ABSTRACT

Title of Dissertation: COMPUTATIONAL STUDIES OF LIPID BILAYERS AND TRANSMEMBRANE PROTEINS

Pouyan Khakbaz, Doctor of Philosophy, 2018

Dissertation directed by: Associate Professor, Jeffery B. Klauda, Chemical and Biolomucular engineering department

Deep understanding of lipid bilayers in three phases at the molecular level could potentially lead us to design a novel artificial membrane. Molecular modeling of bacterial membranes is important as they are cheap and an environmentally friendly candidate to produce fuels. Molecular investigation of transmembrane proteins is crucial as mutations in them were observed in multiple diseases including cancer.

The inner membrane of *Escherichia coli* (*E. coli*) was modeled to include lipid diversity and demonstrate that this is needed to properly probe the interaction of lipids and transmembrane proteins. Molecular dynamics (MD) simulations were used with the all-atom CHARMM36 (C36) force field. Lipid diversity affects the properties of the *E. coli* inner membrane and indicated the importance of including lipids with different head groups and acyl chains. The effect of the growth stage of the *E.coli* colony significantly influenced thicknesses and bulk properties of the membrane.
Phase transitions of fully saturated lipid bilayers, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-phosphocholine (DPPC) and their mixtures were probed for the first time using MD simulations. The phase transitions from fluid (L_α) phase to ripple (P_β) phase and to the gel (L_β) phase were obtained within temperature range in good agreement with experimental phase transition temperature. DMPC and DPPC bilayers in L_β phase resulted in fatty acid chains tilted relative to bilayer normal and with average tilt angle in agreement with experiment. MD simulations revealed molecular-level structural details of the pure DMPC bilayer in P_β phase at a temperature to compare to experimental X-Ray diffraction measurements. The structure of the major and minor arm is in agreement with experiment when enough lipids are used to model this phase.

The final two topics involved collaborations with experimental labs to provide insight into experimental observables. First, MD simulations successfully showed that improved tolerance and production of biorenewables of a metabolically engineered E.coli strain is the result of increased bilayer thickness. Secondly, MD simulations of the homodimerization of plexin A3 were used to probe the association of the transmembrane (TM) and juxtamembrane (JM) domains of this important cell-signaling membrane protein. These simulations indicated multiple dimerization conformations, and suggested importance of extracellular domain residues on strong TM interactions.
COMPUTATIONAL STUDIES OF LIPID BILAYERS AND TRANSMEMBRANE PROTEINS

by

Pouyan Khakbaz

Dissertation submitted to the Faculty of the Graduate School of the University of Maryland, College Park, in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2018

Advisory Committee:
Professor Jeffery B. Klauda, Chair
Professor Amy Karlsson
Professor Srinivasa Raghavan
Professor Sergei Sukharev
Professor Ganesh Sriram
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Dedication

I dedicate my Ph.D. thesis to my father, mother, and brother who supported me throughout my study.
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I would like to acknowledge my supportive advisor Dr. Jeff Klauda, who guided me perfectly throughout my Ph.D. study at University of Maryland. I would also like to acknowledge all of my dear labmates during 5 years of my Ph.D.
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1. Chapter 1: Introduction

1.1. Lipid Bilayer

Lipids are one of the four main building blocks in biology and many are amphiphilic molecules, i.e., they have a portion that likes water and a portion that dislikes water. In general, lipids are molecules that are synthesized by either or both of the two following routes: carbanion-based condensations from thioesters or carbocation-based condensations of isoprene moieties. The function of lipids can be classified into three categories, i.e., energy storage, membrane formers, and biological signaling. Lipids are used by cells to store calories for future use in molecules such as triacylglycerol and steryl esters. The most common and ultimate use for lipids is to form membrane structures in the cell due to their ability to self-associate in aqueous environments. The amphiphilic character of these lipids at the appropriate concentration will result in bilayer formation that encapsulates cells and/or various organelles. The properties of these lipid membranes vary and go beyond cell protection and are important in cell division, membrane protein function, and protein aggregation. Certain lipids in the membrane can also serve as signal transduction and recruiters of proteins. Some proteins are known to respond to signals in the membrane in the transmembrane domains and propagate these to cytosolic portions of a protein. Other proteins are known to selectively bind to certain lipids that are segregated into different cell organelles, domains of sterol-rich membranes, or other unique membrane properties.
Although lipids have the same general properties, there is a wide variety of lipid diversity that exists in cells. These can be classified into eight categories that are associated with common chemical structures, i.e., fatty acyl, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides. In cells, glycerophospholipids, sphingolipids and sterol lipids are the most common but can contain a wide variety of acyl chains and hydrophilic head groups. These head groups can be polar, zwitterionic (having both negative and positive charges), or anionic. The head groups also vary in size, i.e., small (hydroxyl in sterols or glycerols), moderate (phosphocholines and phosphoinositols) or large (glycerolipids with polysaccharides). In addition to head group diversity, there is also a wide range of non-polar chemical structures. Fully-saturated acyl chains and monounsaturated chains are the most common for non-sterol lipids but these can vary in length with 14- to 26-carbons. However, depending on the species and its diet, lipid chains can also exist with branched, polyunsaturated, cyclic or cyclic-containing chains. For sterols, a variety exists in the steroidal fused ring structures and the attached short chain. This wide diversity is used by cells for its function and structural stability.

Eukaryotes have cells that contain an outer plasma membrane (PM) and membranes that enclose various organelles. These organelles are known to have a wide variety of lipids (see Figure 1-1 for a few examples) and concentrations. If we consider yeast, phosphatidylcholine (PC) lipids are the most common in the endoplasmic reticulum, trans-Golgi network (TGN), and mitochondrion (MIT), but a minor lipid in the PM (Figure 1-2). As shown in Figure 1-2, the distribution of lipids is not uniform.
between organelles of yeast. The TGN and PM have the highest amounts of anionic lipids, i.e., phosphatidylinstol (PI) and phosphatidylserine (PS) lipids. The ergosterol levels increase along the secretory pathway of yeast (ER to TGN to PM). The MIT membranes contain cardiolipin, which is common in many membranes with high curvature. Glycosphingolipids, such as, galactosylceramide (GalCer, Figure 1-1) are known to exist at lower concentrations in the ER and TGN. In addition to the uneven distribution of lipids between organelles, many organelles (excluding the ER) have asymmetrical leaflets. For example, the outer leaflet of the PM is enriched in PC, sphingomyelin (SM), and glycopshingolipids, whereas the inner leaflet contains mainly phosphidylethanolamine (PE), PS and PI lipids.

Eukaryotes may have varying lipid compositions in their organelles, but prokaryotes also have a wide array of lipids. In gram-negative bacteria, the viscous periplasm is bound by the inner membrane and a complex outer membrane. The outer membrane is asymmetric with phospholipids in the inner leaflet and mainly lipopolysaccharides (LPSs) in the outer leaflet. The LPS contain a six-chained Lipid A that is connected to a core region of sugars and then attached to a polysachharide chain. Many bacteria also contain unique acyl chain structures, such as iso- and anteiso-branched chains or those with a cyclopropane moiety (Figure 1-3).
In addition to phospholipids, sterols also have a wide variety of chemical structures that exist in nature (Figure 1-3). In mammals, cholesterol is the most common sterol but in yeast ergosterol is the most common. In plants, sterols also come in various forms such as campesterol, sitosterol and stigmasterol. The main difference between these sterols is the unsaturated and branching character of the chain on the steroid ring (Figure 1-3). These slight changes can influence various membrane properties such as lipid domain formation, chain order and membrane
Aside from sterols, plants are a common source for polyunsaturated chains in the mammalian diet. For example, 50-70% of the acyl chains in soy bean seeds are either 18:2 or 18:3 (2 or three double bonds)\textsuperscript{26}.

\textbf{Figure 1-2.} The head group composition of lipids of the ER (endoplasmic reticulum), TGN (trans-Golgi Network) and PM (Plasma Membrane) in Saccharomyces cerevisiae based off experimental measures\textsuperscript{6-9} and ignoring inositol sphingolipids in the PM and TGN.
Figure 1-3. Chemical Structure of various common sterols in nature (left) and the chemical structure of 1-palmitoyl-2-cis-9,10-methylene-hexadecanoic-acid-sn-glycero-3-phospho-ethanolamine (PMPE) with the sn-2 chain numbered 1-16 and the cyclopropane carbon numbered as 17.
1.2. Membrane proteins

Membrane proteins are the proteins, which are associated to lipid bilayers. Membrane proteins functions are critical to maintain normal behavior of the cells since mutations of these proteins are known to play important role in many diseases including cancer\(^\text{27}\). Computational modeling tools and specifically molecular dynamics simulations have been used extensively on membrane proteins to probe wide range of biological interests\(^\text{28, 29}\).

Membrane proteins can be classified to transmembrane proteins and peripheral membrane proteins. Transmembrane (TM) proteins span the hydrophobic part of the cell membranes partially or entirely. Depending on specific function of TM proteins, they can be categorized to ion channels, membrane receptors and membrane transporters. Membrane transporters are responsible for transporting wide range of materials to cytoplasmic side of the cell. Depending on membrane transporter, the transport mechanism is categorized as active or passive transport. Passive transporters facilitate the transport of single substrate by dissipation of its concentration gradient\(^\text{30}\). Active transporters are divided into primary active transporters and secondary active transporters. In primary active transporters, the energy of ATP hydrolysis is used to transport the substrate in to the cell, while in secondary active transporters, the electrochemical gradient of one substrate, which is mainly proton or cation, is used to transport the other substrate against its concentration gradient\(^\text{31}\). Ion channels and transmembrane receptors are biologically important as considerable amount of drugs (almost 60\%) target ion channels and G-protein coupled receptors\(^\text{32, 33}\).
Membrane receptors play important role in a variety of biological pathways including hormonal and neurological responses and signal transduction \(^{34}\). Therefore, there has been a great interest to study this class of transmembrane proteins experimentally and computationally \(^{35}\). Plexins, which are transmembrane receptors involved in signal transduction, will be discussed in detail in Chapter 5.

1.3. *Molecular Dynamics simulation: Background and review*

Dynamics of biomolecular systems takes place in a wide range of time scales. In some cases, experimental techniques can predict the dynamics of the system. For example, spectroscopy could be used to investigate bond vibration. In some cases, we need to study the dynamics of the system in more details that experimental techniques cannot provide us that information and a common alternative way is to use molecular dynamics simulations. All molecular dynamics (MD) simulations require accurate parameters that are known as force fields. The accuracy of simulation highly relies on the accuracy of the force field as it contains all the parameters needed to calculate interactions and potential energy. Potential function contains two distinct interactions: bonded interactions and non-bonded interaction. Bonded interactions include angle-bending, stretching of covalent bonds, torsion potentials, and improper torsion potentials (Figure 1-4) \(^{36}\). The second class of interactions is non-bonded interactions and they involve coulomb electrostatic interactions, dispersion and repulsion. Parameters regarding bonded interactions are usually fixed in the beginning of a MD simulation. Non-bonded interactions are typically calculated from neighbor lists, which are updated during the simulation. A MD simulation is carried out by integrating Newton’s equation of motion with the following formula \(^{36}\):
\[ F_i = m_i \ a_i \]

By having the force for all atoms, the coordinates can be calculated for the next step. The updated coordinates then used to recalculate the potential energy again and this process continues \(^\text{36}\).

**Figure 1-4.** Example of bonded and non-bonded interactions in force field

Molecular dynamics simulation has some limitations and the most important one is timescale. Many of biological events take place beyond nanoseconds to microseconds. With current fore fields the cost will be too high beyond this time scale and in some cases it is impossible to run classical MD simulations because it might take forever to sample configurational and/or phase space. One possible and common approach to overcome the timescale problem is to run coarse-grained MD simulation.
In this case, approximately four atoms are assumed as one interaction site so we can get the results much faster but with less molecular details.

MD simulations have been carried out to study membrane proteins and their interaction with lipid bilayer \(^{37}\). Enhanced conformational sampling algorithms could be incorporated with molecular dynamics simulations to study biological membranes and membrane proteins. Replica-exchange molecular dynamics (REMD) method and replica-exchange umbrella sampling (REUS) are common examples \(^{38}\). MD simulations indicated a mechanism that demonstrated how cell-penetrating peptides are able to translocate across cell membrane in an energy independent pathway \(^{39}\). MD simulations have been carried out on ABC transporters, ion channels, and secondary transporters. Temporal and special resolutions obtained from those simulations were beyond findings based on experimental techniques \(^{40}\). As it can be seen, MD simulation is a powerful tool to study biological membranes and membrane proteins. Throughout this dissertation, extensive amount of molecular dynamics simulations of cell membranes and transmembrane proteins will be discussed.

1.4. **Overview of dissertation**

This dissertation consists of four major chapters applying molecular simulations to probe lipid membranes to membrane-associated proteins. Chapter 2 presents work on the importance of considering lipid diversity in the cell membranes using all-atom molecular dynamics simulations by probing the effect of considering lipids with
different head groups and different fatty acid chains in *E. coli* membrane. The effect of cyclic ring moiety on cell membrane properties was specifically studied across the development cycle of this bacteria. Chapter 3 presents work on probing the phase transition of saturated phospholipid bilayers from fluid or liquid-crystalline phase to the ripple phase and to the gel phase by analyzing extensive molecular dynamics trajectories. Characterizing the condense phases (gel and ripple) of DMPC and DPPC bilayers was the main focus of that study. Chapter 4 presents how engineered head groups in *E. coli* membrane result in more tolerance of the membrane to chemicals used in biofuels and microbe chemical production. Finally, Chapter 5 presents work on different homodimerizations of the transmembrane (TM) domain and juxtamembrane (JM) domain of plexin A3 captured by coarse-grained and all-atom molecular dynamics simulations.
2. Chapter 2: Probing the Importance of Lipid Diversity in Cell Membranes via Molecular Simulation

2.1. Introduction

Although *in vivo* membranes are diverse in their lipid composition, the common practice in experiments with membranes is to simplify this diversity. Model membranes have are used to study the structure and function of membrane proteins. For example, EmrE (a small multi-drug resistance transporter) of *E. coli* maintains a similar structure (measured from NMR) with various PC lipids to those that better model the bacterial membrane (PE, PG and PC) or polar lipid extracts from *E. coli* \(^{41}\). However, the dynamic rate of structural changes is affected by the lipid composition \(^{41, 42}\). For some transmembrane proteins, the use of PC-only membranes can result in a loss of the protein’s native structure \(^{43}\). The lipid-protein interaction for lactose permease is complex and not limited to only the head group. Proper folding of proteins into membranes is also believe to strongly dependent on lipid composition \(^{43}\).

Many careful studies of lipid-protein interaction demonstrate that proper consideration of lipid diversity in membranes is important in *in vitro* experiments.

2.2. Review of Molecular Simulations of Lipid Bilayers

The focus of this section of dissertation is on how molecular simulations can be used to better quantify the importance of lipid diversity on membrane properties. This section also contains a mini-review of typical approaches in modeling membranes in simulations to provide insight on how the latest force field developments have allowed for simulations beyond simple membranes of one or two lipid types. Then, new research on how lipid composition changes of *E. coli* during the colony’s life
cycle will be presented. Model description of the cytoplasmic membrane of *E. coli* from the log phase to the stationary phase will be provided. This section will also describe the molecular dynamics (MD) simulation methods.

As it was stated, the focus of this section is to demonstrate the importance of properly considering lipid diversity in molecular simulations. Therefore, this section will review the approaches used by researchers utilizing MD simulations to model membranes and peptide or proteins that reside in these membranes. The use of computer simulation to understand lipids started roughly in the late 1980s using simple representations of lipids. Then, in the early 1990s MD simulations of lipid bilayers were shown to agree with experiment but at a limited time scale of 100ps. The field continued to progress and by the turn of the century many studies were performed on lipids bilayers that approached the 50ns timescale and investigations of bilayers with cholesterol, small molecules, proteins and DNA. Most all-atom simulation studies of lipid membranes continue to focus on simple models of biological membranes with one to three lipids. Many of these studies have been compared to parallel experimental studies of up to ternary mixtures with applications to lipid domain formation, cholesterol modulation of lipid bilayer properties, and lipid flip-flip. Modeling of the lipid bilayer in simulations of transmembrane proteins and peripheral membrane proteins have typically been simplified to at most 3 lipid types.

Although these simple model membranes have their importance in many studies to understand the biophysical contributions of lipids to structure and dynamics, in some cases the exclusion of important lipids might affect the membrane and also
protein function in the membrane. The development of lipid force fields has progressed quickly the last five to ten years to allow for inclusion of lipid diversity in membrane models. Most all-atom and united-atom force fields have the capability to reasonable represent lipids of varying common head groups (PC, PE, PA, PS and PG) and chain types (saturated and monounsaturated)\textsuperscript{57-62}. The CHARMM36 (C36) all-atom force field has the most diverse set of lipids that have been extensively tested against available experimental data, i.e., cholesterol\textsuperscript{63}, polyunsaturated chains\textsuperscript{64}, branched chains\textsuperscript{65}, chains with a cyclopropane moiety\textsuperscript{66}, cardiolipin\textsuperscript{67}, sphingolipids\textsuperscript{68}, PI\textsuperscript{69}, lipopolysaccharides\textsuperscript{70}, and other lipids are currently under development. The force fields and computational resources are at an exciting stage in which all-atom and united-atom simulations of realistic and diverse membranes of organisms can be developed and compared to simple model membranes that have been previously used.

Our first simulation study to probe the effect of lipid diversity was to model an average representation of the lipids that exist in yeast (average of the organelle distribution shown in Figure 1-2)\textsuperscript{71}. One of the difficulties beyond accurate force fields was developing a robust method to build membranes with multiple lipid types. The CHARMM-GUI Membrane Builder\textsuperscript{67,71-73} has been crucial in allowing for easy building of model membranes with high lipid diversity. For yeast, a six-component membrane was built with cholesterol, DPPC (dipalmitoyl-PC), DOPC (dioleoyl-PC), POPE (palmitoyloleoyl-PC), POPA (PO-phosphatidylamine), and POPS, which was the first MD simulation of a model membrane of yeast\textsuperscript{71}. The component surface area per lipid of DOPC was found to reduce by over 10 Å\textsuperscript{2} in the yeast model membrane. Moreover, this membrane also contained measurable populations of
cholesterol that were parallel to the membrane surface but in the middle of a leaflet surrounded by DOPC. The presence of cholesterol in center of membrane was also observed in experiments 74-77 and coarse-grained simulations 76, 77 with polyunsaturated lipid tails. Upon increase the tail saturation these unique sterol orientations were not present. This first-order membrane model is currently being updated to include the varying diversity of lipids in the ER, TGN and PM. This will allow for studies of proteins that interact with these membranes in this important model organism in the kingdom of fungi.

Modeling of bacterial membranes has been common with multiple applications such as antimicrobial peptides and models for membrane transporters. E. coli membranes have typically been modeled in their simplest form with POPE lipids 52, 78-80. However, the cytoplasmic membrane of E. coli has a lipid head group composition is 70-80% PE, 10-20% PG, and 0-10% cardiolipin 18 with most of the cardiolipin at the polar and septal regions. Therefore, more advanced modeling of the cytoplasmic membrane consist of two-component bilayers of POPE and POPG 81-83. Although this mixed bilayer is improved by including PG lipids, it ignores the lipid chain diversity. E. coli and some other bacteria contain a unique chain structure with a cyclopropane moiety on a chain 84, 85, e.g, cy-C17:0 (Figure 1-3 with PMPE as an example). We were the first to model this unique chain and the MD simulations compared favorably against existing NMR data 84. Then, a model membrane that contained six lipid types was developed with a 4:1 PE to PG ratio and the percentage of the fatty acid chains 15:0, cy17:0, 16:0, 16:1, and 18:1 in the membrane were 7%, 35%, 40%, 8%, and 10%, respectively 66. This cytoplasmic membrane model of E.
**coli** in the late stationary phase differed from POPE/POPG membranes in that the bilayer was thinner, more rigid and a decreased surface density. The change in thickness and rigidity will dramatically influence simulations of membrane proteins, especially mechanosensitive channel proteins. In addition to cyclic-containing chains, some bacteria have branched chains, which are predominately iso- and anteiso-branched chains. *Chlamydia trachomatis* is an example bacteria that contains these branched lipids at levels of 15-20%. Our models of bilayers with branched chains and the cytoplasmic membrane of the elementary body and reticular body form of *C. trachomatis* demonstrate that branching increases the lateral elastic moduli and slows axial lipid relaxation. These models of *C. trachomatis* consisted of nine lipids types with PC, PE and PG lipids with cholesterol concentrations of 24-34%. The elastic moduli for the infectious elementary body form was found to be the greatest (583±83 dyn/cm) and may be important for cell stability as it penetrates the host cell.

Although diversity and unique lipids can be important to the properties of membrane, many organelle membranes have an asymmetric distribution of lipids. Most of the modeling of lipid asymmetry has focused on eukaryote plasma membrane models. Some simple models of 3-4 lipids using united atom force fields have been simulated but these lack some important lipids or domain formations. To allow for longer time scales and larger systems, most simulations have focused on using coarse-grained force fields. For example, lipid composition asymmetry was investigated using the MARTINI lipids of phase separated liquid-ordered (L_o) and liquid-disordered (L_d) domains of PC lipids and cholesterol. Although not
representative of head group asymmetry in natural cells, it was found that interleaflet coupling existed between the \( L_0 \) and \( L_d \) domains, e.g., the \( L_0 \) domains influence the curvature, lipid rotational dynamics and lateral diffusion of lipid in the opposing \( L_d \) domain \(^{89}\). Recently, a complex 63 lipid model for the asymmetric plasma membrane has been simulated with MARTINI lipids \(^{91}\). This system consisted of \( \sim 20,000 \) lipids and run for 40\( \mu \)s and resulted in transient \( L_0 \) domains and nanodomains with gangliosides in the outer leaflet \(^{91}\).

A more extreme case of lipid asymmetry is that of the outer membrane (OM) of gram-negative bacteria. The outer leaflet contains mostly LPS and inner leaflet with PE, PG and cardiolipin phospholipids. Wu et al. were the first to fully simulate bilayers of LPS and confirm that the all-atom C36 lipid force field agrees with NMR experiments \(^{70}\). MD simulations of varying lengths of the LPS were compared, i.e., Lipid A only, LPS with the R1 core, and LPS with 5 and 10 \textit{E. coli} O6 O-antigen repeating units. The addition of the core and longer O-antigens resulted in an increase in the surface area per lipid and decrease in chain order. The hydration of the inner core region of the LPS was found to be critical in maintaining the bilayer structure. A more representative model for the leaflet asymmetry was further studied with/without the presence of the outer membrane phospholipase A (OmpLA) protein \(^{92}\). The hydrophobic thickness of the asymmetric model membrane was found to be 3 Å thinner than a typical phospholipid bilayer. Compared to simple models of OmpLA with DLPC bilayers, the LPS stabilized the OmpLA loop conformations and reduced its dynamics demonstrating the importance of accurately representing the membrane to protein structure and dynamics.
2.3. *Lipid Composition Changes in the Growth Phase of E. coli Culture*

This section will present a new set of MD simulations that focus on the effect of lipid composition changes of the cytoplasmic membrane of *E. coli* as a function of its growth phase in culture. The main change in the lipid composition from the log phase to the stationary phase is in the amount of cyC17:0. The emphasis here will be how these changes in lipid composition influence various bilayer properties.

Lipid compositions of the cytoplasmic membrane of *E. coli* at each state of its life cycle were selected based on the experimental data from matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and tandem mass spectrometry (TOF/TOF) technique. Relative numbers of each lipid in the membranes were selected based on relative peak intensities in mass spectrometry technique (Table 2-1). To allow for a complete understanding of the influence of cyC17:0 on lipid properties, the concentration of this lipid was set to zero for the earlylog stage even though measurements suggest 10%. Life cycle of cytoplasmic membrane of *E. coli* was classified in four stages: early log, midlog, stationary, and overnight (also known as the death stage). The main difference of each of those stages was the overall concentration of chains that contain a cyclopropane ring. Total amount of lipids per leaflet was 78 for all MD simulations, and total amount of lipids, which had cyC17:0 in their chemical structures, were 0, 28, 82, and 110 for early log, midlog, stationary, and overnight stage, respectively. Based on the previous study, Top6 model was used to represent the lipid composition of overnight stage. All membrane models assumed a symmetric membrane composition between leaflets.
Table 2-1. Compositions of lipid membranes used in our MD simulations. The definitions for the lipids are as follows: POPE (16:0-18:1-PE), PYPE (16:0-16:1-PE), OSPE (18:1-16:1-PE), PMPE (16:0-cyC17:0-PE), QMPE (15:0-cyC17:0), PSPG (16:0-16:1-PG), and PMPG (16:0-cyC17:0-PG). The amount of lipids for each type are shown within the parenthesis in this table.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Lipids (number of each lipid)</th>
<th>Water:lipid</th>
<th>Total lipids</th>
<th>Total atoms</th>
<th>%lipids with cy-C17:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early log</td>
<td>OSPE(12), POPE(24), PYPE(90), PSPG(30)</td>
<td>35</td>
<td>156</td>
<td>35226</td>
<td>0</td>
</tr>
<tr>
<td>Midlog</td>
<td>OSPE(12), PMPE(18), PMPG(4), POPE(36), PSPG(26), PYPE(54), QMPE(6)</td>
<td>35</td>
<td>156</td>
<td>35364</td>
<td>18</td>
</tr>
<tr>
<td>Stationary</td>
<td>OSPE(10), PMPE(54), PMPG(12), POPE(12), PSPG(18), PYPE(34), QMPE(16)</td>
<td>35</td>
<td>156</td>
<td>35344</td>
<td>53</td>
</tr>
<tr>
<td>Overnight</td>
<td>OSPE(12), PMPE(74), PMPG(16), POPE(20), PSPG(14), QMPE(20)</td>
<td>35</td>
<td>156</td>
<td>35472</td>
<td>70</td>
</tr>
</tbody>
</table>

2.4. Method

All-atom molecular dynamics (MD) simulations using explicit solvent were carried out for all systems. Initial conformations of *E. coli* membranes were obtained by CHARMM-GUI Membrane Builder. Minimization, equilibration, and isobaric-isothermal ensemble (NPT) MD simulations were carried out using NAMD simulation package. Temperature and pressure of systems were kept at 310.15 K and 1 bar, respectively. Lenard-Jones potential was used to describe Van der Waals interactions, and a forced-based switching function in the range of 8 to 12 Å was chosen. Each simulation after CHARMM-GUI equilibration was run for 300 ns and 2 fs was the time step. Particle Mesh Ewald was used for long-range electrostatics interactions. Hydrogen atoms were constrained by using RATTLE algorithm. CHARMM36 (C36) force field was used, since we have evaluated the accuracy of this in our previous study of the overnight model for the *E. coli* cytoplasmic...
membrane. Langevin dynamics maintained the temperature and the Nosé-Hoover Langevin-piston algorithm \(^{96,97}\) was used to maintain the pressure. For each stage of growth, three replicates using different initial seeds were carried out to obtain better statistics. The relative number of PE:PG lipids was 4.2:1, and the ratio of water molecules to lipids was 35:1 for all \textit{E. coli} membranes.

With these simulations of various stages of \textit{E. coli}’s life cycle, a wide variety of analysis was performed to investigate the effect of diversity of lipids at each stage during the life cycle of the \textit{E. coli} cytoplasmic membrane. The last 100 ns of simulations (after equilibration) was used to perform all analysis. Bulk properties of membranes were evaluated by calculating the surface area per lipid (\(X \cdot Y\) divided by number of lipids of each leaflet) and area compressibility modulus, \(K_A\) was calculated using following formula,

\[
K_A = k_B T \langle A \rangle / \sigma_A^2
\]

where \(k_B\) is the Boltzmann constant, \(\langle A \rangle\) is the average surface area, \(T\) is the temperature, and \(\sigma_A^2\) is the variance of the area. Deuterium order parameters of the acyl chain (\(S_{CD}\)), which is the measure of order in C-H vector, were calculated for all the lipid types using the following equation,

\[
S_{CD} = \langle \frac{3}{2} \cos^2 \beta - \frac{1}{2} \rangle
\]

where \(\beta\) is the angle between the C-H vector and bilayer normal. Generating electron density profile (EDP) was important since we could use that to calculate the bilayer thickness (\(D_B\)), hydrophobic core thickness (\(2D_C\)), and head to head group distance (\(D_{HH}\)). The electron density for each atom in the bilayer was calculated using the CHARMM program \(^{98}\). Then, the SIMtoEXP program \(^{99}\) was used to parse these
densities into groups and obtain the (EDPs). The $D_{HH}$ was calculated by the peak-to-peak maximum distances in the total EDP. $D_B$ is defined as the distance between the midpoints of the volume probably of water and $D_C$ is defined as a half of the distance between the midpoints of bilayer's hydrocarbon acyl chains volume probabilities $^{100}$. The two-dimensional (2D) radial distribution function (RDF) was calculated in order to probe the lateral structure of all membranes. Visual Molecular Dynamics (VMD) $^{101}$ was used to create snapshots and obtain $S_{CD}$.

2.5. Results

Snapshots of the end of two simulations (early log and stationary) are shown in Figure 2-1. The amount of lipids with double bonds increase and the presence of those with cyC17:0 increases between these two systems. Clearly these are in a liquid disordered state with a high amount of lipid chain disorder as seen in the Figure 2-1. Coordinates and NAMD/CHARMM compatible PSF files for all models can be downloaded from $^{102}$.

Figure 2-1. Snapshots of the last frame of the earlylog stage (left) and stationary stage (right) simulations (run 1). OSPE was shown in blue, POPE in red, PSPG in orange, PYPE in green, PMPE in pink, PMPG in cyan, and QMPE in purple (see Table 1 for name definitions). Water molecules were not shown for clarity.
The surface area per lipid was calculated during the course of simulations (Table 2-2). The average surface area per lipid of overnight stage was 63.08±0.17 Å², which was the highest surface area per lipid compared to other stages (Table 2-2). The *E. coli* membrane at the early log stage had the lowest surface area per lipid; therefore it was slightly denser compare to other stages during the life cycle.

**Table 2-2.** Summary of membranes properties, where 2D_C is the hydrophobic thickness, D_B is the bilayer thickness, D_HH is the head-to-head distance, SA is the surface area, and K_A is the area compressibility modulus.

<table>
<thead>
<tr>
<th>Stage</th>
<th>2D_C(Å)</th>
<th>D_B(Å)</th>
<th>D_HH(Å)</th>
<th>SA(Å²/lipid)</th>
<th>K_A(N/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early log</td>
<td>30.62±0.13</td>
<td>38.27±0.12</td>
<td>37.73±0.13</td>
<td>60.77±0.02</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>Midlog</td>
<td>30.36±0.03</td>
<td>38.15±0.03</td>
<td>38.00±0.11</td>
<td>61.26±0.04</td>
<td>0.29±0.01</td>
</tr>
<tr>
<td>Stationary</td>
<td>30.00±0.03</td>
<td>37.07±0.07</td>
<td>37.33±0.07</td>
<td>62.48±0.07</td>
<td>0.31±0.01</td>
</tr>
<tr>
<td>Overnight</td>
<td>30.09±0.13</td>
<td>36.82±0.10</td>
<td>37.00±0.23</td>
<td>63.08±0.17</td>
<td>0.33±0.03</td>
</tr>
</tbody>
</table>

S_CD for the acyl chain was calculated for all stages of *E. coli* membrane. Higher order parameter indicates higher order of chain. Generally, a decreasing order down the acyl chain is expected due to free rotation of the methyl. The S_CDs are known to be inversely related to the surface area per lipid \(^{103}\), i.e., a larger surface area will result in a lower S_CD. However, the difference between the order parameter at each stage did not dramatically change for most of the lipids (Figure 2-2). The sn-1 chain of POPE (Figure 2-2) clearly demonstrates that overnight and stationary stage were more disordered compare to earlylog and midlog stage, which indicates the effect of the cyC17:0 in decreasing the order of chains. This expected trend was also observed for the upper carbons of the sn-2 chain on lipids without a cyC17:0 chain.
Figure 2-2. S\textsubscript{CD} profiles of QMPE, PMPE, and POPE for the (left) sn-2 chain and (right) sn-1 chain.

The S\textsubscript{CD}s for the QMPE and PMPE showed dramatic decreasing of order at C11 position, which was the adjacent carbon atom to the cyclopropane ring carbon atoms. However, that sharp change of order occurred at C10 position for POPE (Figure 2-2). The average order parameter of C10 and C11 in \textit{sn}-2 of POPE at overnight stage was 0.050±0.0007 and 0.083±0.001 respectively, while the average order parameters of C11 in \textit{sn}-2 of PMPE and QMPE were 0.027±0.002 and 0.021±0.002 respectively.

Local decreasing of order was observed for all chains, which have double bonds, for all stages of life cycle. For instance, the average order parameter of C3-C7 in sn-2 of
OSPE was 0.20 at overnight stage. At the position of double bond, C9 and C10, a dramatic decrease of order to 0.06 was calculated.

Overall, the EDPs membranes had the similar shape for total and component electron density profiles. EDPs of the *E. coli* cytoplasmic membrane for each model of the life cycle is shown in Figure 2-3. Slight shift at the peak of total electron density profiles of stationary stage and overnight stage was observed. This was in agreement with decreasing the order parameters of these stages compare to earlylog and midlog stage. The EDP was also generated for combined hydrophobic core and water (Figure 2-3) and demonstrated similar minor changes in these distributions. The CH group (double bond carbons or cyclopropane carbons) EDP was generated to compare positions and densities for the CH group. Interestingly, a higher electron density of cyclopropane was seen at the center of bilayer for stationary stage and overnight stage (Figure 2-4) and demonstrates deeper penetration into the center of the bilayer.

From total electron density profiles the $D_{HH}$ was calculated for all membranes. The $D_B$ and $2D_C$ were calculated based on volume probability profile (see Table 2-2). Bilayer thickness of *E. coli* membrane at overnight stage was 36.82±0.1 Å, which was in a good agreement with thickness of *E. coli* membrane in Top6 model 66. Lower hydrophobic core thicknesses were obtained for stationary and overnight stage for the average value of 30.00±0.03 and 30.09±0.13 Å respectively. Lowest head to head group distance was 37.00±0.23 Å at overnight stage. Overnight stage provided thinner membrane compared to other stages of growth indicating a slight effect of
lipid diversity and cyclopropane ring concentration in the properties of cytoplasmic membrane of *E. coli*.

The area compressibility modulus is the measure of rigidity of the membrane and the previous Top6 model was shown to vary from simple model membranes with just POPE/POPG. Results showed that overnight stage was more rigid compare to earlylog, midlog, and stationary stages (Table 2-2). The average $K_A$ of overnight stage was $0.33\pm0.03$ N/m, while the average $K_A$ of earlylog stage was $0.28\pm0.02$ N/m. This notable but small difference at the overnight stage indicates the effect of lipid diversity and specifically cyclopropane ring concentration on rigidity of cytoplasmic membrane of *E. coli*.

**Figure 2-3.** The total electron density profiles of each state of growth (top). Electron density profile of combined hydrophobic core and water (bottom). These are all symmetrized so zero is the center of the bilayer.
Figure 2-4. Electron density profiles of CH group for different stages of growth with the double bond atoms (top) and the atoms on the chain associated with the cyclopropane (bottom). These are all symmetrized so zero is the center of the bilayer.

The 2D RDFs of phosphate atoms were calculated between PE-PE, PG-PG, and PE-PG and all lipids. In order to show the convergence during the simulation time scale, 2D RDFs were calculated for 20ns blocks and resulted in no dramatic change during the course of the simulation (Figure 2-5). This convergence is much quicker than that reported in Hong et al. with the 15% cholesterol and the C36 force field. Results of the stationary stage showed dramatic difference between PE-PE and PG-PG 2D RDFs (Figure 2-6A). In case of PE-PE RDFs, two strong peaks were found at $r = 5.9$ and 7.7 Å. The amine on PE lipids hydrogen bounds with oxygen on the phosphate on PE and PG around these peak distances (Figure 2.6B-C). The two strong RDF peaks are thus the result of internal phosphate conformations that either
has the bonded phosphate oxygens pointing toward or away from each other. This dual major peak structure appears to be abolished to a single major peak with the presence of 15% cholesterol based on past simulations. In the case of PG-PG and PE-PG 2D RDFs, a slight shift can be seen for the primary and secondary peaks towards slightly longer distances. The PG-PG RDF clearly shows that these like anionic lipids do not associate and the 2D RDF is below the bulk density for most of the distances shown in Figure 8. However, PG does associate with PE lipids in a similar fashion to how PE self-associates. Comparing the 2D RDFs for all lipid pairs, the effect of increasing cyC17:0 has a minimal effect on the lateral organizations of

![Graph](image)

**Figure 2-5.** 2D RDFs for different blocks during the simulations time scale of mid-log stage. <b11-b15> is the average of the last five blocks. 15 are the total number of blocks.
Figure 2-6. 2D radial distribution functions of phosphate atoms for four RDF pairs in the stationary stage. The lipid-lipid RDF is the average for all lipid pairs. B and C. Snapshot of complexed lipids showing hydrogen bonding between amine group and PO and the phosphate-phosphate distance. These were taken from the 300ns of the E. coli stationary phase.

the lipids. As it was mentioned earlier, the main difference in lipid compositions of each stage during the growth cycle was the concentration of lipids with a cyclopropane ring (cyC17:0). Therefore, it would be interesting to correlate some membrane properties like surface area per lipid and bilayer thickness with the percentage of lipids with a cyC17:0 chain. Linear regressions showed linear behavior for SA/lipid, $D_B$ and $2D_C$ as a function of percentage of lipids with a cyC17:0 chain (Figure 2-7 with equations provided in this figure). The P-values for the significance
of regression for SA/lipid, $D_B$ and $2D_C$ were 0.001, 0.017, and 0.072, respectively. $2D_C$ was less sensitive to the change in the amount of lipids with cyC17:0 compared to the $D_B$ and was borderline statistically significant in linearity based on its P-value.

![Graph showing regression analysis](image)

**Figure 2-7.** (top) Average surface area per lipid as a function of percentage of lipids with a cyC17:0 chain. (bottom) Bilayer thickness ($D_B$) and hydrophobic core thickness ($2D_C$) percentage of lipids with a cyC17:0 chain. Equations for linear fits are listed and reported standard errors in fitted slope and intercepts are also shown.

### 2.6. Discussion and conclusion

In summary, our MD simulations demonstrate structural properties of the cytoplasmic membrane of *E. coli* varied between stages of the bacterium life cycle albeit small in most cases. The overnight stage had the highest surface area per lipid, lowest order parameters and thinnest bilayer thickness compared to the earlylog, midlog, and stationary stages. The chain order parameters for the earlylog and midlog stages are
quiet similar, which is also the case comparing the stationary stage and overnight stage. Similar groupings can be observed for thicknesses and overall density profiles. Therefore, the *E. coli* cytoplasmic membrane can be divided into two distinct regimes during its life cycle, i.e., growth stages (earlylog and midlog) and plateau stages (stationary and overnight) with the amount of growth nearly identical to cells that die. However, the surface area per lipid was more strongly dependent on the amount of lipids with cyC17:0 chains (Table 2-2 and Figure 2-5). It appears that this more bulky acyl chain has a minimal effect on the bilayer thickness but modulates the lateral packing (reducing the lipid packing with an increase of the concentration of cyC17:0).

Our past survey of the Orientations of Proteins in Membranes (OPM) database found that the Top6 model agreed well with the hydrophobic thickness of *E. coli* cytosolic membrane proteins of 29.4±1Å. Extending this comparison now to the earlylog through overnight stages demonstrates that our range of 30.0-30.6 Å in the membrane hydrophobic thickness conforms to these protein crystal structures. The small change in this thickness suggests that the transmembrane proteins maintain similar orientations and do not need to tilt or deform the membrane at different life cycle stages. Another key result from our past work, focused on the increase in the $K_A$ for the Top6 overnight model compared to simplistic mixture of POPE/POPG. Although MD simulations here for the overnight model agree with our past simulations, the $K_A$ for other stages are reduced, but only show small changes in the $K_A$ from 0.28 (early log) to 0.33 N/m (overnight) (Table 2-1). The mixed acyl in our models compared to POPE/POPG and also a change in PE:PG ratio (5.3:1 for
POPE/POPG and 4.2:1 for our *E. coli* models) together effect the area compressibility more than our original hypothesis of the cyC17:0 chain alone. However, there is a statistically significant increase in the $K_A$ as the amount chains with cyC17:0 is increased.

Although *E. coli* dramatically increases the amount of cyC17:0 chains through its colony life cycle, the remaining question is why is this bacteria increasing this lipid? Of the properties studied, the largest change was that of the surface area per lipid and $K_A$ (10% increase from earlylog to overnight). Decreases in the lipid packing and increases in the area compressibility modulus are directly related to membrane structural integrity. It is known that upon entry into the stationary phase there is an increased expression of mechanosensitive channels (Msc) proteins that the cell uses to prevent lysis under osmotic stress $^{105}$. Since these are sensitive to the membrane stress, the increase of cyC17:0 might be important to proper function of the Msc proteins $^{105}$. Moreover, the outer membrane also changes in its composition and morphology when entering into the stationary phase $^{106}$ that can reduce the strength of the outer membrane. This increase $K_A$ along with more Msc proteins may provide a level of protection for the bacteria that is modulated by the amount of lipids with the cyC17:0 chain. In addition, it is possible that the cyclopropane ring may have some unknown function or interaction with a transmembrane protein and its increase may have some physiological importance.

Overall, choosing an appropriate mixture of diverse lipids to model organism and organelle membranes is important to accurately represent the properties and associated function of membranes in nature. Our MD simulations of the cytoplasmic
*E. coli* membrane demonstrate changes in membrane properties that significantly differ from past simplistic two-lipid models. The effect of the growth stage of the *E. coli* colony significantly influences a select set of properties of the membrane. What has not been probed in this study is the potential influence of leaflet asymmetry in lipid composition. As described in this chapter, more dramatic changes in lipid composition exist between organelles in eukaryotes and lipid biophysical properties will be dramatically influenced by these changes. The effect of lipid composition should be carefully considered in simulation as well as experimental studies when probing the function of proteins that reside in or interact with the membrane.
3. Chapter 3: Investigation of Phase Transitions of Saturated Phosphocholine Lipid Bilayers via Molecular Dynamics Simulations

3.1. Introduction

Lipid bilayers are essential building blocks for biological membranes. There has been great interest to probe cell membranes as they form the structure to cellular organelles and serve as a barrier to the transport of biological materials into the cytoplasm\(^1\). For the lipids membranes with no cholesterol (or sterols in general), there are three main phases that can exist: fluid (or liquid-crystalline) (\(L_\alpha\)), ripple (\(P_\beta\)) and gel (\(L_\beta\)) (see schematic in Figure 3-1)\(^2\). Fluid or liquid disordered (with mixtures containing sterols) is the most common phase in biology in which the fatty acid chains are completely disordered. This phase can be seen at high temperatures depending on the lipid in the membrane and characteristics of this phase are high lipid mobility and chain flexibility. The \(L_\alpha\) phase has been extensively studied using varying experimental and computational techniques\(^3-7\). However, the focus of this paper is the condensed phases of a lipid bilayer, i.e., \(P_\beta\) and \(L_\beta\). The \(L_\beta\) phase is seen at lower temperatures defined by a gel transition temperature. The acyl chains are more ordered and consist of nearly all in the \textit{trans} configuration\(^8\).

![Figure 3-1](image)

\textbf{Figure 3-1.} Schematic conformations of lipid bilayers at different phases: Head groups are shown in black circles and fatty acid chains are shown in lines, Red and blue were specified to show the interdigitation region in the \(P_\beta\) phase.
In between the L\(_\alpha\) and L\(_\beta\) phase can exist a pre-transition phase that is believe to consist of a wave-like configuration called P\(_\beta\) phase, which does not exist for all the lipids, such as those with unsaturated tails\(^{113}\). Among all lipids, which have P\(_\beta\) phase, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-sn-glycero-phosphocholine (DPPC) are the most common lipids, in which P\(_\beta\) has been studied\(^{114-116}\). The P\(_\beta\) formation can be obtained either from heating up the L\(_\beta\) phase with tilted chains or cooling down the L\(_\alpha\) phase\(^{117}\). For the P\(_\beta\) phase, a thick and thin region can be found, which are mostly defined in the literature as a major and minor arm (Figure 3-1), respectively\(^{114}\). Kink region with interdigitated fatty acid chains might exist between major arm and minor arm domains. The existence of these two arms is known, but details regarding the accurate configuration of lipid head groups and lipid tails and how they differ between the regions is unknown or under debate. Some believe in coexistence of both the L\(_\alpha\) and the L\(_\beta\) in P\(_\beta\)\(^{118}\). i.e., the major arm mostly represent the L\(_\beta\) and there is small disordered region in membrane presents the L\(_\alpha\) phase (minor arm). The stability of the P\(_\beta\) phase depends on temperature range. The thermodynamic stability for P\(_\beta\) has been evaluated using differential scanning calorimetry, X-ray diffraction, and electron microscopy and values are reported in Table 3-1 for PC lipids.

**Table 3-1.** Temperature range of stable P\(_\beta\) obtained from\(^{119-121}\)

<table>
<thead>
<tr>
<th>PC lipids</th>
<th>Temp range (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>di-18:0</td>
<td>321.65 to 330.15</td>
</tr>
<tr>
<td>18:0,16:0</td>
<td>306.25 to 319.75</td>
</tr>
<tr>
<td>16:0,18:0</td>
<td>311.25 to 321.55</td>
</tr>
<tr>
<td>18:0,14:0</td>
<td>290.85 to 305.35</td>
</tr>
<tr>
<td>di-14:0</td>
<td>287.15 to 297.15</td>
</tr>
<tr>
<td>di-16:0</td>
<td>298.65 to 313.15</td>
</tr>
</tbody>
</table>
The phase transitions of lipid bilayers with one lipid type have been studied broadly in experiments. It was shown that insertion of small molecules like n-alkanol and surfactants could perturb the phase transition of bilayers and those perturbations depended on an additive concentration. Addition of a weak polyelectrolyte, poly methacrylic acid (PMA), to a DMPC bilayer resulted in lower main transition temperature and change in morphology in the Lβ phase. The structure of the DMPC bilayer in the Pβ phase was first resolved with high resolution using scanning tunneling microscopy. The ripple wavelength and ripple amplitude were obtained and found to be 13 and 4.5 nm, respectively. Recently, John Nagle and his co-workers used X-ray diffraction to determine the structure of DMPC lipid bilayer in Pβ phase. The ripple wavelength and ripple amplitude were determined as 14.5 and 1.82 nm, respectively. X-Ray diffraction was also used to determine the structure of DMPC bilayer in Lβ phase. Those experimental results indicated the tilted fatty acid chains in the Lβ phase and also in the major arm domain of the Pβ phase. The average of tilt angle of DMPC bilayer in the Lβ phase was measured as 32.3°.

Phase transition of lipid bilayers with multiple lipid types has been studied broadly using the experimental techniques. The differential scanning calorimetry, which is the common technique to measure transition temperatures, was used to generate the phase diagram of DMPC-DPPC and other PC lipid mixtures. Other techniques such as electron spin resonance (ESR) and 2H NMR were also used to generate phase diagram of DMPC-DPPC mixture with and without presence of cholesterol. The spectra intensities obtained from 2H NMR of DMPC-DPPC
mixture with cholesterol indicated complex behavior, which was different from regular binary lipid mixtures.

Studying biological membranes with molecular detail has been of great interest since molecular simulations could lead to detailed understanding of many biological processes \(^{127}\). Molecular simulations have been used broadly to interpret the behavior of cell membranes in different biological pathways \(^{128}\). Molecular dynamics (MD) simulations were widely used to study different phases of lipid bilayers \(^{129-133}\). Coarse-grained (CG) and all-atom (AA) molecular simulations can be used to probe the different phases of lipid bilayers depending on the level of resolution and timescale needed to understand the phase changes \(^{134, 135}\). The AA-MD approach will result in more detail of the structural membrane properties, while CG-MD can probe longer length and timescales. Selecting the proper force field plays an important role to capture phase transitions in a reasonable range of temperatures. The AMBER and CHARMM lipid force fields have been used to study phase transition of lipids in AA-MD \(^{136-138}\), while Martini force field is commonly used in CG-MD \(^{139, 140}\). A previous CG-MD simulation was able to investigate the effect of charged nanoparticles on phase transformation of DPPC bilayer \(^{141}\). Simulations indicated the phase transition from L\(_a\) to L\(_\beta\) by analyzing the surface area per lipid and order of molecules obtained from radial distribution function. Symmetric and asymmetric P\(_\beta\) phases of DPPC bilayer were obtained from rapidly and slowly cooling down from L\(_a\) with Monte Carlo simulations using coarse-grained model \(^{142}\). Interdigitation was not seen clearly in those simulations, and fatty acid chains in thick region of asymmetric P\(_\beta\) phase were not as tilted as in the L\(_\beta\) phase. CG MD simulations were also used to represent
the \( L_\beta \) phase of DPPC bilayer\textsuperscript{143}. The CG model was not able to capture the tilt but the transition temperature from \( L_\alpha \) to \( L_\beta \) was in agreement with experiment.

AA-MD simulation studies of phase transition are rare compared to CG model simulations. AA-MD simulations were performed on DPPC lipid bilayers in order to capture the \( P_\beta \) phase. The \( P_\beta \) phase was captured below the experimental transition temperature, and the interdigitation was clearly seen in minor-arm region \textsuperscript{116}. Atomistic simulations were performed to probe the transition states of DPPC and DPPE bilayer\textsuperscript{144}. Structures of DPPC bilayers at lower temperatures did not represent a uniform tilted fatty acid chains. The \( P_\beta \) phase with comparable features to the experiment was not defined. The interdigitation, which is the challenging concept in the \( P_\beta \) phase of DMPC bilayer, was not predicted in minor arm region of DMPC bilayer based on X-ray scattering \textsuperscript{114}, while in our work, as Marrink and his co-workers showed previously \textsuperscript{116}, high interdigitation and disordered in the minor arm region was obtained.

Past studies have used simulation to probe the structural characteristics of the \( L_\beta \) phase bilayers. MD simulations were performed at 19°C for DPPC bilayers, and the \( L_\beta \) phase with the tilted acyl chains was obtained with fair agreement with experiment \textsuperscript{129}. United-atom force field was used to represent the \( L_\beta \) phase of DPPC \textsuperscript{145}. Their modified force field represented the \( L_\beta \) phase in better agreement with the experiment in terms of tilt and surface area per lipid. MD simulations were performed on DPPC bilayer in the \( L_\beta \) phase and the tilt angle and area compressibility modulus were calculated in good agreement with the experiment \textsuperscript{146}. Recently, AA-MD simulations with Berger united-atom and CHARMM36 force fields were carried out to represent
the L_β phase of DPPE^{147}. Highly ordered bilayer in the L_β phase was obtained only with the biased ordered backbone in the initial setup. Berger force field predicted the main transition temperature 7K to 17K below the experimental result^{148}.

In this chapter, structures of unbiased formation of DMPC and DPPC condensed phases starting structures in the L_α phase to form the P_β and/or L_β phase(s). The phase transition is shown for pure DMPC and DPPC lipid bilayers and their mixtures as well and these transition temperatures are compared to the experiment. The P_β of the pure DMPC bilayer at different system sizes was obtained to demonstrate that CHARMM36 force field could capture key the fundamentals of the P_β phase independent of system size. The ratio of the major arm relative to the minor arm was also studied for different sizes to determine its size dependence. To our knowledge, this work is the first to present the formation of the P_β phase of DMPC bilayer using AA-MD at the temperature, which P_β phase was seen in experiment^{114}. Our results indicated the interdigitation of fatty acid chains in the minor arm domain and gel-like behavior in the major arm domain. Wavelength and other properties of DMPC bilayer obtained from simulations were compared with the experimental results from X-ray diffraction^{114}. In addition to the P_β phase, we calculated chain tilt angles in the L_β phase bilayers and are in fair agreement with experimental values. The phase diagram of DMPC-DPPC mixtures at different molar concentrations of lipids is also presented at the end of this section.
3.2. Method

AA-MD simulations using explicit water molecules were performed for pure and mixed lipid bilayers. The CHARMM-GUI Membrane Builder was used to build the initial configurations of bilayers in the Lα phase\textsuperscript{71, 73}. The NAMD simulation package\textsuperscript{93} was used to carry out minimization, equilibration (following the standardized CHARMM-GUI procedure) and production run MD simulations. Each simulation equilibrated at 310.15K initially using the CHARMM-GUI, and then MD simulations were run at specific temperatures. Simulations were carried out with isobaric-isothermal ensemble (NPT). The pressure was fixed at 1 bar and temperatures varied depending on what system we were studying. Details regarding temperatures will be discussed in next sections. The Lennard-Jones potential was used to describe the van der Waals interactions, and a force-based switching function in the range of 8 to 12Å was used. After equilibration, simulations were carried out from 300ns to 1µs depending upon the system size and specific phase of lipid bilayer. A 2-fs timestep was used for all simulations. The particle Mesh Ewald method was selected for long-range electrostatics interactions. Hydrogen atoms were constrained by using the RATTLE algorithm. Here, the CHARMM36 (C36) force field\textsuperscript{58} was used as we have evaluated the accuracy of force field in previous studies\textsuperscript{149-151}. All systems were fully hydrated and hydration number was constant at 35 waters per lipid. The Nosé–Hoover Langevin-piston algorithm was used to keep the pressure and Langevin dynamics was used to maintain the temperature throughout the simulations\textsuperscript{152, 153}. For each simulation, last 100ns was used for analysis. The total time for all simulations is 28.8 µs. The summary of all systems is found in Table 3-2.
Table 3-2. Simulation details for all membranes. # of lipids is total (both leaflets).

<table>
<thead>
<tr>
<th>lipids</th>
<th>#lipids</th>
<th>time(ns)</th>
<th>#replicates</th>
<th>total time (µs)</th>
<th>Temperature (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC-small</td>
<td>72</td>
<td>500</td>
<td>3</td>
<td>1.5</td>
<td>273.15</td>
</tr>
<tr>
<td>DMPC-small</td>
<td>72</td>
<td>500</td>
<td>3</td>
<td>1.5</td>
<td>275.17</td>
</tr>
<tr>
<td>DMPC-small</td>
<td>72</td>
<td>300</td>
<td>18</td>
<td>5.4</td>
<td>278.15-303.15</td>
</tr>
<tr>
<td>DMPC-medium</td>
<td>288</td>
<td>500</td>
<td>18</td>
<td>5.4</td>
<td>291.15</td>
</tr>
<tr>
<td>DMPC-large</td>
<td>1152</td>
<td>1000</td>
<td>1</td>
<td>1</td>
<td>291.15</td>
</tr>
<tr>
<td>DMPC-DPPC</td>
<td>72</td>
<td>300</td>
<td>27</td>
<td>8.1</td>
<td>283.15-323.15</td>
</tr>
<tr>
<td>DMPC-DPPC(_{1:3})</td>
<td>72</td>
<td>300</td>
<td>15</td>
<td>4.5</td>
<td>293.15-313.15</td>
</tr>
<tr>
<td>DMPC-DPPC(_{1:1})</td>
<td>72</td>
<td>300</td>
<td>15</td>
<td>4.5</td>
<td>293.15-313.15</td>
</tr>
<tr>
<td>DMPC-DPPC(_{3:1})</td>
<td>72</td>
<td>300</td>
<td>15</td>
<td>4.5</td>
<td>293.15-313.15</td>
</tr>
</tbody>
</table>

3.3. Results

3.3.1. Pure DMPC

Three systems with different size were built using CHARMM-GUI Membrane Builder: small (72 lipids), medium (288 lipids), and large (1152 lipids) sizes. For the small DMPC bilayer system, a set of temperatures starting from 273.15K to 303.15K was selected to capture the phase transitions from the L\(_{α}\) to the P\(_{β}\) and to the L\(_{β}\) phase. The fatty acid chains were all disordered as the L\(_{α}\) phase in initial setup obtained from CHARMM-GUI and systems were packed as if they were in the L\(_{α}\) phase. Then, the temperature of interest was selected for each specific run. The temperature range was selected based on known experimental main transition temperature of DMPC bilayer, which is 297.15K \(^{154}\). Simulations at lower temperatures (273.15K and 275.15K) were carried out for 500ns, while the rest were carried out for 300ns. Surface area per lipid, which is X·Y divided by number of lipids of each leaflet, is the first measure to show the phase transition trend (Figures 3-2, 3-3, 3-4). The sharp decrease in surface
area per lipid indicates transition from the $L_{\alpha}$ to the $L_{\beta}$ phase (Figure 3-2) based on the included snapshot structure. Based on Figure 3-2, the average surface area per lipid for that specific run at 275.15K is between the values of the $L_{\beta}$ phase and the $L_{\alpha}$ phase, which might indicate the formation of a fully $P_{\beta}$ structure (see snapshot inset). Confirming the transition to the $P_{\beta}$ phase can be hard to predict just based on surface area per lipid analysis and more analysis is needed to justify the temperature range, which will be discussed later. Table 3-3 shows the average surface area per lipid over the last 100ns of the pure DMPC simulations. Apparently, there is a dramatic change in the surface area that occurred between 288.15K and 293.15K, which indicates a phase transition occurs within this temperature range.

When simulations were run at 275.15K, melting of head group and the interdigitation of fatty acid chains indicated starting point of the $P_{\beta}$ phase formation (Figure 3-5). At 278.15K, the interdigitation still can be seen but chains are more ordered compared to structure at 275.15K (Figure 3-5). The $P_{\beta}$ phase was stable at 283.15K and 288.15K, and chains are tilted with more order compared to the $P_{\beta}$ phase at 275.15K (Figure 3-5). Stable DMPC structure in the $P_{\beta}$ phase until 288.15K is in fair agreement with the experiment as X-ray diffraction resolved the DMPC in the $P_{\beta}$ phase at 291.15K \textsuperscript{114} (more details on ripple in the next sub-section). At 293.15K, the fatty acid chains were highly disordered, which indicated the $L_{\alpha}$ phase of DMPC bilayer (Figure 3-5). Similar results ($L_{\alpha}$ phase) were obtained for higher temperatures of 298.15 and 303.15K (Figure 3-5). Snapshots of all other replicates can be found in Figure 3-6. This main transition temperature to the $L_{\alpha}$ phase obtained from simulations is in agreement with the experimental transition temperature, which is
297.17K$^{154}$. 

**Figure 3-2.** Surface area per lipid vs. time of a single replicas simulations at 273.15K and 275.15K resulted in the L$_\beta$ phase and the P$_\beta$ phase respectively.

**Figure 3-3.** Time series of surface are per lipid of L$_\beta$ phase (273.15K) and P$_\beta$ phase (275.15K) of small DMPC lipid bilayers.
Figure 3-4. Time series of surface are per lipid of small-size DMPC simulations at different temperatures
Table 3-3. Averaged surface area per lipid (±std. dev.) over 3 replicates of last 100ns simulations of pure DMPC (small) bilayer

<table>
<thead>
<tr>
<th>Temp (K)</th>
<th>Å²/lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>273.15</td>
<td>50.3±0.3</td>
</tr>
<tr>
<td>275.15</td>
<td>50.1±1.4</td>
</tr>
<tr>
<td>278.15</td>
<td>51.7±0.3</td>
</tr>
<tr>
<td>283.15</td>
<td>52.3±0.4</td>
</tr>
<tr>
<td>288.15</td>
<td>51.9±0.9</td>
</tr>
<tr>
<td>293.15</td>
<td>58.6±0.1</td>
</tr>
<tr>
<td>298.15</td>
<td>59.5±0.5</td>
</tr>
<tr>
<td>303.15</td>
<td>60.2±0.2</td>
</tr>
</tbody>
</table>

Figure 3-5. Small size DMPC Bilayers at different temperatures. Water and hydrogen atoms were not shown for clarity. Upper and lower leaflets were shown in gray and green, respectively. Phosphate atoms were shown in pink. All images are of just the primary cell.
The following parts of this section describe in detail the properties of the \( L_\beta \) and \( P_\beta \) phases. The focus of this work will be quantifying the structural properties of these condensed bilayer phases. However, due to this freezing behavior many replicas can get trapped in an intermediate gel-like states (Figure 3-2 and Figure 3-6). As will be
described in the following sections, averages of membrane properties are based off well-formed bilayer structures.

### 3.3.1.1. Phase properties of L\(_{\beta}\) phase

In this sub-section, a discussion of the L\(_{\beta}\) phase properties of the DMPC bilayer is presented based on simulations with the small system. The end configuration of a single replica for each temperature can be found in Figure 3-5. Snapshots of other replicates and temperatures can be found in the supplementary materials (Figure 3-5). Figure 3-5 indicates the DMPC bilayer at 273.15K is in the L\(_{\beta}\) phase as nearly all the fatty acid chains are tilted relative to the bilayer normal and chains are arranged in a high order. The order parameter of acyl chain, which is the measure of the order in C-H vector, was calculated using this formula: 

\[
S_{CD} = \langle \frac{3}{2} \cos^2 \beta - \frac{1}{2} \rangle,
\]

where \(\beta\) is the angle between bilayer normal and C-H vector. Order parameters were calculated for both fatty acid chains of the DMPC bilayer in the L\(_{\beta}\) phase at 273.15K. The average order parameters of C2 to C13 of sn-1 and sn-2 were 0.256±0.02 and 0.252±0.02, respectively (Figure 3-7). The \(S_{CD}\) values are not high for the L\(_{\beta}\) phase as one typically sees for liquid-ordered phases\(^{150, 155}\). This is due to tilted fatty acid chains as the order parameters were obtained higher in P\(_{\beta}\) phase, where fatty acid chains are less tilted.
Figure 3-7. Average order parameters of C2-C13 of sn-1 chain (top) and sn-2 chain (bottom) of small DMPC in the L\textsubscript{β} phase, P\textsubscript{β} phase, and L\textsubscript{α} phase.

The tilt angles of fatty acid chains were calculated for all replicates in the L\textsubscript{β} phase at 273.15K-run1, 275.15K-run1, 275.15K-run3, and 288.15K-run2. The averaged value based on the last 100ns of simulation was 32.2±1.6°. This is in good agreement with the experimental tilt angle of DMPC in the L\textsubscript{β} phase obtained from the X-ray diffraction, which is 32.3°±0.6° (Figure 3-8). Third replica at 275.15K represented the L\textsubscript{β} configuration with a lowest tilt angle, where the average during the last 100ns of simulation was 26.27±0.5° (Figure 3-6).
Electron density profiles were generated for all replicates in the L$_\beta$ and the L$_\alpha$ phase using the SIMtoEXP program. The average electron density profiles of both phases are shown in Figure 3-9. The Head-to-head group distance ($D_{HH}$) was calculated from average total electron density profiles of all the L$_\beta$ replicates as 40.4$\pm$0.5Å, which was in fair agreement with 40.1$\pm$0.1Å obtained from the experiment. The Component electron density profiles can be found in Figure 3-10.

In the L$_\alpha$ phase, peak positions of phosphate, choline, and carbonyl groups shifted toward the bilayer center to provide thinner membrane compared to the L$_\beta$ phase. The bilayer thickness, $D_B$, which is defined as the distance between the midpoints of the volume probability of water, and $2D_C$, which is defined as the distance between the midpoints of bilayer’s hydrocarbon chains volume probability, were calculated for the L$_\beta$ phase and they were 40.3$\pm$0.5Å and 29.0$\pm$0.5Å, respectively. This latter result is in good agreement with the experimental result as $2D_C$ was measured as 30.3$\pm$0.2Å by
using the X-Ray diffraction\textsuperscript{112}.

**Figure 3-9.** Average total electron density profiles of small DMPC bilayers in L\textsubscript{\(\beta\)} phase and L\textsubscript{\(\alpha\)} phase.

**Figure 3-10.** Average component electron density profiles of small DMPC bilayers in the L\textsubscript{\(\beta\)} phase (Top) and in the L\textsubscript{\(\alpha\)} phase (Bottom)
3.3.1.2. Quantifying the $P_\beta$ phase of DMPC

In this sub-section, we will present our analysis of the $P_\beta$ phase of DMPC and its comparison with the experiment. This required simulations at varying system size to determine if the $P_\beta$ phase properties depend on the number of lipids. The first part of this section will focus on the results from the small system size of 72 DMPC molecules.

As it was mentioned in introduction, lipid bilayers in the $P_\beta$ phase are believed to have wave-like configuration. Figure 3-11 shows the $P_\beta$ phase conformation by using the periodic images so the wave-like structure of bilayer can be seen clearly. The wave-like behavior varies between the replicas and is periodic in either the Y or X direction depending on the development of the $P_\beta$ phase. Some common features of this phase is a gel-like region with little to none chain interdigitation and a region with significant chain interdigitation between leaflets. These regions likely correspond the major (gel-like region) and minor (thin and interdigitated region) arms.

In order to quantify the minor arm and major arm regions, a contour plot of the difference in the z-position of the end carbons between upper leaflet and lower leaflet was averaged (Figure 3-12). This is a direct measure of the interdigitation but also is related to the membrane thickness, i.e., the more negative a value the thinner the membrane. As the center of bilayers was fixed at zero, negative values in contour plot represents the interdigitation.
Figure 3-11. Snapshots of small size DMPC bilayers in the Pβ phase at different temperatures and views using boundary images.

The contour plots were generated for all wave-like configurations obtained from end of simulations to see if they are actually fully rippled. If so, different properties of the Pβ phase like wavelength, major arm thickness, minor arm thickness and ripple amplitude were calculated. Snapshots indicated the Pβ phase configurations at 275.15, 278.15, 283.15, and 288.15K. The contour plots were generated for all the Pβ phase replicates at these temperatures (Figures 3-12 to 3-17). The periodic boundary images were included to make sure we captured the entire unit cell in the calculation. The contour plots indicated fully rippled structure for 275.15K-Run2, 283.15K-Run1, 288.15K-Run1, and 288.15K-Run3. The Pβ phase in 288.15K-Run3 was formed in a Y direction, while other fully rippled structures were formed in the X direction. The distances greater than zero were selected to identify the major arm region. The Distances between zero and -2Å indicated kink region of the Pβ phase, and distances less than -2Å were specified to identify the minor arm domain of membranes. Therefore, light blue on the contour plots indicated the kink and less interdigitation
area and dark blue indicated the highly interdigitated region in the minor arm domains. The average distances of highly interdigitated area varied from -8Å to -10Å. The light and dark yellow colors showed the major arm domains in all the Pβ phases depending on how distances are close to zero. Average length of the major arm and the minor arm domains were calculated and were reported in Table 3-4.

An early experimental study suggested the major arm thickness of DMPC bilayer to be 75% bigger than the minor arm. Recent X-ray diffraction study suggested the major arm of the Pβ phase to be almost 2.5 times bigger than minor arm. Our simulations of small-size DMPC bilayers are suggesting averaged major to minor arm ratio of 1.75±0.25, which is in good agreement with the early experimental result that was just mentioned above. Reason behind dramatic difference with recent experiment will be discussed in discussion. The Pβ amplitude, which is the difference between maximum and minimum Z-position of phosphate atoms in the major arm, were also calculated for all the fully rippled structures. The contour plots of the position of phosphate atoms of both leaflets were generated (Figure 3-13,3-14) to obtain the minimum and maximum positions of phosphate atoms in the major arm during the last 100ns of simulations. Reported values in Table 6 are based on an averaging from upper leaflet and lower leaflet. High standard error indicated variation of amplitude in leaflets. Wavelength of each Pβ phase was obtained from summation of the minor arm, the kink, and the major arm domains. The average ripple amplitude of the small systems is almost half of the experimental result, which is due to the small system-size and the correct value may be found using larger systems (see discussion section later in this chapter).
Table 3-4. Characteristics of Pβ phase at different temperatures of small DMPC bilayers. Z represents the bilayer thickness of different regions. M represented the Medium-size and L represented the large DMPC with uniform ripple configuration 1 and 2 represents Pβ1 and Pβ2 conformations, which correspond to two ripple conformations in large DMPC bilayer.

<table>
<thead>
<tr>
<th>Temp(k)</th>
<th>Major(Å)</th>
<th>Minor(Å)</th>
<th>Kink(Å)</th>
<th>Wave(Å)</th>
<th>Amp(Å)</th>
<th>Major/Minor</th>
<th>Z-minor(Å)</th>
<th>Z-major(Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>275.15-R2</td>
<td></td>
<td></td>
<td></td>
<td>19.7±1.0</td>
<td>16.9±1.1</td>
<td>4.1±0.5</td>
<td>40.7±0.4</td>
<td>6.8±0.3</td>
</tr>
<tr>
<td>283.15-R1</td>
<td>25.3±0.3</td>
<td>11.3±0.6</td>
<td>3.7±0.2</td>
<td>40.3±0.1</td>
<td>6.6±2.3</td>
<td>2.3±0.1</td>
<td>31.6±0.2</td>
<td>45.3±0.1</td>
</tr>
<tr>
<td>288.15-R1</td>
<td>21.5±1.6</td>
<td>15.0±1.6</td>
<td>4.3±0.4</td>
<td>40.8±0.1</td>
<td>6.8±0.6</td>
<td>1.5±0.3</td>
<td>29.5±0.2</td>
<td>44.5±0.7</td>
</tr>
<tr>
<td>288.15-R3</td>
<td>24.0±0.7</td>
<td>12.4±0.7</td>
<td>3.9±0.7</td>
<td>40.3±0.9</td>
<td>6.9±2.3</td>
<td>2.0±0.1</td>
<td>29.6±2.8</td>
<td>45.0±0.6</td>
</tr>
<tr>
<td>291.15(M)</td>
<td>47.5±4.3</td>
<td>32.4±4.5</td>
<td>4.3±0.4</td>
<td>85.0±0.3</td>
<td>13.7±0.6</td>
<td>1.7±0.4</td>
<td>30.4±0.2</td>
<td>44.1±0.9</td>
</tr>
<tr>
<td>291.15(L1)</td>
<td>30.8±3.1</td>
<td>18.7±1.7</td>
<td>2.1±0.3</td>
<td>51.6±4.2</td>
<td>8.8±1.9</td>
<td>1.7±0.1</td>
<td>30.3±0.3</td>
<td>40±1.5</td>
</tr>
<tr>
<td>291.15(L2)</td>
<td>31.5±0.4</td>
<td>18.7±1.7</td>
<td>1.8±0.2</td>
<td>52.1±1.6</td>
<td>6.4±2.3</td>
<td>1.6±0.1</td>
<td>30.3±0.3</td>
<td>43.5±0.5</td>
</tr>
<tr>
<td>Exp/“</td>
<td>102</td>
<td>43</td>
<td>----</td>
<td>145</td>
<td>18.2</td>
<td>2.4</td>
<td>----</td>
<td>41.8</td>
</tr>
<tr>
<td>291.15(L)</td>
<td>99</td>
<td>44</td>
<td>----</td>
<td>143</td>
<td>2.25</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>

Figure 3-12. Averaged contour plots for the difference in the z-position of the end carbons between upper leaflet and lower leaflet during the last 100ns of simulations. Color bar units are in Å. Periodic images were included in all figures.
Figure 3-13. Contour plots of average Z-position of phosphate atoms at upper leaflet over the last 100ns of simulations of small DMPC bilayers in fully rippled configuration. Color bars are in Å. Periodic images were included.

Figure 3-14. Contour plots of average Z-position of phosphate atoms at lower leaflet over the last 100ns of simulations of small DMPC bilayers in fully rippled configuration. Color bars are in Å. Periodic images were included.
**Figure 3-15.** Averaged contour plots for the difference in the z-position of the end carbons between upper leaflet and lower leaflet during last 100ns of simulations of small DMPC bilayers in NOT fully rippled configuration. Color bars are in Å. Periodic images were included.

**Figure 3-16.** Contour plots of average z-position of phosphate atoms at upper leaflet over the last 100ns of simulations of small DMPC bilayers in NOT fully rippled configuration. Color bars are in Å. Periodic images were included.
Figure 3-17. Contour plots of average z-position of phosphate atoms at lower leaflet over the last 100ns of simulations of small DMPC bilayers in NOT fully rippled configuration. Color bars are in Å. Periodic images were included.

Thickness of bilayer in the minor arm and the major arm domains were calculated from Head-to-head distances obtained from the total electron density profiles (Table 3-4). The average D_{HH} of all the fully rippled small bilayers was 44.9±0.2 Å, which is almost 4 Å higher than our average simulation results of the L_{β} phase, and 3 Å higher than experimental result for the major arm thickness. The first replicate at 288.15K represented fatty acid chains similar to the L_{β} phase in the upper leaflet (Figure 3-5). The overall tilt angle relative to the membrane normal was calculated for upper leaflet and it was 23.0 ± 0.1°. The tilt is lower than average tilt angle obtained from bilayers in the L_{β} phase. This is also reason behind getting higher order parameter for the P_{β} phase compared to the L_{β} phase that was mentioned earlier (Figure 3-7). Average electron density profile of the major arm domains in a fully P_{β} phases was compared
to the average density profile of gel replicates, and similar trend was found in both profiles (Figure 3-18).

**Figure 3-18.** Average electron density profiles of small DMPC bilayers in the major arm domain of the Pβ phase and the Lβ phase.

Simulations with larger system sizes were carried out to probe the effect of amount of lipids in a simulation setup on the Pβ phase properties. Simulations of medium-size and large-size were performed at 291.15K, where the Pβ phase configuration was obtained from X-Ray scattering at that temperature. Time series of surface area per lipid were generated for medium and large system to make sure they both reached the equilibrium condition (Figure 3-19). The average D_{HH} was not affected by the system size and was found to be 42.6±1.2Å for the medium and large system in fair agreement with the small bilayers. To quantify ripple length, the contour plots of average distances of end carbons between upper leaflet and lower leaflet of medium-
size DMPC bilayer was generated (Figure 3-20). The Pβ phase was fully developed for this system size in the X direction, and wavelength was more than twice compared to average wavelength of small systems (Table 3-4). The contour plots of Z-position of phosphate atoms in medium-size DMPC bilayer resulted in 13.7±0.6Å ripple amplitude, which is in much closer to the experimental result (18.2 Å) obtained from the X-ray diffraction compared to small systems114 (Figure 3-21).

Figure 3-19. Time series of Medium-size DMPC bilayer in the Pβ phase, Bottom: times series of surface area per lipid of large-size DMPC bilayer in the Pβ phase
Figure 3-20. Averaged contour plots for the difference in the z-position of the end carbons between upper leaflet and lower leaflet during last 100ns of simulations for the medium-size DMPC bilayer at 291.15K. Color bar units are in Å. Periodic images were included.

Figure 3-21. Contour plots of Average z-position of phosphate atoms over the last 100ns of simulations of medium-size DMPC bilayers. Color bars are in Å. Periodic images were included.
The average major arm and minor arm regions were 47.5±4.3 Å and 32.4±4.5 Å, respectively, which means the major arm is almost 47% percent bigger than the minor arm in this case. The average ripple wavelength was 85.0±0.3 Å, which is lower than the experimental result due to the limit of the system size is under the wavelength.

Configuration of the medium-size DMPC bilayer in the Pβ phase at the end of the simulation could be seen in Figure 3-22. The distinct domains can be clearly seen as it was quantitatively obtained from the contour plot. The interdigitation of acyl chains and lower membrane thickness in the minor arm compared to more ordered region in the major arm could be clearly seen. The overall average tilt angles relative to the membrane normal of the major arm domain were calculated for the last 100ns of simulation and the tilt was 27.9±1.1° and 10.3±3.1° for upper leaflet and lower leaflet respectively. The disparity between leaflets was seen due to different tilt angle relative to the Z-axis. The tilt in upper leaflet represented gel-like configuration more as the experimental result of tilt angle for DMPC in Lβ phase is 32.3±0.6°.112.

Figure 3-22. Medium-size DMPC bilayer in the Pβ phase at 291.15K. Phosphate atoms were shown in pink. Upper leaflet was shown in grey, and lower leaflet was shown in green, hydrogen atoms were not shown for clarity.
The large-size DMPC bilayer simulation at 291.15K indicated the $P_\beta$ phase in both X and Y directions with more clearly ripple feature in a Y direction (Figure 3-23). Time evolution of the $P_\beta$ phase formation could be seen from contour plots generated at different stages of simulation from beginning to middle and toward the end of simulation. The lateral ripple formation has reached an equilibrated state from 600ns. The contour plot of average distances of end carbons between upper leaflet and lower leaflet showed stronger $P_\beta$ phase in a Y direction (Figure 3-24). Despite a fully $P_\beta$ phase of small systems and replicate for the medium size system, the large ripple bilayer showed two major arms and two minor arms in one unit cell (Figure 3-25). Highly interdigitated region is located between two gel-like configurations, which we can count as two major arms. Therefore, we can assume these two major arm domains share highly interdigitated regions. Therefore, half of the average length of interdigitated area obtained from the contour plot was calculated to be the minor arm length of each $P_\beta$ configuration (Table 3-4). The average lengths of the major arm 1 and major arm 2 regions were calculated as 30.8±3.1Å and 31.5±0.4Å respectively. Average length of the minor arm domain for each ripple configuration was 18.7±1.7Å. Therefore, $P_{\beta1}$ and $P_{\beta2}$ configurations had the major arm 64% and 68% bigger than the minor arm respectively. As it can be seen in Figure 3-25, the major arm 1 represented more aligned and tilted fatty acid chains compared to the major arm 2. The total tilt angle relative to the membrane normal of lipids in the major arm 1 was calculated as 31.0±0.6° and 8.9±0.7° for upper leaflet and lower leaflet respectively. The tilt angle of upper leaflet was in good agreement with experimental tilt angle in $L_\beta$ phase, which it was stated earlier. Contour plots of average Z-position
of phosphate atoms in both leaflets were generated to quantify major ripple amplitude (Figure 3-26). In this case the average ripple amplitude for ripple 1 and ripple 2 were 8.8±1.9Å and 6.4±2.3Å respectively. Average amplitudes are in the same range as small systems indicating wavelength could affect the amplitude.

**Figure 3-23.** Multiple views of large-size DMPC bilayer in P$_{11}$ phase at 291.15K

Contour plots indicate multiple directions for ripple formation in the large DMPC bilayer. This is likely due to the system size and relatively short simulation timescale. Although the last 400ns show a converged multiple directions for the ripple, it is
possible that for an order of magnitude increase in simulation time a fully-ripple configuration in one direction, which is Y direction in our simulation, could be obtained. The island of minor arm centered at (+25 Å, -25 Å) might coalesce with the other minor domains. If we instead look at the region of the contour plots where the maximal lengths of the major/minor arms (X near +70 Å), then the major/Minor ratio will be ~2.25 (Figure 3-24). This ratio is in good agreement with the experimental ratio of 2.4. The major arm length, which is one important feature in the Pβ phase, would also be calculated as 99 Å in good agreement with the experimental result from X-Ray diffraction (97 to 102 Å).

These results indicated that formation of the Pβ phase in large DMPC bilayer was not as uniform as previous Pβ structures with a smaller system size. However, the interdigitation of acyl chains could be clearly seen in multiple regions of bilayer suggesting that the existence of interdigitation was not an artifact of system size.

**Figure 3-24.** Averaged contour plots for the difference in the z-position of the end carbons between upper leaflet and lower leaflet during last 100ns of simulations for the large-size DMPC bilayer at 291.15K. Color bar units are in Å. M and m represented major and minor arms respectively. Periodic images were included.
**Figure 3-25.** Two \( \beta \) conformation in one unit cell of large DMPC simulation.

**Figure 3-26.** Contour plots of Average z-position of phosphate atoms over last 100ns of simulations of large-size DMPC bilayers. Color bars are in Å. Periodic images were included.
3.3.2. Pure DPPC

To probe the influence of chain length, simulations were carried out for DPPC on a set of temperatures from 283.15K to 323.15K to capture different phases at different temperature ranges. The range of a phase stability temperatures are higher compared to DMPC, as we know the main phase transition occurs at 314.15K for DPPC bilayer due to longer fatty acid chains, which is 17K higher than main transition of DMPC\textsuperscript{154}. The surface area per lipid was calculated to indicate the temperature range of transition from the L\textsubscript{α} to the P\textsubscript{β} and then to the L\textsubscript{β} phase. Sharp decrease in a surface area per lipid occurred at 308.15K suggesting transition from the L\textsubscript{α} to the P\textsubscript{β} or the L\textsubscript{β} phase below 313.15K (Table 3-5). This means DPPC bilayer is stable in the L\textsubscript{α} phase at 313.15K. Time series of all replicates could be found in Figures 3-27. Snapshots at the end of simulations (Figure 3-28) are used to initially verify formation of the P\textsubscript{β} or the L\textsubscript{β} phases at lower temperatures. At the lowest temperature, 283.15K, MD simulations indicated the sub-gel phase of DPPC lipid bilayer, where the fatty acid chains were tilted but in the opposite direction relative to each other. At 288.15K, most fatty acid chains were still in high order but small portion of chains were disordered. At a higher temperature of 293.15K, some head groups on upper leaflet were melted and the interdigitation of fatty acid chains occurred. At 298.15K, the L\textsubscript{β} phase with almost all tilted chains relative to bilayer normal was obtained. The L\textsubscript{α} phase was obtained at 313.15K, which is in agreement with our surface area per lipid analysis. The L\textsubscript{α} phase was remained stable at 318.15K (Figure 3-28). Snapshots of all other replicates can be found in Figure 3-29. It appears that higher tendency
exists at this system size of DPPC bilayer to maintain the $L_\beta$ configuration, while DMPC bilayer was stable in $P_\beta$ phase more than $L_\beta$ phase at same system size.

**Figure 3-27.** Time series of surface area per lipid of DPPC simulations at different temperatures
Table 3-5. Surface area per lipid, averaged over the last 100ns of DPPC simulations

<table>
<thead>
<tr>
<th>Temp (K)</th>
<th>Å²/lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>283.15</td>
<td>49.5±0.2</td>
</tr>
<tr>
<td>288.15</td>
<td>50.0±0.4</td>
</tr>
<tr>
<td>293.15</td>
<td>51.8±0.9</td>
</tr>
<tr>
<td>298.15</td>
<td>50.1±0.4</td>
</tr>
<tr>
<td>303.15</td>
<td>49.6±0.5</td>
</tr>
<tr>
<td>308.15</td>
<td>50.6±0.5</td>
</tr>
<tr>
<td>313.15</td>
<td>59.1±0.1</td>
</tr>
<tr>
<td>318.15</td>
<td>60.4±0.3</td>
</tr>
</tbody>
</table>

Figure 3-28. Snapshots of DPPC bilayers at the end of simulations.
Figure 3-29. Snapshots at the end of DPPC simulations

The probability of tilt distribution was calculated for eight replicates represented the L_β configuration (Figure 3-30). Two peaks were shown between 30° and 33° of first replicates at 288.15K and 303.15K, which is the close range to the experimental result. Other replicates indicated higher tilts from 34° to 37°. The Sub-gel configurations were not considered in tilt calculations. The average tilt angle over the
last 100ns of simulations was calculated as 36.1±0.6°, which is higher than the experimental tilt angle of DPPC in Lβ phase (32±0.5°) \(^{158}\).

![Probability distribution of tilt angles of DPPC bilayers in Lβ phase.](image)

**Figure 3-30.** Probability distribution of tilt angles of DPPC bilayers in Lβ phase.

The average electron density profiles were generated from all replicates in the Lβ phase and the Lα phase (Figure 3-31). The average Head-to-head group distance (D_{HH}) was 44.7±0.4Å for the Lβ phase. Order parameters of acyl chains were calculated for all the Lβ configurations at 288.15K, 298.15K and 303.15K. The average values from C3 to C15 of sn-1 and sn-2 were 0.217±0.007 and 0.221±0.006, respectively. The average order parameter profiles of DPPC bilayers in different phases can be found in Figure 3-32. The average value of Lβ phase was relatively low and that was due to the high tilted acyl chains during simulations.
Figure 3-31. Average total electron density profile of DPPC bilayers in $L_\beta$ and $L_\alpha$ phases.

Figure 3-32. Average order parameters of DPPC bilayer at different phases, Top: Sn-1 Bottom: Sn-2
As it was mentioned before, third replicate at 293.15K showed ripple characteristics. In order to see if this structure is fully rippled, the contour plot of average distances between end carbons between upper and lower leaflets was generated as same as it was done for DMPC bilayers (Figure 3-33). The distinct regions for the minor arm and major arm could be defined but the minor arm region was not fully developed, as it was the same case for some DMPC replicates in the Pβ phase with small-size system. The average major arm and minor arm thicknesses were $38.7\pm4.7\text{Å}$ and $8.46\pm1.0\text{Å}$, respectively. The major arm to minor arm ratio is too high in this case due to not having a fully developed Pβ phase. As seen in Figure 3-28 a small portion of membrane represented melting of head groups and this ratio is too high for this system size compared to what we obtained for DMPC bilayer with same number of lipids. The average ripple amplitude was $5.2 \pm 0.04 \text{Å}$ based on contour plots of Z-positions of phosphate atoms of two leaflets (Figure 3-34). The ripple amplitude range is similar to what we obtained for DMPC bilayer with a same system size indicating larger system size is needed to reproduce more realistic Pβ conformation. It appears that DPPC bilayer with this system size tends to form the Lβ compared to the Pβ phase.
Figure 3-33. Contour plots of average distances of end carbons between upper leaflet and lower leaflet during last 100ns of simulation of medium-size DPPC bilayer at 293.15K color bar numbers are in Å. Periodic images were included.

Figure 3-34. Contour plots of Average z-position of phosphate atoms over the last 100ns of simulations of DPPC bilayers. Color bars are in Å. Periodic images were included.

3.3.3. DMPC-DPPC simulations

Mixtures of DMPC and DPPC in lipid bilayers were simulated to probe the phase transitions. The Averaged surface area per lipid over the last 100ns of simulations
was calculated for 3 mixtures (Figure 3-35). System with equal molar concentration of DMPC and DPPC had a sharp jump in the surface area per lipid between 298.15 and 303.15K. System with %75 DMPC showed a sharp jump between 293.15 and 298.15K. This is in agreement with the experimental phase transition temperature of DMPC, which occurs around 297.15K\textsuperscript{154}. For system with %75 DPPC, a sharp jump was seen in 2 steps from 303.15 to 308.15K and 308.15 to 313.15K. Therefore, more analysis like visualization of bilayer configurations at the end of simulations is needed to justify the exact range of transition temperature for this bilayer composition. This range of transition temperature is acceptable as experimental main transition temperature of DPPC bilayer is around 314.15K\textsuperscript{154}.

![Graph showing average surface area per lipid of DMPC-DPPC simulations.](image)

**Figure 3-35.** Average surface area per lipid of DMPC-DPPC simulations.

Snapshots at the end of the simulations were generated for all 3 different compositions to show the phase transition (Figures 3-36,3-37,3-38). Figure 3-36 shows the phase transition event for the system with equal molar lipid concentrations.
At 293.15K, two out of three replicates represented the L$_\beta$ configuration, while the remaining replica showed a P$_\beta$ conformation. The average tilt angle of the L$_\beta$ phase over the last 100ns of simulation was 33.8±1.4$^\circ$, which was in good agreement of the experimental tilt angle measurement of pure DMPC and DPPC. At 298.15K, chains were still in gel-like configuration for all replicates. At 303.15K, two out of three replicates represented highly disordered fatty acid chains, while the first replica was still in a L$_\beta$ configuration. This event suggests formation of stable L$_\alpha$ phase at that temperature. This temperature range in a phase transition is in agreement with the surface area per lipid analysis. Apparently at 308.15 and 313.15K, we expect to get the L$_\alpha$ phase as it can be seen in Figure 3-36 for all replicates.

Figure 3-37 represents the configuration of lipids at the end of simulations of DMPC-DPPC$_{1-3}$. At 293.15K all three replicates were in a L$_\beta$ configuration. The average tilt angle over the last 100ns of simulations was calculated as 34.1 ± 1.6$^\circ$, which was slightly higher than the experimental tilt angle of each lipids, and close to the range obtained for DMPC-DMPC$_{1-1}$ at same temperature range, which was between 293.15 and 298.15K.

At 298.15K, the first replicate represents highly interdigitated fatty acid chains between DMPC and DPPC lipids from upper and lower leaflets. Two other replicates were in gel-like configurations. At 303.15K, still higher tendency exists to maintain the gel-like configuration. Third replicate showed melted head groups at lower leaflet but that was not the case for the upper leaflet. At 308.15K, chains were highly disordered, which indicated the L$_\alpha$ phase. The L$_\alpha$ phase was stable at 313.15K for all three replicates.
Figure 3-36. Snapshots were taken at the end of the simulations for system with DMPC-DPPC$_{1-1}$. 

Figure 3-38 represents the phase transition of system with DMPC-DPPC$_{3-1}$ lipid composition. The P$_\beta$ phase was obtained at the end of simulations at 293.15K for all three replicates supporting our earlier results for pure small-size DMPC bilayer simulations and higher tendency to form P$_\beta$ phase at lower temperatures with that system size. In this case, where we have 75% DMPC in mixture, all bilayers were in a P$_\beta$ configurations, while in two other mixtures with more DPPC in bilayers, bilayers were mostly in a gel-like configurations, and this event was also seen earlier in our
pure DPPC simulations with same system size. At higher temperatures starting from 298.15K, all replicates were in L_α phase with highly disordered acyl chains.

Figure 3-37. Snapshots were taken at the end of simulations for system with DMPC-DPPC_{1-3}
Figure 3-38. Snapshots were taken at end of simulations for system with DMPC-DPPC$_{3-1}$.
The total electron density profiles were calculated for the systems to support transition temperature ranges, which were obtained from previous analysis (Figure 3-39). System with equal molar concentration of DMPC and DPPC showed lowest electron density at center of bilayer for the L_β phase at 293.15K (Figure 3-39A). At 298.15K, the shape of EDP is still similar to the one at 293.15K but the electron density was slightly higher at center since there was less tilt angle of fatty acid chains in this gel-like configuration. Dramatic change in EDP can be seen at 303.15K, and profiles at higher temperatures remained in almost same shape, which indicates transition to the L_α phase occurred at temperature between 298.15 and 303.15K. Figure 3-39B shows the EDP for system with DMPC-DPPC_{1-3} at different temperatures. Clearly, the sharp change in profile occurred at 308.15K, which means the transition temperature is within the range between 303.15 and 308.15K. Finally, Figure 3-39C shows EDP for system with DMPC-DPPC_{3-1} at same set of temperatures. The minimum value of electron density at lowest temperature, which is 293.15K, was slightly higher compared to the electron density at same temperature of two other systems. This was due to the P_β phase formation at 293.15K, and the L_β phase was not obtained for this bilayer composition within specified temperature range.
Figure 3-39. Total electron density profiles at different temperatures of A. DMPC-DPPC$_{1-1}$, B. DMPC-DPPC$_{1-3}$, C. DMPC-DPPC$_{3-1}$
3.4. Discussion

In summary of this chapter, our MD simulations predicted the main transition temperature to the L\(_\alpha\) phase of DMPC and DPPC lipid bilayers in a close agreement with the experimental results. In the case of the DPPC bilayer, the main transition was captured between 308.15 and 313.15K, which is close to the experimental result of 314.15K\(^{154}\). The main transition temperature for the DMPC occurs between 288.15 and 293.15K, which was at least 4K below the experimental result but by representing the L\(_\beta\) conformation with better agreement with experiment in terms of all tilted fatty acid chains, which was not obtained in previous simulations\(^{136}\). Although the temperature range is 4K below the experimental value but the end structure at 293.15K was in fully developed L\(_\alpha\) phase suggesting a condition near the phase transition temperature. Our temperature for the main phase transition for DMPC bilayers is closer to experiment compared to past heating simulations which was done at a fast rate of 1 K/ns\(^ {137}\) with CHARMM36 in GROMACs. Moreover, DPPC was found to not have a P\(_\beta\) phase in past heating simulations\(^{137}\), but the predicted main transition temperature was closer to the experiment. There can be a hysteresis in heating and cooling that might explain some difference between our work (cooling) and past work\(^ {137}\) (heating). However, structural analysis for DMPC at 300K which past simulations suggested a P\(_\beta\) phase\(^ {137}\) (above the main transition temperature), might be due to shorter simulations (200ns) that prevented relaxation to a L\(_\alpha\) state. With this in mind, the conclusions made by Pulhackova et al.\(^ {137}\) on phase transitions for CHARMM36 and other lipid force fields may be biased preventing slow structural relaxation near the phase transition temperature.
Our \( L_\beta \) structures of DPPC using the NPT ensemble showed almost 5\(^\circ\) higher tilt angle compared to tilt obtained from biased MD simulations (31.3±0.2\(^\circ\)) using the constant area NPAT ensemble and the C27 lipid FF\(^{146}\). This past work used a fixed surface area at 45.6Å\(^2\), which is lower than average experimental surface area at 292K\(^{158}\). Although our average tilt angle for the \( L_\beta \) phase DPPC bilayer is ~4\(^\circ\) higher than experiment, MD simulations with CHARMM36 could obtain stable \( L_\beta \) phase at multiple temperatures below the main transition temperature. The Probability distribution of tilt angle showed peaks between 30\(^\circ\) and 34\(^\circ\), which was in agreement with previous simulation obtained tilt angle of DPPC bilayer in the \( L_\beta \) phase as 33.6\(^\circ\) at 292.15K\(^{129}\). We were able to capture the \( L_\beta \) phase and the sub-gel phase at different temperatures. The fatty acid chains are nearly all in the trans configuration and co-linear for both leaflets obtained in all replicates at 303.15K. This experimentally-observed chain orientation was not obtained in previous simulations using the lipid14 and GROMOS force fields\(^{138}\). (Note: This past work reported results using CHARMM force field\(^{138}\) simulated in the AMBER program, but different cutoff schemes were used and charge conversions so this was not in line with C36 lipid force field).

Simulations indicated a stable \( P_\beta \) phase for the DMPC bilayer in a temperature range from 275.15 to 288.15K, while the \( P_\beta \) phase was seen just for one replica at 293.15K for DPPC bilayer, which indicates a tendency of DPPC to maintain the \( L_\beta \) conformation at the small system size of 72 lipids. The absence of the DPPC \( P_\beta \) phase could be due to longer fatty acid chains compared to DMPC preventing the ripple formation at this small system size. Although the \( P_\beta \) phase for the DPPC bilayer was
not fully developed at 293.15K, distinct thin and thick regions as a minor arm and a major arm with expected characteristics of these regions were obtained, which was not obtained previously with Monte Carlo simulation using coarse-grained model or past heating simulations.

The $P_\beta$ phase of DMPC was obtained for all systems sizes of lipids (small to large). In addition, the DMPC $P_\beta$ phase was obtained within a reasonable temperature range and consistent with the experiment. Due to system size limitations, the $P_\beta$ wavelength obtained from averages of bilayers of varying size ranged from 40Å to 85Å, which is lower than experimental result (145Å). However, for the large DMPC simulation there was a region that showed longer major and minor arms. Considering this region the major arm and minor arm domains would be 99 and 44 Å, respectively that gives us 143Å for $P_\beta$ wavelength, which is in great agreement with experiment. That wavelength is smaller than our simulation box indicating ripple wavelength does not exceed experimental value by having simulation box bigger than wavelength.

Ripple amplitude in the medium-size was much closer to experimental result indicating the importance of a closer-to-experiment ripple wavelength to obtain the more realistic amplitude, as the ripple amplitudes obtained for $P_\beta1$ and $P_\beta2$ with relatively small wavelengths were similar to what we obtained for small systems. It appears that ripple amplitude has direct relationship with wavelength in $P_\beta$ phase. The ripple amplitude would be much closer to experimental result for unified $P_\beta$ phase in Y-direction of large DMPC bilayer by increasing the order of magnitude in simulation timescale as its effect on having accurate length range of the major arm
and minor arm regions were discussed in result section. Uniform $P_\beta$ phase of large DMPC in Y-direction would result in 143Å wavelength, which is in great agreement with experimental result (145Å)\textsuperscript{114}, and indicated larger system size would result in more realistic ripple configurations. Another important feature of the $P_\beta$ phase is membrane thickness in the major arm. The average $D_{HH}$ obtained from the larger systems (medium and large) was 42.6±1.2Å, which is in good agreement with experimental result (41.8Å)\textsuperscript{114}. Our simulations proposed clear interdigitation of acyl chains in the minor arm domain (Figure 10), which was not observed with X-Ray diffraction \textsuperscript{114}. The reason beyond this might be the fact that minor arm portion in membrane is too small in ripple direction and diffraction was not strong enough to detect interdigitation.

There have been past simulation studies on proposed ripple structures using all-atom MD and CG MD. Previous simulations using CHARMM36 and lipid14 force fields suggested a ripple-like configuration that was obtained at 300K, which is 3K higher than experimental transition temperature of DMPC bilayer \textsuperscript{137}. Moreover, their major arm region did not represent the gel-like characteristics in terms of nearly all tilted fatty acid chains relative to membrane normal as we obtained that in our simulations. One possible reason to not capture the $P_\beta$ phase correctly would be the short simulation timescale as they ran simulations for 200ns for the system size slightly bigger than our medium-sized DMPC bilayer. As mentioned above, the interdigitation of acyl chains was clearly seen in all replicas resulted in fully the $P_\beta$ phase in agreement with previous all-atom MD simulation using 384 lipids, which was bigger than our medium-size DMPC bilayer in this study \textsuperscript{137}. They used the
CHARMM36 force field but they tried to capture the phase transition by heating up the systems from low to high temperatures with relatively fast heating rates (1 K/ns), while we chose cooling from L_α state directly to a temperature of interest and equilibrated for a longer timescale (200 vs. 500ns).

Coarse-grained model also suggested interdigitation of acyl chains in thin region of membrane (minor arm) in agreement with our simulations, while details in major arm region including average tilt angle value were not provided. They used 1800 lipids and stable P_β phase was formed in Y-direction similar to our P_β phase in large DMPC bilayer. They found head group interaction important in P_β phase formation.

In conclusion, the CHARMM36 force field predicts the phase transition from L_α phase to P_β phase and to the L_β phase of pure DMPC, pure DPPC and DMPC-DPPC mixture bilayers within comparable temperature range relative to the experimental results. The predicted fully-developed L_β phase structures of DMPC are in good agreement with the chain tilt and the surface area per lipid. To our knowledge, we are the first to represent the P_β phase of the DMPC bilayer at an all atom level and compare with recent experimental measures from X-ray diffraction. We are also first to characterize details regarding the major arm and the minor arm regions of DMPC bilayer using all-atom molecular dynamics simulations. Based on our MD simulations, the interdigitation of acyl chains exist in the P_β phase formation and system size affects the P_β phase characteristics. Overall, for a lipid force field that was tuned to matching the L_α phase, the CHARMM36 force field accurately predicts the structure of these more ordered phases.
4. Chapter 4: Engineering *Escherichia coli* Membrane
Phospholipid Head Distribution improves Tolerance and Production of Biorenewables

4.1. Introduction

Renewable feedstock is a cheap and good alternative for current fossil fuels as they are much cheaper and environmentally favorable compared to petroleum-based fuels. Wide ranges of microbes have been genetically engineered for biofuels bulk productions. Membrane damage has been a critical topic as a principle mechanism of inhibitor toxicity due to the membrane’s role as a protective barrier. Ethanol was recognized as a chemical leading to leakage of ions and solutes to the cell.

Previous studies indicated that engineering of the saturation level, fatty acids conformations, and length of the fatty acids could improve production and tolerance of membrane damage. However, the effect of phospholipid head groups should not be neglected as those previous studies were only focused on tails of fatty acids. In this chapter, we postulate that engineered head groups of *E. coli* membrane will result in better tolerance and less disruption of membrane using all-atom molecular dynamics simulations. Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) head groups were selected by changing the expression of key phospholipid biosynthesis enzymes PssA. The PssA model will be compared with the native model of *E. coli* membrane to indicate More PE head groups will result in more tolerance and less damage in membrane.

Composition of lipid bilayers in PssA and control membrane models were based on experimental data and details are provided in table 4-1. The total number of lipids
was 160 for both models. Systems were fully hydrated and the hydration numbers for systems with and without ethanol were specified as 50 and 35, respectively.

4.2. Method

All-atom molecular dynamics (MD) simulations using explicit solvent were carried out for all systems. Initial membrane conformations of inner *E. coli* membranes were built by CHARMM-GUI membrane builder \(^{71, 73}\). Minimization, equilibration, and isobaric-isothermal ensemble (NPT) MD simulations were performed using NAMD simulation package \(^{93}\). The temperature and pressure of systems were kept at 310.15K and 1 bar respectively. Lennard-Jones potential was used to describe Van der Waals interactions, and a force-based switching function in the range of 8 to 12Å was selected. Each simulation after equilibration was run for 300ns using a time step of 2fs. The Particle Mesh Ewald method was used for long-range electrostatics interactions. Hydrogen atoms were constrained by using the RATTLE algorithm. CHARMM36 (C36) force field was used since we have evaluated the accuracy of this in our previous studies of the overnight model for the *E. coli* cytoplasmic membrane and the importance of lipid diversities in cell membranes \(^{169}\). Langevin dynamics maintained the temperature and the Nosé–Hoover Langevin-piston algorithm was used to maintain the pressure. Three replicates were carried out for each system. The last 100 ns of each simulation were used for the analysis.

In order to study bulk properties of membranes, surface area per lipid and area compressibility modulus were calculated. Surface area per lipid was calculated by multiplying X and Y dimension of the simulation box divided by number of lipids of each leaflet. Area compressibility (\(K_A\)) was calculated using following formula:
\[ K_A = k_B T \langle A \rangle / \sigma_A^2 \]

where \( k_B \) is the Boltzmann constant, \( \langle A \rangle \) is the averaged surface area, \( T \) is the temperature, and \( \sigma_A^2 \) is the variance of the area. Electron density profiles (EDP) were generated in order to calculate the bilayer thickness (\( D_B \)), hydrophobic core thickness (\( 2D_C \)), and head to head group distance (\( D_{HH} \)). The electron density of each atom was calculated using CHARMM program. Then, the SIMtoEXP \(^{170}\) program was used to parse those densities into groups, so that we could obtain component and total electron density profiles. \( D_{HH} \) was estimated by the peak-to-peak distances in total electron density profiles. \( D_B \) was defined as the distance between the midpoints of the volume probability profile of water, and \( D_C \) was defined as half of the distance between the midpoints of bilayer’s hydrocarbon acyl chains volume probabilities.

Visual Molecular Dynamics (VMD) was used to create snapshots and also \( S_{CD} \)s.

Order parameters of acyl chain (\( S_{CD} \)) were calculated for all lipids. This is a measure of the order in the C-H vector, calculated using the following formula:

\[ S_{CD} = \left\{ \frac{3}{2} \cos^2 \beta - \frac{1}{2} \right\} \]

where \( \beta \) is the angle between C-H vector and bilayer normal.
Table 4-1. Phospholipid composition of the membrane models. Values are the number of lipids, out of a total of 160. DPPE: 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, PMPE: 1-palmitoyl-2-cis-9,10-methylene-hexadecanoic-acid-sn-glycero-3-phospho-ethanolamine, DYEPE: 3-palmitoleoyl-2-palmitoleoyl-D-glycero-1-phosphatidylethanolamine, PMPG: 1-palmitoyl-2-cis-9,10-methylene-hexadecanoic-acid-sn-glycero-3-phosphatidylglycerol, DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine

<table>
<thead>
<tr>
<th>PE headgroup</th>
<th>control</th>
<th>+pssA</th>
<th>+pssA/control</th>
<th>control/+pssA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPE (16:0-16:0)</td>
<td>56</td>
<td>54</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>PMPE (16:0-cyc17)</td>
<td>34</td>
<td>18</td>
<td>42</td>
<td>14</td>
</tr>
<tr>
<td>DYEPE (16:1-16:1)</td>
<td>16</td>
<td>12</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>DOPE (18:1-18:0)</td>
<td>32</td>
<td>64</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>DSPE (18:0-18:0)</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Total PE</td>
<td>146</td>
<td>154</td>
<td>154</td>
<td>146</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PG headgroup</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PMPG (16:0-cyc17)</td>
<td>14</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
</tbody>
</table>

4.3. Results

The experimental analyses showed that increasing the relative abundance of the PE phospholipid head group significantly alters a variety of membrane and cell surface properties. Molecular dynamics simulations were used in order to investigate the change in membrane properties as a function of strain-specific phospholipid composition and concentration of inhibitor. Two membrane models were initially used: one representing the control strain and one representing the +pssA engineered strain. The relative abundance of the head groups and fatty acid tails in these models (Tables 4-1 and 4-2) were based on the experimental data (Table 4-3 and Figure 4-1). Snapshots of the end of the simulations are shown in Figure 4-2a.
Table 4-2. Membrane-related properties. Cells were grown in MOPS+2% dextrose with 10 mM C8, initial pH of 7.0, in shake flasks at 220rpm and 30 °C. Values are the average of at least three biological replicates with the associated standard deviation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Membrane integrity: % of population SYTOX negative</th>
<th>Membrane polarization</th>
<th>Membrane surface negative potential (9-ΑΑ Binding (μmol/g DCW))</th>
<th>Membrane electrochemical potential (ΔΨ, a.u.)</th>
<th>Cell hydrophilicity</th>
<th>Intracellular pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50.2±1.0</td>
<td>0.28±0.01</td>
<td>0.607±0.036</td>
<td>0.70±0.02</td>
<td>64.0±7.8</td>
<td>6.28±0.03</td>
</tr>
<tr>
<td>+pssA</td>
<td>89.3±0.3</td>
<td>0.29±0.01</td>
<td>0.453±0.044</td>
<td>1.53±0.08</td>
<td>93.3±0.1</td>
<td>6.68±0.06</td>
</tr>
</tbody>
</table>

Table 4-3. Distribution (wt%) of phospholipid head groups in engineered strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>PG</th>
<th>CL</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔclsA</td>
<td>12.8±0.4</td>
<td>ND</td>
<td>87.2±0.4</td>
</tr>
<tr>
<td>Control</td>
<td>8.8±0.4</td>
<td>3.3±0.1</td>
<td>87.8±2.4</td>
</tr>
<tr>
<td>+clsA</td>
<td>8.6±0.3</td>
<td>3.7±0.2</td>
<td>87.7±1.3</td>
</tr>
<tr>
<td>+pgsA</td>
<td><strong>12.1±0.3</strong></td>
<td>3.2±0.1</td>
<td>84.7±2.1</td>
</tr>
<tr>
<td>+pssA</td>
<td>3.7±0.2</td>
<td>2.6±0.3</td>
<td><strong>93.7±0.4</strong></td>
</tr>
</tbody>
</table>

All strains were cultured in MOPS+2% dextrose with 10 mM C8 in shake flasks at 220 rpm 30 °C with an initial pH of 7.0. Values are the average of at least three biological replicates with error bars indicating one standard deviation. ND, not detected. Values in bold indicate a significant (P<0.05) difference from the Control strain.

Figure 4-1. Production of octanoic acid (C8) and total fatty acids (TFA) by the Control and +pssA strains in mineral salts media containing 10 g/L glucose and 50% (v/v) corn stover hydrolysate at 30 °C, initial pH 7.0. (A) Growth. (B). Fatty acids titer after 144 h of cultivation. Both strains carry the pJMYEEI82564 plasmid, which harbors thioesterase 10 (TE10). Values are the average of three biological replicates with error bars indicating one standard deviation. Control-acrAB-tolC-TE10: AcrAB-
Figure 4-2. A) Left: Snapshot of simulation for the control membrane model. DOPE is shown in light green, DPPE in grey, DSPE in violet, DYPE in cyan, PMPE in pink, and PMPG in dark green. Right: Last snapshot of simulation for membrane in +pssA model. Same color code was used. B) Left: Simulation snapshot of the control membrane model with 4.64 mol% ethanol. Right: Simulation snapshot of the +pssA membrane model with 4.56 mol% ethanol. Lipids are shown in grey. Hydrogen atoms were not shown for clarity. Ethanol molecules were shown in Van der Waals method. C) Total electron density profiles of +pssA and control models with lowest and highest ethanol concentrations. D) Component electron density profiles of water and hydrocarbons (CH3+CH2+CH) in +pssA and control models with highest ethanol concentration.
In the absence of any inhibitor, the surface area per lipid was found to be statistically identical between the control and +pssA membrane models (Table 4-4). The electron density profiles (EDPs) had a similar shape for both models (Figure 4-2c), but a slight shift toward the center of membrane was seen at the peak point of total electron density profile of control model. The simulated EDPs and volume probability profiles of the lipids and water were used to calculate the head-to-head group thickness (D_{HH}), hydrophobic core thickness (2D_C), and bilayer thickness (D_B) (Table 4-5). For each of these measures of thickness, the +pssA membrane model has a higher value than the control model. Among these thicknesses, D_B and D_{HH} were statistically different between control model and +pssA model (P = 0.06 and P = 0.03, respectively). While our observation of an increase in the average fatty acid tail length in the +pssA strain suggests an increase in membrane thickness, these simulation results provide a more direct measure of membrane thickness and represent the diffusion barrier for chemicals across the membrane.

**Table 4-3.** Predicted bulk membrane properties. Asterisks indicate a significant (P<0.05) difference from the same model in the absence of ethanol.

<table>
<thead>
<tr>
<th>Model</th>
<th>Ethanol (mol%)</th>
<th>Surface Area (Å²/lipid)</th>
<th>K_A (N/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>58.98±0.24</td>
<td>0.175±0.002</td>
</tr>
<tr>
<td></td>
<td>4.64</td>
<td>66.86±0.24*</td>
<td>0.076±0.001*</td>
</tr>
<tr>
<td>+pssA</td>
<td>0</td>
<td>58.99±0.18</td>
<td>0.155±0.010</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>60.53±0.05*</td>
<td>0.152±0.003*</td>
</tr>
<tr>
<td></td>
<td>1.84</td>
<td>62.28±0.26*</td>
<td>0.129±0.006*</td>
</tr>
<tr>
<td></td>
<td>4.56</td>
<td>66.76±0.13*</td>
<td>0.079±0.005*</td>
</tr>
<tr>
<td>+pssA/control</td>
<td>4.67</td>
<td>65.85±0.26</td>
<td>0.096±0.002</td>
</tr>
<tr>
<td>Control/+pssA</td>
<td>4.50</td>
<td>67.78±0.37</td>
<td>0.086±0.003</td>
</tr>
</tbody>
</table>
To compare the fluidity of the two membrane models, the order parameters of fatty acid chains ($S_{CD}$) were calculated as the average of C4 to C6 of sn-1 for all lipids (Table 4-6). These were the same when comparing most lipids in each model, which is in agreement with same average surface area per lipid. The area compressibility modulus ($K_A$) is the measure of rigidity of the membrane and was not statistically different between the two models, i.e., 0.176±0.002 and 0.155±0.01 N/m for the control and +pssA models, respectively ($P = 0.16$ for equality of means hypothesis test). The fluidity measured by membrane polarization is identical between the control and +pssA, which might suggest that this is in agreement with our $K_A$ findings. However, membrane polarization is more a measure of lipid diffusion whereas $K_A$ is an elastic measure of force to laterally stretch the membrane. Thus, while the membrane thickness metrics suggest that the +pssA strain has a thicker membrane than the control, the lateral rigidity of these two membranes is predicted to be similar.

In addition to analyzing the control and +pssA membrane in the unperturbed condition, we also simulated these membranes during challenge with up to ~5 mol% ethanol. Ethanol was selected as a representative membrane-damaging compound due to it being a simple alcohol and because the +pssA engineered strain showed an approximately 10% increase in specific growth rate during challenge with 2% (v/v) ethanol $^{168}$. Ethanol penetration into the bilayers of both membrane types is evident in the snapshots in Figure 4-2b. Consequently, the surface area per lipid increased as the ethanol concentration increased (Table 4-4). For both models, this value increased from approximately 59 to 67 $\text{Å}^2$/lipid. Also, for both models, the total electron density
profile peaks were shifted towards the center of the bilayer as the ethanol concentration increased (Fig 4-2c, Figure 4-3). Specifically, at the highest ethanol concentration the lowest head-to-head group thickness was predicted. For the model of the wild-type membrane, the presence of ethanol substantially decreased $D_B$, $D_{HH}$ and $2D_C$ (Table 4-5). Similar changes were observed for $D_B$ and $D_{HH}$ in the $+pssA$ model. These decreases in membrane thicknesses were significantly different as ethanol concentration increased ($P < 0.001$). However, for the $+pssA$ model (Figure 53), the impact of ethanol on the $2D_C$ value was substantially dampened relative to the wild-type strain. Specifically, the control model had a $2D_C$ value of $30.4 \pm 0.7$ during ethanol challenge, while the $+pssA$ model had a $2D_C$ value of $31.9 \pm 0.4$. This result indicates that the $+pssA$ model has a thicker membrane than the wild-type control model and that its hydrophobic core thickness is more resistant to perturbation by the presence of up to 5% ethanol.

**Table 4-4.** Thicknesses of lipid bilayers for the control and $+pssA$ models generated here. The Top6-mid log model is shown for comparison. $D_B$ indicates the bilayer thickness, $D_{HH}$ is the head-to-head group thickness and $2D_C$ is the hydrophobic core thickness. In red are significant ($P<0.05$) difference from control with 0% ethanol and in blue significant differences from $+pssA$ with 0% ethanol. For the last two rows, X/Y indicates X=the head group model composition and Y=represents the fatty acid chain model composition.

<table>
<thead>
<tr>
<th>Model</th>
<th>$D_B$ (Å)</th>
<th>$D_{HH}$ (Å)</th>
<th>$2D_C$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control +0% ethanol</td>
<td>38.67±0.07</td>
<td>40.6±0.11</td>
<td>31.77±0.14</td>
</tr>
<tr>
<td>Control +4.64% ethanol</td>
<td>34.86±0.13</td>
<td>37.33±0.18</td>
<td>30.4±0.70</td>
</tr>
<tr>
<td>$+pssA$ +0% ethanol</td>
<td>39.03±0.12</td>
<td>41.0±0.0</td>
<td>32.02±0.11</td>
</tr>
<tr>
<td>$+pssA$ +0.62% ethanol</td>
<td>38.63±0.03</td>
<td>40.4±0.2</td>
<td>31.73±0.13</td>
</tr>
<tr>
<td>$+pssA$ +1.84% ethanol</td>
<td>37.73±0.18</td>
<td>39.68±0.13</td>
<td>31.6±0.28</td>
</tr>
<tr>
<td>$+pssA$ +4.56% ethanol</td>
<td>35.57±0.17</td>
<td>37.47±0.24</td>
<td>31.88±0.39</td>
</tr>
<tr>
<td>Top6-midlog +0% ethanol</td>
<td>38.15±0.03</td>
<td>38±0.11</td>
<td>30.36±0.03</td>
</tr>
<tr>
<td>$+pssA$ / control +4.67% ethanol</td>
<td>35.43±0.09</td>
<td>37.93±0.18</td>
<td>30.95±0.03</td>
</tr>
<tr>
<td>control / $+pssA$ +4.50% ethanol</td>
<td>35.13±0.22</td>
<td>37.53±0.18</td>
<td>31.46±0.5</td>
</tr>
</tbody>
</table>
Table 4-5. Order parameters $S_{CD}$ (average of C4-C6) of all lipids. Asterisks (*) indicate a significant (P<0.05) difference from the same model in the absence of ethanol. Bold font indicates a significant difference from the control model in the same condition. This analysis was not performed for the 0.62 and 1.84 mol% ethanol simulations for the +pssA model.

<table>
<thead>
<tr>
<th>Model</th>
<th>Ethanol (mol%)</th>
<th>DOPE</th>
<th>DPPE</th>
<th>DSPE</th>
<th>DYPE</th>
<th>PMPE</th>
<th>PMPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.215±0.003</td>
<td>0.258±0.002</td>
<td>0.251±0.006</td>
<td>0.212±0.002</td>
<td>0.235±0.001</td>
<td>0.238±0.008</td>
</tr>
<tr>
<td></td>
<td>4.64</td>
<td>0.177±0.0007*</td>
<td>0.213±0.003*</td>
<td>0.211±0.004*</td>
<td>0.184±0.002*</td>
<td>0.197±0.002*</td>
<td>0.205±0.001*</td>
</tr>
<tr>
<td>+pssA</td>
<td>0</td>
<td>0.217±0.003</td>
<td>0.260±0.002</td>
<td>0.262±0.006</td>
<td>0.219±0.004</td>
<td>0.237±0.004</td>
<td>0.247±0.001</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>0.211±0.001*</td>
<td>0.249±0.001*</td>
<td>0.244±0.005*</td>
<td>0.209±0.003*</td>
<td>0.229±0.001*</td>
<td>0.235±0.001*</td>
</tr>
<tr>
<td></td>
<td>1.84</td>
<td>0.203±0.001*</td>
<td>0.240±0.002*</td>
<td>0.235±0.007*</td>
<td>0.208±0.002*</td>
<td>0.221±0.003*</td>
<td>0.215±0.005*</td>
</tr>
<tr>
<td></td>
<td>4.56</td>
<td><strong>0.184±0.0006</strong>*</td>
<td>0.217±0.003*</td>
<td>0.217±0.006*</td>
<td>0.182±0.005*</td>
<td>0.199±0.005*</td>
<td>0.206±0.007*</td>
</tr>
</tbody>
</table>

For both models, the upper chain $S_{CD}$ decreased as the ethanol concentration increased (Table 4-6), which is consistent with the thickness and surface area per lipid results. For +pssA with highest ethanol concentration, specifically for DOPE, the $S_{CD}$ is statistically higher than the control with highest ethanol concentration (P < 0.001), which may contribute to the increase in hydrophobic core thickness. For both models, the area compressibility modulus $K_A$ decreased to approximately 0.08 N/m in the presence of roughly 5 mol% ethanol (Table 4-4). However, since the wild-type control model had a higher $K_A$ value than the +pssA model in the control condition, 0.175 N/m relative to 0.152 N/m, respectively, the change in the membrane $K_A$ was larger for the control membrane than for the +pssA membrane. Specifically, the wild-type control model showed a $2.30 \pm 0.04$-fold decrease in $K_A$ during ethanol challenge while the +pssA model showed a $2.0 \pm 0.02$-fold decrease. Therefore, the +pssA membrane is more resistant to ethanol effects and ultimately results in a thicker bilayer that may decrease permeation of membrane-damaging compounds, such as ethanol, into the cytoplasm.
Figure 4-3. A, B, and C represent the component electron density profiles of the Phosphate, Carbonyl, and ethanol respectively in +pssA membrane models.

The penetration of ethanol into these membrane was quantified with component electron density profiles (Figure 4-4). The electron density peaks for both the phosphate and carbonyl groups decreased as the ethanol concentration increased, due to increases in the surface area per lipid and penetration of ethanol into the head group region. These results indicate that ethanol penetrates deeper into the control membrane relative to the +pssA membrane (Figure 4-4).
As described above, the $+\text{pssA}$ strain had an altered distribution not only of the phospholipid head groups, but also an altered distribution of the phospholipid fatty acid tails. In order to separate the impact of the head group change from the impact of the fatty acid tail change, we built two hybrid membrane models (Table 4-1). The $\text{pssA}/\text{control}$ hybrid model had the same head group composition as the $+\text{pssA}$ model, while maintaining the fatty acid chains composition of control model. The $\text{control}/\text{pssA}$ hybrid model had the same head group composition as the control strain and the fatty acid chains from the $+\text{pssA}$ model. Both of these hybrid models were assessed at ethanol concentrations of roughly 5 mol%.

A statistically significant increase in bilayer thickness ($D_B$) was predicted for the hybrid $+\text{pssA}/\text{control}$ model relative to the control model ($P = 0.03$). The hybrid $\text{control}/+\text{pssA}$ model did not show a significant difference in bilayer thickness relative to the control model. Therefore, simply changing the head group distribution without
changing the fatty acid tail distribution is sufficient to increase the bilayer thickness in the presence of ethanol (Table 4-5).

However, the membrane in which only the head groups are changed has a significantly thinner hydrophobic core relative to the control model (Table 4-5). The hydrophobic core thickness of the model in which only the fatty acid tails are changed is unchanged relative to the control and +pssA models. Overall, we can conclude that the changes in the distribution of both the fatty acid tails and in the head groups both contribute to the thicker membrane. Specifically, the change in the head group distribution influences the bilayer thickness \( D_B \) while the change in the acyl chains influences the hydrophobic core thickness \( D_C \). These changes in the +pssA strain act together but independently to increase the permeation pathway for chemicals across the membrane.

4.4. Conclusion

Our molecular dynamics simulations indicated more tolerance of E. coli membrane and less leakage by including more PE head groups in membrane. +pssA membrane model, which had more PE groups compared to control model, resulted in thicker and less compressible membrane. In overall, our simulations using the CHARMM36 force field were able to suggest structural reasons for less cell membrane damage and more production of biorenewables with the engineered \( E. coli \) membrane.
5. Chapter 5: Interplay of Specific Trans-and Juxtamembrane interfaces in Plexin A3 Dimerization and Signal Transduction

5.1. Introduction

Plexins (plxns) are a group of Type I transmembrane (TM) receptors involved in the guidance of neurons, vascular, and lymphatic vessels during development as well as zebrafish fin regeneration. Plxns also serve a putative role in cancer metastasis, with altered expression levels or mutations to plxns observed in melanomas and breast, lung, pancreatic, and prostate cancers. Investigations with plxns’ semaphorin (sema) ligands and neuropilin (nrp) co-receptors have also implicated the plxn-nrp-sema pathway as influential to cancer metastasis, with overexpression of SEMA3F inhibiting cancer metastasis in a mouse melanoma model. As such, understanding the mechanisms necessary for activation of the plxn-nrp-sema signaling pathway may provide insight into design of novel therapeutics as well as their role in a myriad of developmental processes.

Plxns contain an extracellular domain involved in ligand binding, a glycine-rich single-spanning TM domain, and a cytosolic domain (CYTO) involved in signal transduction. Activity is characterized by CYTO GTPase-activating protein (GAP) activity. An early immunohistological observation that NRP1 and PLXNA1 cluster in regions of high local concentration upon SEMA3A addition in a chick dorsal root ganglion collapse assay led to the premise that receptor dimerization or clustering confers activity. Indeed, a RapGAP activity assay on purified CYTO domains of *Mus musculus* PLXNA1, PLXNA2, PLXNA4, and
PLXNC1 CYTO suggests plxn CYTO domains dimerized through N-terminal fusions exhibit enhanced activity over the monomeric CYTO domains \(^{187}\).

Purified murine PLXNA2 extracellular domains and murine PLXNA1, PLXNA3, and human PLXNB1 CYTO domains exhibit only weak homomeric tendencies in solution, however \(^ {176, 185-188}\). Removal of the *Mus musculus* PLXNA1 sema-binding domain or extracellular domain confers sema- and nrp-independent collapse activity in a COS-7 growth cone model, indicating PLXNA1 exists in an autoinhibited conformation, and that in the absence of the sema-binding domain, the PLXNA1 TM + CYTO is sufficient for receptor activation \(^ {183}\). Expression of the mouse PLXNA1 CYTO domain alone or with a myristoylation signal fails to induce collapse, though replacement of the human PLXNB1 TM domain with a membrane-anchored CD2 fusion followed by cross-linking enables cellular contraction \(^ {183, 189, 190}\).

Previous studies indicated a heptad repeat in the CYTO juxtamembrane region (JM) modulates homomeric interactions in the full-length receptor, with mutations to the JM of *Drosophila* PLXNA and *Danio rerio* PlxnA3 only showing partial activity in axonal guidance assays \(^ {185, 191}\). Additionally, the human PLXNA1 TM domain alone exhibits a weak tendency to dimerize in a bacterial adenylate cyclase two-hybrid assay \(^ {184, 192}\). Coarse-grained molecular dynamics simulations of the isolated human PLXNA1 TM domain suggest a glycine-rich segment in the TM region largely conserved across Class A plxns may modulate human PLXNA1 homomeric interactions \(^ {184}\). In particular, this conserved glycine-rich region contains two motifs capable of packing via small-x\(_3\)-small interfaces \(^ {184}\). The small-x\(_3\)-small motif is a highly conserved sequence-structure motif overrepresented in a wide range of helical
TM protein dimers such as glycophorin A and the plexin co-receptor NRP1, in which small residues such as glycine, serine or alanine are placed along one face of the TM helix, creating a specific, ridge-and-groove packing structure that contributes to dimer stability \(^{192-194}\). The relative importance of TM versus JM and interrelationship between TM and JM interactions in PlxnA3 dimerization and signal transduction remain open questions.

In this chapter, we examine the role of the glycine-rich region of the *Danio rerio* PlxnA3 TM domain on homodimerization and the interplay of TM and JM interactions in PlxnA3 dimerization and signal transduction.

### 5.2. Method

PlexinA3 dimer structures obtained from coarse-grained (CG) simulations \(^{35}\) were used as initial configurations of all-atom (AA)-MD simulations. CG to all AA conversion was carried out using a backmapping tool that uses a library of mapping definitions which encode geometrical reconstruction.\(^{195}\) Dimers were put in POPC explicit bilayer using the CHARMM-GUI Membrane Builder.\(^{67, 71-73}\) 100 lipids were selected per leaflet and 15 Å was used as water buffer on the top and bottom of the system (including the peptide). The NAMD simulation package was used to run all simulations. Temperature and pressure were fixed at 310.15 K and 1 bar respectively. Lenard-Jones potential was used to describe van der Waals interactions, and a forced-based switching function in the range of 8 to 12 Å was chosen.\(^{94}\) Hydrogen atoms were constrained by using RATTLE algorithm. Langevin dynamics maintained the temperature and the Nosé–Hoover Langevin-piston algorithm \(^{96, 97}\) was used to
maintain the pressure. Three replicates using different initial CG structures were carried out for wild-type and second mutation conformation, while two runs were performed for the first mutation simulation. Simulations were run for 300 ns and 2fs was the time step and 2-3 replicas were run to provide better statistics with dimerization. The CHARMM36 (C36) force field was used for the protein and lipids and TIP3P for water. The CHARMM program was used to calculate the distances between residues, and then contact maps were generated using MATLAB. All images were made using visual molecular dynamics (VMD).\textsuperscript{101}

5.3. \textit{Results}

5.3.1. TM, JM dimerization

As described in the methods, the homology modeled PlexinA3 TM-JM domains were separated initially in a POPC bilayer and CG simulations with the MARTINI force field were performed (CG MD was run by Dr. Indrani Bera, postdoc in Dr. Klauda’s lab and I briefly summarize this work here). In less than 100 ns, the dimers associated and remained tightly packed for the 10 µs simulation. The contact maps (Figure 5-1A-C)\textsuperscript{35} and snapshots (Figure 5-2)\textsuperscript{35} show some variations between replicas but consistently show an interacting TM and JM domain. Replica1 (Figure 5-1A) shows a weaker TM domain packing and a slip in dimer interaction with an off diagonal contact; in other words, a residue of Plexin A3 TM helix one is not in contact with the same residue on the second Plexin A3 TM helix. The JM domain in replica2 (Figure 5-1B) is interacting weaker but the TM domain associates strongly. The overall dimer character of replica3 (Figure 5-1C) is similar to replica1 but the
contact maps are on the diagonal; in other words, a residue on Plexin A3 TM helix 1 is in close contact with the same residue on the second Plexin A3 TM helix. There is a slightly weaker interaction for the TM domain residues that range from 1240-1255 for replica3. Based on these CG-MD simulations, there are slight changes in the strength of the TM and JM interactions, but lack examples of complete dissociation of these domains except the end of the TM region. Thus, for wild-type Plexin A3 TM-JM, multiple, independent TM and JM interactions, each of which contain a specific interface, are consistently observed; both the TM and JM interfaces contribute to a stable Plexin A3 TM-JM homodimer.

Since the MARTINI CG-MD simulations lack the ability to change secondary structure from the initial model, the main motif is a helix for this TM-JM region. To relax this constraint, end configurations form the 10-µs simulations were used to setup and run 300-ns AA-MD. The results from these more detailed simulations show more diversity in TM-JM association for Plexin A3 (Figure 5-3D-I). Specifically, replica1 (Figure 5-3D and G) shows a strongly tilted helix in the membrane with reduced association in the cytoplasmic leaflet; the main association is in the G-x3-G motif region of the TM domain. Also, consistently across independent replicas there is a tendency to form a cross JM motif interaction. Replica2 completely separates in the TM but maintains JM interaction at a weaker extent (Figure 58-3E and H). Replica3 has no association in the periplasmic half of the TM domain but an increased association for the cytoplasmic half (Figure 5-3F and I); a similar JM association is observed for replica1 as well. Overall, these results suggest a strongly
interacting JM region and a weaker interacting TM region with multiple dimeric conformations for PlexinA3 (Figure 5-4).

Figure 5-1. Results from CG- and AA-MD for the wild-type PlexinA3 TM/JM domain. The top three panels are contact maps for three CG runs (A-C) with the residue numbers starting at 1240 in the full-length protein numbering. The middle three panels (D-F) are for the respective AA-MD simulations that correspond to the CG starting configurations. The color bar is in nm and maps are averages over the last 2.5 µs and 100 ns of simulation for the CG and AA-MD, respectively. End snapshots from the 300 ns AA-MD runs are shown in the bottom panels with non-polar, polar, acid and basic residues are color-coded in white, green, red and blue, respectively. The phosphate atoms are colored in gold.
Figure 5-2. End snapshots of the 10 µs wildtype CG-MD simulations with replicas 1-3 shown in panels A-C. The non-polar, polar, acid and basic residues are color-coded in white, green, red and blue, respectively. The phosphate atoms are colored in magenta.

MD simulations were performed for the disruptive JM mutant M1281F to probe how this non-polar, aromatic substitution within the hydrophobic JM coiled-coil motif influences JM association. From the CG-MD of the wild-type, the strongest JM association was with JM residues 1285-1300 (Figure 5-1A-C). However, mutant M1281F abolishes this preference and moderately promotes the association for JM residues 1300 and greater (Fig. 5-3A and data no shown); this is consistent with the expected result that removing hydrophobic residues in the core of the coiled-coil destabilize JM association. Furthermore, the M1281F mutant also appears to enhance the TM region association, again consistent with a model in which loss of JM association can be compensated for through independent TM domain interactions. However, the enhancement of TM domain interactions at the expense of reduced JM
interactions is in conflict with our AA-MD (Figure 5-3C and E), which shows an increased interaction for JM residues >1300 (for one helix) and a slight decrease in the residues near the mutation M1281F, yet a complete loss of interactions in the TM region this result can also be seen for second replicate of this mutation (Figures 5-5).

**Figure 5-3.** Results from CG- and AA-MD for the mutant PlexinA3 TM/JM domain. The top two panels are sample CG-MD contact maps with the residue numbers starting at 1240 in the full-length protein numbering. The middle two panels are contact maps for AA-MD. A, C, and E are the M1281F mutant, B, D, and F are for the L1252G/I1254G double mutant. The color bar is in nm and maps are averages.
over the last 2.5 µs and 100 ns of simulation for the CG and AA-MD, respectively. End snapshots from the 300 ns AA-MD runs are shown in the bottom panels with non-polar, polar, acid and basic residues are color-coded in white, green, red and blue, respectively. The phosphate atoms are colored in gold.

**Figure 5-4.** Snapshots of wild-type dimer in last 100 ns of all-atom molecular simulations. Red to white to blue is the color scheme for the images to represent changes during this 100-ns time period. A-C are for replicas 1-3.

This is likely due to the weaker interaction in the TM region as well as the inability of the CG MARTINI model to kink the helices, thus maintaining an extended, rod-like structure that promotes helix association in the TM domain. Collectively, these results indicate that M1281F causes a specific disruption of the JM coiled-coil dimer with a variable and weaker TM association observed.

Additionally, simulations were performed for the TM double mutant L1252G/I1254G, which places an additional glycine in registry with each of the small-x3-small motifs (G1244+G1248 and G1246+G1250) present in Plexin A3. Thus, this TM double mutant is hypothesized to be a mutant that enhances TM-mediated homodimerization. For CG-MD, there was a slight reduction in the JM
domain association, excluding replica 1 (data not shown). However, with this double mutant there is an increased contact for TM residues 1240-1255, which encompasses the region containing both small-x3-small motifs. The first replicate of AA-MD shows a more crossed TM and JM motif (Figure 5-3F) with strong association in the TM region (Figure 5-3D). This crossed-TM motif results in a more upright dimer compared to those observed in the wild-type (Figure 5-1G and I). Replicas 2 and 3 resulted in weaker interaction in JM domain with a more parallel motif and a similar motif in TM region (Figures 5-6 and 5-7). Thus, these results indicate mutations L1252G/I1254G that enhance TM small-x3-small packing motifs do enhance overall TM-mediated homodimerization relative to wild-type, and can do so at the expense of the JM coiled-coil homodimer.

**Figure 5-5.** Snapshots of the M1281F dimer in last 100 ns of all-atom molecular simulations. Red to white to blue is the color scheme for the images to represent changes during this 100-ns time period. A-B are for replicas 1-2.
Figure 5-6. Snapshots of the double mutant (L1252G/I1254G) dimer in last 100 ns of all-atom molecular simulations. Red to white to blue is the color scheme for the images to represent changes during this 100-ns time period. A-C are for replicas 1-3.

Based on our AA MD simulations, there is a plasticity to structures of homodimerization. Therefore, probability distributions of distances between pair residues in small-x3-small motifs and JM domain were calculated for all dimers to quantify this better (Figure 5-8). For the first replica of the wildtype dimer, highest probability was seen <2.2 Å for JM domain during the last 100 ns of simulation, but the other two replicas show a high probability to be <2.2 Å (0.997, 0.502, and 0.891 for replicas 1-3, respectively). Arg47 (from helix1) hydrogen bonds with Glu45 (helix2) significantly during last 100 ns of the replica1 simulation (Figure 5-9A). Considering the distance for the small-x3-small motif and replica1 of the M1281F mutant, distances above 10 Å (Figure 5-8E) were the most probable, which supports the weak interaction of TM domains with this mutation. On the other hand, in case of JM domain, distances near 2 Å are significant for second replicate (Figure 5-8B), showing strong association in JM domain.
Figure 5-7. Contact maps of AA-MD for the L1252G/I1254G double mutant.

As with the wildtype, hydrogen bonding between Arg47 and Glu45 stabilizes the JM association (Figure 5-9B). For TM double mutant L1252G/I1254G, there is more conformational flexibility in the TM and JM region compared to the wild-type (Figure 5-8C,F). The crossed TM/JM motif is stable throughout the simulation.
(replica1), whereas the other replicas show no crossed TM motif and have more flexibility in the association distance (Figure 5-8F).

Figure 5-8. This figure contains the probability mass functions for distances for the three replicas in our AA MD (Blue: replica1, Red: replica2 and Yellow: replica3). (A-C) JM domain for wild-type, M1281F mutant, L1252G/I1254G double mutant, respectively. Gly8 was picked as the residue for each homodimer. (D-F) TM domain for wild-type, M1281F mutant, L1252G/I1254G double mutant, respectively. The distance pairs were selected as follows to focus on closest interaction regions: Arg47 (helix1) and Glu45 (helix2) for the wild-type and Arg47 (helix2) and Glu45 (helix1) for the M1281F mutant. For the L1252G/I1254G double mutant, pairs were chosen for each replica: Replica1. Lys24 (helix1) and Thr27 (helix2) and Replica2. Lys26 (helix1) and Thr27 (helix2) and Replica3. Thr27 (helix1) and Thr27 (helix2).
Figure 5-9. (A) Wild-type simulation snapshot (replica1) (B) M1281F mutant simulation snapshot (replica2). JM association due to hydrogen bonding between Glu45 and Arg47.

5.3.2. Extended EC, TM, JM dimerization

Extended residues from extracellular (EC) domain were added to transmembrane domain to investigate if stronger interaction in TM domain could be obtained, which was not seen in our MD simulations discussed earlier in this chapter. For plexin A3 with extended EC domain, 30 more resides were included (residues 1211-1310) and 3 replicates were run. Same procedure was followed as the previous simulations were performed. CG-MD simulations were carried out first and then the end-structure was used to run AA-MD simulations for 300ns. Snapshots at the end of the simulations and contact maps could be seen in Figure 5-10. Replica 1 showed strong interaction in TM domain and replica 3 indicated close interaction in extended EC domain as it can be clearly seen from contact maps. Probability distributions of distances were generated to support strongest interaction in TM domain obtained from replica 1 (Figure 5-11).
Figure 5-10. A. Contact map averaged from last 100ns of AA-MD simulation of replica1. B. Contact map averaged from last 100ns of AA-MD simulation of replica2. C. Contact map averaged from last 100ns of AA-MD simulation of replica3. D. Snapshot at the end of simulations of replica1. E. Snapshot at the end of simulations of replica2. F. Snapshot at the end of simulations of replica3.

Figure 5-11. A. Probability distributions of distances averaged over last 100ns of replica 1 simulation. B. Probability distributions of distances averaged over last 100ns of replica 2 simulation. C. Probability distributions of distances averaged over last 100ns of replica 3 simulation.

As it can be seen from Figure 5-11.A, the highest probability of distances between Gly1244 from Helix A and Gly 1249 from Helix B occurs at distance close to 3Å,
which is not the case for replica 2 and replica 3. It should be noted that interactions in TM domain for replica 2 and replica 3 are still stronger than replicates obtained with shorter sequence (70 residues), which complete loss of interaction in Gly-rich region was observed. Overall, extended residues in extracellular domain showed stronger interaction in TM domain compared to previous results.

In order to verify the importance of extended domain on strong interaction in the TM domain obtained from first replicate, strong nonpolar mutations were done in EC domain. Gly1221 from Helix A and Gly 1227 from Helix B both mutated to Phenylalanine (PHE). Contact map averaged from last 100ns of simulations and snapshots at the end of simulation indicated total loss of interaction in TM domain (Figure 5-12).

![Figure 5-12](image_url)

**Figure 5-12.** A. Contact map of replica1-mutated averaged over last 100ns simulation. B. Snapshot at the end of replica1-mutated simulation.
5.4. Conclusion

Overall, our AA-MD simulations for wild-type plexin A3 resulted in strong interaction in JM domain and weaker interaction in TM domain by having multiple homodimer conformations. MD simulations of disruptive JM mutant M1281F resulted in complete loss of interaction in TM domain, while simulations with double mutant in TM domain, L1252G/L1254G indicated crossed TM/JM motif and strong interaction in TM region. Including extended EC domain resulted in stronger TM interaction compared to replicates using shorter sequence.
6. Chapter 6: Conclusion and Future Work

In this dissertation, an extensive amount of MD simulations were performed on cell membranes and membrane proteins using the CHARMM36 force field. The CHARMM36 force field was able to demonstrate the importance of having diversity in fatty acid chains of *E. coli* membrane in order to obtain more realistic membrane. Choosing an appropriate mixture of diverse lipids to model organism and organelle membranes is important to accurately represent the properties and associated function of membranes in nature. Our MD simulations of the cytoplasmic *E. coli* membrane demonstrate changes in membrane properties that significantly differ from past simplistic two-lipid models. The effect of the growth stage of the *E. coli* colony significantly influences a select set of properties of the membrane. What has not been probed in this study is the potential influence of leaflet asymmetry in lipid composition. As described, more dramatic changes in lipid composition exist between organelles in eukaryotes and lipid biophysical properties will be dramatically influenced by these changes. The effect of lipid composition should be carefully considered in simulations as well as experimental studies when probing the function of proteins that reside in or interact with the membrane. Potential future work on this project would be considering other bacteria models and try to consider all the lipid types as it was obtained from experimental result and probe the properties of membrane interest.
Phase transitions of saturated PC lipids were successfully probed and excellent agreement with experiments were obtained in terms of temperature range of each transition. For the first time, the DMPC bilayer in ripple phase was comprehensively quantified using AA-MD and the major arm and minor arm domains properties were compared with recent experiments. Two ripple conformations were obtained per unit cell for large DMPC bilayers (with 1152 lipids), which one wavelength matched that of simulations with less lipids and another that matches experiment. We expect that longer simulation time scales will result in a single ripple confirmation that agrees favorably with experiment. Our contour plots of average distances between end carbons indicated the major arm and minor arm domains in excellent agreement with experiment by assuming unified major arm and minor arm regions. The ripple amplitude seemed to be dependent on the wavelength as the amplitude of medium-size bilayer with wavelength almost 2 times bigger than small-size was almost 2 times bigger than the ripple amplitude of small bilayers. Our simulations for small-size of pure and mixture of DMPC and DPPC bilayers demonstrated more tendency of DMPC and DPPC bilayers to be in ripple phase and gel phase respectively. DPPC bilayer in gel phase was quantified in molecular details and the tilt angle of fatty acid chains was obtained in much better agreement with experimental results compared to previous computational studies. Future work in this area could be pursued in multiple directions. One interesting aspect would be focused on running MD simulations for longer timescale on the large DMPC bilayer to see if we can capture unified conformation and compare membrane properties with most recent experimental study. Other approach would be to start from gel phase at low temperature and heat
the system at certain rate until we get the main transition to fluid phase. It will be interesting to see if we are able to capture ripple phase as a pre-transition and then liquid-crystalline phase as a main transition in a reasonable temperature range and if there is any significant difference with our simulation results, which they were discussed here.

Simulations with the CHARMM36 force field were also able to model E. coli membrane with better tolerance and less leakage of chemicals into membrane. Simulations indicated that membrane with an engineered head group ratio (increase in the PE/PG ratio) resulted in thicker and less compressible membrane. The future work in this area would be focused on two different directions. One would be building dual-membrane systems in order to have more accurate model to investigate chemical leakage to the membrane for +pssA and control models where a concentration gradient is imposed. The other option will be studying the effect of other chemicals like furfural and octanoic acid on membrane properties. Therefore, simulations with different concentrations of furfural and octanoic acid could be carried out for single and dual membranes of +pssA and control models to pursue that goal.

Finally, all-atom molecular simulations were able to demonstrate multiple homodimer conformations of plexin A3 in wild-type and with mutations. Disruptive mutant in JM domain and wild-type simulations resulted in weak interaction in TM domain, while simulations with double mutant in TM region indicated crossed motif in TM/JM regions and strong interaction in TM region. First replica of simulations with extended extracellular domain attached to transmembrane domain indicated
strong interaction in TM domain, which was not obtained in our wild-type simulations with just TM and JM domains (70 residues). Third replica indicated comparably stronger interaction in extended EC domain and JM domain, while second replica indicated total loss of strong interaction in each domain. Disruptive nonpolar mutations in EC domain resulted in complete loss of TM interaction that was started based on the dimerized structure of the first replicate suggesting importance of including extended EC domain to obtain stronger interaction in TM domain specifically in small glycine-rich region. Future work in this area would be focused on doing multiple mutations in TM, EC, and JM domains to capture important features to obtain stable dimerization. This work also requires close collaboration with experimental group as we did that for current study. Dr. Bryan Berger’s lab at University of Virginia will perform these of experimental procedures and our simulation results will be compared with their findings.
7. Chapter 7: Bibliography


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Publications and Conferences

Publications


Conferences


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