(4,5)\(P_2\) and binding of Ca\(^{2+}\) significantly increases this effect. When the surface area decreases are normalized by the average surface area of PtdIns(4,5)\(P_2\) in water with Na\(^+\), the trend from the simulations compares favorably with the experimental results (Figure 2.14a). These observations hint that the binding of Ca\(^{2+}\) to PtdIns(4,5)\(P_2\) may lead to desolvation of the ion and displacement of waters that hydrogen bond with the PtdIns(4,5)\(P_2\) phosphomonoester groups.

PtdIns(3,5)\(P_2\) has a much larger area per molecule compared with PtdIns(4,5)\(P_2\) and is much less affected by the addition of divalent ions. Ca\(^{2+}\) and Mg\(^{2+}\) both decrease the area per molecule
by $< 5 \text{Å}^2$. We compare these results with simulations involving PtdIns(4,5)$P_2$ and PtdIns(3,5)$P_2$ packed in a bilayer in chapter 4 using an independent method for judging the size of the molecules.
Figure 2.12: Top: The distance of the QM waters to the nearest PtdIns(4,5)P₂ atom in the presence of Ca²⁺ and Mg²⁺. Middle: The average number of hydrogen bonds between QM waters and PtdIns(4,5)P₂ in the presence of Ca²⁺ and Mg²⁺. Bottom: e, a snapshot of Ca²⁺ and the QM waters surrounding PtdIns(4,5)P₂; f, motion of the atoms during the simulation with Ca²⁺ drawn as lines every 0.1 ps; g, a snapshot of Mg²⁺ and the QM waters surrounding PtdIns(4,5)P₂; h, motion of the atoms during the simulation of Mg²⁺ drawn as lines every 0.1 ps, highlighting movement of the Mg²⁺ ion and QM waters.
Figure 2.13: The free energy of spreading the phosphate groups of PtdIns(4,5)P_2 in the presence of K^+, Ca^{2+}, and Mg^{2+}. The insets show Mg^{2+} on the red curve, with smaller blue spheres representing the oxygen atom of the first shell of water molecules, and Ca^{2+} on the blue curve. The shading is the difference in computed free energy using WHAM when analyzing only the first half of each window and using the entire trajectory.

Figure 2.14: Left: The average head-tail angle of PtdIns(4,5)P_2 and PtdIns(3,5)P_2 in the presence of monovalent and divalent ions. Middle: The average area per molecule of PtdIns(4,5)P_2 and PtdIns(3,5)P_2 in the presence of monovalent and divalent ions. Right: The average area per molecule of PtdIns(4,5)P_2 normalized by the area with Na^+ compared to experimental results. Error bars on the left show the 25th and 75 quartile, and the range is marked by horizontal lines. Error bars on the middle panel represent the standard deviation of the area measurement at every frame in the trajectory.
The effect of divalent ions on the protonation of PtdIns(4,5)P$_2$

We also investigated whether the presence of Ca$^{2+}$ or Mg$^{2+}$ affected the free energy, and thus likelihood, of having a proton bound to the 5-phosphate group of PtdIns(4,5)P$_2$. We used one-dimensional QM/MM US, as before. After surmounting a barrier of $\sim 5$ kcal/mol to pull the proton more than 1.5 Å away from its covalently bound oxygen, the presence of Ca$^{2+}$ makes proton dissociation favorable. The final state, with the proton dissociated from PtdIns(4,5)P$_2$ and bound to a hydronium ion is more favorable than the initial state by approximately 8 kcal/mol. Compare Figure 2.15 with Figure 2.10 which is reproduced by its side for comparison.

Figure 2.15: Left: the free energy for deprotonating PtdIns(4,5)P$_2$ in the presence of non-interacting Na$^+$. Right: The free energy for dissociating a proton from the 5-phosphate group of PtdIns(4,5)P$_2$ in the presence of Ca$^{2+}$ and Mg$^{2+}$. The shading is the difference in computed free energy using WHAM when analyzing only the first half of each window and using the entire trajectory.

Full deprotonation in the presence of Mg$^{2+}$ has not been achieved. Rather, the presence of Mg$^{2+}$ during the US induced proton hopping from the 5-phosphate oxygen to the 4-phosphate ester oxygen. This result is analogous to Figure 2.9d-f, where proton transfer from the 5-phosphate to the 4-phosphate ester oxygen in the presence of Na$^+$ was shown to be unfavorable by upwards of 20 kcal/mol. The presence of Mg$^{2+}$ instead of Na$^+$ decreases this penalty by about half. Taken together, these data suggest that PtdIns(4,5)P$_2$ is protonated on the 5-phosphate group of the inositol ring in pure water, with non-interacting monovalent ions, or with Mg$^{2+}$ bound, but deprotonated with
Our results are consistent with observations that Mg$^{2+}$ is only about one-third as effective as Ca$^{2+}$ ions in reducing the surface potential of mixed PtdIns(4,5)$P_2$ vesicles (Toner et al., 1988), leading to the implication that the intrinsic association between Mg$^{2+}$ and PtdIns(4,5)$P_2$ is weaker than the intrinsic association between Ca$^{2+}$ and PtdIns(4,5)$P_2$. The protonation state of PtdIns(4,5)$P_2$ appears to follow the same trend as phosphatidic acid, whose phosphate group becomes doubly deprotonated at physiological pH in the presence of divalent or trivalent ions, particularly Ca$^{2+}$, due to electrostatic correlations (Wang et al., 2012a). Our results for ion binding to solvated PtdIns(4,5)$P_2$ can be extended to the membrane environment by following the methodology of Loew, et al. 2013 and will be discussed in chapter 4.

2.4 Conclusions

In this chapter, we used quantum level calculations to find the optimal geometry and charge distribution of PtdIns(4,5)$P_2$ and PtdIns(3,5)$P_2$. We determined the free energy of having a proton on the 5-phosphate, the 4-phosphate, or being completely deprotonated. We analyzed the interaction of PtdIns(4,5)$P_2$ and PtdIns(3,5)$P_2$ with water molecules in the presence of counterions and how these ions displace at least one water from hydrogen bonding with PtdIns(4,5)$P_2$ and PtdIns(3,5)$P_2$. We also showed how Mg$^{2+}$ and Ca$^{2+}$, in particular, significantly decrease the area of PtdIns(4,5)$P_2$ whereas PtdIns(3,5)$P_2$ is mostly unaffected by these ions.

Our calculations are consistent with estimates of the charge on PtdIns(4,5)$P_2$ and PtdIns(3,5)$P_2$ under physiological conditions, and the noted ability of Ca$^{2+}$ to induce phase transitions in monolayers and membranes containing PtdIns(4,5)$P_2$, with a reduced effect on PtdIns(3,5)$P_2$. Moreover, we predict that PtdIns(4,5)$P_2$ carries a negative charge of -4 at physiological pH but the addition of Ca$^{2+}$ is able to induce deprotonation upon binding. This reduces the increases the negative charge of PtdIns(4,5)$P_2$ to -5 but is compensated by the +2 charge of Ca$^{2+}$ giving rise to a complex that carries net charge of -3. Mg$^{2+}$ is unable to bind as tightly and does not lead to deprotonation of
PtdIns(4,5)P$_2$. In the next chapter, we discuss the nature of Ca$^{2+}$ and Mg$^{2+}$ binding to PtdIns(4,5)P$_2$ and PtdIns(3,5)P$_2$ and the free energy of association.

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Divalent ion binding to PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$

In this chapter, we delve deeper into the interactions between PtdInsP$_2$ and physiological counterions with the goal of describing the strength of these interactions, which interactions may be dominant in a cellular context, and how these interactions modulate the properties of PtdInsP$_2$ we calculated in chapter 2. In the process of evaluating these goals, we will have the opportunity to evaluate several hypotheses, including but not limited to:

**Hypothesis 1** Cellular divalent ions, Ca$^{2+}$ and Mg$^{2+}$, bind more tightly to PtdIns(4,5)P$_2$ and PtdIns(3,5)P$_2$ than cellular monovalent ions, Na$^+$ and K$^+$.  

**Hypothesis 2** Ca$^{2+}$ and Mg$^{2+}$ have preferred equilibrium binding orientations and distances to PtdIns(4,5)P$_2$ and PtdIns(3,5)P$_2$; Ca$^{2+}$ and Mg$^{2+}$ bind more tightly to PtdIns(4,5)P$_2$ over PtdIns(3,5)P$_2$.

**Hypothesis 3** The free energy of binding cellular divalent ions is negative (favorable) for both PtdIns(3,5)P$_2$ and PtdIns(3,5)P$_2$, but there are two distinct forms of binding: direct (tight) binding and water-mediated (weak) binding.

**Hypothesis 4** Binding and forming a complex with Ca$^{2+}$ or Mg$^{2+}$ affects the physical chemistry of
PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\) (judged here using single molecule simulations).

The results of all-atom classical simulations of single molecules integrated with umbrella sampling techniques, analogous to the simulation setups in the previous chapter, have revealed new insights about specific and non-specific interactions between PtdIns\(P_2\) and cellular ions that may be found in complex together in the cell. In particular, cellular divalent ions that undergo fluctuations in their concentration near the membrane interface – \(Ca^{2+}\) and \(Mg^{2+}\) – bind to an unprotonated phosphate group very tightly, whereas \(Na^+\) and \(K^+\) do not. Tight binding by divalent ions is hugely favorable, in terms of free energy, and will occur spontaneously yet neutralization by several monovalent ions is often mediated by one or more water molecules and does not result in direct interactions. \(Ca^{2+}\) binds particularly tightly to PtdIns(4,5)\(P_2\), as two phosphate oxygen atoms displace two waters from its first hydration shell; this does not occur for \(Mg^{2+}\) and as expected, the free energy difference is not as favorable. The presence of the divalent ions affects the protonation state and the charge of the lipid, and thus, its molecular area as we previously demonstrated. It is plausible to expect the size of individual molecules to influence the packing of lipids inside small clusters containing high concentrations of PtdIns\(P_2\). We show that the unique pairing of the 4-phosphate group of PtdIns(4,5)\(P_2\) and \(Ca^{2+}\) leads to a chain of consequences, including deprotonation of the 5-phosphate, pulling the two vicinal phosphate groups closer, decreasing the molecular footprint, and we hypothesize this enables the packing of even more pairs of PtdIns(4,5)\(P_2\) and \(Ca^{2+}\) forming highly concentrated clusters or domains of PtdIns(4,5)\(P_2\) that has been seen \textit{in vitro} and \textit{in vivo}.

3.1 Unconstrained and unbiased divalent ion distributions around PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\)

Our first goal was to characterize the way ions, free to diffuse in a water sphere containing a single molecule of PtdIns(4,5)\(P_2\) or PtdIns(3,5)\(P_2\), distribute around the lipids. The same water sphere setup from the hybrid QM/MM simulations was employed, and waters on the edge were held fixed to
prevent the sphere from expanding. The simulations were completed in the NVT ensemble using Langevin dynamics at 300 K. However, instead of using a quantum functional to evaluate a portion of the system energy, these simulations were run entirely with the CHARMM C36 classical force field, allowing the simulations to access much longer time scales and sample a greater region of phase space.

The simulations carried out for PtdIns(4,5)P₂ and PtdIns(3,5)P₂ had slightly different simulation setups and thus, I will describe them separately. Note that, to some extent, the simulations are sensitive to the initial configuration insofar as an ion seeded close to a lipid will finds its equilibrium position faster than an ion that is not. The data presented here underwent different amounts of equilibration, due to changing computational procedures between the work on PtdIns(4,5)P₂ and the work on PtdIns(3,5)P₂, and therefore, the dynamics of how quickly an ion reaches its equilibrium position should not be interpreted as the kinetics of the event, in my opinion. An additional difference is the inclusion of multiple Ca²⁺ or Mg²⁺ ions in the simulations containing divalent ions and PtdIns(3,5)P₂ to see if ions in a bound state would exchange during the lifetime of the simulation. In the simulations containing Ca²⁺ or Mg²⁺ and PtdIns(4,5)P₂, a single divalent ion was free to diffuse while monovalent Na⁺ ions were fixed at the edge of the water sphere for charge neutrality.

Multiple variants of these simulations were started, as this aspect of the project began before the results of chapter 2 were fully clear and it was not known to us the charge distribution and complete parameter set for PtdIns(4,5)P₂. If a proton is included on the 4-phosphate group of PtdIns(4,5)P₂ instead of on the 5-phosphate group, transient binding to the 4-phosphate group is seen for 2-3 ns before tight binding to the 5-phosphate group takes over. This observation suggests that even a protonated phosphate group at charge -1 is capable of attracting a divalent ion for at least 2-3 ns yet it remains to be seen whether a thermal fluctuation in could cause the divalent ion to dissociate at a later time if it did not bind strongly to the 5-phosphate. If neither phosphate group of PtdIns(4,5)P₂ is protonated, and the charge per lipid is set at -5, then two Ca²⁺ or two Mg²⁺ bind to the lipid at around
4 Å each. That is, two Ca$^{2+}$ or two Mg$^{2+}$ concurrently seem to bind in the weak form to PtdIns(4,5)$P_2$ carrying a net charge of -5. However, this simulation utilized the parameters of (Lupyan et al., 2010) discussed extensively in the last chapter, and should therefore be considered with that in mind. Replacing K$^+$ with Na$^+$ results in very similar distributions, but here K$^+$ was chosen as it has a higher intracellular concentration than Na$^+$ by roughly a factor of 10.

Ca$^{2+}$ and Mg$^{2+}$ exhibit distinct preferred binding distances for PtdIns(4,5)$P_2$ when a single copy of either ion is in a water sphere with PtdIns(4,5)$P_2$ and neutralizing Na$^+$ ions at the edge of the water sphere. Both Ca$^{2+}$ and Mg$^{2+}$ localize to the more negative 4-phosphate group, yet Mg$^{2+}$ has six waters in its first hydration shell on average when bound and Ca$^{2+}$ has four. One of the waters in the first hydration shell for Mg$^{2+}$ is typically positioned in between the ion and the phosphate oxygen atom, which results in an equilibrium binding distance of approximately 4 Å. Hereafter I refer to this as the “weak” form of divalent ion binding. There are no intervening waters when Ca$^{2+}$ is bound (the “tight” form of divalent binding); the four waters in its hydration shell are all oriented on the face furthest from the phosphate oxygen atom and the equilibrium distance for this configuration is approximately 2 Å.

These simulations were seeded from the same input as the QM/MM simulations in Figure 2.12 and the ions were already close to the lipid. A modest energy barrier must be overcome for partial dehydration of the ions and the fact that each ion reached a stable binding position within a couple nanoseconds of equilibration is evidence that the input was close to a local minimum on the energy landscape. Notably, the bound position of Mg$^{2+}$ in the QM/MM simulations is not the same as the bound position in the classical simulation (compare Figure 2.12g vs. Figure 3.1a). One explanation is that the classical force field does not faithfully allow dehydration of the Mg$^{2+}$ ion; this possibility is discussed below where we have used umbrella sampling to overcome the energy barrier associated with stripping waters from the hydration shell. A second, more likely possibility, is that the position of Mg$^{2+}$ in the QM/MM simulations is a local minimum and the extremely short simulation time does not allow significant diffusion.
Although the K$^+$ ions may come within $\sim 5$ Å of an oxygen, the ions leave freely and without always being immediately replaced by another ion (Figure 3.1c). The K$^+$ interaction is always mediated by at least one water molecule (weak), and rarely closer than 4 Å. On some levels, this result is unexpected. One might expect several K$^+$ ions to come as close as possible to the phosphate groups; although the phosphate groups themselves are “multivalent” in a sense, each of the phosphate oxygens not bound to a proton is more precisely described as being a monovalent anion, carrying a partial atomic charge between -0.5 and -1.0. There are at least three mechanisms working against a direct 1:1 interaction of the oxygens and the K$^+$ ions. First, each Coulombic force acting on the K$^+$ ions from the phosphate oxygens is only approximately half as strong as it is on the divalent ions at the same distance (the difference between (+1) and (+2) in the numerator of the Coulomb equation). This interaction is balanced with the thermal energy of the system. The Bjerrum length is likely around 7 Å as discussed in the introduction; if the Coulombic force, screened by water, is not strong enough to attract the K$^+$ ions closer than $\sim 7$ Å then diffusion is just as likely to determine the position of the ions as is the electrostatics. A second factor preventing multiple K$^+$ ions from binding to several oxygens in a single phosphate group or multiple phosphate groups is the mutual repulsion between K$^+$ ions. The free energy for a (+2) ion to bind to two (-1) atoms, connected through a covalent bond, is more negative than for two (+1) atoms to bind to two (-1) atoms in close proximity because although the repulsion from the (-1) atoms is mitigated by their covalent linkage, the same is not true for the mobile (+1) ions. Third, if the Coulombic interaction is not strong enough to dehydrate the K$^+$ ions, then the effective size of each hydrated K$^+$ is significantly larger than a phosphate group, preventing the colocalization of multiple K$^+$ ions to a phosphate group. There are also other unfavorable entropic effects that would occur with the confinement of multiple (+1) atoms instead of a single (+2) atom.

The simulations with Ca$^{2+}$ or Mg$^{2+}$ and PtdIns(3,5)$P_2$ were performed with either two mobile Ca$^{2+}$ or two mobile Mg$^{2+}$, unlike the simulations above where a single divalent ion was mobile. Here, both Ca$^{2+}$ and Mg$^{2+}$ exhibit different behavior than they do with PtdIns(4,5)$P_2$. A single Ca$^{2+}$ binds
Figure 3.1: The equilibrium distances between divalent ions (a) and monovalent ions (b) to PtdIns(4,5)\(P_2\). Ca\(^{2+}\) binds close to PtdIns(4,5)\(P_2\) (tight) whereas the interaction with Mg\(^{2+}\) is mediated by a water molecule (weak). Multiple K\(^{+}\) ions fluctuate between 8-16 Å away (c) from the phosphate groups of PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\) (b, c, and e). While Ca\(^{2+}\) forms a water-mediated bond with PtdIns(3,5)\(P_2\) at 4 Å, (d and f), Mg\(^{2+}\) does not seem to reach a stable equilibrium distance. In all graphs, the dashed lines are at exactly 2 Å and 4 Å. The distances are measured to the 4-phosphate phosphorus atom for PtdIns(4,5)\(P_2\) and to the 3-phosphate phosphorus atom for PtdIns(3,5)\(P_2\).

to the unprotonated 3-phosphate of PtdIns(3,5)\(P_2\) but in a partially weak form. There are five waters in the first hydration shell of Ca\(^{2+}\) but they are not completely distributed on the far side. Interestingly, the bound Ca\(^{2+}\) has fluctuations of \(~2\) Å away from the partially weakly bound position whereas the tight binding of Ca\(^{2+}\) to PtdIns(4,5)\(P_2\) showed no fluctuations over 12 ns. Perhaps the fluctuations of the Ca\(^{2+}\) position in this case are coupled to the motion of the phosphate groups. The second Ca\(^{2+}\) is never closer than \(~10\) Å when the first Ca\(^{2+}\) is bound and is likely not interacting at all. Curiously, Mg\(^{2+}\) does not show strong, stable interactions – either tight or weak – with PtdIns(3,5)\(P_2\). There

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are instances where a Mg$^{2+}$ ion comes within 4 Å of the phosphate groups, but it does not remain there (dark red line). Between 18 ns to 35 ns, a Mg$^{2+}$ spends most of the interval around 8 Å from the phosphate groups, but it is not bound in any conventional sense. Although the Mg$^{2+}$ represented by the dark red line does not form a bond with the lipid, the presence of this ion between 8 and 24 Å seems to exclude the second Mg$^{2+}$ from that region (light red line). The distribution of the K$^{+}$ ions is similar for PtdIns(3,5)$P_2$ and PtdIns(4,5)$P_2$.

To continue this work, seeding a simulation with both Ca$^{2+}$ and Mg$^{2+}$ would be a clear extension. It is reasonable to expect that if Ca$^{2+}$ binds first, Mg$^{2+}$ will be attracted to the remaining negative charge on the lipid and localize closer than the Bjerrum length, but no closer than 4 Å. Mg$^{2+}$ is an order of magnitude more concentrated inside the cell than Ca$^{2+}$ and does not undergo the same fluctuations in concentration as Ca$^{2+}$ if Mg$^{2+}$ binds first at 4 Å, whether Ca$^{2+}$ could displace the Mg$^{2+}$ or not would be difficult to predict. The competition between Ca$^{2+}$ and Mg$^{2+}$ binding to charged lipids has been investigated experimentally from shortly after the isolation of PtdIns(4,5)$P_2$, by determining the partitioning of radioactive $^{45}$Ca$^{2+}$ in PtdIns(4,5)$P_2$-containing solutions or pH titration in the presence of divalent cations (Dawson, 1965; Hendrickson and Fullington, 1965). In those early experiments, the apparent preference for Ca$^{2+}$ over Mg$^{2+}$ was found to be 1 to 2.5 times. Later studies using monolayers or membranes containing PtdIns(4,5)$P_2$, found that the preference may be up to 21 times (Toner et al., 1988). Wang (2013) quantitatively calculated the competitive binding of Ca$^{2+}$ and Mg$^{2+}$ by measuring the Langmuir adsorption of divalent cations to PtdIns(4,5)$P_2$-containing monolayers. The apparent dissociations constants for Ca$^{2+}$ and Mg$^{2+}$ are $4.6 \pm 1.3 \mu M$ and $7.7 \pm 1.8 \mu M$, respectively. Whether this preference holds for PtdIns(3,5)$P_2$ as well has not been tested experimentally and our results suggest the preference, if any, for Ca$^{2+}$ over Mg$^{2+}$ binding to PtdIns(3,5)$P_2$ is slight.

The distribution of both unconstrained divalent ions, either Ca$^{2+}$ or Mg$^{2+}$, in the PtdIns(3,5)$P_2$ simulations were grouped at every time step so the relative probability of having a divalent ion at any given distance could be computed (Figure 3.2a). The cumulative frequency, as the integral of
the relative frequency, reveals information about the probability of finding both divalent ions within a
given radial distance from the PtdIns(3,5)P₂ (Figure 3.2b). The probability of finding both Ca²⁺ ions
within 10 Å is nearly 0.5, indicating the probability of finding one Ca²⁺ within 10 Å is nearly 1. On
the other hand, the probability of finding both Mg²⁺ ions within 10 Å is only 0.2, less than half the
probability for Ca²⁺. When Ca²⁺ is bound to the 3-phosphate, it oscillates between two phosphate
oxygens (Figure 3.2c and d) with a half-life time of around 18-19 ns.

Figure 3.2: The relative (a) and cumulative (b) probability distribution of divalent ions around
PtdIns(3,5)P₂. A single Ca²⁺ binds to the 3-phosphate group and hops between two oxygens (c)
with a half-life of around 18 ns (d).
3.2 The free energy of PtdIns(4,5)P$_2$ and PtdIns(3,5)P$_2$ to bind Ca$^{2+}$ and Mg$^{2+}$

The unconstrained simulations in the previous section reveal the preference for binding, but do not have enough sampling to determine the free energy differences between slightly different binding positions. Using umbrella sampling, we can bias the simulations to conformations of interest and assess the free energy difference between the states. One of the first free energy calculations with divalents that we performed was measuring the preference for Ca$^{2+}$ to bind solely to the 4-phosphate (far away from the 5-phosphate) or to both the 4-phosphate and the 5-phosphate of PtdIns(4,5)P$_2$ at the same time. That is, we were trying to answer whether bound Ca$^{2+}$ is chelated by one or two phosphate groups of PtdIns(4,5)P$_2$.

Two dimensional umbrella sampling was used with a priori reaction coordinates as the distance between oxygens on the 4-phosphate and 5-phosphate groups of PtdIns(4,5)P$_2$. The simulation began with Ca$^{2+}$ bound to the 4-phosphate ($\sim 2$ Å, as shown above) and $\sim 7$ Å from the 5-phosphate (Figure 3.3c, orange). As the distance to the 5-phosphate was decreased, while remaining 2 Å from the 4-phosphate (Figure 3.3c, green), there was no increase in free energy. Next, the distance to the 4-phosphate was increased while the distance to the 5-phosphate was held fixed around 3.3 Å. This process produced an increase in free energy and as the Ca$^{2+}$ was brought closer to both phosphates in conjunction (Figure 3.3c, red), the free energy remained significantly higher than binding to the 4-phosphate alone.

This calculation does not answer the question of the free energy that it takes to bring in Ca$^{2+}$ or Mg$^{2+}$ from bulk and alternatively, the free energy to dissociate a bound divalent ion from PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$. To answer that, we performed a separate set of calculations using the distance between the 3-phosphate of 4-phosphate of PtdIns(3,5)P$_2$ or PtdIns(4,5)P$_2$, respectively, and Mg$^{2+}$ or Ca$^{2+}$ as the a priori umbrella sampling reaction coordinate. The simulations began with divalent
Figure 3.3: The preference for Ca$^{2+}$ to bind to just the 4-phosphate or the 4-phosphate and 5-phosphate of PtdIns(4,5)P$_2$ at the same time. The free energy contours (a) and the thermodynamic sampling trajectory (b) with insets (c) showing renderings of the state marked by the red, green, and orange circles on the sampling path.

ions positioned at their equilibrium binding positions and a series of umbrella sampling windows were constructed to force the divalent ions away from those positions. A biasing potential of 50 kcal/mol/Å$^2$ with windows of 0.25 Å was used. At the beginning of each umbrella sampling window, two preliminary trajectories were completed to move the divalent ion to the new constraint and to equilibrate the system at the new constraint. The simulations were run in a water sphere with Langevin dynamics, identical to the unconstrained simulations in the last section.

The starting positions and snapshots showing the divalent ions during throughout the US windows are shown in Figure 3.4b,c,e, and f. To compare free energy differences between simulations, we offset the curves so the free energy in the final widow is zero. Ca$^{2+}$ binding to the 4-phosphate group of PtdIns(4,5)P$_2$ is an overwhelmingly favorable event by about 80 kcal/mol (Figure 3.4a, c, and h). An ionic bond between two monovalent atoms is on the order of 4-7 kcal/mol (e.g., NaCl) but it takes 50 to 150 kcal/mol to break a covalent bond. If a Ca$^{2+}$ binds to PtdIns(4,5)P$_2$ in vivo, it is
difficult to imagine that the ion would dissociate without being replaced by something that is able to stereospecifically coordinate the phosphate oxygen atoms just as well. Coming in from bulk is almost entirely energetically downhill and associated with stripping a single water from the hydration shell of Ca\(^{2+}\) around \(\sim 4.5\ \text{Å}\) and a second water around \(\sim 2\ \text{Å}\) (more discussion of the hydration in section 3.3). Ca\(^{2+}\) binding to the 3-phosphate group of PtdIns(3,5)\(P_2\) is favorable by approximately 5 kcal/mol although there an energy barrier greater than the free energy difference between the fully bound and the fully free state (Figure 3.4b,c, and g). This energy barrier, most intense between 2 and 4 Å, is consistent with unfavorable solvent interactions of the Ca\(^{2+}\) with 5 water molecules in its first hydration shell. A similar rise in free energy is seen when Ca\(^{2+}\) moves from 2 Å to 3.5 Å away from PtdIns(4,5)\(P_2\), but in that case, the Ca\(^{2+}\) ion is transitioning from 4 water molecules in its first hydration shell to 5.

Figure 3.4: The free energy to dissociate divalent ions from PtdIns(3,5)\(P_2\) (b, e, f, g) or PtdIns(4,5)\(P_2\) (c, h). Panel (a) compares Ca\(^{2+}\) binding to PtdIns(3,5)\(P_2\) and PtdIns(4,5)\(P_2\). Panel (d) compares two Mg\(^{2+}\) binding positions to PtdIns(3,5)\(P_2\). Some curves were reproduced for clarity and comparison. The blue line in panel (a) is the blue line in panel (g). The purple line panel (a) is the blue line in panel (h). The red line in panel (d) is the red line in panel (g).
The unconstrained simulations showed Ca\(^{2+}\) binding to PtdIns(3,5)\(P_2\) around 4 Å away from the 3-phosphate group (Figure 3.1d), placing it always on the far side of the barrier in the energy landscape (Figure 3.4g). Thus our intuitive approach of assigning two different bond modes — tight and weak — reflects fundamental differences in the underlying energy landscapes: in one case, the energy landscape is completely downhill and the tight binding state is freely accessible to the ion, whereas in the other case, the energy landscape contains a hill that obscures the tight binding state, leaving only the weak state as a local minimum.

The energy landscape for Ca\(^{2+}\) and Mg\(^{2+}\) to bind PtdIns(3,5)\(P_2\) are similar to each other, although the energy difference between even the weak binding state and being in bulk is not significant for Mg\(^{2+}\). This explains why Mg\(^{2+}\) tends to not display an obvious preference for binding to the weak state over being further away at 8 Å or more (Figure 3.1d) and a cumulative probability of only 40% for one Mg\(^{2+}\) to be within 10 Å (Figure 3.2b).

We hypothesized that Mg\(^{2+}\) might prefer to bind between the two phosphate groups of PtdIns(3,5)\(P_2\), due to its large hydrated radius and the increased spread between the non-vicinal phosphate groups of PtdIns(3,5)\(P_2\). Umbrella sampling simulations targeted to the 4-hydroxyl group of PtdIns(3,5)\(P_2\) revealed an energetically unfavorable result, with the upturn in energy beginning around 4 Å. Over the course of this simulation, the first hydration shell of Mg\(^{2+}\) maintained six waters. The electrostatic attraction of the phosphate groups in this geometry was insufficient to induce partial dehydration and the large size of the fully hydrated cation was not able to be accommodated in the space between the phosphate groups.

The attraction of Mg\(^{2+}\) to the 4-phosphate of PtdIns(4,5)\(P_2\) is extremely interesting and unexpected (Figure 3.4h). Until about 5 or 6 Å away from the 4-phosphate group, Mg\(^{2+}\) and Ca\(^{2+}\) appear similar. At this point, their energies sharply diverge. Ca\(^{2+}\) loses a water from its hydration shell but Mg\(^{2+}\) does not. Forcing Mg\(^{2+}\) to come closer without decreasing its effective radius by reducing the number of waters in its hydration shell is the same unfavorable event that occurred as Mg\(^{2+}\) was forced between the two phosphate groups of PtdIns(3,5)\(P_2\). Eventually, as
Mg$^{2+}$ comes to within about 2 Å of the 4-phosphate group it loses a water is the potential of mean force drops over 25 kcal/mol within 0.5 Å. This dip makes the binding of Mg$^{2+}$ at 2 Å slightly more favorable than being in bulk phase but, crucially, not as favorable as being between 4 and 8 Å. It should be noted that the minimum for Mg$^{2+}$ between 4 and 8 Å is shallow and broad, so many conformations can fit into this well and the true probability of being in that region of phase space may be underrepresented on this one-dimensional landscape.

Umbrella sampling was also carried out with other distance constraints in the presence and absence of a proton on the 5-phosphate group of PtdIns(3,5)$P_2$. Those are not discussed here, but in general, both with and without a proton (charge states -4 and -5), it was unfavorable for Ca$^{2+}$ or Mg$^{2+}$ to bind to the 4-hydroxyl.

### 3.3 Hydration of Ca$^{2+}$ and Mg$^{2+}$ during approach to PtdIns(3,5)$P_2$ and PtdIns(4,5)$P_2$

To accurately interpret the potentials of mean force in terms of the hydration of the ions, we determined how many waters in the hydration shells of Ca$^{2+}$ and Mg$^{2+}$ by averaging over the states in the unbiased simulations when the ions were not bound to the lipid (i.e., not stripped of any waters) but still likely feeling in the influence of the lipid. We find the hydrated radius, that is, the distance from the “center” of the divalent ion to the tail of the probability distribution of water oxygens, of Mg$^{2+}$ in the CHARMM C36 force field to be $\sim$ 2.28 Å and Ca$^{2+}$ to be 2.60 Å. This contrasts with experimental data from a variety of techniques that the bare ionic radius of Mg$^{2+}$ (67 pm to 90 pm) is smaller than Ca$^{2+}$ (99 pm to 140 pm) but the hydrated radius of Mg$^{2+}$ (300 pm to 700 pm) is larger than Ca$^{2+}$ (260 pm to 630 pm) (reviewed in [Wang, 2013](#Table 3-1)). Due to the variability among different measurements (whether crystallized, in solvent, in an electric field, or other non-biological context) and the uncertainty of the individual measurements, the exact difference in hydrated radius of Ca$^{2+}$ and Mg$^{2+}$ is not clear.
The coordination number for Ca\(^{2+}\) ions ranges from 6 to 8, but in crystal structures that have Ca\(^{2+}\) present, fewer waters are bound to Ca\(^{2+}\) than Mg\(^{2+}\) at the ratio of 1.5 to 2.2, suggesting the waters are more weakly bound to Ca\(^{2+}\) when interacting with biological molecules (i.e., when forming a Ca\(^{2+}\)–O bond with a protein) \cite{Katz1996}. *Ab initio* quantum calculations at the Hartree-Fock MP2/6-31G* (and above) computational level, similar to our single molecule geometry optimizations, revealed the average bond length for the first hydration shell of Ca\(^{2+}\) with six waters to be 2.443 Å compared with 2.097 for Mg\(^{2+}\) with six waters in an octahedral geometry \cite{Katz1996, Bock1994}. These data are consistent with the peak values in Figure 3.5a to within less than 0.5 Å. The net charge on Ca\(^{2+}\) with six coordinating waters is +1.57 compared with just +1.18 for Mg\(^{2+}\). Although this effect is not directly captured in the classical force field, the difference in hydration structure implicitly modulates the effective attraction between the divalent ion and anions due to differences in the charge separation.

Determining the hydration enthalpy of these ions, and the more detailed quantity of the energy required to strip just one or two water molecules from the first hydration shell, without confounding factors is not a trivial task experimentally or computationally. As a caveat, like the hydrated size of the ions, values for the hydration enthalpy range considerably. The computational studies of \cite{Katz1996, Bock1994} report it takes \(~ 4\) kcal/mol to dehydrate Mg\(^{2+}\) from six to five waters and \(~ 9\) kcal/mol to go from five to four. For Ca\(^{2+}\), it takes \(~ 10\) kcal/mol to go from six to five waters but only an additional 3 kcal/mol to go from five to four. That this second step, from five coordinating waters to four coordinating waters, is different by a factor of two for Ca\(^{2+}\) and Mg\(^{2+}\) may be crucial. Further, as Ca\(^{2+}\) is dehydrated its hydrated radius decreases and the charge on the ion increases; the magnitude of these changes are larger for Ca\(^{2+}\) than for Mg\(^{2+}\).

During umbrella sampling, the number of coordinating waters to the ions was tracked (Figure 3.5b), using the distances in Figure 3.5a. The results are particularly striking for the attraction between the ions and PtdIns(4,5)P\(_2\). It is clear that Mg\(^{2+}\) undergoes dehydration from six to five waters at 2 Å, corresponding to the peak in Figure 3.4h. The broad well between 3 and 8 Å on
that figure likely reflects a rearrangement of the coordinating waters during approach, rather than dehydration. Ca$^{2+}$ undergoes two dehydration events, one at 5 Å and one at a distance of a little less than 3 Å from the 4-phosphate group of PtdIns(4,5)$P_2$. These two events line up with the two large decreases in the potential of mean force in Figure 3.4 and qualitatively the drops are proportional to the differences noted by (Katz et al., 1996; Bock et al., 1994).

The results in the presence PtdIns(3,5)$P_2$ are less clear but in line with the observation that the potential of mean force to bind Ca$^{2+}$ and Mg$^{2+}$ are more similar than different. At large separations from PtdIns(3,5)$P_2$, both ions have seven, not six, coordinating water molecules. A natural followup to this analysis is to quantify the hydration number of Ca$^{2+}$ and Mg$^{2+}$ in simulations with pure water, without any lipid, to see if seven or six water in the first hydration shell is more likely. Mobile counterions (e.g., Cl$^-$) would be required for charge neutrality, but a sufficiently dilute solution could
be used to decrease their influence on the divalent ions. Estimating the difference in successive water binding energy becomes complex at this level, especially for Mg$^{2+}$, where second shell effects may become important, at least when performing calculations at the B3LYP level (see Rodriguez-Cruz et al., 1999, Table 2), but may be on the order of $\sim 20$ kcal/mol for Mg$^{2+}$ and $\sim 10$ kcal/mol for Ca$^{2+}$. On average, between 3 Å and 2 Å away from the 3-phosphate group of PtdIns(3,5)$P_2$, both divalent ions are stripped of two waters, bringing their coordination number down to five, aligned with the rise and drop in free energy (Figure 3.4b).

### 3.3.1 Distances to phosphate oxygen atoms when Ca$^{2+}$ and Mg$^{2+}$ bind PtdIns(3,5)$P_2$ and PtdIns(4,5)$P_2$

The next piece of the puzzle is to look at what happens when waters leave the hydration shell of Ca$^{2+}$ and Mg$^{2+}$. The distance between the divalent ions and each phosphate oxygen atom is tracked in Figure 3.6. As discussed above, when Ca$^{2+}$ and Mg$^{2+}$ bind to PtdIns(3,5)$P_2$, two water molecules leave their first hydration shell between 3 and 2 Å away from the 3-phosphate group but this is not associated with a significant decrease in free energy (i.e., the energetic cost to dehydrate the ions is barely compensated for by binding to the oxygens). In these graphs, the red line corresponds to the a priori reaction coordinate for umbrella sampling and indeed, binds tightly to both Ca$^{2+}$ and Mg$^{2+}$. The two other 3-phosphate oxygen atoms stay around 4 Å away, in the so-called water-mediated “weak” binding regime. Hopping between phosphate oxygens is not seen here, as in the unconstrained simulations, due to the 50 kcal/mol/Å$^2$ constraint.

In contrast, when Ca$^{2+}$ binds to PtdIns(4,5)$P_2$, two waters are stripped from its hydration shell and it binds tightly to two phosphate oxygens atoms. This exchange is extremely favorable. The third oxygen on the 4-phosphate is in the weak binding regime. Notably, the 5-phosphate oxygens (recall that two are unprotonated [more negative] and one is protonated) are just as far (or farther) from Ca$^{2+}$ bound to the 4-phosphate of PtdIns(4,5)$P_2$ than they are when Ca$^{2+}$ is bound to 3-phosphate of PtdIns(4,5)$P_2$. That is, the separation between the phosphate groups of PtdIns(4,5)$P_2$ are farther
apart than the phosphate groups of PtdIns(3,5)P_2 when Ca^{2+} is bound, despite an extra C–C bond length on the inositol ring between the phosphate groups of PtdIns(3,5)P_2. The large splay in the phosphate groups of PtdIns(4,5)P_2 can be seen by comparing the renderings in Figure 3.4b and c.

As Mg^{2+} approaches PtdIns(4,5)P_2 there is broad energy well from 8 to 3 Å and a sole dehydration event at 2 Å, with another drop in energy. When the a priori reaction coordinate is between 8 and 3 Å, all three 4-phosphate oxygen atoms are approximately the same distance away from Mg^{2+}. When the reaction coordinate drops to 2 Å, a single oxygen atom is bound, with the other two 4-phosphate oxygens weakly binding. The three 5-phosphate oxygens are closer to Mg^{2+} than they are when Ca^{2+} is bound, supporting the hypothesis that Ca^{2+} and Mg^{2+} induce different conformations of PtdIns(4,5)P_2.

Thus far we have evaluated the first three hypotheses on page 54 that we set out to address in this chapter. In unconstrained simulations, divalent ions show preferential and closer binding compared to monovalent ions. Ca^{2+} and Mg^{2+} exhibit two characteristic binding modes corresponding to direct or water-mediated binding. Ca^{2+} binds extremely tightly to PtdIns(4,5)P_2 after losing two water molecules from its hydration shell whereas Mg^{2+} binds with similar affinity to either PtdIns(3,5)P_2 or PtdIns(4,5)P_2. Many of the properties we discussed in chapter 2 depend on the protonation state of the molecule. Now that the binding modes have been established, we can revisit the free energy of protonation in the presence of monovalent or divalent ions (Figure 2.15) and demonstrate how the energy landscapes can be used to predict molecular properties like the logarithmic acid dissociation constant, pKa, which has consequences for the size and shape of the lipid and its ability to cluster.
Figure 3.6: The distance between Ca$^{2+}$ and Mg$^{2+}$ ions binding to all six inositol phosphate oxygens on PtdIns(3,5)P$_2$ (a, b) and those ions binding to all six inositol phosphate oxygens on PtdIns(4,5)P$_2$ (c, d). The time scale does not reflect actual dynamic events; more sampling was performed for certain umbrella sampling simulations to ensure smoothness of the energy landscape (i.e., this is evidenced by changes in the slope of the distance corresponding to the reaction coordinate). As long as there is overlap of the reaction coordinate between adjacent sampling windows, the WHAM technique will robustly converge on a smooth energy landscape (convergence tolerance and number of bins are the only adjustable parameters).

3.4 pKa of protonation in the presence of Ca$^{2+}$ and Mg$^{2+}$

It is possible to calculate pKa values from the free energy landscapes for protonation in the presence of Na$^+$, Ca$^{2+}$ or Mg$^{2+}$, presented Figure 2.15. To do this, we consider the canonical definition of the association constant, $K_a$, between a ligand, $L$, and its binding partner, $R$, in terms of the ratio of the probabilities of being not bound or bound. In this case, we take the ligand to be a proton and its binding partner to be PtdIns(4,5)P$_2$ although this calculation would work equally well with
PtdIns(3,5)P₂. So we write,

\[ K_a = \frac{1}{[L]} \times \frac{P_{\text{bound}}}{P_{\text{unbound}}} \]

where \( P_{\text{bound}} \) and \( P_{\text{unbound}} \) can be determined by integrating over the degrees of freedom and the potentials of mean force. pKa is related to \( K_a \) using the following relationship:

\[ \text{pKa} = \log_{10} \left| K_a \times \frac{1}{1661 \text{ Å}^3} \right| \]

using the standard concentration of 1 mol/liter = 1/1666 Å³.

Note that the larger the pKa, the smaller the extent of dissociation at a given pH. In some conventions, \( K_a \) is defined with the unbound state in the numerator and the bound state in the denominator, in which case, the pKa expression would have a negative sign.

We can perform the pKa calculation in two ways. In the first method, we integrate the bound state a distance we call \( r = r^* \) which is the point where the proton leaves the domain of influence of the oxygen to which it was originally bound (snapshots of our choice of \( r^* \) are shown in Figure 3.7b). This is judged by viewing the simulation trajectories and looking at the minima of the potentials of mean force. The unbound state is integrated from \( r = r^* \) until the final point in the energy landscape, offsetting all the potentials of mean force so the last window has a reference free energy of 0.

In the second method, we integrate the bound state until \( r = r^* \) as before. To evaluate the unbound state, we integrate from \( r = r^* \) until the next metastable minimum at \( r = r^{**} \). In this technique, events that we categorize as dissociation, such as proton transfer to other atoms on PtdIns(4,5)P₂, may not correspond to dissociation events judged from experiments. That is, dissociation of the proton from a specific oxygen and rebinding to a different oxygen might not be detected in experiments that have reported the pKa of the ionizable groups on charged lipids. Values for \( r^* \) and \( r^{**} \) and the results of these calculations are shown in Figure 3.7. Error bars in the pKa calculation represent the outcome of changing the value of \( r^* \) by 0.25 Å or approximately the width of a well in the potential of mean force.

We stress that the absolute value of these numbers will not exactly match the results of
experiments due to the approximations necessary in obtaining absolute free energy curves from the potentials of mean force calculated along a single reaction coordinate. (Kooijman et al., 2009) calculate the pKa of PtdIns(4,5)$P_2$ in lipid vesicles, which is different from our case in several respects (e.g., the effect of a negative surface charge density of the vesicle membrane) so a direct comparison is not strictly valid. Moreover, the pKa values reported by Kooijman et al. correspond to PtdIns(3,5)$P_2$ and PtdIns(3,4,5)$P_3$ which could have different ionization properties due to the positioning of the phosphate groups. In fact, for PtdIns(4,5)$P_2$, Kooijman et al. note that a biphasic pH-dependent ionization behavior that cannot be explained by a Henderson-Hasselbach equation was obtained. Our calculations are consistent with this; our free energy landscape does not simply represent two states (bound or unbound) but displays intermediate metastable states. These states correspond to sharing of the proton between the vicinal phosphomonoester groups. Hence, our pKa should be regarded as an apparent pKa for proton transfer from the host oxygen to the next energetically favorable state (rather than a free unbound state).

The subtleties caused by a complex (rugged) free energy landscape and non-independence of proton binding to vicinal sites cause problems in defining an absolute pKa; moreover, the apparent pKa is subject to the definitions of vicinal binding sites as metastable states (or our choice of $r^\star\star$). Another complexity is a computational one. In the QM/MM potentials of mean force, the energy landscape is only accurate for short excursions from the initial bound state along the reaction coordinate. For longer excursions, the wave functions from the QM region are spread over larger volumes causing an imbalance in basis set superposition. Also for these large distances along the reaction coordinate, close interaction between classical charges and the QM wave function become significant. Recall that Na$^+$ is not interacting with the lipid in these QM/MM simulations (and not in the QM region) and so the state is also referred to as just pure water.

In general, we find that the pKa for protonation of PtdIns(4,5)$P_2$ in the presence of Ca$^{2+}$ is the smallest, implying strong dissociation of the proton. The pKa in the presence of Mg$^{2+}$ is similar to that of a pure water environment, where we find protonation of PtdIns(4,5)$P_2$ on the 5-phosphate
group to be the most stable conformation.

Figure 3.7: A method to calculate the pKa for protonation of PtdIns(4,5)P₂ in the presence of monovalent and divalent ions (a) and renderings (b) of the system when the proton (black sphere) is at r = r* and has left the domain of influence of its originally bound oxygen. Ca²⁺ is blue and Mg²⁺ is pink.

3.5 Conclusions

In this chapter we set out to extend our knowledge of PtdIns(3,5)P₂ and PtdIns(4,5)P₂, established from single molecule simulations in chapter 2 to describe how strongly divalent ions interact, which ones are dominant, and the implications for the physical chemistry of PtdIns(3,5)P₂ and PtdIns(4,5)P₂. We have demonstrated that divalent ions bind more strongly than monovalent ions. Ca²⁺, in particular, binds so tightly to PtdIns(4,5)P₂ that any free Ca²⁺ within 10 Å of a patch of plasma membrane rich in PtdIns(4,5)P₂ is likely to lead to association with a biologically significant lifetime, yet Mg²⁺ has a much higher intracellular concentration and is probably constitutively bound to PtdIns(4,5)P₂ and PtdIns(3,5)P₂ through a water molecule. Tight and weak binding affects the protonation state of PtdIns(3,5)P₂ and PtdIns(4,5)P₂ which modulates the net charge and
distribution of charges, head-tail angle, splay of the phosphate groups, and the hydrogen bond
network these lipids form with water. To assess how changes in a single molecule translate to
changes in the properties of the plasma membrane, we move on to discuss simulations of model
membranes containing PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$ with various lipid compositions, charge
states, cholesterol content, and counterions.
Simulations of model membranes containing \( \text{PtdIns(3,5)}P_2 \) and \( \text{PtdIns(4,5)}P_2 \)

This chapter represents our most advanced attempt to address the central theme of this thesis, the hypothesis that the biological role of PPIs is expressed through their effects on the biophysical chemistry of membranes. Planning for this chapter began several years ago and yet the project is so rich with data that I am only able to present a few of the salient results that we have discovered so far. In sum, these simulations are the result of more than 500,000 CPU hours on our local cluster and three high performance computing machines on the XSEDE network (Lonestar4, Kraken, and Trestles). Although not intended, the work for this chapter entailed as much methods development as original research.

The main logistical challenge we faced is one of data structure and organization. I would be remiss if I did not mention that most of the work spent while these simulations were running went into the creation of a python data class that is able to read in short simulation trajectories, stitch them together, and store all the relevant degrees of freedom for a given analysis, and the results of those analyses in a custom object. The workspace and analysis modules can be saved and restored to arbitrary points. This work would not be possible without the assistance of Ryan Bradley. He had a vision to unify our code at a time when I thought it was impossible. As of May 20th, the code base
is over 50,000 lines and is hosted on a public GitHub repository. About a dozen different analysis scripts are complete, using a dictionary lookup that stores key information about each membrane system, and another dozen routines are in various states of completion and debugging.

Through the ingenuity of Ryan, our code is able to interface with coarse-grained membrane simulations and continuum free energy models to extend the scale of our work from Ångstroms to microns. Besides the direct scientific conclusions of this thesis, one tangible outcome of this work is the data structures that have been put in place; these can be used to continue this project and extend it in new directions taking advantage of a pre-built set of algorithms and routines to extract quantitative properties from future simulations. In the coming months and years, the true dividends of these simulations and our codebase will become clear. As more simulation variants with different lipid compositions reach maturity, and perhaps simulations with embedded proteins, our code will be able to tackle more complex questions and automatically compute differences among all the systems.

4.1 Revisiting old data and new questions for simulations of model membranes

These simulations open up investigations on two fronts: first, we want to compare how our single molecule results from chapters 2 and 3 translate into a bilayer setting, and second, new questions that can only be asked when PtdIns(3,5)$_2$ and PtdIns(4,5)$_2$ are embedded in a bilayer. As a blend of returning to old questions and pursuing new ones, we formulated an initial list of questions we would like these simulations to address:

1. What is the size distribution of the lipids?
   
   (a) How does the presence of ions affect the size of PtdIns(3,5)$_2$ and PtdIns(4,5)$_2$ in a bilayer?
   
   (b) How does the charge of PtdIns(4,5)$_2$ affect its size in a bilayer?
(c) What is the difference in size between PtdIns(3,5)P_2 and PtdIns(4,5)P_2 in a bilayer?
(d) Is there a relationship between area per molecule and head-tail angle in a bilayer?

2. How are the lipids distributed within the plane of the membrane and does this distribution change during our simulations?

3. Do different lipid species move at different rates?
   (a) What is the diffusion rate of PtdIns(4,5)P_2?
   (b) How is the diffusion of PtdIns(4,5)P_2 affected by ion binding?

4. Where do ions bind to lipids in the membrane? Do different ions prefer to bind a single charged lipid or in between charged lipids?

5. What is the equilibrium distribution of ions normal to the charged and neutral membrane surfaces?

6. Do ions near the membrane move faster than ions further away? How does the diffusion rate of ions vary as a function of their distance from the membrane surface?

7. What are the mechanical properties of the membrane?
   (a) Does the presence of PtdIns(3,5)P_2 or PtdIns(4,5)P_2 affect the stiffness of a bilayer?
   (b) Is it important to have PtdIns(4,5)P_2 asymmetry for these properties?

8. What is the free energy for PPIs to associate with each other? How likely is it for two lipids to cluster?

The first set of questions can be summarized by the overarching question: what can our simulations say about the structure of physiological membranes? The second set of questions can be categorized around the central theme of: how does the presence of a charged membrane affect the surrounding solvent, as mimics of the plasma membrane and the cytosol? Finally, the last group of questions are centered around: how does lipid heterogeneity affect the bending rigidity and fluctuations of a physiological bilayer?
We have been able to answer many of these questions, but often the analyses involved more subtleties than anticipated and spawned additional questions and more coding. I will discuss our results and challenges below at a level that should aid anyone following this work. Unfortunately, the final question on the list, the free energy for lipids to form clusters, has not been answered by our simulations thus far. Over 100+ ns of production runs, we did not see the formation of higher order lipid structures beyond a dimer, and to accurately answer the question, a separate umbrella sampling simulation using the distance between lipids as the a priori reaction coordinate would be necessary. The configuration for such a simulation is complete, but the results are still pending and depend on very lengthy computations. Before addressing specific results, it is necessary to describe the systems in detail.

4.2 Simulation setup

Construction of each system began with the generation of a random square 200 × 200 grid to place the lipids. A variety of lipid types were arranged along the grid, forming a monolayer or single leaflet of a bilayer. Each membrane bilayer was composed of two monolayers that were independently seeded (i.e., even for symmetric bilayer systems, each monolayer was created independently). We measured the area of each leaflet using values from simulations of pure lipid systems to ensure there was no significant excess area or spontaneous curvature in either leaflet. The two leaflets are placed together and the lipid-only system is minimized with restraints in the z direction, perpendicular to the bilayer, in vacuum first. Water is added at approximately 100 waters per lipid, and the system is equilibrated again; counterions are added (randomly) and the system equilibrated once more. This amount of water is about three times as much as is necessary to fully hydrate the bilayer, yet the extra water is included due to the high charge of our system and the large number of counterions. We used a steepest descent minimization and equilibration run of 500,000 steps with a time step of 1 fs (0.5 ns per equilibration) in the NVT ensemble.
In the first set of simulations, we varied the symmetry of the bilayer leaflets, length of acyl chains, the counterions, the charge state of PtdIns(4,5)P$_2$, substituting PtdIns(3,5)P$_2$ for PtdIns(4,5)P$_2$, and the presence or absence of cholesterol (but not all of the combinations were tested independently). Every leaflet starts with exactly 400 molecules (if a molecule flipped/flopped between leaflets it was usually accompanied with an instability in the simulation). In the absence of cholesterol, there are 800 lipids per system whereas with cholesterol present, there are 600 lipids per system. The systems typically have about 300,000 total atoms and their compositions are listed in Tables 4.1 through 4.7. The most complete systems have reached 100 ns of production simulation time.

<table>
<thead>
<tr>
<th>Internal name</th>
<th>membrane-v509</th>
<th>membrane-v510</th>
<th>membrane-v511</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecule</td>
<td>Symmetric leaflet composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PtdCho</td>
<td>72% DOPC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PtdSer</td>
<td>18% DOPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PtdEtn</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PtdInsP$_2$</td>
<td>10% PtdIns(4,5)P$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ions</td>
<td>150 mM NaCl</td>
<td>150 mM MgCl$_2$</td>
<td>150 mM CaCl$_2$</td>
</tr>
</tbody>
</table>

Table 4.1: Membrane composition for three symmetric membrane systems with different counterion species.

<table>
<thead>
<tr>
<th>Internal name</th>
<th>membrane-v514</th>
<th>membrane-v515</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecule</td>
<td>Symmetric leaflet composition</td>
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</tr>
<tr>
<td>PtdCho</td>
<td>72% DOPC</td>
<td></td>
</tr>
<tr>
<td>PtdSer</td>
<td>18% DOPS</td>
<td></td>
</tr>
<tr>
<td>PtdEtn</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PtdInsP$_2$</td>
<td>10% PtdIns(4,5)P$_2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>charge -3</td>
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<tr>
<td></td>
<td>10% PtdIns(4,5)P$_2$</td>
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<tr>
<td></td>
<td>charge -5</td>
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</tr>
<tr>
<td>Cholesterol</td>
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</tr>
<tr>
<td>Ions</td>
<td>150 mM NaCl</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2: Membrane composition for two symmetric membrane systems with a non-protonated form of PtdIns(4,5)P$_2$ and a doubly-protonated form of PtdIns(4,5)P$_2$.

We chose lipid profiles based on three constraints: using the most recent and advanced lipidomic data from mammalian cells, choosing molecules that were already parameterized for the CHARMM C36 force field with published simulation results, and a desire to include a measurable number of PtdInsP$_2$ residues (1% mole fraction of a 400 lipid leaflet is only 4 molecules). Lipid composition was
based on data from analysis of mammalian cell membranes. Prior to polarization of Madin-Darby canine kidney cells (MDCK), PtdCho was found to be approximately 30% of the total lipid content, PtdSer about 5%, PtdEtn about 10-15 %, PtdIns about 5%, and cholesterol about 20-25%, among many other minor species (e.g., ceramides, plasmalogen, sphingomyelin) (Sampaio et al., 2011). The average chain length, as the sum of the two acyl chains, was found to be 36; the two most common acyl chain lengths for PtdCho are 16:0 / 18:1 (25% of total) and 18:1 / 18:1 (15% of total) where the first number is the carbon length and the second number is the number of double bonds. Of those acyl chains for the glycerophospholipids, approximately 30% contained a single double bond and 20% contained two double bonds. After transitioning to a polarized epithelial phenotype, up to 50% of the glycerophospholipid acyl chains in the apical membrane contained a single double bond and only 15% of the chains contained two double bonds (Gerl et al., 2012). There are few fully saturated glycerophospholipids in natural membranes, although most of the sphingolipids are.

In the symmetric membrane systems, we chose to use a majority of PtdCho with dioleoyl acyl chains (18:1; DOPC) and a minority of PtdSer with dioleoyl acyl chains (DOPS) and PtdIns(4,5)P\textsubscript{2} with 1-stearoyl (18:0) 2-arachidonyl (20:4) acyl chains, the most abundant form for cellular PPIs and a combination of acyl chains that is more common in synaptic membrane compartments than epithelial cell lines (Brockman et al., 2003) (refer to Table 4.1 through 4.2 for symmetric membrane compositions). For asymmetric membrane systems, we placed PtdCho in the “outer” leaflet (N.B., what we call “outer” is arbitrary but is consistent across all asymmetric systems) with 1-palmitoyl-2-oleoyl acyl chains (16:0 / 18:1) and cholesterol. The “inner” leaflet consisted of PtdSer with dioleoyl acyl chains, PtdEtn with dioleoyl acyl chains, PtdIns(4,5)P\textsubscript{2} with 1-stearoyl-2-arachidonyl acyl chains, and cholesterol. Tables 4.5 through 4.7 list the detailed membrane compositions. The charge density on the inner leaflet of the asymmetric systems is about −1 e nm\textsuperscript{−2}, about the same surface charge density of DNA or microtubules (Janmey et al., 2014), and in line with estimates of the charge density of some cellular membranes (McLaughlin, 1977).

The simulations were implemented using the same CHARMM C36 force field in chapters
but the Gromacs simulation package (Pronk et al., 2013) was used to drive these simulations. Recently, several membrane bilayer systems have been simulated using coarse-grained force fields, which are able to use more molecules and reach much longer timescales to collect data on long-range dynamic quantities, like diffusion. These have been very successful at capturing unique events, such as the production of curvature upon protein binding to membranes. However, these results come at the expense of details: the specific chemical interactions between molecules that are grouped together in a coarse-grained bead cannot be disentangled and compared to all-atom results. In the latest iteration of these force fields, the inositol ring of PtdIns(4,5)P$_2$ is a triangle and each phosphate group is a single bead hanging off the triangle. There is no way to resolve the detailed interactions of PtdIns(4,5)P$_2$ with different divalent cations from those simulations and there is no reason to expect that meaningful differences will be manifest, as the goal of parameterizing those force fields was not to properly reproduce the quantities we are interested in here. Connecting and extending our work – perhaps using the knowledge gained about the detailed chemical interactions from these simulations to tune and tweak coarse-grained beads for PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$ – using coarse-grained simulations is an exciting avenue that I expect to be explored within the next year.

As a caveat, the graphs presented in this chapter represent an evolving cycle of analysis. As more simulation time is gathered, the graphs are updated every time we run the code. The simulations involving PtdIns(3,5)P$_2$ have accumulated the least simulation time and are most prone to changing in the coming months. For some questions, we have computed all possible comparisons and for other questions, we have only analyzed a subset of the systems. I have attempted to present the results in way that highlights the salient points with sufficient context to inform relative differences between lipid types, ion species, membrane symmetry or other factors.
### Table 4.3: Membrane composition an asymmetric membrane system with Na\(^+\) counterions.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Leaflet</th>
<th>Outer</th>
<th>Inner</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdCho</td>
<td>75% POPC</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PtdSer</td>
<td>–</td>
<td>15% DOPS</td>
<td></td>
</tr>
<tr>
<td>PtdEtn</td>
<td>–</td>
<td>50% DOPE</td>
<td></td>
</tr>
<tr>
<td>PtdIns(_P_2)</td>
<td>–</td>
<td>10% PtdIns(4,5)(_P_2)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>25% cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ions</td>
<td>150 mM NaCl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4.4: Membrane composition an asymmetric membrane system with Mg\(^{2+}\) counterions.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Leaflet</th>
<th>Outer</th>
<th>Inner</th>
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<tr>
<td>PtdCho</td>
<td>75% POPC</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PtdSer</td>
<td>–</td>
<td>15% DOPS</td>
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</tr>
<tr>
<td>PtdEtn</td>
<td>–</td>
<td>50% DOPE</td>
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</tr>
<tr>
<td>PtdIns(_P_2)</td>
<td>–</td>
<td>10% PtdIns(4,5)(_P_2)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>25% cholesterol</td>
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<td></td>
</tr>
<tr>
<td>Ions</td>
<td>150 mM MgCl(_2)</td>
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<td></td>
</tr>
</tbody>
</table>

### Table 4.5: Membrane composition an asymmetric membrane system with Ca\(^{2+}\) counterions.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Leaflet</th>
<th>Outer</th>
<th>Inner</th>
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<tr>
<td>PtdCho</td>
<td>75% POPC</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PtdSer</td>
<td>–</td>
<td>15% DOPS</td>
<td></td>
</tr>
<tr>
<td>PtdEtn</td>
<td>–</td>
<td>50% DOPE</td>
<td></td>
</tr>
<tr>
<td>PtdIns(_P_2)</td>
<td>–</td>
<td>10% PtdIns(4,5)(_P_2)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>25% cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ions</td>
<td>150 mM CaCl(_2)</td>
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</tbody>
</table>
Table 4.6: Membrane composition an asymmetric membrane system with PtdIns(3,5)P_2 and Mg^{2+} counterions.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Leaflet</th>
<th>Outer</th>
<th>Inner</th>
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<tr>
<td>PtdCho</td>
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<tr>
<td>PtdSer</td>
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<td>15% DOPS</td>
</tr>
<tr>
<td>PtdEtn</td>
<td>-</td>
<td></td>
<td>50% DOPE</td>
</tr>
<tr>
<td>PtdInsP_2</td>
<td>-</td>
<td></td>
<td>10% PtdIns(3,5)P_2</td>
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<tr>
<td>Cholesterol</td>
<td>25% cholesterol</td>
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</tr>
<tr>
<td>Ions</td>
<td>150 mM MgCl_2</td>
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</tbody>
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Table 4.7: Membrane composition an asymmetric membrane system with PtdIns(3,5)P_2 and Ca^{2+} counterions.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Leaflet</th>
<th>Outer</th>
<th>Inner</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdCho</td>
<td>75% POPC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PtdSer</td>
<td>-</td>
<td></td>
<td>15% DOPS</td>
</tr>
<tr>
<td>PtdEtn</td>
<td>-</td>
<td></td>
<td>50% DOPE</td>
</tr>
<tr>
<td>PtdInsP_2</td>
<td>-</td>
<td></td>
<td>10% PtdIns(3,5)P_2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>25% cholesterol</td>
<td></td>
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</tr>
<tr>
<td>Ions</td>
<td>150 mM CaCl_2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2.1 Equilibrium measurements

Production runs were performed with a 2 fs time step, the trajectory written every 1000 steps (2 ps), and the center of mass motion was removed every 10 steps (20 fs). Most of these options follow standard MD conventions but are listed here for completeness; appendix A shows an example file that could be used to continue these simulations. Linear constraints were placed on bonds with hydrogen atoms. Neighbor lists of atoms within 1.2 nm were updated every 10 fs. The Lennard-Jones potential was smoothly shifted to zero between 0.8 nm and 1.2 nm, and no dispersion correction was used (dispersion correction is recommended only for homogenous liquids where the radial distribution function is exactly 1 outside the Lennard-Jones cutoff). The PME method of electrostatics was used with cubic interpolation (order 4) between grid points with a grid spacing of 1.2 Å and cubic periodic boundary conditions in all directions. Temperature coupling was performed using velocity rescaling with a stochastic term and time constant of 0.5 ps at 310 K. This is similar to the Berendsen thermostat, but the stochastic term here ensures the simulation is in the canonical ensemble unlike with Berendsen coupling (Bussi et al., 2007). Pressure coupling was performed using the semi-isotropic Parrinello-Rahman algorithm that links the periodic box vectors to an equation of motion such that the motion in the $x$ and $y$ direction is equal but different from the motion of the $z$ direction (i.e., the bilayer can deform in the (x,y) plane independent of the $z$ direction box vector). The time constant for coupling was 2.0 ps, the reference pressure was exactly 1.0 bar for both (x,y) and $z$, and compressibility was set to $4.5 \times 10^{-5} \text{ bar}^{-1}$ (equal to the compressibility of water at 1 bar and 300 K).

We tried to match many of our simulation parameters to simulations that were used to parameterize the lipids so we could make connections between our systems and simpler ones. Even on these large systems of 300,000 atoms, standard measures of equilibration converge quickly, within the first 10 ns (Figure 4.1). The box vectors (i.e., the dimensions of the system along each axis) play a dominant role in controlling other system properties (e.g. average area per molecule).
The box vectors reach their equilibrium value by 6-10 ns per system, but we generally did not analyze the first 20 ns of the production runs, out of an abundance of caution.

4.3 The structural properties of membranes containing PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$

This section focuses on our attempt to characterize the distribution of lipids in the membrane systems and various aspects of their static properties that do not seem to change once the system reaches equilibrium (e.g., from 40 to 100 ns of simulation time). These properties are broadly represented by the spacing between lipid types (distribution functions), how large the lipids are (area per molecule), how bent the lipids are (head-tail angle), and how stiff the membrane is (bending rigidity). Collectively, comparing these properties between systems will provide insight into how physiological membrane
models are affected by PPIs and ionic conditions.

4.3.1 Lipid-lipid distribution functions

We used a two-dimensional planar radial distribution function, $g(r)$ to describe the distances between lipid pairs. The radial distribution function is most often used to describe how density varies in a fluid (although it is useful for describing any phase of matter), by plotting the probability a particle of type $j$ will be found a given distance $r$ from a particle of type $i$, generally. The result of this calculation provides information about the average density of the fluid, or in our case, the average density of a particular molecular species (or contacts between two different species when $i \neq j$). If the position of the particles depended only on the potential energy $U_{ij}(r)$ between the particles at a given distance, then the radial distribution function would be given by the Boltzmann distribution, such as in an ideal gas; if $U_{ij}(r) = 0$, then $g(r)$ would be exactly 1 everywhere.

For the membrane systems, it is important to keep in mind that the position of the particles (i.e., lipids) is not simply a function of the potential energy between the lipids. The time scale for the movement of the lipids is on the same order as the time scale for the simulations. That is, the lipids do not move more than one or two lipid-distances during the course of the simulation. Therefore, these distributions should be viewed not as specific arrangements of lipids set up by an underlying potential field but rather as average distributions between pairs of lipids. Deviations from 1 at a given value of $r$ means there is excess density of that interaction pair at $r$ relative to bulk and at small distances, $g(r) \rightarrow 0$ because two atoms cannot occupy the same space. Using the nomenclature of a liquid environment, the first peak would correspond to the first hydration shell of the liquid (refer to Figure 3.5a). Figure 4.2 gives an overview of the calculations and examples for early and late time averages for two different pair interactions, POPC–POPC and DOPS–DOPS.

The radial pair distribution function (RDF) for PtdIns(4,5)P$_2$–PtdIns(4,5)P$_2$ contacts reflects the likelihood two PtdIns(4,5)P$_2$ molecules will be separated at a given distance relative to bulk. The roughness of the function is due to the limited number of PtdIns(4,5)P$_2$ particles in the asymmetric
Figure 4.2: a. An illustration of measuring the pair distribution function using the phosphodiester phosphorus atoms in a leaflet. b. The radial distribution function for POPC–POPC at early and late time points. c. The radial distribution function for DOPS–DOPS at early and late time points. Code for these calculations used the scipy and matplotlib libraries in python.

bilayers (and with a small number of particles there is a larger, but still small, number of distances between particles); with more particles, there are more statistics for each level of separation. In the presence of Ca^{2+} there is an increased probability two PtdIns(4,5)_{P2} molecules will be spaced around 1.5 nm apart relative to simulations with Na^+ or Mg^{2+}, using their phosphodiester phosphorus atoms as the point to which distances are measured (Figure 4.3a). We used the phosphodiester phosphorus atom as proxy for the membrane surface in these calculations as it is present in every lipid type in our simulations and provides an easy comparison between systems. The center of mass could also be used but it is more computationally intensive, is also influenced by motions of the acyl chains, and is not as planar as the phosphodiester phosphorus atoms.
We must be careful not to interpret these RDFs as an emergent, dynamic quantity. It is not that PtdIns(4,5)P₂ molecules arrange themselves as a crystal with spacing of 1.5 nm when Ca²⁺ is added. In fact, there is no macroscopic rearrangement of the molecules at all. A quick calculation illustrates how subtle rearrangements of molecules are manifest on these plots.

If we assume the (x,y) dimensions of the membrane plane are 14 nm × 14 nm, then there is approximately 4.9 nm² available to each PtdIns(4,5)P₂ (there are 40 of them). So a single PtdIns(4,5)P₂ can sit, on average, in a box with sides of 0.7 nm. For two PtdIns(4,5)P₂ molecules sitting in the center of adjacent boxes, their separation would be 0.7 nm and that is approximately the location of the first green peak in Figure 4.3a. Of course, two molecules in adjacent boxes will not sit in the exact center of their boxes at all times. The distribution of molecule-to-molecule distances as the molecules move around the center point of their boxes gives the other green peaks between 1 and 2 nm. The observation that Ca²⁺ produces a strong peak at a specific r means that PtdIns(4,5)P₂ in adjacent boxes have a preferred distance between them and the peak comes at the “expense” of reduced probability at other distances relative to Na⁺ (i.e., where the curve for Na⁺ peaks at 0.7 nm and 2 nm, the curve for Ca²⁺ is much smaller, even below its bulk value).

PtdIns(4,5)P₂–DOPS contacts also seem to have a preferred distance in the presence of Ca²⁺ that is slightly different in the presence of Na⁺ or Mg²⁺, although the difference is not as clear as for PtdIns(4,5)P₂–PtdIns(4,5)P₂ contacts (Figure 4.3b). Another interesting observation is that PtdIns(4,5)P₂–DOPE contacts in the presence of Ca²⁺ seem to be sharply peaked between about 0.5 nm and 1.1 nm with a dip in the middle, whereas the region between 0.5 nm and 1.1 nm is roughly equally probable in the presence of Na⁺ or Mg²⁺ (Figure 4.3c).

Mg²⁺ seems to increase the density of contacts under 1 nm between PtdIns(4,5)P₂ and cholesterol, although this calculation may be affected by which atoms on cholesterol are selected (we chose a carbon atom that is near the corresponding phosphorus atom in the lipids, but have not checked how the results would change with other atoms). In preliminary results, we also analyzed the formation of hydrogen bonds between PtdIns(4,5)P₂ and cholesterol. We did not find a significant
number of hydrogen bonds between cholesterol and PtdIns(4,5)P₂ in any ionic condition, however a full analysis is necessary, especially given how important cholesterol has been shown to be in modulating PtdIns(4,5)P₂ cluster formation.

Another method of looking at the distribution of the lipids in the membrane is the structure factor. The structure factor can be related to the radial distribution function via a Fourier transform, assuming an isotropic and uniform distribution (which is likely not an acceptable approximation here). However, the structure factor can also be computed directly and compared to experimental results from e.g., neutron or X-ray diffraction. We plan to compute the structure factor of these systems. We have also started to look at the tilt of the lipids, which affects the energetics of the membrane, influencing
phase behavior and fluctuations, but these results have not been finalized.

### 4.3.2 Area per molecule and head-tail angle

Most simulations of bilayers report an average area per molecule by dividing the box vectors (i.e., the dimensions of the system along each axis) in the plane of the membrane by the number of molecules. This works for a homogenous system of a single lipid species but it does not give lipid-resolution for heterogenous systems, such as ours. We measured “area” in two ways. First, we used a Voronoi tessellation to divide the area in the plane of the phosphodiester phosphorus atoms. Given a specific phosphodiester phosphorus atom and an interpolated rectilinear grid between phosphorus atoms, each tile (or cell) in the Voronoi tessellation contains points that are closer to the specific phosphorus than any other (Figure 4.4). Note that the specific phosphorus atom does not have to be in the center of the Voronoi tile to satisfy this condition. Under most conditions, Voronoi tiles are also referred to as “geometrically stable” meaning that a small change in the original markers, the position of phosphorus atoms in our case, results in a small change in the Voronoi tessellation.

The second method we used to determine the area of PtdIns(4,5)P$_2$ molecules specifically, is the same technique implemented in chapter 2 and shown in Figure 2.14. This method reports the square of the maximum distance between phosphate oxygen atoms on the inositol ring and is a lowest-bound for the area of a square that would wholly encapsulate the head group of PtdIns(4,5)P$_2$. In our experience, these areas tend to be larger than those produced by the Voronoi tiling; the Voronoi areas tend not to vary very much between lipid species or ionic conditions.

The distribution of Voronoi cell areas for both leaflets of a single membrane system is shown in Figure 4.5. On average, cholesterol takes up a little less than 40 Å$^2$ of area in the plane of the membrane, defined by the phosphodiester phosphorus atoms. The average area of the other lipid species ranges from 50 to 60 Å$^2$, with a long-tailed distribution. The average area of the Voronoi cells in different ionic conditions is plotted in Figure 4.6. Curiously, Ca$^{2+}$ tends to decrease the area of all lipid types, by shifting the centroid of the distribution to the left, except significantly for PtdIns(4,5)P$_2$. 

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Instead, the average Voronoi cell area for PtdIns(4,5)P$_2$ with Ca$^{2+}$ is slightly larger owing to the decrease in the number of areas between 40 and 60 Å$^2$ at the same time as an increase in cells with an area of 80 to 100 Å$^2$.

One issue with the Voronoi tessellation is that the cell areas are sensitive to a small extent to whether cholesterol is included in the interpolation of the membrane surface. Without the inclusion of cholesterol, all cells are bigger, though the trends remain consistent. The cell areas in the presence of Na$^+$ or Mg$^{2+}$ are similar to each other, and Ca$^{2+}$ seems to lead to extra density at smaller areas for most of the lipids.

To interpret these results, it is important to keep in mind that these area measurements are not the geometric area of a molecule due to the three-dimensional spatial arrangement of its atoms but
Figure 4.5: The area of Voronoi tessellation cells from an asymmetric membrane containing cholesterol (included in the interpolation of the membrane plane here) with Ca\(^{2+}\) counterions. The “surface” is defined according to the phosphodiester phosphorus atom positions, shown as small orange spheres.

rather the proportion of points in a grid that are closer to a specific molecule than any other molecule, by definition of the Voronoi tessellation. For a given leaflet, if Ca\(^{2+}\) reduces the average cell area for most lipid types but increases the average cell area for PtdIns(4,5)\(P_2\), it means the area of points closer to PtdIns(4,5)\(P_2\) grows as a tradeoff with the area of points closer to other lipids (since their sum is constant). There are several potential explanations for this observation, but one interpretation may be that PtdIns(4,5)\(P_2\) is accessing different conformations in the presence of Ca\(^{2+}\), compared with the other ions, and this disrupts the packing of the lipids adjacent to PtdIns(4,5)\(P_2\).

The second method we used to measure area, using the spread of the phosphate group oxygens, produced results that are more vivid (Figure 4.7). Here, Ca\(^{2+}\) seems to lock in a specific value of the area of PtdIns(4,5)\(P_2\) around 55 Å\(^2\) whereas Mg\(^{2+}\) induces two, distinct values of the area around 50 Å\(^2\) and 60 Å\(^2\), and Na\(^+\) supports an even probability of having an area between 50 and 60 Å\(^2\) (Figure 4.7a). The difference in area between different charge states is not as apparent, although both charge states of -3 and -5 seem to prefer more specific areas than the charge state of -4 (Figure 4.7b). It is also very clear that PtdIns(4,5)\(P_2\) and PtdIns(3,5)\(P_2\) have different areas in a bilayer context using this method. Recall from Figure 2.14, the average area per molecule from
Figure 4.6: The area of Voronoi tessellation cells from an asymmetric membrane containing cholesterol (included in the interpolation of the membrane plane here) for POPC (a), PtdIns(4,5)P$_2$ (b), DOPS (c), and DOPE (d) in the presence of various counterions. The average area is in parentheses.

Single molecule simulations is about 66.8 Å$^2$ with Na$^+$, 50.3 Å$^2$ with Ca$^{2+}$, 58.5 Å$^2$ with Mg$^{2+}$ for PtdIns(4,5)P$_2$. The current results are smaller than the data from single molecule simulations due to the additional constraints imposed by neighboring lipids, but match reasonably well. A key difference is the lower peak in area for Mg$^{2+}$, even lower than the main peak for Ca$^{2+}$, which was not discovered in the single molecule simulations. We have yet to visualize this conformation in our simulations, but it will be very interesting to see the orientation of the molecule and the binding location of the divalent ion. The average area per molecule from single molecule simulations of PtdIns(3,5)P$_2$ did not change much in the presence of Ca$^{2+}$ and Mg$^{2+}$, which we also confirm here. Although much larger than PtdIns(4,5)P$_2$, the area of PtdIns(3,5)P$_2$ is less sensitive to ionic conditions and the
composition of the surrounding lipids.

Figure 4.7: The area per molecule in bilayers measured by the spread of the phosphate group oxygens for PtdIns(4,5)P₂ with various counterions (a), different charge states (b), and compared to PtdIns(4,5)P₂ (c). The average area is in parentheses.

We also correlated the average area using this method with the head-tail angle of PtdIns(3,5)P₂ and PtdIns(4,5)P₂ in Figure 4.8. One peak can be seen for Na⁺ and Ca²⁺ with PtdIns(4,5)P₂ in symmetric and asymmetric systems whereas the two separate area peaks can be seen for Mg²⁺. In all cases, the head-tail angle for PtdIns(4,5)P₂ in these systems is between 100 and 120 degrees, indicating the inositol ring is lying mostly flat along the plane of the membrane with a slight extension into the solvent environment. A recent paper, published while this thesis was being prepared, observed the tilt angle of the inositol ring of PtdIns(4,5)P₂ sampling a range between 90 and 180 degrees with a normal distribution, although only a single PtdIns(4,5)P₂ molecule is included in their bilayers. [Wu et al. 2014]. Changing the charge of PtdIns(4,5)P₂ does not seem to change the
head-tail angle and moving the 4-phosphate to the 3 position does not seem to change the head-tail angle either.

Other computational methods of measuring area per molecule include simulations of bilayers in the NPT ensemble or with varying levels of surface tension $\gamma$ in the NP$_\gamma$T ensemble. Early simulations measured the surface area of DOPC between 60.17 and 62.15 Å$^2$ with $\gamma = 35$ mN m$^{-1}$, though this reference notes that the large difference in area per molecule between simulations of $\gamma = 35$ mN m$^{-1}$ to 45 mN m$^{-1}$ indicates the difficulty of simulating at specific values of surface tension (Feller and Pastor, 1999). This is very close to the area of DOPC measured experimentally, around 62.9 Å$^2$ using X-ray and neutron scattering from Nagle and colleagues over several years (Braun et al., 2013; Kučerka et al., 2005; Akabori and Nagle, 2014; Mills et al., 2008; Tristram-Nagle et al., 2010). In other simulations using the same force field as us (CHARMM C36), the area of POPC in pure POPC membranes was found to be around 64.7 Å$^2$ compared to the experimental area of 68.3 Å$^2$; the area of POPE in pure POPE membranes was smaller at around 59.2 Å$^2$ compared to the experimental area of 59.75 Å$^2$ (Klauda et al., 2010). With the exclusion of cholesterol from the membrane plane, the Voronoi cell areas are larger, and reasonably match these results, although we do not expect the area of these lipids in pure systems to be exactly the same as in an asymmetric and heterogeneous environment such as ours. Truncation of long-range Lennard-Jones forces does affect these results and the authors advise using PME electrostatics with a real-space cut-off of 10 or 12 Å, which we followed (Klauda et al., 2010). It is disappointing, and perhaps a sign that further improvement in biomolecular force fields is still necessary, that parameters which produce accurate experimental values for bilayer simulations result in monolayers that have too little surface tension at a given area per molecule compared with experimental measures (e.g., Langmuir troughs). Area compressibility and head group–head group spacing from simulations can also be compared to experimental data (Klauda et al., 2012), although the experimental measurements for these quantities may depend on the orientation of the samples and whether they are unilamellar or multilamellar vesicles.
4.3.3 Bending rigidity of membranes containing PtdIns(4,5)P$_2$ and PtdIns(3,5)P$_2$

We estimate the bending rigidity of our simulated bilayers by analyzing its height fluctuations and mapping them onto a continuum description of bilayer undulations. On length scales much larger than the thickness of the bilayer, the Helfrich Hamiltonian (Helfrich [1973]) describes the free energy per area of a closed lipid bilayer with the following expression:

$$H_{el} = \int \left[ \frac{\kappa}{2} (2H(x) - H_0(x))^2 + \kappa K + \gamma \right] dx. \quad (4.3.1)$$

This equation describes the free energy of a bilayer with a uniform bending rigidity $\kappa$ according to the mean curvature $H$, spontaneous curvature $H_0$, Gaussian curvature $K$, and surface tension $\gamma$ (proportional to excess surface area of the membrane) over an area defined by the two-dimensional real space vector $x$. Our simulations are tensionless by definition of the NPT ensemble (i.e., the membrane is free to change its area), and hence set $\gamma = 0$. We also neglect the spontaneous curvature since our bilayers have equal numbers of lipids in each leaflet, and are expected to have nearly equal monolayer areas, and ignore the contributions of the saddle-splay modulus $\kappa$ and Gaussian curvature because the Gaussian curvature contributes a constant unless the bilayer ruptures (and hence changes topology). There is a large corpus of literature on various alterations of this scheme – for purely theoretical methods, simulations, and experiments – and it is outside the scope of this section to completely review the current state of the field (see volume 208 of the journal Soft Matter for papers just discussing recent applications of the Helfrich Hamiltonian).

Under these assumptions, we rewrite equation 4.3.1 in the Monge gauge, in which the membrane surface is described by its height $z$ at a position $(x, y)$ according to the following equation:

$$H_{el} = \kappa \int dxdy (\nabla^2 z(x, y))^2. \quad (4.3.2)$$
We characterize bilayer undulations by taking the Fourier transform of the bilayer heights \( z(x, y) \) over a projected area \( A_P \) using the reciprocal-space analog \( q \) of the two-dimensional real-space vector \( x \) according to

\[
h_q = \int dx \exp(iq \cdot x)h(x),
\]

and we apply the equipartition theorem to generate the bilayer height fluctuation spectrum,

\[
\langle z_q z_{-q} \rangle = \frac{k_B T}{A_P \kappa q^{-4}}.
\]

We compute the bending rigidity from the height-height autocorrelation function of the bilayer midplane. The surface of each leaflet is assigned according to the phosphodiester phosphorus atoms in each lipid, excluding cholesterol (refer to Figure 4.4). The membrane surface is interpolated between the phosphorus positions using a grid spacing of 4 Å, at every frame of the trajectory. The bilayer midplane is then computed by averaging the monolayer surface heights. After shifting the midplane to an average height of \( z = 0 \), we compute the height-height autocorrelation function via fast Fourier transform (FFT) functions and perform the subsequent analysis with our custom python code and FFT and fitting functions provided by scipy 0.13 [Hunter, 2007; Oliphant, 2007]. Our procedure follows the convention discussed in the literature [Brandt et al., 2011; Lindahl and Edholm, 2000].

The bilayer undulation spectra over the magnitude of the wavevector \( q \equiv |q| = \sqrt{q_x^2 + q_y^2} \) are plotted in Figure 4.9. We extract the bending rigidity \( \kappa \) by fitting a power law with slope \(-4\) to the low wavevector portion of the spectrum, where \( q \ll 1 \text{nm}^{-1} \). The Helfrich formulation models the bilayer as a smooth surface with a negligible thickness. Given that our system has an extent of roughly \( 15 \times 15 \text{nm} \), and the bilayer is approximately 6 nm thick, it is clear that height fluctuations must not follow the description given in equation 4.3.4 exactly. The high-\(q\) regime where \( q \ll 1 \text{nm}^{-1} \) is below the correlation length that separates undulation modes from protrusion modes [Lindahl and Edholm, 2000]. In the high-\(q\) regime, the fluctuations scale as \( q^{-2} \) (or \( q^{-1} \) at very high \( q \)) due to a
restoring force similar to a microscopic or intrinsic surface tension. Even though transition between undulations and protrusions is continuous, we see a fairly clear separation of these regimes, and extract the bending rigidity by fitting to a $-4$ power law only on the low-$q$ undulation modes, which have wave vectors below $1\text{nm}^{-1}$.

Due to our system size of approximately $\sim 15\text{nm}$, we are only able to access a handful of undulation modes, making our estimates of $\kappa$ somewhat less certain. Because of the scaling laws described here, it would require larger system sizes to obtain better estimates, at which point coarse-grained simulation is the preferred method.

For symmetric systems with no cholesterol, the bending rigidity of membranes with Na$^+$ or Mg$^{2+}$ is on the order of $20\ k_b\ T$ whereas the bending rigidity of membranes with Ca$^{2+}$ is on the order of $30\ k_b\ T$ (Figure 4.9b). In vitro, the bending rigidity has been measured to be on the order of $\sim 20k_b\ T$ for SOPC vesicles with cholesterol, but is highly dependent on membrane composition (Song and Waugh, 1993). Micropipette aspiration has measured the bending rigidity of vesicles with mixtures of DOPC, DPPC, and cholesterol to be between $15$ and $25\ k_b\ T$. Moreover, the bending energy of a charged membrane may be greater than that for a neutral membrane due to the presence of the electric double layer containing the counterions and then the coions, respectively (Slochower et al., 2014). The electrostatic contribution to the bending energy depends on the induced osmotic potential, which in turn depends on the Debye length, the ion concentrations (number and valence), and the surface potential of the membrane (Winterhalter and Helfrich, 1988). The difference in bending energy between a flat, neutral membrane and GUVs containing greater than 15% PtdSer may be up to $10\ k_b\ T$, measured through micromanipulation (Vlkova et al., 2004).

Changing the charge of PtdIns(4,5)$P_2$ also results in a noticeable change in the rigidity of the membrane systems (Figure 4.9b). Either reducing the negative charge of PtdIns(4,5)$P_2$ to -3 or increasing the negative charge of PtdIns(4,5)$P_2$ to -5 causes an increase in stiffness of the membrane. An increase in $\kappa$ can be thought of as resulting from higher energy molecular-level interactions that suppress long-range fluctuations (i.e., the membrane is in a higher energy state). In
particular, PtdIns(4,5)\(P_2\) at charge state -3 results in an extremely stiff membrane, a more than 50% increase over charge state of -4. It is possible that the different pattern of counterion binding to the various charge states of PtdIns(4,5)\(P_2\), or the difference in inter-PtdIns(4,5)\(P_2\) hydrogen bonding when the phosphomonester groups are variably protonated, is responsible for the decreased flexibility of the membranes in those cases. This will be a very interesting avenue to pursue.

In asymmetric systems, membranes with PtdIns(3,5)\(P_2\) and either Mg\(^{2+}\) or Ca\(^{2+}\) are stiffer than membranes with PtdIns(4,5)\(P_2\) and Na\(^+\) but less stiff than membranes with PtdIns(4,5)\(P_2\) and Mg\(^{2+}\) or Ca\(^{2+}\). As expected, membranes containing PtdIns(3,5)\(P_2\) with Mg\(^{2+}\) and Ca\(^{2+}\) have similar properties, just as the molecular size and head-tail angle of PtdIns(3,5)\(P_2\) is very close with either counterion, and the single molecule PMF for PtdIns(3,5)\(P_2\) to bind these ions follows the same general pattern. The asymmetric membrane systems also have cholesterol whereas the symmetric systems do not. Previous studies have shown that cholesterol depletion (by not more than 50%) increases membrane stiffness measured by a decrease in the deformability of certain cell types (Byfield et al., 2004). Yet other studies have shown that the presence of cholesterol increases the resistance to area dilation of pure SOPC membranes (Song and Waugh, 1993). In general, we see that the membrane systems with cholesterol are stiffer (all bending rigidities are above 20 \(k_B T\)), although the cholesterol-depleted symmetric membrane with Ca\(^{2+}\) is just as stiff as the asymmetric, cholesterol-containing membrane. As cholesterol is not the only variable different between the systems, the effect of cholesterol on the bending rigidity is convolved with the effect of the ions.

The finite and non-negligible height of our membranes relative to their length might increase the apparent bending rigidity; as membrane size increases, more low frequency (long wavelength), low energy modes become accessible. An interesting extension of this work might be to simulate membrane systems of the same composition with increasing sizes and compare the bending rigidity of the all-atom systems with coarse-grained systems and compare the coarse-grained systems to simulations using continuum methods.

Another related calculation is to analyze the fluctuations in the peristaltic motion of the membranes.
That is, instead of tracking the correlated movement of the membrane midplane, the separation of the two leaflets is visualized by not averaging the position of the two leaflet surfaces. We have implemented code to perform this calculation, but have not completed the analysis on all the systems yet.

In conclusion, bilayers containing PtdIns(4,5)P₂ have a bending rigidity around 20-30 $k_B T$, similar to experimental measurements of other membranes. Decreasing or increasing the charge of PtdIns(4,5)P₂ increases the bending rigidity, suggesting that deviations from the physiological charge state of PtdIns(4,5)P₂ alters the stiffness of biological membranes. In the presence of Ca$^{2+}$, the bending rigidity of membranes containing PtdIns(4,5)P₂, but not PtdIns(3,5)P₂, is increased to close to 30 $k_B T$ from 20 $k_B T$ in the presence of Na⁺.
Figure 4.8: The correlation between molecule area reported using the second method and head-tail angle. In our convention, 90 degrees means the inositol ring is lying flat against the membrane surface and 180 degrees means the inositol ring is perpendicular to the membrane surface.
Figure 4.9: a. A schematic of the bending rigidity calculation Monge gauge. b. Fluctuation spectra for symmetric membranes with Na\(^+\), Mg\(^{2+}\), or Ca\(^{2+}\). c. Fluctuation spectra for symmetric membranes with Na\(^+\), and PtdIns(4,5)\(P_2\) at charge -3, -4, or -5.
Figure 4.10: a. The fluctuation spectra for asymmetric membranes containing PtdIns(4,5)P$_2$ and Na$^+$, Mg$^{2+}$, or Ca$^{2+}$. b. The fluctuation spectra for asymmetric membranes containing PtdIns(4,5)P$_2$ or PtdIns(3,5)P$_2$ with Mg$^{2+}$ or Ca$^{2+}$. 
4.4 Ion binding to membranes containing PtdIns(3,5)P₂ and PtdIns(4,5)P₂

The behavior of counterions near charged biological membranes has not been analyzed conclusively. Many different types of experiments contribute to our knowledge that ions bind to charged membranes (e.g., electrophoretic mobility of vesicles, spectroscopy of binary mixtures, surface pressure measurements, X-ray scattering of membranes in the presence of divalent cations, etc.) but they are unable to distinguish to which lipids in a highly heterogenous membrane ions bind, where exactly the ions might bind to the lipids, what is the structure of the ion distributions within the plane and away from the plane of the lipids, whether there is localized over-charging in certain regions, and so forth. There a number of theoretical models that have been developed over the past hundred years building complexity upon each other, but they usually rely on many assumptions that do not hold in our case, including the assumption that membranes carry a uniform charge density and counterions are monovalent. Here we able to report the results for highly charged lipid species distributed throughout a heterogeneous membrane with both monovalent and divalent cations.

4.4.1 Lipid-ion distribution functions

In a similar manner to the lipid-lipid distribution functions, lipid-ion distribution functions can be computed. Here we used a three-dimensional radial distribution function (RDF) to look at the average density of ions near each lipid type. For each lipid type, the distance (from the phosphodiester phosphorus atom) to Na⁺, Mg²⁺, or Ca²⁺ between r and dr is computed and plotted in Figure 4.11. The RDF shows a peak for PtdIns(4,5)P₂ with Ca²⁺ that is larger and earlier than the peaks corresponding to PtdIns(4,5)P₂ simulations with Na⁺ and Mg²⁺ (Figure 4.11b). A second, smaller peak for the Ca²⁺ curve can be seen. In a classical fluid, the second peak on the g(r) plot is the second solvation shell. Here, however, since we are measuring from the phosphodiester phosphorus
atom, the first peak < 0.5 nm likely represents direct binding to the phosphodiester group (carrying charge -1) and the second peak between 0.5 and 1.0 nm represents binding to the inositol ring phosphomonoester groups (carrying charges between -1 and -2). If the graphs are interpreted with this in mind, then it seems Ca\(^{2+}\) binds much more strongly to the phosphodiester group than Na\(^{+}\) or Mg\(^{2+}\) and about equally well to the phosphomonoester groups as Na\(^{+}\). This is a very surprising result, as Ca\(^{2+}\) is thought to bind much better to the inositol phosphates than monovalent ions from our single molecule work. Also surprising is that the Mg\(^{2+}\) curve, instead of being sharply peaked like Ca\(^{2+}\), displays increased probability (relative to bulk) throughout the range from 0.4 to around 1.3 nm. This might suggest that Mg\(^{2+}\) does not have a strongly preferred binding location, and thus distance, relative to the phosphodiester phosphorus. This is supported by the broad and shallow well in the PMF for Mg\(^{2+}\) to bind PtdIns(4,5)\(P_2\) we calculated with umbrella sampling in Figure 3.4h.

It is also important to keep in mind these graphs are normalized by the number of atoms of a given type. Therefore a peak in the Ca\(^{2+}\) curve that is the same height as a peak in the Na\(^{+}\) curve means the same number of both ions are present, but there is twice as much positive charge at that location in the Ca\(^{2+}\) simulations.

If the net charge of PtdIns(4,5)\(P_2\) is -3, then the Na\(^{+}\) curve is more strongly peaked below 0.5 nm, close to the phosphodiester phosphorus (Figure 4.11b). Recall that this net charge is achieved by setting the charge on both phosphomonoester groups and the phosphodiester group to -1. The broad peak between 0.4 and about 1.3 nm represents binding to both phosphomonoester groups, and when this broad peak is integrated, it is probably about equal to the integral of the first peak, suggesting an equal affinity of Na\(^{+}\) to each phosphate group. However, when the net charge of PtdIns(4,5)\(P_2\) is -5, binding to the phosphomonoester groups, carrying a charge of -2, is stronger than binding to the phosphodiester group, carrying a charge of -1.

The distances between the phosphomonoester groups and the phosphodiester group are slightly different in PtdIns(3,5)\(P_2\), which partially explains the shift in the peaks in Figure 4.11c. The curves for PtdIns(3,5)\(P_2\) with Mg\(^{2+}\) and Ca\(^{2+}\) resemble each other, as we expect based on the single
Figure 4.11: a. The radial distribution function for PtdIns(4,5)\(P_2\) to various counterions in asymmetric membranes. b. The radial distribution function for PtdIns(4,5)\(P_2\) to Na\(^+\) under different charge states. c. The radial distribution function comparing PtdIns(3,5)\(P_2\) and PtdIns(4,5)\(P_2\) to Mg\(^{2+}\) and Ca\(^{2+}\).

molecule PMF calculations. Moreover, the curves for PtdIns(3,5)\(P_2\) resemble a hybrid of the curves for PtdIns(4,5)\(P_2\) with Ca\(^{2+}\) and Mg\(^{2+}\). Instead of having a equal probability between 0.4 and 1.3 nm, the closer peak is larger, but the probability does not return to bulk (or below) between the peaks. However, the peaks for PtdIns(3,5)\(P_2\) with either ion are larger (and about equal in width) than for PtdIns(4,5)\(P_2\) with Mg\(^{2+}\), implying that the probability that either ion is within 1.0 nm of PtdIns(3,5)\(P_2\) is greater than the probability Mg\(^{2+}\) is within 1.0 nm of the phosphodiester phosphorus of PtdIns(4,5)\(P_2\). We have started to decompose these plots into separate “lateral” (x,y) and “normal” (z) distances, but have not completely tested the code yet. Determining the extent to which Ca\(^{2+}\) binds to a single molecule or acts as a “bridge” between two molecules is an upcoming calculation and one of the unique pieces of data to emerge from these bilayer simulations.
4.4.2 Ion distributions normal to the membranes

We calculated the equilibrium distribution of ions perpendicular to our membrane systems measured from the center of the membrane. These distributions are usually established by 10 ns and do not change considerably over the course of the simulation. Asymmetric membranes, in particular, present a telling story. In the asymmetric membrane systems, we have plotted the cations and anions separately in Figure 4.12a and b. Consistent with the results in the previous section, and somewhat contrary to our belief that Ca$^{2+}$ binds much more strongly than Na$^+$ to PtdIns(4,5)P$_2$, both Ca$^{2+}$ and Na$^+$ show strong peaks at the anionic membrane surface ($\sim +25$ Å) of roughly equal height (where height represents number of ions). Again, the positive charge in this region is twice as great for the simulations with Ca$^{2+}$ than Na$^+$. The peak for Mg$^{2+}$, however, is half as large as the peak for Ca$^{2+}$, although the level of Mg$^{2+}$ remains higher than the level of Ca$^{2+}$ after the peak. On the far side of the peak, the concentration of Ca$^{2+}$ drops below its bulk value whereas Mg$^{2+}$ and Na$^+$ smoothly return to their bulk values. The peak in Cl$^-$ comes later (i.e., further from the surface) than the peak in Ca$^{2+}$, forming a double layer of: anionic membrane surface, cationic layer due to attraction of Ca$^{2+}$, and anionic layer of Cl$^-$ due to the layer of Ca$^{2+}$.

Ca$^{2+}$ is also attracted to the opposite leaflet, which is neutral, containing the zwitterionic (i.e., neutral but containing positive and negative charges in equal proportion) POPC and cholesterol. At the moment, the origin of this attraction is unclear. Figure 4.11a demonstrates that Ca$^{2+}$ binds strongly to the phosphodiester group of PtdIns(4,5)P$_2$. Presumably, Ca$^{2+}$ also binds strongly to the phosphodiester group of POPC and this is the origin of the peak, but it does not explain why this does not occur for PtdIns(3,5)P$_2$ (Figure 4.13). Due to the attraction of Ca$^{2+}$ to the neutral leaflet, the Cl$^-$ on this side of the membrane is peaked as well, instead of rising smoothly to its bulk concentration.

The total negative charge of the anionc leaflet is -260 e. The total number of Ca$^{2+}$ ions in the peak rises to nearly 200, indicating a positive charge of +400 by 40 Å from the center of the membrane. In
Figure 4.12: a. Cation and anion distributions perpendicular to an asymmetric membrane containing PtdIns(4,5)P$_2$, unscaled. b. Cation and anion distributions perpendicular to an asymmetric membrane containing PtdIns(4,5)P$_2$, scaled according to the bulk concentration (far from the membrane surface). Inset shows the offset of the peak concentration for Ca$^{2+}$ and Cl$^{-}$. The surface of the charged leaflet is around +25 Å, the surface of the neutral leaflet is around -25 Å.

In fact, before the centroid of the peak at $\sim$ 25 Å, the negative charge of the leaflet has been neutralized. Visualizing the growth and stability of these peaks over the course of the simulation would provide an illuminating example of how surfaces become overcharged.

Comparing results from membranes containing PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$ confirms that the behavior of Ca$^{2+}$ with PtdIns(4,5)P$_2$ is unique (Figure 4.13). One key difference is that in simulations with PtdIns(3,5)P$_2$, Ca$^{2+}$ does not bind to the neutral leaflet. This suggests that binding of Ca$^{2+}$ to the oppositely charged leaflet is a consequence of Ca$^{2+}$ binding so strongly to the charged leaflet. Future work will determine if these results for membranes containing PtdIns(3,5)P$_2$ hold up after more simulation time or if the PtdIns(3,5)P$_2$ molecules rearrange so
that Ca$^{2+}$ binding to the phosphodiester group becomes more likely. These ion distributions affect the electrostatic environment at the membrane interface at least as much as the charges of the membrane components. As the inner leaflet of the plasma membrane is negatively charged, proteins that localize to the membrane interface must respond to both regions of negative and positive charge density, with very different dielectrics.

An extension of this technique is to decompose the distributions into (x,y) components, so that it would be clear to see if the ions within a particular horizontal slice are localized to specific (x,y) regions, where PtdIns(4,5)$P_2$ may be. As we have the complete set of coordinates for our simulations, this is possible without much difficulty.
4.5 Some dynamic properties of membranes containing PtdIns(3,5)\(P_2\) and PtdIns(4,5)\(P_2\)

This section describes features of the membrane simulations that are not static in equilibrium, such as the motion of the lipids and the ions. Experimental measures of diffusion typically rely on some form of optical measurement using a tag that is attached to the molecules. Whether or how these probes disrupt the diffusion of the lipids has been debated in much the same way that it is unclear whether visualizing the location of PPIs in cells by using fluorescently tagged PPI-binding proteins alters the positioning of the lipids through recruitment to protein domains.

The main advantage of our work is that we can directly measure the diffusion of PtdIns(3,5)\(P_2\) and PtdIns(4,5)\(P_2\) in our model membranes along with the motion of the other lipids. Alas, the main disadvantage is that it takes a very long time to generate enough data for the (relatively) slow moving lipids to diffuse significant amounts. An illustrative example is shown in Figure 4.14. Over the course of an entire simulation, the lipids diffuse over an area the order of just a few nm\(^2\), compared to an average separation between molecules of roughly 0.7 nm. Our results will certainly leave out some very desirable features, such as the aggregation of multiple PtdIns(4,5)\(P_2\) into clusters (if that were to occur) but nevertheless, we have been able to determine how the diffusion of various lipid species is affected by in the ionic environment, cholesterol, and whether PtdIns(3,5)\(P_2\) or PtdIns(4,5)\(P_2\) is present. Future work will be able to study diffusion on the order of 10 nm\(^2\).
4.5.1 Diffusion of lipids in membranes containing PtdIns(3,5)P$_2$ and PtdIns(4,5)P$_2$

To complete these calculations we calculated the two-dimensional diffusion coefficient of the center of mass of lipids according to the Einstein diffusion relation:

$$\langle r^2 \rangle = 2dDt^\alpha,$$

(4.5.1)

where $r$ is the position of the particle (i.e., lipid), $d = 2$ is the dimensionality, $D$ is the diffusion coefficient, $t$ is the interval over which the displacement is calculated, and $\alpha \rightarrow 1$ in the limit the
displacement is “normally” diffusive ($\alpha > 1$ is superdiffusion and $\alpha < 1$ is subdiffusion). The transition from superdiffusive ballistic motion to normally diffusive behavior occurs in lipid systems before $10^{-2}$ ns. We fix $\alpha = 1$ and fit the slope of the mean squared displacement (MSD) on a log-log plot with the interval time (Figure 4.15, left).

Each lipid in a system generates one diffusion curve, which we fit together to obtain an average diffusion coefficient for each lipid type. We find that if a membrane is subject to Ca$^{2+}$, diffusion of all lipid species in the membrane is generally slower relative to Na$^+$, whereas if a membrane is subject to Mg$^{2+}$, diffusion of all lipid species in the membrane is close to diffusion with Na$^+$ or faster (Figure 4.15, right). It is very interesting that Ca$^{2+}$ exerts its effect on the entire membrane structure – on all lipids together – although it only binds tightly to PtdIns(4,5)P$_2$. The uncertainty in the values, judged from the variance of the slopes of the individual curves that are averaged together for each lipid type, are typically less than 0.1 $\mu$m$^2$/s$^{-1}$, suggesting coherent motion for a given lipid type within a simulation system.

Previous measurements of diffusion of PtdIns(4,5)P$_2$ in GUVs (mixed 5/15/80% with cholesterol and SOPC) or on the inner leaflet of the plasma membrane using the autocorrelation of fluorescently tagged PtdIns(4,5)P$_2$, have reported diffusion coefficients in the range $1 \mu$m$^2$/s$^{-1}$ to $20 \mu$m$^2$/s$^{-1}$ (Wang et al., 2012b; Golebiewska et al., 2008). Wang 2012b reports that 10 $\mu$M Ca$^{2+}$ slows the diffusion of PtdIns(4,5)P$_2$ to $0.8 \pm 0.4 \mu$m$^2$/s$^{-1}$, close to our results with Ca$^{2+}$, concomitant with observations of rounded fluid-like PtdIns(4,5)P$_2$-rich clusters. The addition of 1 mM Mg$^{2+}$ slows the diffusion to $\sim 5 \mu$m$^2$/s$^{-1}$, which is also close to the effect we see, and about the same level as 10 $\mu$M Zn$^{2+}$.

Cholesterol tends to diffuse at about the same rate as the lipids, but seems to be affected less by Ca$^{2+}$ than the lipids. Of note, the diffusion rate of POPC is decreased in the presence of Ca$^{2+}$. As of now, it is not known whether this is due to the binding of Ca$^{2+}$ to the neutral leaflet of the membrane or if the diffusion rate of POPC is decreased simply because the diffusion rate of the lipids in the opposite monolayer are decreased.

We can also compare the difference between the asymmetric and symmetric systems (Figure 4.15, right).
One difference between the systems is the presence of cholesterol. Other studies have shown that cholesterol depletion decreases the lateral diffusion of transmembrane proteins, and cholesterol increases phase separation with distinct diffusivities (Levental et al., 2009). Diffusion of PtdIns(4,5)P_2 in presence of Mg^{2+} or Ca^{2+} is decreased in the presence of cholesterol, although we do not observe any phase separation and this effect is qualitatively different from two populations diffusing at different rates. Nevertheless, the difference between the systems for Na^+ is only marginally significant (the variance in the slopes is around 0.1 \mu m^2 s^{-1}) so the slowing effect of cholesterol might be plausibly be related to divalent ion binding.

Comparison of the diffusion coefficients between membranes containing PtdIns(4,5)P_2 and PtdIns(3,5)P_2 is illuminating (Figure 4.17). First, PtdIns(3,5)P_2 moves faster than PtdIns(4,5)P_2 in...
the presence of either ions. This makes sense in light of the fact that these ions do not bind as tightly to PtdIns(3,5)P2. The difference between the diffusion rates of PtdIns(4,5)P2 in the presence of Ca2+ is 4.06 µm² s⁻¹. In other words, the diffusion of PtdIns(3,5)P2 in the presence of Ca²⁺ is three times the rate of the diffusion of PtdIns(4,5)P2 in the presence of Ca²⁺.

### 4.5.2 Mobility of ions adjacent to membranes containing PtdIns(3,5)P2 and PtdIns(4,5)P2

We have measured the diffusion of ions in two-dimensional slabs adjacent to the membrane surface. When viewing the trajectories, it is clear that some ions are bound for the duration of the simulation and some ions are completely free and sample the entire box volume multiple times (Figure 4.17a). A minority of ions, at least in simulations with Ca²⁺, transition from a bound state to a free state and vice versa. The three-dimensional individual diffusion curves for each Ca²⁺ ion are seen in Figure

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Figure 4.16: The diffusion of PtdIns(4,5)P2 with and without cholesterol in asymmetric and symmetric membrane systems, respectively.
Figure 4.17: The diffusion of PtdIns(3,5)P₂ and PtdIns(4,5)P₂ with divalent ions.

4.18b: the free ions sample an area about 10² as large as the bound ions in 50 ns.

The diffusion of free Ca²⁺ ions in water has been determined to be between $1.2 \times 10^4 \mu m^2 s^{-1}$ to $1.8 \times 10^4 \mu m^2 s^{-1}$, a little greater than the maximum diffusion seen in our simulations; the diffusion coefficient for Mg²⁺ ions is within a couple percent \cite{Hollingshead1941, Harned1949, Levy1949}. Note that the diffusion of the free ions is 1000 fold faster than the lipids and the stuck ions seem to have the same diffusion coefficient as the lipids, in the range $1 \mu m^2 s^{-1}$ to $10 \mu m^2 s^{-1}$.

Most of the movement in the (x,y) plane seems to be directed by the motion of the lipids. Separating the movement in a plane parallel to the membrane from a plane perpendicular to the membrane can answer questions about the likelihood an ion might travel between neighboring proteins bound to the membrane.

Next, we divided the system into equal height zones parallel to the membrane surface, extending away from the membrane, and calculated an effective mean squared displacement (MSD) for the duration an ion stays in the zone. Then for each zone, we calculated an apparent “diffusion” coefficient using the MSDs for the durations an ion was in that zone (coefficients were only computed...
for durations when ions stayed in the zone the whole time). Here we should note that the particles (i.e., ions) are not undergoing free diffusion because in addition to thermal motion there is the electric potential set up by the membrane. Since this potential varies as a function of height above the membrane, the potential is different in each zone. Therefore what we are plotting is just an effective mobility.

We see that there is a spatial dependence of the mobility (Figure 4.18), which generally collapses into two main regions, corresponding to the free and stuck ions in panel b. The transition matrix between zones can also be computed to give the probability that an ion in one zone (e.g., bound) will transition to another zone (e.g., free). From the transition matrix, we can calculate the first passage time, enabling us to perform calculations like the average time an ion spends in the zone closest to the membrane as a proxy for how long ions may stay bound. We plan to use this to calculate the average residence time for ions bound to lipids.

The diffusion seems to reach bulk value about 2-2.5 nm from the membrane midplane (about 1.5-2 nm from the surface of the membrane, top right of panel c). Recall from Figure 1.4, theoretically and under ideal conditions, the electrostatic potential away from a membrane composed of monovalent negative charges packed in hexagonal tiles with area 68 Å² is relatively flat by 6 Å and down to -25 mV by 12 Å. A potential of 25 mV is about equal to $RT/F$ for $R$, the universal gas constant, $F$, the Faraday constant, and $T = 293$ Kelvin.

We also recapitulate that some Ca²⁺ ions are bound – and stuck – to the neutral leaflet, represented by the blue dots in the lower left corner of panel c, consistent with the equilibrium ion distributions. The ions closest to the charged leaflet, are mostly bound (red dots in the lower left corner of panel c). The difference in mobility between stuck and free ions is two orders of magnitude, with relatively few intermediate values. Ions that are stuck in the z direction are generally stuck in the (x,y) direction, as well. It has been suggested that counterions near charged surfaces may be constrained in their movement away from the surface but not along it. Our results so far do not support that hypothesis.
Figure 4.18: a. Snapshots of the position of two ions (black and white dots) over the course of several ns during a simulation. b. The diffusion curves for ions clearly separate into stuck (or bound) and free populations. c. The diffusivity tensor of Ca$^{2+}$ ions above the neutral and charge leaflet of the membrane. Stronger color (blue or red) is closer to the surface.

4.6 Conclusions

Simulations of bilayers profoundly expand our ability to draw conclusions about the biological role of PtdIns(3,5)$P_2$ and PtdIns(4,5)$P_2$. In addition to confirming many of our hypotheses and results from single molecule QM/MM and classical all-atom simulations, the simulations of model membranes provide new insights. The area per molecule and head-tail angle are in line with estimates from chapters 2 and 3. The bending rigidity calculations reveal that in addition to binding to PtdIns(4,5)$P_2$, Ca$^{2+}$ stiffens the membranes, suppressing fluctuations on the order of nanometers. Membranes containing PtdIns(3,5)$P_2$ with divalent ions are about as stiff as membranes
containing PtdIns(4,5)P₂ with Mg²⁺, approximately 20% less stiff than with Ca²⁺. Using the whole lipid, instead of just a fragment, we see from the lipid-ion distributions that Ca²⁺ is able to bind the phosphodiester phosphate group of PtdIns(4,5)P₂ quite strongly, Mg²⁺ binds with equal probability between the phosphodiester and inositol phosphomonoester groups, and Na⁺ binds only to the inositol phosphomonoester groups.

Another novel result from these simulations is the observation that the concentration of Ca²⁺ near the charged leaflet of an asymmetric membrane is more than 16 times its bulk value and the concentration of Ca²⁺ near the neutral leaflet is about 5 times its bulk value. This effect does not happen for Mg²⁺ or Na⁺, which rise to only 6 or 7 times their bulk value near the charged leaflet and do not rise above their bulk value around the neutral leaflet. In contrast, Ca²⁺ and Mg²⁺ bind to membranes containing PtdIns(3,5)P₂ identically to how Mg²⁺ binds to PtdIns(4,5)P₂.

The addition of Ca²⁺ to asymmetric membranes slows the diffusion of most lipid types, including PtdIns(4,5)P₂, to half its value in the presence of Na⁺. Moving from a symmetric membrane without cholesterol to an asymmetric membrane with cholesterol slows the diffusion of PtdIns(4,5)P₂ with divalent ions. Strikingly, the diffusion of PtdIns(3,5)P₂ in the presence of Ca²⁺ is three times the diffusion rate of PtdIns(4,5)P₂ with Ca²⁺. The mobility of Ca²⁺ ions near the membrane demonstrate there are effectively two states, free and stuck, in simulations with asymmetric charged membranes.

Why are these results interesting? Take together, these data transport the single molecule simulations from an isolated environment into a biological context. To our knowledge, these simulations represent some of the most physiologically accurate membrane simulations to date. We have determined estimates for the physical properties of PtdIns(3,5)P₂ and PtdIns(4,5)P₂ in a model of the plasma membrane that matches predictions from experiments in monolayers and vesicles. Our analysis of fluctuations provides insight into the energetics necessary to deform realistic membranes, where there is reason to suspect a concentration of 1-10 % mole fraction PtdIns(4,5)P₂ might be present in those regions. Although coarse-grained and continuum theory models provide estimates of the energy to deform membranes on the order of a couple µm, they
do not capture the subtle differences between systems that we have. Our results agree with experimental measurements taken during micropipette aspiration and tether pulling of quasiflat membranes containing mixtures of DOPC, DPPC, and cholesterol or pure SOPC. Equilibrium distributions of the ion concentrations characterize the vastly different electrostatic environments that proteins may encounter upon approach to a charged membrane, such as when the intracellular concentration of Ca$^{2+}$ may be high (e.g., in an excitatory cell) relative to a resting state of high Mg$^{2+}$. For proteins that insert alpha helices or hydrophobic peptides into the membrane, the diffusion rates of the lipids provides an intuition about the motion of those proteins, and the lipid-lipid RDF curves can be used to compare how the lipids pack before and after the insertion of peptides.

The results presented in this chapter are just a sliver of what can be extracted from the bilayer simulations so far. Many more exciting calculations are in various levels of planning and preparation. In the next chapter, I briefly mention some of the calculations which we expect to come to fruition shortly.
I would like to take this chapter to mention extensions of this work that will be realized in the near future. We continue to plan simulations around biological questions. Below, I’ve listed four primary questions and some progress we have made to approach the answer through simulations.

**What happens inside PtdIns(4,5)P₂-rich clusters?** We initiated simulations of an asymmetric membrane containing 80% PtdIns(4,5)P₂ in a single leaflet but were unable to reach stability during initial equilibration. One approach that might be helpful is to place the ions according to the electrostatic potential established by the huge charge of the lipids instead of allowing the ions to reach their equilibrium distribution during the simulation. A simulation of this type would be able to answer many new questions: what is the diffusion rate inside PtdIns(4,5)P₂-rich clusters? What is the diffusion rate of the cluster as a whole? What happens in the opposite leaflet from the cluster? What is the pattern of ion binding at the edge of the cluster?

**What is the free energy for lipids to associate?** We have designed a free energy simulation using the distance between a pair of PtdIns(4,5)P₂ molecules as the *a priori* reaction coordinate for umbrella sampling, but we have not run this simulation yet. This simulation will start as a branch of a previous simulation so we will be able to monitor changes in the membrane system that occur as the two lipids come together. If we repeat this simulation in multiple systems we can ask: does it cost more or less free energy for two PtdIns(4,5)P₂ molecules to come
together in PtdCho or with PtdSer and PtdEtn? If we continue the umbrella sampling, we can ask: does the addition of a third molecule to a dimer of PtdIns(4,5)\(P_2\) cost the same amount of energy as the formation of the dimer, and so forth?

**How tightly do ions bind to lipids?** We have created the initial configuration for a set of umbrella sampling windows for pulling an ion away from the charged membrane surface to determine the potential of mean force to displace an ion. This simulation will also determine when a bound ion leaves the influence of a lipid and is replaced by another ion. Does the replacement ion come from bulk or another bound ion? Does the replacement ion come from an intermediate zone of weakly bound ions? How do proteins, such as those that contain PH domains, bind to PtdIns\(P_2\) in the presence of tightly bound ions?

**Can we fit the ion distribution curves and make a connection to theory?** We are able to fit the ion distribution curves for Na\(^+\) ions to classical theories using a mean field but there are two issues with fitting the curves for our other systems: theories require a low surface potential and monovalent ions. There is no fully analytic solution for divalent ions and indeed, it is clear that a different functional form is necessary to capture the behavior of Ca\(^{2+}\) in our systems (particularly the overcharging and subsequent depletion zone). We have been working to test the approximations of classical theories and appropriateness for our simulations. We want to be able to address the question: when is a mean field description of a charged membrane an accurate approximation and when is the stereo-specific chemistry of PPIs necessary to describe their interactions?

In the end, until experiments are able to monitor the inner working of cells with atomic precision, molecular modeling will have its role making predictions of biological interactions and specific circumstances of interest to be investigated exhaustively. The inevitable increase of hardware capabilities and force field parameters will improve the accuracy of these predictions as simulation time scales on the order of microseconds and milliseconds become commonly available in coming
years.
Sample simulation input code for bilayer simulations

Code A.1: input-in.mpd (ASCII file)

```plaintext
integrator = md
nsteps = 50000
dt = 0.002

nstxout = 50000
nstvout = 50000
nstxtcout = 1000
nstenergy = 1000
nstlog = 500

continuation = yes
gen_vel = no

constraint_algorithm = lincs
constraints = h-bonds
lincs_iter = 1
```
lincs_order = 4

ns_type = grid
nstlist = 5
rlist = 1.2
rcoulomb = 1.2
rvdw = 1.2
vdwtype = switch
rvdw_switch = 0.8
coulombtype = PME
pme_order = 4
fourierspacing = 0.12
pbc = xyz

tcoupl = V-rescale
tc-grps = Other Water_and_ions
tau_t = 0.5 0.5
ref_t = 310 310

pcoupl = Parrinello-Rahman
pcoupltype = semiisotropic
tau_p = 2.0
ref_p = 1.0 1.0
compressibility = 4.5e-5 4.5e-5

dispcorr = no
nstcomm = 10

comm-mode = Linear

comm-grps = Other Water_and_ions
Bibliography


