The Pennsylvania State University
The Graduate School
Department of Bioengineering

MOLECULAR SCALE SPECTROSCOPY AND SIMULATION OF
STRESSED LIPID BILAYERS

A Dissertation in
Bioengineering

by
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Lipid-lipid interactions play a very important role in mediating membrane protein activity, through alterations in membrane physical properties or lateral phase separation. However, the underlying physical principles governing lipid organization remain unclear and there are very few experimental tools to measure organization and structural properties on the molecular scale. In this work, the effect of molecular packing on some of the key membrane properties including bilayer thickness, acyl chain conformational flexibility, diffusive mobility, and electrostatic potential, and dynamics of DiI, was characterized using atomistic computational simulations. Lateral tension increases acyl chain interdigitation, and lateral/rotational mobility, and decreases hydrophobic thickness and electrostatic potential. DiI dynamics indicated that it is likely to be a good reporter for changes in bilayer fluidity. Combining the diffusion coefficient and fluorescence lifetime of DiI, the membrane microviscosity near the headgroup and tail regions was measured. Further, it is shown for the first time that fluorescence lifetime of DiI can report free-area in lipid bilayers to a precision of 1 Å². This property was used to measure thermal undulations in giant vesicles and nanoliposomes from fluctuations in lifetime of DiI. The methods developed here enable characterization of membrane mechanics, which may aid in identifying the role of membrane in endothelial mechanotransduction. Finally, a decrease in hydrophobic thickness induced by non-lipid amphiphiles was found to promote raft formation, and vice versa, emphasizing the role of hydrophobic mismatch. This finding brings a new perspective to how non-lipid amphiphiles regulate raft formation in cell membranes.
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Chapter 1

Introduction

Lipids form the underlying structural matrix (~4 nm thick) of cellular membranes, which help cells maintain the impermeable barrier between cell cytosol and extracellular material and compartmentalize internal organelles. Biological membranes harbor many proteins (nearly 30% of the genes expressed in animal cells\(^1\)) that play essential roles in cellular function including maintaining ion gradients across the membrane (e.g. ion channels), transduction of information from outside the cell to the cytosol through receptor activation (e.g. G-protein signaling), formation of structural connections between cell cytoskeleton and extracellular matrix (e.g. integrins), and communication between cells through junctional proteins (e.g. cadherins and connexins). In the famous fluid-mosaic model,\(^2\) Singer and Nicholson defined the lipid bilayer as a two-dimensional viscous solution with integral proteins. Since then our understanding of the lipid bilayer structure and its active role in regulating protein activity has developed significantly.\(^3,4,5\) Lipids are no longer considered as the passive structural elements of cell membranes. They govern membrane protein activity through direct action on proteins, through structural properties of the bilayer, and through lipid metabolism. Understanding the interrelationship between structure and function of lipid bilayers and downstream signal transduction is key to developing novel biotherapeutic agents\(^6\) against diseases that find their origins in lipid-protein interaction.
1.1. Lipid bilayer and its role in signal transduction

1.1.1. Lipid bilayer structure and organization

Structural membrane lipids in eukaryotic cells can be broadly classified into two groups: glycerolphospholipids and sphingolipids. The majority of the lipids in the eukaryotic cell membranes belong to the class of glycerophospholipids, which can be further classified into phosphatidylcholines (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), of which PC is the major class comprising more than 50% of the total lipid content in cells. These lipid types have a common glycerol backbone and differ in the chemical nature of the headgroup. As a general feature, all lipids are amphiphilic molecules with a polar (hydrophilic) headgroup region and a non-polar (hydrophobic) hydrocarbon tail region (schematic shown in Figure 1-1). Due to the amphiphilic nature, most lipids spontaneously aggregate to form double layers (or bilayers) under aqueous conditions resulting in a two-dimensional planar structure. Depending on the geometry of the molecule (e.g. diacyl versus monoacyl lipids), they might also form micellar structures (Figure 1-1).

Another key molecular component of biological membranes is cholesterol. Cholesterol is unique to eukaryotic cell membranes and is the principal sterol synthesized in animal cells. Cholesterol is also an amphiphilic molecule that is largely hydrophobic with a rigid ring structure and a polar hydroxyl headgroup (Figure 1-1). Cholesterol plays several structural and functional roles in biological membranes. Cholesterol regulates membrane protein activity through specific sterol-protein interactions, by altering the bilayer’s physical properties, and through self-organization of the bilayer into domains.
Specific cholesterol binding sites have been identified for several membrane proteins including G-protein coupled receptors$^{10,11,12}$ and ligand-gated ion channels.$^{13}$ Specific sterol-protein interactions are thought to modulate the functioning of these membrane proteins.$^{8,9}$ Cholesterol can also indirectly modulate protein activity by modulating the physical properties of the lipid bilayer. For example, membrane cholesterol content has been shown to modulate volume-regulated anion current in endothelial cells, and this effect was reversed by replacing cholesterol with its chiral analogues,$^{14,15}$ indicating

Figure 1-1: Chemical structures of phospholipids, sphingolipids, and cholesterol. Lipids adopt lamellar or micellar phases depending whether their shape is optimal for planar or highly curved membranes as shown in the bottom panel.
modulation of protein activity through bilayer fluidity changes. Similar results were reported for inward-rectifier K\(^+\) channels in endothelial cells.\(^{16}\) Finally, cholesterol is an essential ingredient for lateral organization of lipid membranes into membrane domains (or rafts).

Even though, macroscopically, the lipid bilayer is thought of as a two-dimensional continuous fluid, microscopically, it exhibits high asymmetry in both the lateral and the transverse dimensions. The inner and outer leaflets have asymmetric distribution in lipid composition with the outer leaflet enriched in phosphotidylcholines and sphingomyelins and the inner leaflet enriched in phosphotidylserines and phosphotidylethanolamines.\(^{17}\) Cell membranes also exhibit highly complex self-organization in the lateral dimension, with regions enriched in sphingomyelin and cholesterol and regions enriched with poly-unsaturated phosphotidylcholines and depleted of cholesterol.\(^{18}\) In this thesis, we will focus on the lateral self-organization of lipid bilayers into phase domains.

Lipid bilayers occur in various crystalline phases including gel, ripple, and liquid (disordered and ordered).\(^{21}\) In the gel phase, the hydrocarbon chains are arranged in an all-trans state, whereas in the liquid state they undergo fast trans to gauche transformations resulting in the high fluidity. The transition of the gel phase to liquid phase happens at a specific temperature, commonly referred as phase transition temperature. The phase transition temperature of a lipid is governed by its chemical structure and is predominantly affected by the length and degree of saturation of the hydrocarbon chains. For example, the phase transition temperature of DPPC (16:0 PC) is 42\(^\circ\)C and that of DMPC (14:0) is 24\(^\circ\)C. Thus a decrease in chain length by two carbon
atoms decreases the melting temperature by 18°C. In a mixture of two or more lipids with different phase transition temperatures, the non-ideal mixing behavior of the lipids results in heterogeneous phase domains.\textsuperscript{19,20} The miscibility of a mixture is typically represented by phase diagrams (as shown in Figure 1-2). Depending on the composition and temperature, the mixture can result in solid/solid, liquid/liquid, or solid/liquid, coexistence regions. Coexisting liquid-liquid phase regions are typically observed in ternary mixtures of two lipids and cholesterol. These regions are referred to as “liquid-disordered” (cholesterol-poor) and “liquid-ordered” (cholesterol-rich), and are thought to resemble lipid rafts in cell membranes. While the existence of lipid domains under physiological conditions is still in debate, recent state of the art measurement techniques confirm their presence in cell membranes with sizes on the order of few tens of nanometers.\textsuperscript{22} Moreover, these domains are transient in nature, forming and disappearing on the timescales of few milliseconds.\textsuperscript{23}

![Figure 1-2: Phase diagram of a binary mixture of DPPC and Cholesterol (Sankaram et al.,)](image)
Giant unilamellar vesicles prepared from simple lipid mixtures (two or three components) exhibit microscopically observable phase domains, permitting their characterization under regular fluorescence microscopy. These simplified model membrane systems closely mimic realistic biological membranes and thus provide a platform on which to build fundamental physical and chemical principles for lipid-lipid interactions. One step closer to the biological membranes would be to study giant plasma membrane vesicles derived from cells that include all the membrane proteins. In fact, recent studies report microscopic lateral phase separation in such giant plasma membrane vesicles and several membrane proteins show preferential partitioning to these domains.

1.1.2. Modulation of protein activity by the lipid bilayer

In the bilayer form, lipids exhibit high mobility in the lateral dimension and restricted mobility in the normal direction (i.e. leaflet to leaflet, also called as “flip-flop”) and so is classified as a two-dimensional fluid structure. The fluidity of the membrane is governed by the packing of lipids in the lateral dimension, which in turn primarily depends on the conformational flexibility of the hydrocarbon chains and to some extent on the nature of the headgroup (size, charge, and hydrogen bonding). Lipids with saturated acyl chains exhibit lower mobility due to tight packing and those with unsaturated acyl chains (presence of a kink) exhibit higher mobility due to looser packing.

Phase diagram of a ternary mixture of DOPC, DPPC, and Cholesterol. (Veatch et al., 2003)
of the chains. Thus, physicochemical properties of lipid bilayers are governed by several lipid structural features: acyl chain length, chain saturation/unsaturation, headgroup size/charge, and hydrogen bonding.

Membrane-mediated protein activity can be classified into two categories: 1) specific interactions of lipids with proteins inducing conformational changes, and 2) non-specific interaction in which lipid bilayer physical properties modulate protein conformational changes by indirectly altering the energetic state of proteins. Various physical properties of the lipid bilayers including viscosity, hydrophobicity, compressibility, curvature, and lateral pressure, are thought to play essential role in modulating integral membrane protein activity. The most important property of the lipid bilayer that has a strong influence on the protein conformation is its hydrophobic thickness. Matching the hydrophobic thickness of the lipids to that of the embedded proteins avoids the energetic costs associated with exposing to water the hydrophobic side chains of these integral proteins. Mismatching the hydrophobic thickness of the lipid bilayer to the protein can result a change in the lipid or protein’s conformation or both. Hydrophobic mismatch has been shown to influence the opening and closing of transmembrane stretch-activated ion channels. Moreover, such hydrophobic mismatch can also drive aggregation or oligomerization of the membrane proteins. Interesting, lipid-lipid mismatch can also drive membrane segregation into domains, which in turn can facilitate segregation of membrane proteins. Another well-studied property of the lipid bilayer is its fluidity (i.e. inverse of viscosity). Fluidity of the lipid bilayer can be modulated through compositional changes or through application of external mechanical forces. Though changes in fluidity cannot directly influence the
activation barrier for proteins\textsuperscript{29}, it could potentially alter the kinetics of ligand-receptor interactions or protein-protein interactions in the membrane.\textsuperscript{40,41} Nicolau et al.\textsuperscript{42} have shown that the diffusion characteristics of raft and non-raft regions can also determine the residence time of the protein in raft regions and thus the protein concentrations in rafts. Moreover, several anesthetics and drugs are known to alter the cellular function through interactions with the plasma membrane and alteration in membrane fluidity.\textsuperscript{43}

While bilayer thickness and fluidity have been investigated for a long time, more recent studies show that other properties of the lipid bilayer such as curvature and lateral pressure profiles might also influence protein sorting and activity.\textsuperscript{44,45} Unlike hydrophobic mismatch that acts on the entire protein hydrophobic core, alteration of depth-dependent lateral pressure can provide a means to induce conformational changes non-uniformly along the transverse direction of the protein.\textsuperscript{46} So far, this mechanism has been investigated only theoretically or through the use of computational molecular dynamics, as lateral pressure profiles cannot be obtained experimentally. Membrane curvature is well-observed phenomenon that is critical for membrane budding and fusion, and plays a very important role in intracellular trafficking. Bilayer curvature affects lipid-protein interactions and vice versa. Lipid curvature-induced protein sorting and protein induced lipid curvature are highly interlinked phenomena.
1.2. Motivation of this work

1.2.1. Endothelial cell mechanotransduction

Endothelial cells (ECs) lining the inner walls of the blood vessels play a key role in regulating vascular function, e.g. vascular tone, homeostasis, coagulation, and inflammation. Endothelial dysfunction can lead to various diseases like atherosclerosis, thrombosis, and hypertension. Unlike, most other cell types, ECs are under constant influence of hemodynamic forces exerted by blood in the lumen. Mechanotransduction is the process by which these cells convert mechanical stimuli into biochemical signals. Extensive research in this area has uncovered several mechanotransduction mechanisms involving plasma membrane, focal adhesions, cytoskeleton, cell-cell junctions, ion channels, and glycocalyx (Figure 1-3). Also recent studies suggest that mechanically-induced stresses trigger signaling pathways (e.g. g-protein signaling, receptor tyrosine kinases) in specialized membrane domains (i.e. lipid rafts).

As a repository for many cell signaling proteins, the plasma membrane plays a very important role in endothelial signal transduction. Signaling molecules such as integrins, potassium channels, stretch-activated calcium channels, and g-protein coupled receptors, residing in the plasma membrane, have been shown to be activated upon application of mechanical stimuli. Also, studies have shown that shear stress increases membrane fluidity. We have discussed in the previous section how such fluidity changes can affect protein activity in the membrane. Segregation of proteins into specialized microdomains is also thought to facilitate specific protein-protein interactions and also act as a signal amplifier. However, to date very little is understood about how
shear stress-induced membrane tension (or fluidity) alters the dynamics of lipid rafts in intact cells, due to the lack of sensitive experimental techniques. So, it is essential to develop novel biophysical and biochemical tools to probe different membrane phase domains in intact cells under physiological conditions.

Figure 1-3: Molecular mechanisms of shear-induced endothelial cell mechanotransduction.56

1.2.2. Characterization of lipid packing at the molecular level

Understanding the relationship between molecular dynamics and membrane structure is key to assessing membrane function. “Area-per-lipid” is the most
fundamental property of lipid bilayers because it captures the packing of the lipids, which
in turn determines the bilayer's fluidity, hydrophobic thickness, electrostatic potential,
and other important properties. (discussed in detail in chapter 2). Also, computational
molecular dynamics models of lipid bilayers are developed by critically comparing the
simulated area-per-lipids to experimentally determined values. Quantitative
measurements of lipid packing at the molecular level are accessible to techniques such as
x-ray scattering, deuterium NMR, and neutron scattering. While, these techniques
provide accurate quantitative measurements, they cannot be readily applied in vitro due
to the high complexity of biological membranes, and so are limited to pure bilayer
systems such as model membranes. On the other hand, there is significant interest in
developing fluorescence-based methods, as they enable organelle targeting and provide
high sensitivity and applicability to in vitro and in vivo studies. Fluorescence
techniques such as fluorescence correlation spectroscopy and fluorescence anisotropy
have been instrumental in measuring lipid bilayer mobility. These are often limited to
a single-point in the cell or take very long data acquisition times in scanning modes. Due
to the long acquisition times, these techniques cannot access dynamic changes in cell
membranes occurring in the order of less than a second, which is the case for lipid rafts.
Also, while the diffusion coefficient is indicative of lipid packing, the exact quantitative
values for packing, such as area-per-lipid, cannot be obtained accurately. An alternate
strategy to this problem is to develop novel probes or identify the spectroscopic quantities
of probes that show great sensitivity to changes in membrane structure. Several probes
have been identified using this strategy. For example, Haidekker et al. have used the
DCVJ molecular rotor to detect shear induced changes in membrane fluidity. Also,
generalized polarization of Laurdan has been used to detect changes in membrane structure as this probe is thought to be sensitive to water structuring near the lipid-water interface.\textsuperscript{64} Once again, these techniques are excellent for qualitative but not for quantitative measurements.

Single-molecule fluorescence spectroscopic measurements of lipoid dyes, such as DiI are widely used to assess membrane dynamics through measurements of lateral and rotational diffusion.\textsuperscript{66} In this thesis, we will introduce two novel methods based on the fluorescence lifetime of DiI to quantitatively determine molecular packing and its dynamic changes arising from fluctuations in packing due to thermal undulations of the bilayer. These two methods are discussed in detail in Chapters 3 and 4. Understanding the nature of lipid-dye interactions is key to proper interpretation of these data. Interpretation of fluorescence spectroscopic data demands detailed atomistic understanding of the dye’s behavior, for example fluorescence lifetime of the dye depends on the location and orientation of the transition dipole moment of the dye molecule. This information is largely inaccessible to experiments. Molecular dynamics simulations of popular membrane dyes such as DPH, NBD, and pyrene have resulted in significant atomistic insights into these dye’s behavior in lipid bilayers.\textsuperscript{68,69,70,71} Intercalation of these dyes even at very low concentrations can perturb the structure of the lipid bilayer. Previously, our group has studied the effect of DiI-C\textsubscript{18} on lipid bilayer structure and dynamics using molecular dynamics simulation.\textsuperscript{72} Here, we used atomistic molecular dynamics simulation of lipid bilayers with embedded DiI to characterize the influence of lipid packing on the dye’s location and dynamics (covered in chapter 1).
1.3. Outline of the thesis

The overarching goals of this thesis are to understand membrane dynamics at a molecular scale and how these interactions can mediate force sensation by cells. We pay special attention to how membrane lateral tension influences lipid bilayer structure and dynamics, using computational molecular dynamics simulations (chapter 2). These results provide atomistic insights into shear stress-induced changes in lipid molecular interactions. Further, we developed sensitive single-molecule fluorescence techniques to quantitatively determine membrane microviscosity, lipid packing and membrane thermal undulations (chapters 3, 4 and 5). Finally, we focus on how certain membrane perturbants that act as rigidifying/fluidizing agents modulate lateral phase separation in lipid bilayers. Using atomistic simulations of lipid bilayer with various membrane perturbants, we dissected the molecular interactions responsible for their role as rigidifying/fluidizing agents.

1.4. References


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Chapter 2

Effect of lateral tension on membrane physical properties

Foreword

The following chapter is from the manuscript: “Atomistic simulations of lipid and DiI dynamics in membrane bilayers under tension”, Hari S. Muddana, Ramachandra R. Gullapalli, Evangelos Manias, and Peter J. Butler, submitted to Physical Chemistry & Chemical Physics, 2010

2.1. Introduction

Mechanical forces modulate cell growth, differentiation, signal transduction, transport, and migration, through biochemical signaling pathways which may be related to membrane molecular organization and dynamics. For example, lateral membrane tension causes conformational changes in integral membrane proteins, affects membrane permeability, lipid lateral diffusion, and organization of lipid rafts. These effects are believed to be mediated by bilayer thickness changes that result in hydrophobic mismatch between the lipid acyl chains and transmembrane region of proteins, leading to distortion of the lipid bilayer and concomitant protein conformational changes.

Despite the importance of lipid dynamics in cell signaling, to date the only experimental studies quantifying the relationship between lipid dynamics and force have been conducted in sheared endothelial cells and in hair cells. In these studies,
lipid dye, such as 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), 9-(Dicyanovinyl)-julolidine (DCVJ), or di-8-ANEPPS, was used to infer lipid dynamics from fluorescence intensity or fluorescence recovery after photobleaching (FRAP). Because these studies probed lipid dynamics indirectly and because the precise membrane tensions, at the molecular level, were unknown, there is a need to quantify directly the relationship between membrane tension and lipid dynamics.

The most prominent methods to assess lipid dynamics, including FRAP, fluorescence correlation spectroscopy (FCS), fluorescence anisotropy, and fluorescence lifetime imaging probe membrane lipid dynamics by analyzing the dynamics of lipophyllic fluorescent dyes (e.g. DiI, 1,6-diphenyl-1,3,5-hexatriene (DPH), and Laurdan). In particular, DiI is popular because of its structural similarity to phospholipids and its ability to selectively partition into different lipid phases (gel or fluid) depending on the matching between the length of its alkyl chains and the lipid acyl chain length. Spectroscopic investigations employing DiI have been used to study membrane organization and dynamics. The fluorescence lifetime of DiI depends on the accessibility to water and on the viscosity of the local microenvironment, offering a useful tool to detect lipid rafts in cells and phase separation in model membranes. However, proper interpretation of these fluorescence measurements requires precise knowledge of location, orientation, and interactions of dye with lipids and water, which are difficult to obtain experimentally. Examples of the utility of using molecular dynamics (MD) simulation as a tool to answer these questions include predictions of the location of drug-like small molecules in lipid bilayers along with validation by small-angle neutron scattering experiments.
The aim of this computational modeling study was to determine the effects of membrane tension on mechanotransduction-related structural and dynamical properties of the bilayer. In addition, we wished to understand the fidelity with which DiI, a popular membrane probe, reflects lipid dynamics, so that DiI photophysics could be used as a readout for tension effects on stressed membranes. To accomplish this goal, we performed a series of atomistic molecular dynamics (MD) simulations of fluid-phase dipalmitoylphosphatidylcholine (DPPC)/DiI bilayers under various physiological tensions. The main readouts from this study are as follows. First, we characterized the effects of tension on bilayer thickness, acyl chain packing, interdigitation, and electrostatic potential. Second, we determined the relationship between area-per-lipid and lipid lateral diffusion, and compared these results to predictions from free-area theory. Third, we compared the DiI probe dynamics to the dynamics of the native lipids, leading to an analysis of the relationship between lipid packing and fluorescence lifetime of DiI in terms of hydration and local viscosity.

2.2. Simulation Methodology

Force field parameters for DPPC and DiI-C\textsubscript{18} were identical to Berger et al.\textsuperscript{26} Bond lengths and bond angles of the DiI’s headgroup were obtained from X-ray crystallography data of a structurally similar carbocyanine dye.\textsuperscript{27} Simple point charge (SPC) model was used for water.\textsuperscript{28} The partial charges for the DPPC molecules were identical to those described by Chiu et al.,\textsuperscript{29} whereas, the partial charges on the DiI molecule were obtained by performing \textit{ab initio} quantum mechanical calculations using
Gaussian 03 software package with the Hartree-Fock method and the 6-31G basis set using the charge partitioning scheme of Merz-Kollman.\textsuperscript{22,29}

A simulation box of well-equilibrated pure DPPC bilayer consisting of 128 DPPC molecules and 3655 water molecules was obtained from Tieleman, \textit{et al.}\textsuperscript{28} The DiI-C\textsubscript{18} dye was incorporated in the DPPC bilayer by replacing two DPPC molecules with a single DiI-C\textsubscript{18}, in each leaflet. To ensure electrical neutrality, two chloride ions were added to the system. To make sure that the equilibrium position of the dye was independent of its initial position, the dye headgroups were placed at random z-locations (above and below the lipid-water interface) and the system was equilibrated. Initial configurations of the simulation box with varied area-per-lipid ($\alpha$) were constructed by scaling the original system, while maintaining constant volume, and the systems were equilibrated as described below. Membranes were simulated at seven different area-per-lipid values ranging from 0.635 to 0.750 nm$^2$, corresponding to tensions ranging from -2.6 to 15.9 mN/m. Final production runs were performed in NP$\gamma$AT ensemble. This ensemble was chosen in order to conveniently apply tension by prescribing area per lipid. Other ensembles such as NPT or N$\gamma$T ensembles could have been chosen and these would give equivalent structural and dynamical properties.\textsuperscript{28,30}

Molecular dynamics simulations were carried out using the GROMACS software package (version 3.3.2).\textsuperscript{31,32} Pre-equilibration of energy minimized structures was performed under NVT conditions, at 323 K, for a simulation time of 1 ns, allowing for removal of any overlaps or defects caused by DiI placement and the application of tension. Subsequent equilibration of the structures was performed under NP$\gamma$AT conditions, at 323 K and 1 bar normal pressure, for a total simulation time of 100 ns.
Final production runs were carried out under NP$_x$AT conditions for an additional 100 ns for each system configuration. Periodic boundary conditions were applied in all three coordinate dimensions. The temperature of the production runs was 323 K, which is above the gel-to-liquid phase transition temperature, $T_m$, of DPPC (~315 K). Temperature and pressure were controlled using Berendsen’s weak coupling method with the time constants set to 0.1 ps and 1.0 ps respectively. Semi-anisotropic scaling was used for pressure coupling with zero compressibility in xy-plane to maintain the area constant. The LINCS algorithm was used to constrain the bond lengths, allowing for larger time steps than if the bonds were unconstrained. The Particle-Mesh Ewald (PME) method was used for electrostatic interactions, with a direct-space cutoff of 1 nm, and cubic interpolation (PME order = 4) for the calculation of long-range interactions in reciprocal space, with a Fourier transform grid of 0.12 nm maximum. Despite its computational cost, PME was chosen because it allows for proper electrostatics in systems with charged molecules and ions. The Lennard-Jones interactions were cutoff (shifted and truncated) at 1.0 nm. A time-step of 2 fs was used with a leap-frog integration algorithm for the equations of motion, accommodating bond constraints and weak coupling to constant T and P baths.

2.3. Results and Discussion

In this study, we addressed how membrane tension or, equivalently, how lipid packing affects structural and dynamical properties of a fluid-phase DPPC bilayer by performing a series of united-atom molecular dynamics (MD) simulations. Various
physicochemical properties of the bilayer were analyzed as a function of area-per-lipid ($\alpha$). We included DiI-C$_{18}$ in our simulations to facilitate the interpretation of lipid packing and dynamics from single-molecule fluorescence measurements of lateral and rotational diffusion, and fluorescence lifetime of DiI, a popular membrane probe. Pertubative effects of DiI on the lipid bilayer were studied previously by Gullapalli et al.\textsuperscript{22} Results are divided into five subsections: structural changes of lipid bilayer under tension, tension-induced changes in lipid order, electrostatic potential and lipid/water dipole ordering, lipid lateral diffusion and free-area theory, and sensitivity of DiI dynamics to membrane tension.

2.3.1. Tension induces bilayer thinning and interleaflet interdigitation

Surface tension was estimated from the pressure tensor, as described in ref.\textsuperscript{38}. As expected, the surface tension increased linearly with an increase in area, from -2.6 mN/m at $\alpha = 0.635$ nm$^2$ to 15.9 mN/m at $\alpha = 0.750$ nm$^2$, above which rupturing of the bilayer was observed (rupture data not shown). While this rupture tension is in good agreement with values from micropipette aspiration of lipid vesicles (ranging from 10 to 20 mN/m,\textsuperscript{5}) MD simulated rupture and experimental rupture tensions often differ because rupture/pore tension depends strongly on the loading rate, which is effectively larger in MD simulations.\textsuperscript{39} Thus within the range of tensions simulated in this study, pore formation or rupture cannot be observed in the size/time scales studied here.\textsuperscript{40} Zero surface tension corresponded to $\alpha = 0.646$ nm$^2$, close to the experimental value of 0.64 nm$^2$ for DPPC.\textsuperscript{41} In addition, the area compressibility modulus calculated from the
tension-area plot\textsuperscript{38} was 105 mN/m, in good agreement with the previous simulation value of 107 mN/m for DPPC bilayer at 50\textdegree C.\textsuperscript{38} Experimentally, compressibility modulus values of 145 mN/m\textsuperscript{42} and 234 mN/m\textsuperscript{43} were reported for DPPC at 50\textdegree C and DMPC at room temperature, respectively. Using an identical force field to the current simulations, Lindahl \textit{et al.}\textsuperscript{44} reported a simulated value of 250-300 mN/m for a larger membrane patch (1024 lipids), suggesting that the lower value in the current study is likely due to the finite-size effect. Considering the empirical nature of the force field parameters, these results indicate that the simulation methodology is sufficiently accurate in determining the microscopic and macroscopic properties of the lipid bilayer over an extended range of simulated tensions.

Bilayer thickness, defined as the distance between water and lipid density crossover points on either side of the bilayer, was directly computed from the mass density profiles (figure 2-1).\textsuperscript{23} The bilayer thickness decreased linearly with increases in area-per-lipid, consistent with volume-incompressibility (Table 2-1). The density profile of the bilayer is highly reminiscent of a confined film\textsuperscript{36,37} – rather than a constant density bulk fluid – and thus changes in bilayer thickness are expected to result in structural reorientations within the bilayer.\textsuperscript{45} In support of this interpretation, it was observed that increasing the surface area resulted in a decrease of the lipid density at the headgroup region and a concurrent increase in the local density at the mid-plane of the bilayer (figure 2-1). This indicates increased interdigitation of the acyl chains of the opposing leaflets due to extension of the chains beyond the bilayer mid-plane. Increased interdigitation has physiological implications; for example, acyl interdigitation has been proposed to result in the formation of membrane micro-domains.\textsuperscript{42} Also, interdigitation
of the acyl chains can alter the hydrophobic interactions and lateral pressure profile of the bilayer, which in turn can alter protein conformation.\textsuperscript{46,47} Spreading of the acyl chains also takes place upon decrease in bilayer thickness (next section).

Figure 2-1: Mass density profiles of lipid (solid), water (dashed), and DiI-C\textsubscript{18} (dotted) across the lipid bilayer at selected values of area-per-lipid (the center of the bilayer was set at $z = 0$, DiI density is at 20x for clarity). A snapshot of the simulation box is also shown (DPPC – grey, DiI – red, water – purple, and DPPC phosphorous atoms are shown in green).

Table 2-1: Summary of structural and dynamical properties of the lipid bilayer as a function of area-per-lipid. \textsuperscript{a}Reported standard error means (S.E.M.) are upper limits of all the simulations. \textsuperscript{b}95% confidence intervals obtained from curve-fitting.

<table>
<thead>
<tr>
<th>Bilayer property</th>
<th>Area-per-lipid, $\alpha$ (nm\textsuperscript{2})</th>
<th>0.635</th>
<th>0.650</th>
<th>0.675</th>
<th>0.687</th>
<th>0.700</th>
<th>0.725</th>
<th>0.750</th>
<th>S.E.M.\textsuperscript{a}</th>
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<td>Surface Tension</td>
<td>[mN/m]</td>
<td>-2.62</td>
<td>0.86</td>
<td>5.24</td>
<td>6.85</td>
<td>9.19</td>
<td>13.49</td>
<td>15.87</td>
<td>0.32</td>
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<tr>
<td>Volume per lipid</td>
<td>[nm\textsuperscript{3}]</td>
<td>1.233</td>
<td>1.234</td>
<td>1.236</td>
<td>1.237</td>
<td>1.238</td>
<td>1.239</td>
<td>1.240</td>
<td>0.001</td>
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<td>Bilayer thickness</td>
<td>[nm]</td>
<td>4.20</td>
<td>4.13</td>
<td>4.00</td>
<td>3.94</td>
<td>3.86</td>
<td>3.76</td>
<td>3.63</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Average $trans$ fraction of chains</td>
<td>[%]</td>
<td>76.8</td>
<td>76.5</td>
<td>76</td>
<td>75.9</td>
<td>75.7</td>
<td>75.4</td>
<td>75.2</td>
<td>0.2</td>
</tr>
<tr>
<td>End-to-End tail vector angle</td>
<td>[$^\circ$]</td>
<td>36</td>
<td>40</td>
<td>43</td>
<td>48</td>
<td>50</td>
<td>53</td>
<td>57</td>
<td>1</td>
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<td>Electrostatic potential</td>
<td>[mV]</td>
<td>615</td>
<td>614</td>
<td>596</td>
<td>608</td>
<td>605</td>
<td>597</td>
<td>589</td>
<td>10</td>
</tr>
<tr>
<td>Electrostatic potential barrier</td>
<td>[mV]</td>
<td>759</td>
<td>770</td>
<td>727</td>
<td>722</td>
<td>704</td>
<td>677</td>
<td>--</td>
<td>--</td>
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<tr>
<td>Mean square displacement ($\tau = 200$ps)</td>
<td>[Å\textsuperscript{2}]</td>
<td>5.41</td>
<td>5.74</td>
<td>6.57</td>
<td>7.00</td>
<td>7.19</td>
<td>8.02</td>
<td>8.85</td>
<td>0.06</td>
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<td>Lateral diffusion coefficient</td>
<td>[$10^{12}$ m\textsuperscript{2}/s]</td>
<td>8.16</td>
<td>9.35</td>
<td>9.80</td>
<td>11.71</td>
<td>12.16</td>
<td>16.72</td>
<td>23.29</td>
<td>0.72</td>
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<td>Rotational relaxation time $\tau_1$</td>
<td>[ns]</td>
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<td>0.30</td>
<td>0.24</td>
<td>0.22</td>
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<td>0.17</td>
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<td>$\pm 0.01$\textsuperscript{b}</td>
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<td>Rotational relaxation time $\tau_2$</td>
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<td>3.57</td>
<td>2.75</td>
<td>2.40</td>
<td>2.23</td>
<td>1.92</td>
<td>1.67</td>
<td>$\pm 0.03$\textsuperscript{b}</td>
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</table>
2.3.2. Tension reduces lipid acyl chain packing and order

The effect of membrane tension on the lipid chains was determined by computing the order parameter of sn-1 and sn-2 chains separately, as a function of the chain carbon atom number, as an indicator of the lipid membrane order in accordance to our previous publication. The value of $S_{CD}$ can vary from -0.5 (perfect ordering along the plane of the bilayer) to 1 (perfect alignment along the normal to the bilayer). An $S_{CD}$ value of zero indicates an isotropic orientation (i.e., a random orientation of the CH bonds). Order parameter profiles for the sn-1 and sn-2 chain as a function of area-per-lipid are shown in figure 2-2 (a&b). For $\alpha = 0.635 \text{ nm}^2$, $S_{CD}$ versus carbon chain number exhibited the typical trends with a plateau region near the headgroup (with an average order of 0.2 ± 0.02) which gradually dropped to zero near the terminal methyl groups of the tails. These values are in good agreement with the values reported previously from experiments and simulations. Increasing the surface area caused a significant decrease in the order parameter values of both sn-1 and sn-2 chains throughout the length of the carbon chain. Changes in the order parameter with tension were smallest (~30%) near the headgroup region and largest (~50%) at the terminal tail region.

Changes in the $S_{CD}$ order parameter resulted from the combined effect of tension on the acyl chain dihedrals and on the chain tilt angle. To determine the relative magnitude of tension-induced changes in these parameters, we calculated the average $trans$ fraction of the chain dihedrals, and the average angle between the end-to-end acyl chain vectors. In general, the average $trans$ dihedral fraction was largest near the headgroup region and decreased towards the terminal tail region (a strong drop was
observed in the last dihedral of both the acyl chains). Although measurable decreases in the average trans fraction of the chain dihedrals were observed with increased area-per-lipid, these changes were small (< 3%, shown in figure 2-2 (c)) and there was no change in the qualitative trend. This result strongly suggests that the acyl chain configuration is not markedly affected by the application of tension. On the other hand, when we analyzed the distribution of angles between the end-to-end sn-1 and sn-2 chain vectors, shown in figure 2-2 (d), the peak angle gradually increased from 36 degrees (for $\alpha = 0.635 \text{ nm}^2$) to 57 degrees (for $\alpha = 0.750 \text{ nm}^2$), with applied tension. This result clearly denotes that a significant “spreading” of the chains takes place with increases in the area-per-lipid. We
can conclude that the tension-induced decrease in the \( S_{CD} \) order parameter is primarily due to an increase in the spreading of the acyl chains, \( i.e., \) changes in the acyl chain orientation within the bilayer, rather than due to any changes in the acyl chain conformations (\( i.e. \) trans and gauche fractions). Similar molecular shape changes of lipid were simulated previously in the outer leaflet of high curvature liposomes.\(^{50}\)

2.3.3. Tension reduces the electrostatic potential barrier through lipid/water dipole reordering

The electrostatic potential at the lipid-water interface results from the orientation of the water and lipid dipoles. To determine this electrostatic potential, the ensemble-averaged charge density along the \( z \)-axis was computed. Figure 2-3 (a) depicts the individual contributions of lipids and water to the total charge density. Due to symmetry, only half of the simulated membrane is shown in the figure. Compensation of the dipole potential of the lipid molecules by water resulted in a negative potential in the bulk water with respect to the bilayer interior. The corresponding electrostatic potential with respect to the center of bilayer was computed by double integration of the time-averaged charge density, \( \rho(z) \), using Poisson’s equation:

\[
V(z) = \varphi(z) - \varphi(0) = -\frac{1}{\varepsilon_0} \int_0^z dz' \int_0^{z'} \rho(z'')dz''
\]

where \( \varphi(z) \) and \( \rho(z) \) are the time-averaged dipole potential and the charge density, respectively, as a function of the distance normal to the bilayer, and \( \varepsilon_0 \) is the permittivity of vacuum. \( \varphi(0) \) is the dipole potential at bilayer center that acts as a reference point. The resulting potential profiles for different values of the area-per-lipid, are shown in figure
In the case of $\alpha = 0.635 \text{ nm}^2$, the potential difference between the center of the bilayer and water was -615 mV. This value is consistent with previous simulations of DPPC bilayers.$^{32}$ Experimentally, values for the potential difference vary from -200 to -575 mV for various phosphocholine-water interfaces.$^{35}$ With increasing lateral tension, the following effects in the electrostatic potential profiles were observed (illustrated in figure 2-3 (a)): First, the potential difference between the bilayer interior and bulk water decreased from 615 mV (for $\alpha = 0.635 \text{ nm}^2$) to 588 mV (for $\alpha = 0.750 \text{ nm}^2$). Similar results have been reported by Skibinsky et al., where lowering of surface tension by addition of trehalose induces an increase in electrostatic potential.$^{51}$ Second, the positive potential barrier at the lipid-water interface, which originates from the strong ordering of water molecules around the phosphoryl groups of the lipids, was reduced in magnitude and shifted in position away from the bilayer center. The height of the potential barrier with respect to bulk water is shown in Table 2-1. The potential barrier reduced by $\sim$80 mV at the highest tension simulated. These tension induced changes in electrostatic potential at the lipid-water interface may have physiological significance, as a decrease in the electrostatic potential of $\sim$30 mV can cause significant increase in ion transport across the lipid bilayer.$^{52,53}$

The ordering of the water dipole was quantified by calculating the time-averaged projection of the water dipole unit vector onto the interfacial normal (z-axis, Figure 2-3 (b)). A value of $\langle \cos \theta \rangle = -0.5$ corresponds to perfect ordering of the water dipoles parallel to the membrane normal, whereas a value of $\langle \cos \theta \rangle = 0$ corresponds to random water dipole orientation (disordered state). In general, high ordering of the water dipoles along the membrane normal is observed near the phosphorous atoms of the lipids.$^{35}$
Figure 2-3: (A) Charge density profile of the lipid bilayer is shown on the left, including the contributions from lipid and water. On the right are the respective electrostatic potential profiles as a function of area-per-lipid. (B) Ordering of water dipoles with respect to bilayer normal at different area-per-lipid. (C) Angular distribution of DPPC P-N vector with respect to the normal of the bilayer. In figures (A) and (B) the abscissa is normalized by the size of the simulation box for each $\alpha$, and only half of the simulation box is shown, due to symmetry ($z = 0$ denotes the center of the bilayer).
Away from the interface, the ordering persists in the z-axis until the point where the lipid density becomes zero. Increasing the membrane tension resulted in decreased water dipole ordering around the lipids, as seen by an increase in the $\langle \cos \theta \rangle$ minimum. This decreased water dipole ordering correlates well with the reduced potential barrier at the lipid-water interface and explains the previously observed changes in the electrostatic potential profile at the lipid-water interface. Also, a slight shift was observed in the location of the water dipole where ordering towards the membrane surface was highest (figure 2-3 (b)). This shift correlates well with the spatial shift observed in the electrostatic potential profile.

The ordering of the lipid dipoles at the lipid-water interface was quantified by calculating the angular distribution of the P-N vector with respect to the bilayer normal (figure 2-3 (c)). In general, the peak of the P-N vector angle was around 90°, indicating that the P-N vector was aligned parallel to the lipid-water interface. Increasing the area-per-lipid resulted in a slight decrease of the peak angle value, as evidenced by the peak shifts of the PN vector from 90° (for $\alpha' = 0.635 \text{ nm}^2$) to 82° for the largest tension simulated (for $\alpha = 0.750 \text{ nm}^2$) (figure 2-3 (c)). That is, increases in tension resulted in a tilt in the PN vector towards the bilayer normal. Considering both the water and lipid dipole orientations, we can conclude that both water and lipid dipole reordering contribute to the observed changes in electrostatic potential profiles with tension. Based on these results we predict that mechanical stretching of the lipid bilayer can result in significant changes in small-molecule diffusion through the bilayer, due to reduced resistance to diffusion accompanying reductions in electrostatic potential. This novel
mechanism of mechanosensitivity of cell membranes is worthy of further simulation and experimental testing.

2.3.4. Moderate tension increases lipid lateral diffusion by increasing free-area, but free-area theory does not hold for large tensions

Lateral diffusion coefficients ($D$) were computed from the mean-squared displacement (MSD) of the center-of-mass (COM) motion of the molecules. The MSD was ensemble averaged and calculated for multiple time-origins, and $D$ was quantified through Einstein’s equation:

$$D = \lim_{t \to \infty} \frac{1}{2dt} \langle [\vec{r}_i(t + t') - \vec{r}_i(t')]^2 \rangle$$

where, $r_i$ are the $x,y$ positions of the center of mass of a lipid $i$ at a given time $t'$ and after a time interval $t$ (i.e., at time $t+t'$); $d$ is the dimensionality of the motion considered (here $d=2$ for the in-plane lateral diffusion); the brackets denote ensemble average (over molecules and time) and also over multiple time origins $t'$. The MSDs were corrected for the COM motion of the membrane (i.e. removing any net leaflet translation). MSDs of DPPC at different area-per-lipid values are shown in figure 2-4 (a).

Lipids exhibit two different types of in-plane motion, a “rattling-in-cage” motion at short time-scales ($< 1$ ns), and translation via “hopping” diffusion at longer time scales ($> 10$ ns), as shown in the inset of figure 2-4 (a). Long-time diffusion coefficients were quantified by fitting the MSD curves to a linear function at long times (10 to 70 ns). Experimental values of the lateral diffusion coefficient of lipids in fluid-phase membrane bilayers range from $1.5 \times 10^{-11}$ m$^2$/s to $6 \times 10^{-12}$ m$^2$/s depending on the method.
Figure 2-4: (A) Mean square displacements (MSD) of lipid molecules under different tensions. Representative $xy$-trajectories of DPPC and DiI molecules are shown in the inset ($\alpha = 0.635 \text{ nm}^2$). (B) The plot of $\ln(D)$ vs. $1/a_f$, where two different linear regimes were identified, represented by solid lines, with slopes $\beta$. Error bars represent standard errors, $n = 124$. 
simulation-measured diffusion coefficient of DPPC at $a = 0.635$ nm$^2$ was $8.1 \times 10^{-12}$ m$^2$/s, which is close to the values obtained using fluorescence correlation spectroscopy.$^{20}$ According to the free-area theory,$^{55}$ lipid hopping from one cage to another depends on the availability of a void space (larger than a critical size) next to the molecule. Opening up of a void space occurs occasionally due to random density fluctuations. Free-area theory of lipid lateral diffusion has been shown to fit well with experimental diffusion data obtained as a function of temperature and cholesterol concentration in lipid bilayers.$^{56,57}$ However, the predictive ability of the model has been challenged because of the large number of fitting parameters required.$^{58}$ Lipid lateral diffusion coefficient ($D$) according to free-area theory is given by the equation,

$$\ln(D) = \ln(g \ d \ u) - \frac{\gamma a_c}{a_f}$$  \hspace{1cm} (3)

where, $g$ is a geometric factor ($\sim 1/4$), $d$ is diameter of the cage, $u$ is the gas kinetic constant, $\gamma$ is the free area overlap factor (0.5 to 1), $a_c$ is the critical area required for lipid diffusion, and $a_f$ is the free area defined as the difference between average molecular area ($a$) and the van der Walls area of the lipid ($a_0$). Note that the critical area is not the same as the van der Walls area. Fitting parameters include maximum diffusion coefficient (i.e. $D_{\text{max}} = gdu$, from eq. 3), critical area of the lipid, and free-area overlap factor ($\gamma$). Typical values of $a_0$ and $a_c$ for phosphocholines are 0.42 nm$^2$ and 0.48 nm$^2$ respectively.$^{59}$ The $D_{\text{max}}$ can be approximated by $K_B T/f$, where $f$ is the friction coefficient given by $4\pi\eta R$ for a spherical particle of radius $R$ in a medium of viscosity $\eta$.

According to equation 3, free-area theory predicts a linear relationship between $\ln(D)$ and $1/a_f$ with a constant slope for lipid monolayers in the area-per-lipid range of
0.50 nm² to 0.90 nm². From our simulations, however, the plot of ln(D) vs. 1/af (figure 2-4 (b)) over the range of area-per-lipid tested, resulted in two distinct linear regimes. The slope of the curve, β = -γ·a_c, measured at smaller area-per-lipid (0.635 to 0.700 nm²) was -0.34, which is comparable to that measured in various fluid-phase lipid monolayers (summarized in Table 1 of reference 59). However, the slope of the curve at larger area-per-lipid (0.700 to 0.750 nm²) was -1.2. Since there is no no evidence of phase transition, explanation of a non constant slope must be due to changes in interleaflet friction and/or molecular shape. Indeed, experimental studies showing that lipid diffusion is substantially higher in monolayers (D ~ 20 x 10⁻¹² m²/s) compared to bilayers (D ~ 6 x 10⁻¹² m²/s), as measured using FCS 20;59 suggest that changes in interleaflet interactions explain discrepancies between low and high tension slopes.

We now explore the idea of non-constant friction and molecular shape further. First, fitting of diffusion data to free-area theory is typically done by assuming that the D_{max} is constant for a given lipid, i.e. the friction coefficient is assumed constant through the entire range of area-per-lipid values. However, D_{max} obtained from extrapolating the linear fits to 1/af = 0 in figure 2-4 (b) shows that D_{max} is not constant and increases at high tensions. This suggests that the friction on the lipid molecules is decreased significantly at high tensions. This is supported by other observations in this study, namely, increase in disorder of the water molecules at the lipid headgroups (figure 2-3 (c)) and increased disorder of terminal tail regions of lipid acyl chains (figure 2-2 (a&b)). The decrease in headgroup and inter-leaflet friction might account for the dramatic increase in diffusion coefficient at higher tensions compared to the values predicted by free-area theory. Second, a significant increase in the slope of ln(D) vs. 1/af at high
tensions also indicates an increase in critical area of the molecule and/or the free-area overlap factor. However, changes in these parameters are not sufficient to explain changes in diffusion. Specifically, although free-area theory can be used to fit the diffusion data at large tensions, the fits result in a $\beta$ of -1.2. To obtain this slope either the overlap factor ($\gamma$) or the critical area ($a_c$) must be significantly greater than 1. But the overlap factor, by definition, lies between 0 (no overlap of free area) and 1 (complete overlap). Similarly, the critical area, the minimum area needed for diffusion, is normally held constant (at 0.48 nm$^2$) but could increase if the molecular shape changes. Increase in critical area of the molecule at high tensions is evident from the observation that significant spreading of the acyl chains occurs at high tensions (figure 2-2 (d)) but it will never increase sufficiently to yield a $\beta$ of -1.2. Other local-density/free-area theoretical approaches, which have been developed for polymer and oligomer systems, can yield quantitative agreement with the $D$ values obtained from the simulations$^{60,61}$, however these models introduce additional adjustable parameters, which may be difficult to define properly for the lipid bilayer system. We conclude that a new theory for lipid diffusion is needed that takes into account changing friction and molecular shape with tension.

2.3.5. Changes in lipid packing are reflected in changes in DiI diffusion and rotation

Experimentally, membrane dynamics are often assessed using measurements of dynamics of fluorescent probe molecules.$^{15,17,19,20}$ Such spectroscopic measurements assume that the probe molecules faithfully reflect lipid dynamics. Interpretation of the obtained data necessitates knowledge of the microenvironment factors such as hydration
and viscosity, which are dictated by the location and orientation of the chromophore. We discuss the sensitivity of fluorescence dynamics of DiI to lipid packing and compare DiI dynamics to the native lipid dynamics. Key properties pertaining to DiI are summarized in Table 2-2.

Table 2-2: Location, orientation, hydration, and dynamics of DiI.

<table>
<thead>
<tr>
<th>α (nm²)</th>
<th>Dil Location¹</th>
<th>Dil Orientation²</th>
<th>MSD at τ = 200ps</th>
<th>Rotational Relaxation</th>
<th>Hydration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nm)</td>
<td>(degrees)</td>
<td>(Å²)</td>
<td>τ₁ (ns)</td>
<td>τ₂ (ns)</td>
</tr>
<tr>
<td></td>
<td>± 0.01</td>
<td>± 0.20</td>
<td>± 0.06</td>
<td>± 0.03⁷</td>
<td>± 0.25⁷</td>
</tr>
<tr>
<td>0.635</td>
<td>0.62</td>
<td>4.74 (56.24)</td>
<td>3.73</td>
<td>1.09</td>
<td>9.69</td>
</tr>
<tr>
<td>0.650</td>
<td>0.54</td>
<td>5.5b⁵</td>
<td>--b⁴</td>
<td>--b⁴</td>
<td>--b⁴</td>
</tr>
<tr>
<td>0.675</td>
<td>0.57</td>
<td>2.14 (54.88)</td>
<td>4.34</td>
<td>0.95</td>
<td>8.07</td>
</tr>
<tr>
<td>0.687</td>
<td>0.62</td>
<td>-2.56 (55.14)</td>
<td>4.65</td>
<td>0.75</td>
<td>7.56</td>
</tr>
<tr>
<td>0.700</td>
<td>0.60</td>
<td>6.05 (56.71)</td>
<td>4.46</td>
<td>1.13</td>
<td>7.09</td>
</tr>
<tr>
<td>0.725</td>
<td>0.53</td>
<td>3.22 (61.08)</td>
<td>5.19</td>
<td>0.89</td>
<td>6.14</td>
</tr>
<tr>
<td>0.750</td>
<td>0.54</td>
<td>2.07 (52.90)</td>
<td>5.49</td>
<td>0.82</td>
<td>7.19</td>
</tr>
</tbody>
</table>

¹Positive values indicate DiI location below the lipid-water interface. ²A bimodal distribution of dye orientation was observed, with one dye molecule temporarily trapped in a metastable configuration with an average orientation of around 60 degrees. For this reason, the lateral and rotational dynamics are not reported. ³The values in parenthesis indicate full width half maximum (FWHM) of the distribution. ⁴95% confidence intervals obtained from curve-fitting.

The lateral diffusion coefficient of DiI has been shown to be in the same range but slightly lower than that of DPPC. In this study, we could not test the sensitivity of long-time lateral diffusion coefficient of DiI to lipid packing due to lack of sufficient statistics; there exist only two DiI molecules in the simulation box compared to 124 DPPC molecules. Alternatively, we computed the short-time diffusion (cage diffusion) of DiI and DPPC as a function of tension, and compared their MSDs at time t = 200 ps (Table 2-1 and Table 2-2, respectively). The MSD of DiI was lower than that of DPPC at all tensions. The free area of the DPPC bilayer is smaller near the lipid headgroup region and increases considerably in the hydrophobic region of the bilayer. Despite the fact that DiI
was located near the lipid acyl chain region (Table 2-2). DiI exhibited slower diffusion than DPPC. This is most likely due to the rigid and bulky structure of the DiI headgroup. Nevertheless, MSD of both DPPC and DiI scaled linearly with increases in tension and exhibited equal sensitivity. Considering that DiI exhibits hopping translation similar to DPPC at long time scales (inset of Figure 2-4 (a)), diffusion of DiI at longer times will likely also scale similarly to DPPC. Direct evidence for this would require simulating substantially larger systems, which is beyond the capabilities of our computational facilities. Nevertheless, based on the above observations, we conclude that the lateral diffusion mechanism of DiI is similar to that of the native lipid and that tension induces increases in DiI diffusion that are quantitatively similar to lipid diffusion.

We further assessed the rotational dynamics of DiI and DPPC by computing the rotational autocorrelation function of DiI’s orientation vector (vector joining the two indole rings) and of the P-N vector of DPPC, respectively. On average, the orientation vector of DiI was parallel to the lipid-water interface to within a few degrees, and was independent of tension-induced changes in lipid packing (Table 2-2). The rotational correlation function, \( C(\tau) \), is defined as \( C(\tau) = \langle P_2(\cos(\theta(\tau))) \rangle \), where \( \theta(\tau) \) is the angle between the orientation vectors separated by a time interval ‘\( \tau \)’, \( P_2 \) is the second Legendre polynomial, and \( <> \) represents the ensemble average. Rotational autocorrelation curves of DPPC and DiI for different values of tension are shown in figure 2-5 (a). In most cases, the rotational correlation of DiI did not decay completely to zero suggesting that either DiI does not undergo isotropic rotation or, more probably, our simulation time was not sufficient to reach the asymptotic limit. A double-exponential function of the form \( F(t) = F_0 + \sum_{i=1}^{2} e^{-t/\tau_i} \), commonly used to fit the experimental
rotation data, was used here to determine the rotational relaxation times\textsuperscript{63} (Table 2-2). The fast and slow relaxation times represent the wobbling motion and overall rotation, which were in the range of 0.8 to 1.1 ns and 6.1 to 9.7 ns, respectively, for DiI. These

Figure 2-5: (A) Rotational autocorrelation curves of DiI (dotted) and DPPC (solid) under different tensions. (B) The corresponding rotational relaxation times of DiI and DPPC, $\tau_1$ (fast) and $\tau_2$ (slow), obtained from fit with bi-exponential decay functions. Solid lines are linear fits of the data. 95\% confidence interval of $\tau_1$ and $\tau_2$ of DiI are $\pm$ 0.03 and $\pm$ 0.25 ns respectively. 95\% confidence interval of $\tau_1$ and $\tau_2$ of DPPC are $\pm$ 0.01 and $\pm$ 0.25 ns respectively.
results are in good agreement with experimentally-determined rotational correlation times of DiI-C\textsubscript{12} (shorter chain length than DiI-C\textsubscript{18}) in fluid-phase DOPC bilayer.\textsuperscript{64} In comparison, the fast and slow relaxation times of DPPC were on the order of 0.1 to 0.3 ns and 1.7 to 3.8 ns, respectively (Table 2-1), and were smaller compared to DiI by a factor of 2 to 3. Both slow and fast relaxation times of DPPC and DiI decreased linearly with increased area-per-lipid with equal sensitivity, as inferred from the linear fits shown in figure 2-5 (b). These observations show that even though the rotational relaxation times of DiI do not match those of DPPC, their trend with membrane tension suggests they are a sensitive indicator of lipid packing.

2.3.6. **DiI sensitivity to membrane tension may be revealed in fluorescence lifetime measurements**

Although the present classical molecular dynamics simulations cannot simulate fluorescence, which is a quantum mechanical process, they do enable one to assess the local physical factors that govern fluorescence. In general, fluorescence lifetime of carbocyanine chromophores is sensitive to water accessibility and to the local microviscosity. Cyanine dyes exhibit weak fluorescence in water and a dramatic increase in quantum yield upon incorporation into lipid membranes.\textsuperscript{21} Viscosity-dependent fluorescence lifetime of cyanine dyes has been shown to be related to changes in the trans-cis photoisomerization dynamics of the central methine bridge.\textsuperscript{65,66} Moreover, Packard and Wolf have shown that fluorescence lifetime of DiI increases with an increase in order of the lipid acyl chains.\textsuperscript{19}
We assessed chromophore hydration by counting the average number of water molecules in the first shell of DiI’s headgroup. This hydration number can be accurately computed from the radial pair-distribution function of DiI-nitrogens and water-oxygens. Hydration of the chromophore under different membrane surface areas varied between 4.7 and 5.6 water molecules with no particular trend with tension (Table 2-2). This observation is in concert with the other trends observed here; namely, since the location of the dye with respect to lipid-water interface does not change with increased area-per-lipid (Table 2-2) and water does not penetrate appreciably beyond lipid headgroups (Figure 2-1), no marked change in the DiI hydration is expected. On the other hand, measurable decreases in the rotational relaxation times of DiI were observed with increased tension, which is indicative of decreased viscosity near the DiI’s headgroup region. These results indicate that changes in fluorescence lifetime of DiI due to membrane order\textsuperscript{19} are most likely due to changes in the viscosity near the headgroup, rather than due to changes in hydration. In summary, these results suggest that fluorescence lifetime of DiI may be a sensitive indicator of tension-induced decreases in lipid packing in membranes.

2.4. Conclusions

Lateral tension-induced changes in membrane organization and dynamics play an important role in transforming mechanical signals into biochemical signals at the cell surface. Despite the known significance of membrane tension, very little is known at a molecular level about the effects of tension on membrane organization. The goal of this
study was to provide insights into the effects of membrane lateral tension on lipid structure and dynamics through atomistic molecular dynamics simulations of a fluid-phase lipid bilayer under a broad range of tensions (from zero to values just below rupture tension). Quantitative agreement of the simulation findings with available experimental values indicate that the simulation methodology used was robust and accurate in determining equilibrium properties of the lipid bilayer.

Key findings from the simulations are as follows. First, physiologically relevant tensions in the range of 0-15 mN/m caused decreases in bilayer thickness in a linear fashion consistent with volume-incompressibility. Second, tension induced a significant increase in acyl chain interdigitation and a decrease in lipid order. Third, tension induces a significant decrease in electrostatic potential barrier (up to 80mV at the highest tension), due to decreased ordering of both water and lipid dipoles. Fourth, the observed lateral diffusion coefficient of DPPC cannot be described satisfactorily using the free-area theory, across all tensions applied, due to a significant change in molecular shape and friction at high tensions. Finally, Dil has systematically lower lateral and rotational diffusion coefficients compared to DPPC, but the increase in each with tension is quantitatively similar for Dil and DPPC. Similarly, fluorescence lifetime of Dil, which depends on lipid order near the headgroups, appears to be a good indicator of tension in membranes.

These results have potential physiological implications. For instance, hydrophobic mismatch between lipids and proteins causes opening and closing of transmembrane stretch-activated ion channels. Alternatively, forces may cause changes in the electrostatic potential of the bilayer, which in turn affects membrane channel
conductance, ion and water transport through the lipid bilayer, protein conformation, and kinetics of membrane-bound enzymes. For example, decrease in dipole potential leads to a decrease in dissociation of gramicidin channel dimers leading to increased sodium ion permeability. Altered lipid mobility, due to force-induced changes in lipid packing, can also lead to changes in protein molecular mobility and change the kinetics of enzymatic reactions that require protein complex formation (e.g. dimerization).

Force-induced changes in lipid mobility are also associated with regulation of mitogen activated protein (MAP) kinase activity. To explain the relationship between lipid mobility and membrane protein-mediated signaling, Nicolau et al. proposed that a local decrease in lipid viscosity, reflected in lipid mobility, temporarily corrals membrane proteins and increases their residence time and interaction kinetics leading to initiation of MAPK signaling pathways once a threshold residence time is reached. Studies on model membranes have demonstrated that membrane tension promotes formation of large domains from microdomains in order to minimize line tension developed at microdomain boundaries, and there exists a critical pressure at which lipid phase separation into liquid-ordered and liquid-disordered domains is observed. Taken together, these studies point to changes in bilayer structure and dynamics as a mechanism of force-induced biochemical signaling.

Future research will be needed to develop a new theory for tension-diffusion relationship that takes into account frictional and molecular shape changes. The current simulations not only provide additional quantitative insights into some of the well-studied bilayer properties (e.g. bilayer thickness, diffusion coefficient), but also lead to novel
hypotheses related to membrane-mediated mechanotransduction in cells (e.g. interdigitation and electrostatic potential) that can be tested experimentally.

In addition, we tested which DiI fluorescence spectroscopic properties have potential as reporters of membrane tension effects on lipids. We observed that although DiI exhibited slower lateral and rotational diffusion compared to DPPC, its lateral and rotational diffusion increased with tension in a manner quantitatively similar to DPPC. This suggests that changes in DiI dynamics are good indicators of membrane tension. We also showed that hydration of the dye does not vary with packing, whereas the local viscosity experienced by the dye changes significantly. These results support the utility of DiI as a reporter of lipid packing and validate the use of DiI to label membrane cellular microdomains based on underlying heterogeneity in lipid order. Thus these findings offer new insights into the interpretation of fluorescence dynamics of DiI and lipids in lipid bilayer systems.

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Chapter 3

Single-molecule spectroscopic characterization of membrane microviscosity

Foreword

Part of this manuscript is published in the article: “Endothelial cell membrane sensitivity to shear stress is lipid domain dependent”, Tristan Tabouillot, Hari S. Muddana, and Peter J. Butler, Cellular and Molecular Bioengineering, 2010. DOI: 10.1007/s12195-010-0136-9.

3.1. Introduction

Cell plasma membrane microviscosity (i.e. microfluidity) plays an important role in many physiological (e.g. angiogenesis, neurotransmission) and pathophysiological processes (e.g. atherosclerosis and hypertension). The role of membrane microviscosity in these processes is often associated with activation of transmembrane proteins such as ion channels, G-protein coupled receptors, growth factor receptors, regulation of membrane-cytoskeleton interactions, and membrane permeability. The notion of microviscosity as related to the original Singer-Nicholson membrane model has been refined in light of observations that the cell plasma membrane is a highly heterogeneous system composed of a wide variety of lipids (charged/uncharged, saturated/unsaturated, and phospholipids/sphingolipids/sterols), with a lipid to protein mass ratio of 1:1, or approximately 50 lipids per protein. Through
lipid-lipid and lipid-protein interactions, the membrane is segregated into liquid-disordered (enriched in unsaturated phospholipids) and liquid-ordered (enriched in sphingolipids, saturated phospholipids, and cholesterol) regions. These liquid-ordered regions, termed as “membrane rafts”\textsuperscript{16} that are 10 to 100 nm in size, are thought to act as a platform for initiating downstream signaling pathways. The plasma membrane is not only complex, but is highly dynamic with submicroscopic lipid reorganization occurring on the millisecond time scale.\textsuperscript{17} Understanding the dynamics of these liquid-ordered and liquid-disordered regions is vital for elucidating the role of membrane compartmentalization in cell signaling.

The term “lipid raft” originated from the fact that certain membrane constituents were resistant to non-ionic detergents (detergent resistant membranes (DRM)) and floated to the top of a detergent column. However, it has become clear that these biochemical techniques artificially induce domain formation.\textsuperscript{18} Thus, there is a significant interest in developing new optical techniques and ultrasensitive imaging probes for investigating membrane domain microviscosity under physiological conditions that avoid perturbation and which can capture its dynamic nature.\textsuperscript{19} Typically, membrane microviscosity is estimated from measurements of translational or rotational diffusion of fluorescent probes obtained using fluorescence recovery after photobleaching (FRAP), fluorescence anisotropy, and fluorescence polarization microscopy, or fluorescence correlation spectroscopy (FCS).\textsuperscript{6,20,21} Due to complications in using theoretical diffusion models for heterogeneous systems (e.g. Saffman-Delbruck model and free-area model for lateral and rotational diffusion) determining microviscosity from lipid mobility is often qualitative. An alternate strategy is to use fluorescence probes whose intrinsic fluorescence properties
such as quantum yield, fluorescence lifetime, excitation and emission spectra, are sensitive to the local microenvironmental factors such as hydration and viscosity.

Dialkyl indocarbocyanine (DiI-Cn) belongs to the general class of cyanine dyes and is used quite extensively for studying membrane organization and dynamics due to its structural similarity to phospholipids. Moreover, DiI exhibits selective partitioning into different lipid membrane phases (ordered vs. disordered) depending on matching of the alkyl chain lengths with the lipid acyl chains. Generally, shorter chain length (n < 14) DiI partitions into liquid-disordered phase and longer chain length (n > 16) partitions into liquid ordered phase. In addition to its high extinction coefficient and fluorescence quantum yield, the excitation and emission spectra can be tuned by altering the length of the central methine bridge or the hetero atoms of the indole rings. As the trans-cis photoisomerization of the central methine bridge acts as the predominant non-radiative decay pathway, cyanine dyes exhibit strong sensitivity to viscosity of the microenvironment. Restriction of intramolecular rotation results in an increased fluorescence lifetime, a phenomenon that was used previously to detect DNA and RNA binding events.

In this study, we demonstrate the applicability of fluorescence lifetime of DiI for characterizing microviscosity of model and cell lipid membranes, both qualitatively and quantitatively. Due to the selective partitioning ability of different chain-length DiIs, we studied the fluorescence lifetime properties of DiI-C12 (short chain) and DiI-C18 (long chain). Control experiments in solution were conducted to determine the predominant solvent factors that can influence the lifetime of DiI in the lipid membrane system.
Moreover, solution controls served as a calibration for quantifying relationship between fluorescence lifetime and microviscosity. Lifetime of DiI was measured in homogeneous lipid vesicles in different phases, namely, liquid-disordered (Ld), liquid-ordered (Lo), and gel (S0). Partitioning ability of these two dyes was tested in multi-component vesicles with phase-coexisting regions. Finally, our results demonstrate the suitability of this technique to selectively label membrane phase domains in endothelial cells and possibly track the dynamic microviscosity changes occurring in the membrane, under the influence of a chemical or mechanical stimulus.

3.2. Materials and Methods

3.2.1. Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), egg sphingomyelin (ESM), and cholesterol (CHOL) were purchased from Avanti polar lipids, Inc. (Alabaster, AL) and used without further purification. Fluorescent probes 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI-C18, λ<sub>ex</sub> = 549 nm; λ<sub>em</sub> = 565 nm), 1,1’-didodecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI-C12) and 3,3’-dilinoleyloxycarbocyanine perchlorate (DiO-C18, λ<sub>ex</sub> = 484 nm; λ<sub>em</sub> = 501 nm) were purchased from Invitrogen, Inc. (Carlsbad, CA).
3.2.2. Preparation of giant unilamellar vesicles

Lipid/dye mixture solutions were prepared to a concentration of 0.25 mg/ml with dye concentration of ~10 nM for FCS and 100 nM for epifluorescence imaging. Giant unilamellar vesicles (GUVs) were prepared using electroformation. A custom-built electroformation chamber consisting of two transparent #1 ITO coated coverslips (SPI Supplies, Inc., West Chester, PA) with conductive sides facing each other were used to apply AC electric fields to the lipid film. The coverslips were separated by a silicone spacer of 1.6 mm thickness. 3 to 5 μl of the lipid/dye solution was deposited on the coverslip (conductive side) and dried initially under argon for 10 minutes and then vacuum dried for 2 hours. The chamber was filled

![Figure 3-1: Epi-fluorescence image showing the typical yield of DiI-C12 labeled giant unilamellar vesicles (GUVs) prepared from DOPC using our custom-built electroformation chamber. Scale bar is 10 microns.](image)
with 100 mM sucrose solution preheated to 10°C above the highest lipid phase transition temperature and moved immediately to a baking oven which was constantly maintained at a temperature of 10°C above the highest lipid phase transition temperature. AC electric fields were then applied across the ITO electrodes using a LabVIEW controlled A/D board (National Instruments, Austin, TX). Applied voltage at 10 Hz frequency was increased from 0.1 V to 1.6 V at a rate of 30 mV/min, followed by a constant voltage of 1.6 V applied for 3 hours. Frequency was then reduced to 5 Hz and maintained for 1-2 hours to detach the vesicles. A single run of electroformation typically resulted in hundreds of GUVs with sizes ranging from 5 to 100 μm (Figure 3-1).

3.2.3. Fluorescence spectroscopy instrumentation

Single molecule diffusion and fluorescence lifetime measurements were conducted using a time-correlated single photon counting (TCSPC) instrument developed in our lab, described in detail elsewhere. Briefly, the TCSPC instrument was based on an Olympus IX71 inverted microscope (Tokyo, Japan). Excitation source was a water-cooled 80 MHz, 5.4 ps pulse width, 75 mW power, pulsed solid state laser (λ = 532 nm, High-Q Laser, Hohenems, Austria). The laser output coupled to an optical fiber was cleaned by an excitation filter and expanded to slightly underfill the back aperture of the objective in order to ensure a Gaussian illumination profile. The laser power as measured at the back aperture of the objective was adjusted between 50 and 100 μW to avoid significant photobleaching and triplet state formation. Laser light was reflected by the dichroic mirror and focused in the sample using a 60X water immersion objective.
Fluorescence emission from the sample passed through the dichroic mirror, a set of high quality emission filters, and a polarizer fixed at the magic angle of 54.7°, before focusing onto a 50 μm/0.22NA optical fiber that served as the confocal pinhole. The optical fiber was coupled to a GaAsP photomultiplier tube (H7422-40P, Hamamatsu, Tokyo, Japan) with a peak quantum efficiency of 40% at 580 nm wavelength. Photon arrival times were recorded using the SPC-630 module from Becker and Hickl (Berlin, Germany).35

3.2.4. Fluorescence correlation spectroscopy

Diffusion coefficients were determined by autocorrelation of the fluorescence fluctuations arising from diffusion of chromophores into and out of the observation volume.36,37 Autocorrelation of the intensity signal is defined by \( G(\tau) = \frac{\langle \delta F(t+\tau) \delta F(t) \rangle}{\langle \delta F(t)^2 \rangle} \), where \( F(t) \) is the fluorescence intensity at time \( t \), \( \delta F(t) = F(t) - \langle \delta F(t) \rangle \), \( \langle \rangle \) represents the ensemble average, and \( \tau \) is the autocorrelation time. Diffusion coefficients in lipid bilayers were determined by fitting the autocorrelation curves to single-component two dimensional diffusion model, given by the following equation,38

\[
G(\tau) = \frac{1}{N} \left[ \frac{1}{1 + (\tau/\tau_D)} \right]
\]

where \( N \) is the average number of diffusing fluorophores in the confocal observation volume, \( \tau \) is the autocorrelation time, and \( \tau_D \) is the characteristic diffusion time of the fluorophore with diffusion coefficient \( D \) crossing a circular area of radius \( r \).
Autocorrelation curves were fit using Levenberg-Marquardt nonlinear least-squares regression algorithm with the aid of Origin software (Originlab, Northampton, MA). The quality of the fitted curves was evaluated based on chi-square ($\chi^2$) values. Radius of the observation volume determined using the known diffusion coefficient of Rhodamine 6G ($D = 2.8 \times 10^{-10}$ m$^2$/s in water) was ~400 nm. Structure factor $w$ defined as the ratio of height to width of the illumination profile was between 4 and 6 for all the experiments. Positioning of the membrane (model and cell membranes) along the optical axis was achieved by maximizing the fluorescence intensity while performing an intensity scan.

3.2.5. Fluorescence lifetime

Fluorescence decay curves were extracted from histograms of photon arrival times relative to the laser pulse start time. Decay curves were fit to a bi-exponential decay model to obtain the decay time constants. Curve fitting was done using Fluofit software (PicoQuant Gmbh, Berlin, Germany) by a process of iterative reconvolution given by,

$$I(t) = \int_{-\infty}^{t} IRF(t') \sum_{i=1}^{n} A_i e^{-\frac{(t-t')}{\tau_i}} dt'$$

where $I(t)$ is the experimental decay function, $IRF(t)$ is the instrument response function, $A_i$ is the amplitude of $i^{th}$ lifetime, $\tau_i$ is the $i^{th}$ lifetime, and $n$ is the number of exponents. The instrument response function (IRF) was collected from a sample of dilute scattering solution, prior to the experiment. The full width at half maximum (FWHM) of the IRF was calculated to be 330 ps. The quality of the fitted curves was evaluated based on $\chi^2$ values. Fitting the decay curves to a decay model with more than two exponentials did
not improve the goodness-of-fit. The intensity-weighted average lifetimes $\langle \tau \rangle$ were calculated as,

$$\langle \tau \rangle = \frac{\sum_{i=1}^{n} A_i \tau_i^2}{\sum_{i=1}^{n} A_i \tau_i}$$

Due to the use of linearly polarized excitation source, fluorescence emission was passed through a polarizer oriented at magic angle ($54.7^\circ$) to eliminate effects of rotational anisotropy of the chromophore on fluorescence lifetime. Polarizer angle was calibrated by comparing fluorescence lifetime of Rhodamine 6G to the literature reported value of 4.1 ns.40

### 3.3. Results and discussion

#### 3.3.1. Solvent effects on fluorescence lifetime of DiI

In general, fluorescence lifetime is sensitive to various solvent factors including polarity, viscosity, and hydrogen bonding, through changes in radiative and non-radiative decay pathways.39 Thus, it is often difficult to interpret fluorescence lifetime data without the knowledge of the dominant solvent factors affecting the dye. Generally, relaxation of cyanine dyes from excited singlet state to ground state occurs through fluorescence, triplet state formation, internal conversion, and *trans-cis* photoisomerization of the central methine bridge.41 Chemical structure of DiI is shown in figure 3-2 (a), where the headgroup structure is identical to Cy3. While quantum yield of triplet state formation is
Figure 3-2: (A) Chemical structure of DiI-C₉. (B) Fluorescence lifetime of DiI-C₁₂ (black) and DiI-C₁₈ (red) as a function of % water content in binary mixture solutions of water and ethanol. (C) Intensity weighted average fluorescence lifetime of DiI-C₁₂ and DiI-C₁₈ as a function of viscosity measured in binary mixture solutions of ethanol and glycerol. Note that both X- and Y-axes are in log scale.
negligible, 0.3 \%,^{42} and internal conversion is a solvent-independent process, the quantum yield of \textit{trans-cis} photoisomerization is nearly 90\%,^{43} for structurally similar carbocyanine dyes. \textit{Trans-cis} photoisomerization dynamics is influenced by both viscosity and polarity of the solvent.\textsuperscript{41} Below, we analyze the sensitivity of DiI lifetime to solvent polarity and viscosity in binary solution mixtures. Further, we analyze the effect of alkyl chain-length on lifetime, by comparing DiI-C\textsubscript{12} (short chain) and DiI-C\textsubscript{18} (long chain).

Change in fluorescence lifetime as a function of solvent polarity was studied in binary mixtures (v/v) of water and ethanol up to 40\% water content. The range of water content was chosen based on our previous observation through MD simulations that the dye headgroup is located below the lipid-water interface and hydration of the chromophore in fluid-phase DPPC lipid bilayer is at approximately 40\% of the full hydration (refer to chapter 2). The average fluorescence lifetime of both the dyes in 100\% ethanol was \textasciitilde0.25 \text{ns}, consistent with the previously reported values.\textsuperscript{44,45} No significant change in the lifetime of either dyes was observed with increase in water content (figure 3-2 (b)), except for a slight increase in lifetime of DiI-C\textsubscript{18} at 40\% water, which was most likely due to aggregate formation (as evidenced by sudden bursts in fluorescence intensity). This result is in line with the previous observation by Efimova et al.\textsuperscript{46} that the fluorescence lifetime of DiI incorporated in surfactant micelles is unaffected by the \% water content of the medium. Also, Packard et al.\textsuperscript{47} have reported that neither the polarity of the lipid headgroup nor the presence of charged lipids (e.g. phosphatidic acid and stearylamine) had a significant effect on fluorescence lifetime of DiI-C\textsubscript{12} or DiI-C\textsubscript{18}. 
Previously, Nakashima et al.\textsuperscript{48} reported that the fluorescence quantum yield of DiI increases dramatically (up to 100 fold) when incorporated in lipid membranes, and attributed this to the reduction in \textit{trans-cis} photoisomerization due to membrane viscosity. Moreover, it has been shown that the lifetime of DiI increases with increase in order of the membrane by adding cholesterol.\textsuperscript{47} In the current study, change in lifetime as a function of solvent viscosity was studied in binary mixtures of ethanol and glycerol. In an effort to develop a calibration curve, we measured the lifetime of DiI-C\textsubscript{12} and DiI-C\textsubscript{18} in solutions with viscosities ranging from 1 cP to 185 cP (viscosities measured using a cone-plate viscometer), covering the entire range of lifetimes observed in membrane systems. DiI exhibited a bi-exponential decay behavior in all solutions.\textsuperscript{47} The physical basis for these two components is not well understood. Thus, we use the intensity-weighted average lifetime ($\tau_{av}$). Figure 3-2 (c) shows the average fluorescence lifetime ($\tau_{av}$) of DiI-C\textsubscript{12} and DiI-C\textsubscript{18} at different viscosities. While the fast component showed an initial increase and saturated at around 10 cP, the slow component showed a steady increase up to 185 cP. Average lifetime of both the dyes increased from 0.22 ns at 1 cP to 1.33 ns at 185 cP. Ideally, the fluorescence lifetime can increase up to 3 ns when the central methine bridge is completely rigidified through chemical modifications.\textsuperscript{30} An important observation is that there was no significant difference between the average lifetime of DiI-C\textsubscript{12} and DiI-C\textsubscript{18}, indicating that acyl chain length has a negligible effect on \textit{trans-cis} photoisomerization dynamics of the headgroup. In the next section, we discuss the use of the plot in figure 3-2 (c) to estimate the effective viscosity of different membrane phases in model and cell plasma membranes.
3.3.2. Microviscosity measurements in model membranes

In order to estimate the microviscosity of different membrane phases, liquid-disordered (Ld), liquid-ordered (Lo), and gel-phase (So), we measured the fluorescence lifetime of Dil-C_{12} and Dil-C_{18} in giant unilamellar vesicles (GUVs) prepared from DOPC (Ld), 1:1 DOPC:CHOL (Lo), 1:1 DPPC:CHOL (Lo), and DPPC (So) at room temperature.

Figure 3-3: (A) Representative fluorescence lifetime traces of Dil-C_{12} in giant unilamellar vesicles prepared from DOPC (Ld), DOPC:Chol (1:1, Lo), DPPC:Chol (1:1, Lo), and DPPC (So). (B) Average fluorescence lifetimes of Dil-C_{12} and Dil-C_{18} in the above-mentioned lipid compositions. (C&D) Snapshot of Dil-C_{18} in a fluid- and gel-phase DPPC bilayer from atomistic molecular dynamics simulations. Color legend: Water – Pink, DPPC – Grey, DPPC phosphorous atoms – Red, and Dil-C_{18} – Purple. Note that the Dil location is below and above the lipid headgroups in fluid- and gel-phase respectively.
temperature. See references 49,50 for phase information. We observed homogeneous single-phase vesicles in all the lipid compositions, as evidenced by fluorescence imaging, consistent with the previously reported phase diagrams.50,51 By virtue of single-molecule sensitivity of TCSPC, we used very low concentrations of the dye in all our experiments, which has been shown to cause no perturbation to the bilayer structure or dynamics.52

Representative lifetime traces of DiI-C12 in the above-mentioned lipid compositions are shown in figure 3-3 (a). Similar to the solution data, no significant difference was observed in lifetime of DiI-C12 and DiI-C18, indicating that chain length does not affect the headgroup dynamics even in lipid membranes. The average fluorescence lifetime (τ_{av}) of both the dyes increased from 0.82 ns in DOPC (L_d) to 1.27 ns in 1:1 DPPC:CHOL (L_o) (shown in figure 3-3 (b)), indicating that the dye headgroup experiences higher viscosities with increase in membrane order. This result is in agreement with Packard et al.47 and also with our previous observation that the DiI headgroup is located below the lipid-water interface and is thus affected by changes in lipid acyl chain order.52 Also, it is well-established that cholesterol is located below the lipid-water interface and aligned with the lipid acyl chains.53,54,55,56 This correlation in location of DiI and cholesterol might explain the observed increase in lifetime. However, interestingly, an exception to an increase in lifetime was observed in the gel phase, where the lifetime of both the dyes decreased significantly. Since, we did not observe a decrease in lifetime of DiI in solution at high viscosities, we hypothesized that location of DiI headgroup in gel-phase might be different from that in fluid-phase, i.e., more specifically, the DiI headgroup might be located above the lipid-water interface exposing itself to water in the gel-phase bilayer. Since, it is very challenging to test this hypothesis
experimentally, we conducted molecular dynamics simulation of DiI-C\textsubscript{18} incorporated in fluid- and gel-phase DPPC bilayers. Fluid-phase data reported here is from chapter 2. Molecular dynamics simulation methodology used here is identical to our previous work\textsuperscript{52} except that the DPPC bilayer was simulated at an area-per-lipid of 0.48 nm\textsuperscript{2} and temperature 280 K, to represent gel-phase.\textsuperscript{57} In all simulations of fluid phase DPPC, the DiI headgroup moved to below the lipid-water interface irrespective of its initial position (figure 3-3 (c)). However, in gel-phase, DiI-headgroup was located partially or completely above the lipid-water interface (figure 3-3 (d)), consistent with our hypothesis. Positioning of DiI above the lipid-water interface in gel-phase is most likely due to the tight packing of lipids and thus lack of sufficient free volume to accommodate the rigid/bulky DiI headgroup. This result suggests that at least a sub-population of DiI molecules might be positioned such that the headgroup is exposed to water resulting in a decrease in average fluorescence lifetime.

To estimate the membrane microviscosity, we fit the solution data (averaged for both dyes) shown in figure 3-2 (c) to the equation of the form, $\tau_{av} = \alpha \eta^\beta$, where $\alpha$ and $\beta$ are the fitting parameters, and use it as our calibration curve. The functional form of this equation is similar to that observed between quantum yield and viscosity for molecular rotors undergoing rotational relaxation.\textsuperscript{22} The values of $\alpha$ and $\beta$ obtained from least-squares non-linear fit were 0.228 and 0.333 respectively. Estimated microviscosities of DOPC, 1:1 DOPC:CHOL, and 1:1 DPPC:CHOL GUVs obtained from the calibration curve were 46.1, 68.3, and 174.1 cP, respectively. Note that the viscosity calculations were averaged over both DiI-C\textsubscript{12} and DiI-C\textsubscript{18}. It is important to note that the viscosity estimated here refers to the viscosity experienced by the DiI while moving within a
diffusion cage. Typically, travel time from one cage to another is much less ( < 100 ps) compared to the time it spends within the cage (10 to 20 ns).\textsuperscript{58} Thus, changes in friction during hopping translation contribute negligibly to the measured fluorescence lifetime and estimated microviscosity. Moreover, the estimated viscosities refer to the viscosity experienced by the headgroup of the dye.

We further estimated the effective viscosity of the same GUVs from the lateral diffusion coefficient measured at a single-molecule level using FCS. Unlike fluorescence lifetime, viscosities estimated from lateral diffusion coefficient represent the average viscosity experienced by the whole molecule. Typical autocorrelation traces of Dil-C\textsubscript{12} and Dil-C\textsubscript{18} are shown in figure 3-4 (a). Lateral diffusion coefficient in gel-phase DPPC bilayers could not be measured since the dye molecules were immobile and underwent rapid photobleaching.\textsuperscript{59} A single-component diffusion function fit the FCS traces very well, indicating homogeneity of the membrane. Average diffusion coefficients are
reported in figure 3-4 (b). Diffusion coefficients were in the following order: DOPC > DOPC:CHOL > DPPC:CHOL > DPPC (immobile). Diffusion coefficients reported here are in quantitative agreement (± 1 x 10⁻⁸ cm²/s) with previous studies using FCS.²⁰,⁶⁰ No significant difference between diffusion coefficient of DiI-C₁₂ and DiI-C₁₈ was observed (Figure 3-4 (b)), indicating that the chain length does not affect diffusion coefficient significantly. This result also suggests that the friction on the DiI alkyl chains at the bilayer midplane due to interleaflet interactions does not depend on the chain length of the dye.

Qualitatively, it is seen in this study that a decrease in diffusion coefficient (i.e., increase in viscosity) is correlated well with increase in fluorescence lifetime. Here, we estimate the effective viscosity of the membrane bilayer from the lateral diffusion coefficient using the free-area theory of lipid diffusion. Unlike a pure Brownian particle, lipids exhibit two different modes of in-plane motion, a “rattling-in-cage” motion at short time-scales (< 1 ns), and translation via a “hopping” diffusion at longer time-scales (> 10 ns).⁵⁸ Thus, the Stokes-Einstein relationship for Brownian diffusion is inappropriate for lipid diffusion. Lipid diffusion is best described by the free-area model developed by Galla et al.⁶¹ According to free-area theory, lipid hopping from one cage to another depends on the availability of a void space (larger than a critical size) next to the molecule. Opening up of a void space occurs occasionally due to random density fluctuations. Free-area theory of lipid diffusion has been shown to fit well with experimental diffusion data obtained as a function of temperature and cholesterol concentration in lipid bilayers.⁶²,⁶³ We estimate the effective viscosity from the lateral diffusion coefficient using the free-area model equation given by,
\[ D_{Dil} = \beta \left( \frac{K_B T}{f} \exp \left( \frac{-\gamma a_c}{a_f} \right) \right) \]

where \( K_B \) is the Boltzmann constant, \( T \) is absolute temperature, \( f \) is frictional coefficient, \( \gamma \) is free area overlap factor (0.5 to 1), \( a_c \) is critical area required for lipid diffusion, and \( a_f \) is the free area defined as the difference between average area-per-lipid and van der Walls area of the lipid. Note that the critical area is not the same as the van der Walls \( (a_0) \) area. Typical values of \( a_0 \) and \( a_c \) for phosphocholines are 0.42 nm\(^2\) and 0.48 nm\(^2\) respectively.\(^64\) The value of \( \gamma a_c \) was assumed to be 0.26 nm\(^2\).\(^64,\)\(^65\) Due to a slight difference in diffusion coefficient of DiI and DPPC observed in previous MD simulations, we apply a correction factor \( (\beta) \) equal to 0.9.\(^52\) The friction coefficient takes the same form as that of a pure 2D Brownian particle, \( i.e. 4\Pi \eta R_h \), where \( \eta \) is the viscosity and \( R_h \) is the van der Walls radius of the lipid (~0.4 nm, assuming cylindrical geometry).\(^63\) It is important to note here that the friction coefficient refers to the friction experienced by the lipid while traveling within the diffusion cage, and is thus modeled as a the friction coefficient on a Brownian spherical particle. This friction is contributed by both the lipid-water interactions and inter-leaflet lipid interactions. Assuming an area-per-lipid of 0.72 nm\(^2\) for DOPC, obtained from ref.\(^66\), we calculate the effective viscosity for pure DOPC membrane from the above equation to be 39.6 cP. Mathai et al.\(^67\) have previously reported the area-per-lipid values in DOPC vesicles as a function of cholesterol up to 40%. Approximating the area-per-lipid of 0.64 nm\(^2\) at 50% cholesterol, we estimated the effective viscosity of 1:1 DOPC: Chol membrane to be 46.1 cP. Effective viscosity of 1:1 DPPC:Chol membrane could not be estimated because free-area information at room temperature has not yet been reported in the literature.
As earlier noted, effective viscosity estimated through fluorescence lifetime refers to the viscosity near the dye headgroup and viscosity from lateral diffusion coefficient is the average effective viscosity over the entire molecule. Moreover, the free-area is lower near the headgroup and increases significantly at the terminal tail region. In light of this, it is reasonable that the effective viscosities estimated from diffusion coefficient were less compared to that from fluorescence lifetime. Differences between these measurements highlight the inhomogeneity in membrane viscosity along the membrane normal. More specifically, viscosity at the acyl chain terminal regions can be significantly lower from that at the headgroup. Through a simple approximation that the viscosity experienced by the whole molecule is an average of the viscosity at the headgroup and the bilayer mid-plane, i.e. \( \eta = (\eta' + \eta'') / 2 \), where \( \eta, \eta' \), and \( \eta'' \), are average viscosity, headgroup viscosity, and mid-plane viscosity, respectively, we can estimate the microviscosity at the mid-plane. Mid-plane microviscosity of pure DOPC membrane was 33.1 cP and that of the 1:1 DOPC:Chol membrane was 23.9 cP. This indicates that incorporation of cholesterol reduces the inter-leaflet friction, which is in line with previous observations that cholesterol increases the bilayer thickness and reduces the lipid density at the mid-plane. In summary, we showed here that fluorescence lifetime of DiI is very sensitive to the microviscosity at the headgroup region and can be used to detect lipid/cholesterol mediated microviscosity changes. In combination with lateral diffusion measurements, we also showed that one could quantitatively measure the mid-plane microviscosity which is an indicator of interdigitation. In the next section, we demonstrate the applicability of fluorescence lifetime of DiI for probing phase domains in vitro.
3.3.3. Probing membrane phase domains \textit{in vitro}

Both short-chain (DiI-C\textsubscript{12}) and long-chain (DiI-C\textsubscript{18}) Dil’s exhibited identical fluorescence lifetimes in homogeneous environments, \textit{i.e.}, solution and single-phase giant unilamellar vesicles. However, the potential advantage of different chain-length Dils for \textit{in vitro} cell studies is the differential partitioning of DiI-C\textsubscript{12} to liquid-disordered regions (L\textsubscript{d}) and DiI-C\textsubscript{18} to liquid-ordered (L\textsubscript{o}) regions. Klausner et al.\textsuperscript{28} have originally demonstrated that Dil with chain-length < 14 preferentially partitions to L\textsubscript{d} regions,

![Image](https://example.com/image.png)

\textbf{Figure 3-5}: (A&B) Fluorescence image of domain-forming vesicles stained with DiO-C\textsubscript{18} and DiI-C\textsubscript{12} respectively, at high concentrations. Colocalization of DiO-C\textsubscript{18} (green) and DiI-C\textsubscript{12} (red) is shown in figure C. (D&E) staining with DiO-C\textsubscript{18} and DiI-C\textsubscript{12} at low concentrations is shown in figure D & E respectively. Colocalization of these dyes is shown in figure F.
whereas DiI with chain-length > 16 preferentially partitions to L₀ regions. Despite the widely used assumption that DiI-C₁₈ partitions to the lipid rafts in cells, recent studies showed that DiI-C₁₈ partitioning in model membranes is lipid-mixture dependent, with very little partitioning in L₀ regions in ternary mixtures of sphingomyelin, phosphocholines, and cholesterol. Also, Loura et al. have reported that DiI-C₁₈ is localized at the domain interphase region in large unilamellar vesicles with coexisting gel and liquid phases. Thus, partitioning of these dyes in cells cannot be solely determined from model membrane studies, and emphasizes the importance of knowing the DiI partitioning in vitro cell studies. Also, the effect of dye concentration on lipid packing and partitioning is often not assessed in these imaging studies.

To test the effect of concentration on DiI partitioning, we imaged GUVs prepared from DOPC:DSPE:Chol (2:1:1) simultaneously labeled with DiI-C₁₂ and DiO-C₁₈ (variant of DiI-C₁₈ that excites at 484 nm and emits at 501 nm) at room temperature. Colocalization images of DiI-C₁₂ and DiO-C₁₈ at high (0.05 mol% dye to lipid ratio) and low concentrations (0.005 mol% dye to lipid ratio) are shown in figure 3-5. At both high and low concentrations, DiI-C₁₂ partitioned to the L₉ region, almost exclusively. However, DiO-C₁₈ partitioned more to the L₀ region at low concentration, and partitioned nearly equally in L₉ and L₀ regions at high concentrations. This result indicates that low concentrations of these dyes must be used when labeling lipid rafts in cells, and demonstrates the advantage of using single-molecule fluorescence techniques over traditional fluorescence imaging. Though GUVs are excellent model membrane systems, the discrepancies in DiI partitioning depending on lipid-mixture and dye concentration necessitates testing these dyes in in vitro cell studies. Partitioning of these dyes in bovine
aortic endothelial cells (BAECs) was tested by measuring the fluorescence lifetime of DiI-C_{12} and DiI-C_{18}. We hypothesized that DiI-C_{18} would exhibit higher fluorescence lifetime compared to DiI-C_{12} due to partitioning in the L_0 regions. Cells were stained with DiI and immediately positioned on the microscope for fluorescence lifetime measurements, to avoid internalization of the dye in cell membrane organelles. Typical fluorescence images of DiI labeled cells show predominant localization of the dye in cell plasma membrane. Measurements were obtained from a single point in the cell plasma membrane. The size of the laser beam (radius ~400nm) is much larger than the proposed size of lipid rafts which are 10 – 100 nm. Intensity-weighted average fluorescence lifetimes obtained from fitting were 1.26 ns and 1.43 ns for DiI-C_{12} and DiI-C_{18} respectively. Lifetime of DiI-C_{18} was significantly different from that of DiI-C_{12} indicating that these dyes probe different membrane phase regions. Moreover lifetime of DiI-C_{18} was higher compared to that of DiI-C_{12}. This result suggests that DiI-C_{18} spends more time in liquid-ordered regions compared to DiI-C_{12}. It is important to note here the dye molecules do not necessarily stay in a particular domain the entire time, rather a continuous exchange of dye molecules between different phase domains is expected. We also observed that lifetime of DiI-C_{12} in cells was significantly higher compared to that in pure fluid-phase DOPC membrane, indicating the substantial presence of cholesterol/protein even in the liquid-disordered regions of the cell. With the dye’s headgroup located just below the lipid-water interface and aligned with cholesterol location, lifetime can be very sensitive to subtle changes in microviscosity of these domains that can be caused due to segregation of proteins/cholesterol.
3.4. Summary and conclusions

In summary, we showed here that fluorescence lifetime of DiI is a sensitive indicator of microviscosity. Through extensive calibration in solution, we determined the microviscosity in lipid bilayer in different phases, liquid-disordered, liquid-ordered, and gel-phases. Since, the dye headgroup is located near the lipid-water interface (chapter 2), the measured microviscosities reflect the microviscosity near the headgroups. Through lateral diffusion measurements and using free-area theory, we determined the microviscosity of the lipid bilayer averaged along the membrane normal. Using simple approximations, we were able to determine the microviscosity near the tail-region (i.e. bilayer midplane). These results confirm the applicability of using DiI for qualitative and quantitative measurements of microviscosities in model and cells membranes. Further, to enable studying differential changes in microviscosities of phase domains, we studied the effects of alkyl chain length on the dye’s photophysics, thus demonstrating that measured differences in reported lifetimes in cells is due solely to differences in environments probed by C12 and C18 dyes. Similarly, increase in chain length from 12 to 18 had no effect on the fluorescence lifetime or lateral diffusion coefficient. However, these dyes showed differences in partitioning in lipid bilayers with coexisting phases. The short chain length DiI-C12 preferred the liquid-disordered regions, whereas the long chain length DiI-C18 preferred the liquid-ordered regions. Interestingly, we also noticed that partitioning of the DiI-C18 is concentration dependent. At high concentrations, DiI-C18 showed equal partitioning between both phases, and at low concentrations it preferred the ordered region. Based on these studies, we measured the fluorescence lifetime of
these dyes in endothelial cell membranes. Based on the fluorescence lifetime, we found strong evidence that DiI-C12 prefers the liquid-disordered phase and the DiI-C18 prefers the liquid-ordered phase. These results combined with those of Tabouillot, et al. demonstrate the utility of using DiIs to characterize differential sensitivity of these domains to externally applied mechanical forces.

3.5 References


Chapter 4

Spectroscopic characterization of molecular packing in lipid bilayers

4.1. Introduction

Interfacial area-per-lipid is a key structural parameter for lipid bilayers from which several other bilayer physical properties can be predicted. Previously (in chapter 1), we have systematically investigated how lipid packing is related to acyl chain conformations, lateral/rotational diffusion, electrostatic potential, bilayer thickness, and other membrane structural parameters. Experimentally, area-per-lipid can be accurately measured using X-ray/NMR/or neutron scattering techniques. These techniques are considered the gold standard for measurements of membrane thickness and order parameters and few such studies reported in the literature have had as significant an impact on our understanding of the bilayer structure, as studies using these techniques. They also form the basis for validation of computational molecular dynamics models of lipid bilayers. However, studies reporting the area-per-lipid are limited to very ideal experimental conditions (e.g. lipid composition, temperature). Moreover, these methods cannot be readily applied to intact cells due to the fact that lipid identity and structure needs to be known a priori in order to interpret data. The cellular membrane, on the other hand, has such high compositional and structural complexity that knowing its composition and relating that to a given measurement operating on endogenous molecules is impossible. But accurate measurements of areal changes and the associated
thickness changes in cellular membranes would open up several new avenues for understanding the role of hydrophobic mismatch and fluidity in regulating membrane protein activity.\textsuperscript{6} In addition, changes in lipid packing under the influence of an externally applied stimulus are rapid and transient and these spatial and temporal properties are vital to control of cellular physiology by the membrane. For example, in a recent study, we have showed that shear-induces a transient increase in membrane fluidity in endothelial cells that occurs on the order of 10 to 20 seconds,\textsuperscript{7} and subsides even when the shear stimulus remains on. X-Ray or NMR would be unable to capture such transient and spatially varying structural changes.

While measurements of endogenous molecules in membranes provides direct information, it is possible that measurement of exogenous molecules such as lipophillic fluorescent probes could generate equivalent information on membrane structure, provided the relationship between fluorescence measurements and bilayer structure were known. Fluorescent probes can target certain organelles and even particular membrane compartments through creative syntheses. Fluorescence spectroscopy takes advantage of the knowledge of probe structure to infer the relationship between fluorescent readouts and membrane structure. For example, quantitative measurements of membrane mobility at very high spatial resolution are possible through single-molecule techniques such as fluorescence correlation spectroscopy (FCS), fluorescence anisotropy, and more recently stimulated emission depletion (STED)-FCS.\textsuperscript{8,9,10} Measurements of dye dynamics, however, requires long data acquisition times in order to obtain sufficient data for a good fit to theoretical models, and are thus not ideal for studying dynamic responses. An alternate strategy is to identify probes whose intrinsic fluorescence properties (e.g.
quantum yield, absorption/emission spectra, fluorescence lifetime, and intensity) would be sensitive to lipid packing, such as in the case of Laurdan\textsuperscript{11} and DCVJ\textsuperscript{12}. Through proper quantification, one could achieve high spatial and temporal resolution using this strategy.

Through extensive characterization in solution and lipid bilayers, we report here that fluorescence lifetime of DiI (a popular membrane probe) can be used to quantitatively determine the free-area in giant vesicles and nanoliposomes. The method is sensitive to subtle changes in lipid packing on the order less than 1 Å\textsuperscript{2}. As a proof-of-principle, we measured the area-per-lipid values in bilayers of different composition and compared it to literature values obtained from NMR/X-ray/ or neutron scattering. We further characterized free-area changes induced by temperature and lateral tension in nanoliposomes, to determine their thermal expansivity coefficients and free-area compressibility modulus. These measurements can be obtained reliably and very quickly, involving no sophisticated sample preparation. The technique presented here might accelerate our understanding of the role of membrane mechanics in cellular functioning. Moreover, such rapid and automated measurements of membrane mechanical properties might aid in optimization of liposome formulations for drug delivery.
4.2. Materials and Methods

4.2.1. Materials

N-(hexadecanoyl)-sphing-4-enine-1-phosphocholine (ESM; source: Egg), 1,2-distearyl-sn-glycerol-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, and cholesterol (CHOL; source: ovine wool) were purchased from Avanti polar lipids, Inc. Fluorescent probes 1,1′-didodecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-C12; \( \lambda_{\text{ex}} = 549 \) nm; \( \lambda_{\text{em}} = 565 \) nm) and 1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-C18, \( \lambda_{\text{ex}} = 549 \) nm; \( \lambda_{\text{em}} = 565 \) nm) was purchased from Invitrogen, Inc. All the materials were obtained at their highest purity available and used without further purification. Working lipid solutions were prepared at a concentration of 0.5 mg/ml in chloroform with dye concentration of 10 nM for fluorescence spectroscopy and 100 nM for wide-field fluorescence microscopy.

4.2.2. Fluorescence lifetime and fluorescence correlation spectroscopy

Refer to Chapter 3 for details of these methods.
4.2.3. Preparation of giant vesicles and nanoliposomes

Preparation of giant unilamellar vesicles is described in detail in chapter 3. Nanoliposomes were prepared using the extrusion method.\textsuperscript{13} 1 mg of lipid in chloroform was deposited in a round bottom flask and dried under argon for 5 minutes and vacuum dried for an hour. 1 ml of water was then introduced into the flask and maintained at a temperature of 70\degree C. The flask was frequently agitated for up to an hour. This resulted in a cloudy suspension of liposomes, which are likely to be multilamellar and polydisperse. This liposome suspension was then extruded several times ( > 15) through a polycarbonate membrane filter of 100 nm pore size using the mini-extruder from Avanti lipids, Inc. This typically resulted in a homogeneous liposome sample with sizes on the order of 100 to 150 nm diameter, as verified by dynamic light scattering and fluorescence correlation spectroscopy.

4.2.4. Absorption, emission spectra, and quantum yield measurements

Absorption and emission spectra of DiI was measured in solution at a concentration of ~3 µM. Absorption spectra were obtained using the Agilent 8453 UV-Visible spectrophotometer (Agilent Technologies, Inc.), with spectra bandwidth of 1 nm. Emission spectra were obtained using RF-5301PC spectrofluorometer (Shimadzu, Inc.), with the spectra bandwidth of 1.5 nm. Absorption spectra were corrected for the baseline value and the absorption at 532 nm was recorded. The emission spectra were collected at an excitation wavelength of 532 nm. Emission spectra were fitted to two Voigt area functions using Peakfit software (Systat Software, Inc.) and the integrated area of the
spectra was calculated from the fitted curves. Viscosities of the different solvents were measured using a cone-plate viscometer (RotoVisco 1, Rheology Solutions, Inc.). Refractive index of binary mixtures of ethanol and glycerol were determined from the measured viscosity using the empirical relationship reported by Alkindi et al. Quantum yield ($\Phi_S$) was calculated with reference to Rhodamine 6G ($\Phi_R = 0.95$ in ethanol), using the following equation:

$$\Phi_S = \Phi_R \frac{IE_S A_R (n_S)^2}{IE_R A_S (n_R)^2}$$

where the subscripts $R$ and $S$ refer to Rhodamine and the unknown sample, respectively, $IE$ is the integrated emission intensity, $A$ is absorbance at 532 nm, and $n$ is the refractive index. All the measurements were obtained in triplicate.

### 4.3. Results and Discussion

#### 4.3.1. Relationship between fluorescence lifetime and free-volume in solution

In order to determine the precise quantitative relationship between fluorescence lifetime and free-volume, we systematically characterized the radiative and non-radiative decay rates under different solvent conditions. In general, radiative decay rate is predominantly affected by the dielectric constant (or refractive index) of the medium, and these quantities are related to each other by the Strickler-Berg equation. Moreover, radiative decay rate is often independent of the excitation wavelength and the emission spectrum, since the vibration relaxation occurs very fast in solution and the emission arises from the lowest vibrational level of the excited state. On the other hand, non-
radiative decay rate depends on many solvent factors including viscosity, polarity, and hydrogen bonding. In this study, we will particularly focus on the radiative and non-radiative decay rates of DiI, an indocarbocyanine dye that belongs to the cyanine dye family. Previously (in chapter 3), we have shown that fluorescence lifetime of DiI is insensitive to the solvent polarity within the range expected in lipid bilayers, so we restrict our discussion here to the effect of solvent viscosity. Extensive characterization of Cy3 and Cy5 have demonstrated that the non-radiative decay rate of cyanines is strongly influenced by the *trans-cis* photoisomerization from the first excited state, caused by carbon-carbon bond rotation about the central methine bridge.\(^{18,19}\) This is schematically represented in figure 4-1 (a), adopted from the kinetic scheme proposed by Widengren et al.\(^{18}\) The *trans* conformation is the most preferred conformation in the ground state for cyanine dyes.\(^{20}\) The molecule in the *trans* excited state can deactivate to the *trans* ground state through emission of a photon (i.e. fluorescence). Alternatively, it can undergo isomerization to the *cis* conformation. While the exact nature of the intermediate conformation is not well understood, it is thought that the molecule goes through a partially twisted state that is very unstable.\(^{18}\) In the partially twisted state, the molecule can deactivate quickly in a non-radiative fashion to the *trans* or *cis* ground states. The effective rate of this deactivation depends on the activation barrier associated with partial twisting of the central methine bridge. The activation barrier for torsional rotation of the molecule is thus determined by the steric constraints, in other words microviscosity, and temperature. Increasing the viscosity of the solvent increases the activation barrier, thus reducing the rate of *trans-cis* isomerization. Because photoisomerization competes significantly with fluorescence, most cyanine dyes exhibit very low quantum yields when
Figure 4-1: (A) Schematic diagram of the energy surface of the ground and excited state of DiI. (B) Absorption of emission spectra of DiI in solutions of different viscosities prepared from binary mixtures of ethanol and glycerol. (C) Experimental validation of the relationship between non-radiative decay rate (i.e. trans-cis photoisomerization rate) and the viscosity. The relationship between quantum yield and fluorescence lifetime as a function of viscosity is shown in the inset.
in environments allowing substantial intramolecular rotation, whereas quantum yield losses due to photoisomerization are inhibited by rigid microenvironments. Restriction of intramolecular rotation results in increased quantum efficiency and fluorescence lifetime, a phenomenon used to detect when cyanine dyes bind to DNA or RNA. Deactivation of the excited state can also occur through intersystem crossing or internal conversion, however the decay rates associated with these pathways have been shown to be negligible.

Previously, Loutfy and Arnold (1982) have determined the effect of viscosity and temperature on torsional relaxation of several molecular rotors. According to their theory, the torsional relaxation rate is related to the free-volume of the solvent by the following equation:

\[ K_{nr} = K_{nr}^o \cdot \exp \left( x \cdot \frac{V_0}{V_f} \right) \]  

(1)

where \( K_{nr} \) is the non-radiative decay rate, \( K_{nr}^o \) is the torsional rate in the free-state which is obtained in a low viscosity solution, \( x \) is a molecule-specific constant, and \( V_o \) and \( V_f \) refer to the hard-core volume and the free volume, respectively. Equation (1) combined with the Doolittle equation for solvent viscosity as a function of free-volume, i.e. \( \eta = A \cdot \exp \left( \frac{V_0}{V_f} \right) \), where \( A \) is a constant that depends on the nature of the liquid, gives the relationship between torsional relaxation rate and solvent viscosity, as follows:

\[ \ln K_{nr} = C - x \cdot \ln(\eta) \]  

(2)

where \( C \) is an empirical constant. To determine whether DiI behaves as a molecular rotor described by equation (2), we measured the absorption and emission spectra of DiI in binary mixtures of ethanol and glycerol at different solvent viscosities (Figure 4-1 (b)).
from which we calculated the fluorescence quantum yield ($\Phi$). While no significant change in the absorption spectra was observed, emission intensity increased by several fold with increase in viscosity, within the full range of viscosities studied (1.15 cP to 255 cP). As discussed earlier, increase in viscosity increases the activation barrier for trans-cis isomerization resulting in a decrease in non-radiative decay rate. In order to determine the radiative and non-radiative decay rates, we measured the fluorescence lifetime in the above solutions. Radiative ($K_r$) and non-radiative ($K_{nr}$) decay rates are related to the fluorescence quantum yield ($\Phi$) and fluorescence lifetime ($\tau$), according to the following equations:

$$K_r = \frac{\Phi}{\tau}; \quad K_{nr} = \frac{1 - \Phi}{\tau}$$

(3)

Figure 4-1 (c) is a plot between the measured viscosity and the non-radiative decay rate. The data was fit to equation (2), and we find a good fit to the Loutfy’s theory. Moreover, from the theoretical fit we obtained the DiI-specific empirical constant $x$ to be 0.33. Since it is not practical to measure non-radiative decay rates in biological samples under microscopic conditions, using the definition of fluorescence lifetime, we rewrite the equation (1) as follows:

$$V_f = \frac{x \cdot V_o}{\ln(K_{nr}^0) - \ln \left(\frac{1}{\tau} - K_r\right)}$$

(4)

This equation directly relates free-volume with experimentally measured fluorescence lifetime. Since radiative decay rate was independent of the solvent viscosity and the slight variations observed showed no particular trend, it is reasonable to assume that the
radiative rate is constant. In the following, we simplify the above model for a two-dimensional case, and apply it to measure free-area in lipid bilayers.

4.3.2. Measuring free-area, thermal expansivity coefficient, and free-area compressibility modulus

Under homogeneous solvent conditions, the dye molecules are expected to exhibit isotropic rotation, and the torsional relaxation time is related to the free-volume of the solvent. However, in lipid bilayers, the excitation dipole vector (that lies along the vector joining the two indole rings) is oriented parallel to the membrane surface and is constrained to the plane of the bilayer.\(^{23}\) Due to this constrained motion of the dye molecule, we simplify the above three-dimensional free-volume model to a two-dimensional free-area model, by expressing the non-radiative decay as a function of free area. Assuming that the height of the lipid is ‘L’, we can write the hard-core volume and free volume as, \(V_o = L \cdot A_o\), and \(V_f = L \cdot A_f\), respectively. Thus, equations (1) and (4), can be simplified for the 2-dimensional case by replacing \(V_o\) and \(V_f\) with \(A_o\) and \(A_f\), respectively, given by the equation:

\[
A_f = \frac{x \cdot A_o}{\ln(K_{nr}^o) - \ln \left(\frac{1}{\tau} - K_r\right)} \tag{5}
\]

It is important to note that, while using molecular rotors such as DiI for measuring free-area in lipid bilayers, we have to make sure that the chromophore is located within the membrane core, i.e., below the lipid-water interface. Also, it is essential to know exactly where the molecule might be located within the bilayer, since free-area profile of the
bilayer in the membrane normal direction is highly heterogeneous. Location of the DiI molecule within the bilayer has been previously identified through molecular dynamics simulations.\textsuperscript{23} Also, sensitivity of lateral and rotational diffusion coefficients of DiI to membrane packing (Chapter 2) implies that the rate of photoisomerization of the dye might be sensitive to free area too.

Using the above 2-D model for torsional relaxation rates, we wished to test whether subtle changes in lipid packing could be sensed by fluorescence lifetime. We measured the fluorescence lifetime of DiI embedded in nanoliposomes (sizes in the range of 100 to 150 nm) prepared from different lipids, including saturated and unsaturated lipids. Also, the fluorescence lifetime in these liposomes was measured at different temperatures within the range of 25°C to 50°C. Fluorescence lifetime values were converted to free-areas using equation (5). Lipid packing is often quantified by the parameter “area-per-lipid”, which is the area occupied by the lipid including both hardcore van der Waals area and the free-area. In order to compare our measurements with the published area-per-lipid values, we estimated the area-per-lipid from free area using the equation, \( A_L = A_o + A_f \), where \( A_o \) is the hard core van der Waals area of the lipid that is approximately 0.42 nm\(^2\) for phospholipids, and \( A_f \) is the free area. The above approximation might be valid only when the lipids adopt a perfectly cylindrical geometry, since lipids exhibit high spatial heterogeneity in free-area along the membrane normal. Free-area near the lipid headgroups is slightly lower compared to that near the terminal tail regions. Based on the location of DiI obtained from molecular dynamics simulation, we infer that the free-area measured from fluorescence lifetime reflects free-area in the plane of the lipid bilayer just below the lipid-water interface. Estimated area-
per-lipid values were compared to previously published X-ray and NMR data (Figure 4-2 (a)). Comparison of our data with previously published data is tabulated in Table 4-1. In general, for saturated lipids, our results were in good agreement with the published values. However, area-per-lipid estimated for nanoliposomes prepared from DOPC and POPC (unsaturated) deviated significantly from the X-ray data. It is important to note that area-per-lipid values reported through X-ray and NMR techniques correspond to the average along the length of the acyl chains, in contrast to our measurements here which report directly the free area surrounding the dye chromophore. As the unsaturated lipid molecules deviate from the ideal cylindrical geometry and adopt an inverted-cone like geometry, our results for unsaturated lipids cannot be compared directly to that of NMR and X-ray data. The area-per-lipid estimated for unsaturated lipids is underestimated by the fluorescence lifetime measurements. Nevertheless, the measured fluorescence lifetimes still reflect the free-area near the lipid headgroup.

In order to determine the sensitivity of fluorescence lifetime to subtle changes in lipid packing, and to demonstrate the applicability of this method to measuring membrane physical properties, we measured the fluorescence lifetime changes as a function of temperature and osmotic tension. Temperature induced changes in free-area are shown in figure 4-2B. In fluid-phase bilayers, free-area gradually increased with increasing temperature, which was phenomenologically fit to a quadratic function. In gel-phase bilayers (e.g. DPPC, and DSPC), fluorescence lifetime deviated from this quadratic behavior near the phase transition temperature. Also, no sharp change in free-area was observed at the phase transition temperature, which is consistent with the idea that predominant changes in molecular ordering during phase transition occurs near the acyl
Figure 4-2: (A) Area-per-lipid of nanoliposomes prepared from different chain-length saturated lipids at multiple temperatures. Compositions are indicated by a vertical dotted line. Our results (blue circles) are compared to the published values (red solid circles). The dotted line shows the relationship between fluorescence lifetime and area-per-lipid derived from the solution data. (B) Area-per-lipid determined by scanning the fluorescence lifetime through a range of temperatures between 25°C and 50°C for different compositions. The expansion coefficient was estimated as the derivative of the curve at 25°C.
chain terminals, and so chain length and degree of saturation are the key determinants of the phase transition temperature. Fractional change in free-area plotted as a function of temperature was phenomenologically fit to a quadratic function, and thermal expansivity coefficients were determined by obtaining the slope of this curve at 25°C. The values for thermal expansion coefficients ranged from 0.002 °C⁻¹ to 0.005 °C⁻¹ depending on the chain length and degree of unsaturation. Increasing chain length in saturated lipids showed a decrease in thermal expansivity coefficient, likely due to the tighter packing of the lipids. The coefficient of thermal expansion of DMPC was 0.005 °C⁻¹, which is in agreement with 0.005 °C⁻¹ obtained through x-ray scattering and 0.0042 obtained through micropipette aspiration method.²,³⁴ The close agreement of our data with that

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<th>Lipid</th>
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<th>Lit., Å² (°C)</th>
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<td>Pabst et al. (2000)³³</td>
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measured using X-ray scattering indicates the high sensitivity of the current technique. Despite the long acyl chain, thermal expansivity of DOPC and POPC were lower than DMPC, implying higher conformational flexibility of the acyl chains due to presence of kinks. A similar decrease was observed for monounsaturated lipid, SOPC, whose coefficient of thermal expansion was 0.0033 °C⁻¹.

Finally, we demonstrate the applicability of this method to measure the free-area compressibility modulus of lipid bilayers. A schematic diagram of the experimental scenario is shown in figure 4-3 (a). Nanoliposomes prepared in 500 mM NaCl solution
were dispersed in diluted concentrations of NaCl to induce solute concentration gradients across the membrane. This results in an isotropic membrane tension that can be calculated from the Laplace’s Law, $\gamma = \Delta P \cdot r / 2$, where $\gamma$ is the isotropic membrane tension due to osmotic pressure gradient, $P$ is the pressure across the membrane, and $r$ is the radius. Pressure across the membrane can be obtained from the osmotic pressure difference, given by $\Delta P = 2i \Delta CRT$, where $\Delta C$ is the concentration gradient in moles/liter, $R$ is the gas constant, and $T$ is the absolute temperature. Substituting this in Laplace’s law gives:

$$\gamma = \frac{2i \Delta CRT}{r}$$

where $i$ is the van ‘t Hoff factor (2 for NaCl). Average radius of the nanoliposomes was determined using fluorescence correlation spectroscopy. Fluorescence lifetimes as a function of osmotic tension was measured in nanoliposomes prepared from DOPC, DPPC, and DMPC. Fractional change in free-area as a function of osmotic tension for these liposomes is shown in figure 4-3 (b-d). Free-area compressibility modulus was calculated from the slope of this curve. It has to be noted that the free-area compressibility modulus cannot be directly compared with the compressibility modulus measured through micropipette aspiration methods, as the free-area compressibility modulus does not account for the molecular compressibility. The gel-phase lipid DPPC showed the highest free-area compressibility modulus of 283 mN/m. A decrease in chain length by two carbon atoms (DMPC) lowered the free-area compressibility modulus to 220 mN/m. Interestingly, the unsaturated lipid DOPC (18:1) had a higher free-area compressibility modulus compared to the saturated 14 chain-length lipid (DMPC; 14:0). This is similar to the previous observation where the coefficient of thermal expansion
was higher for DMPC. Also, Evans et al. have reported the compressibility modulus SOPC bilyaers of 199.6 mN/m, which is higher than that of DMPC (144.9 mN/m).\textsuperscript{34} We also observed that the areal expansion saturated in DOPC liposomes at about 10 mN/m, saturation of areal expansion at about 10 mN/m in DOPC liposomes, which is indicative of pore formation tension. Pore formation at high tensions will immediately equilibrate the solute concentration across the membrane until the point where the pores are sealed back. Pore formation tension of \(~\text{10 mN/m}\) for DOPC is also in close agreement with 9.9 mN/m reported by Olbrich et al.\textsuperscript{35} In conclusion, our results indicate that mechanical properties of nanoliposomes of \(~\text{100 nm}\) in size can be accurately measured using fluorescence lifetime of DiI, in a non-contact fashion.

### 4.4. Conclusions

Previously, we have discussed how different membrane physical properties are related to lipid packing. For the first time, we report here the direct measurements of free-area in lipid bilayers using single-molecule fluorescence spectroscopic methods. Moreover, this is the first time such measurements have been reported for nanoliposomes. Through solution measurements, we derived the precise quantitative relationship between fluorescence lifetime of DiI and free-volume. This 3D model has been simplified to a 2-dimensional case for measuring free-area in lipid bilayers. Area-per-lipid estimated from these free-area values in nanoliposomes prepared from different liposomes are good agreement with published X-ray and NMR data. In order to demonstrate the applicability of this method for measuring lipid bilayer mechanics, we measured the thermal
expansivity coefficients and free-area compressibility modulus of different nanoliposomes. The technique presented here will enable rapid and automated measurements of membrane mechanical properties, which might benefit optimization of nanoliposome formulations for drug delivery and other biomedical applications. This will also enable a rapid progress in the field of membrane biophysics.

4.5. References


5.1. Introduction

Cellular membranes exhibit thermal undulations with frequencies of 1 Hz or more and magnitudes ranging up to tens of nanometers. While it is well understood that these undulations are governed by the membrane bending rigidity and lateral tension, they are also thought to be influenced by other cellular factors including protein inclusions, cytoskeletal attachments, and metabolically through adenosine triphosphate (ATP). Unlike membrane extensions like filopodia and lamellipodia that operate on a characteristic timescale of tens of seconds to minutes, rapid membrane undulations operate on the timescales of milliseconds to a second. A new perspective has recently emerged that hypothesized that membrane undulations might act as cellular sensors for probing the surrounding microenvironment. Membrane undulations can generate forces in the order of tens of piconewton, which is large enough to cause conformational changes in integral or peripheral membrane proteins. For example, a 2 pN force applied on Talin molecule can expose cryptic binding sites for Vinculin, leading to focal adhesion remodeling. Membrane undulations can also mediate the strength of adhesion to the substrate through clustering/oligomerization of the adhesion proteins. Additionally, undulations are also thought to regulate lipid/protein sorting, protein mobility/aggregation, and vesicle fusion. These mechanisms might play a general role in...
a variety of mechanically activated cellular processes including focal adhesion activation, G-protein signaling, calcium response, and mechanosensitive channel activation. Despite the well-understood importance of membrane undulations, to date it remains very challenging to detect these undulations in complex cell membranes such as in endothelial cells.

Since the discovery of erythrocyte membrane flickering by Browicz in 1890,¹¹ there has been a significant interest in developing high-resolution optical techniques for registering these flickering motions in intact cell membranes (reviewed by Kononenko¹²,¹³). Optical techniques based on reflection interference, laser scattering, and phase video microscopy, have been very successful in capturing flickering in erythrocytes. As a limitation, these methods are capable of detecting undulations only in optically homogeneous cells. Moreover, the accessible range of frequencies for these methods is in the order of few tens of Hertz due to camera exposure/acquisition time limitations. Membrane undulations in complex cell membranes (e.g. nucleated cells) on the apical surface could be detected by atomic force microscopy (AFM)¹⁴ or scanning ion conductance microscopy (SICM).¹⁵ To our knowledge, detection of high-frequency membrane undulations using these methods has not been demonstrated yet.

Fluorescence spectroscopic methods provide high signal to noise ratio with data acquisition times reaching picoseconds using methods such as time-correlated single photon counting. Specific labeling of the membrane with the dye also restricts signals arising from other sources, which is critical for detecting undulations in optically heterogeneous cells such as endothelial cells (or any nucleated cell). In this study, we introduce for the first time a fluorescence technique to detect undulations in giant
unilamellar vesicles and nanoliposomes. The technique is based on detecting fluctuations in fluorescence lifetime of DiI (a popular membrane probe). Fluorescence lifetime of typical chromophores such as rhodamine and cyanine are in the range of few nanoseconds. At a typical count of $10 - 100 \text{ K photons/sec}$, reliable lifetime measurements can be obtained on the sub millisecond timescale. Fluctuation analysis of these lifetime measurements acquired continuously can report the dynamic changes in the microenvironmental factors affecting the dye’s behavior. Haw Yang and coworkers have elegantly demonstrated this idea by detecting rapid conformational changes in proteins, using a photon-by-photon correlation method.\textsuperscript{16} In that work, fluctuations in fluorescence lifetime arose from resonance energy transfer between the fluorophore and a respective acceptor molecule. Here, we have adapted that idea by identifying a fluorescent probe (DiI) that resides in the membrane and is sensitive to subtle changes in membrane areal packing or the associated thickness. By capturing the fluctuation spectrum of the dye’s fluorescence lifetime, in principle, we will be able to capture dynamic changes occurring in the membrane packing. In a recent study by our group (described in chapter 5), we have reported that ensemble-averaged fluorescence lifetime of DiI can quantitatively report the average free-area in lipid bilayers. Furthermore, we determined thermo-mechanical properties (thermal expansivity, area compressibility modulus) of a variety of lipid bilayers using ensemble lifetime measurements. In this study, we extend this idea by hypothesizing rapid fluctuations of fluorescence lifetime of DiI in lipid bilayers reports fluctuations in free-area caused by thermal undulations. We demonstrate that fluctuation frequencies from 10Hz up to 1KHz can be detected using this fluorescence technique.
5.2. Materials and Methods

Refer to section 4.2.3. for nanoliposome preparation and section 3.2.5. for fluorescence lifetime measurements.

5.3. Results and Discussion

5.3.1. Fluorescence lifetime autocorrelation spectroscopy

Fluorescence lifetime of DiI is highly sensitive to the local microviscosity, as the trans-cis photoisomerization pathway dominates the non-radiative decay (chapter 4). We have shown that the ensemble-averaged fluorescence lifetime of DiI in lipid bilayers reflects the average free-area. Based on these results, we hypothesized here that fluctuations in the fluorescence lifetime would then naturally report the fluctuations in the free-area of the lipid bilayer. The two experimental scenarios, planar lipid bilayer and spherical nanoliposomes, investigated in this study are schematically shown in figure 5-1 (a). Compression/stretching induced in the lipid bilayer due to undulations results in fluctuation of free-area, which in turn result in fluctuations of DiI’s fluorescence lifetime. Experimental realization of the fluorescence lifetime fluctuations is schematically shown in figure 5-1 (b). Using the single-photon counting technique, the arrival time of the emission photons with respect to the excitation pulse (micro time) and with respect to the start of the experiment (macro time) are recorded for each detected photon. Microtimes of individual photons are binned into 100µs macrot ime intervals. Assuming that the microtimes in each bin are distributed following a single exponential function, we obtain
Figure 5-1: (A) Schematic diagram illustrating the experimental scenarios investigated in this study. Fluctuations in fluorescence lifetime of DiI due to membrane undulations are indicated by double-headed arrows. (B) (top panel) Single-photon counting technique was used to detect micro/macro times of the detected emission photons. Microtimes of detected photons were binned in 100 µs macrotime interval bins and averaged to obtain the lifetime per bin. (bottom panel) Fluctuations in fluorescence lifetime over the course of the experiment on a DOPC GUV are shown. Ensemble averaged lifetime is shown with the red line.
the fluorescence lifetime of each bin as the average of microtimes of all the detected photons in that bin. Binning time of 100 µs was chosen to ensure that each bin collected at least tens of photons. In principle, binning time limits the maximum frequency (10 KHz) of fluctuations that can be detected. Typical measurements involved collecting this photon-by-photon data for up to several minutes for each sample. While fluorescence lifetime fluctuation autocorrelation method was originally proposed for immobilized molecules,\textsuperscript{16} recently Gopich and Szabo have proposed an extension to this method for freely-diffusing molecules.\textsuperscript{17} In the case of freely diffusing molecules, the true lifetime fluctuations due to undulations are overlaid with the intensity fluctuations due to inhomogeneous (Gaussian) illumination profile. True lifetime fluctuation autocorrelation after correcting for the intensity autocorrelation can be obtained using the following equation:

\[
G_{\tau}^{\text{real}} = G_{\tau}^{\text{exp}} / G_{I}^{\text{exp}}
\]

where \(G\) represents the autocorrelation function, subscripts \(I\) and \(\tau\) denote intensity and lifetime respectively, and the superscript ‘\text{exp}’ denotes experimentally measured correlation function and ‘\text{real}’ denotes corrected correlation function. Since the dye molecules are freely diffusing in the plane of the bilayer, maximum diffusion time limits the lowest frequency that can be detected. Characteristic diffusion times of DiI in lipid bilayers are in the order of 100 ms, thus limiting the lowest undulation frequency that can be detected to 10 Hz.
5.3.2. Detecting undulations in giant vesicles and nanoliposomes

Fluorescence lifetime autocorrelation analysis was performed on giant unilamellar vesicles (GUVs; 20 to 50 µm diameter) prepared by electroformation method. GUVs were stained with nanomolar concentration of DiI, with a DiI:Lipid ratio of 1:20000 molecules. The low concentration of dye used in this study ensures that intercalation of the dye does not perturb the membrane mechanical properties, which was previously confirmed through molecular dynamics simulations. Photon data was collected by positioning the laser beam on the top surface of the GUV. Raw photon data was analyzed using a custom written program to calculate both intensity and lifetime correlation functions. Figure 5-2 (a) shows the normalized autocorrelation of the fluorescence lifetime fluctuations (after correction) in GUVs prepared from (i) DOPC, (ii) DOPC with 50 mol% cholesterol, and (iii) DOPC nanoliposomes (~150 nm). As a negative control we measured the lifetime fluctuations in water (also shown in figure 5-2 (a)). Molecular packing fluctuations in a homogeneous solvent environment such as in water would occur on the time scale of sub-nanoseconds, and thus are not expected to exhibit any fluctuation relaxation within the time scales measured here. Since undulations exhibit a broad range of frequencies, the autocorrelation curves were fit to a stretched exponential function to determine the characteristic relaxation time constant and spread of relaxation times. Fluctuation correlation in DOPC giant vesicles showed a characteristic relaxation time of 29.3 ms (34.1 Hz), with a spread factor of $\beta = 0.7$. Increase in bending rigidity by adding cholesterol showed reduced relaxation time of 15.3 ms (65.4 Hz) in DOPC with 50% cholesterol, with a spread factor of $\beta = 0.67$. This is as expected, since, increase in
bending rigidity results in a decrease in wavelength and an increase in frequency of the undulations. No significant fluctuation correlation was detected in gel-phase DPPC bilayers (data not shown). Fluctuation correlation analysis on 150 nm nanoliposomes

Figure 5-2: (A) Fluorescence lifetime aurocorrelation data in DOPC (red), DOPC+50% cholesterol (blue), DOPC nanoliposomes (green), and water (black). (B) Average fluorescence lifetime and mean-square fluctuation in lipid bilayers of varying bending rigidities. Error bars represent standard deviations. N = 5.
showed a characteristic relaxation time of 2.6 ms (384.6 Hz), with a spread factor of $\beta = 0.49$. A significant decrease in spread factor indicates an increase in the range of undulation frequencies. Unlike giant vesicles which can be treated as infinite bilayers, undulations in nanoliposomes are limited in wavelength by the size of the liposome. This restrains the undulations to very high frequencies.

While it is a common practice to determine the undulation frequency spectrum to determine the membrane bending rigidity, it is not practical to do so under dynamic conditions such as in cells. Moreover, this requires data acquisition over long times. Additionally, poor signal-to-noise at high frequencies limits the characterization in bilayers that exhibit high bending rigidity (e.g. DPPC gel-phase bilayers). In order to quantitatively characterize bending undulations under dynamic conditions, we introduce the parameter ‘mean-squared lifetime fluctuation’, which is same as $G_t^{real}(0)$ from the autocorrelation curve. Mean square fluctuation of the lifetime will depend on the number of photons recorded per bin, i.e., higher the number of photons collected per bin, lower will be the fluctuation. Since each data set differs in the emission photon count, now we bin such that each bin has equal number of photons (typically 100 photons per bin). Then, mean-squared fluctuation is calculated from all the bins in a dataset. Mean-square fluctuations measured in different GUVs are shown in figure 5-2 (b). Clearly, increasing the bending rigidity by either increasing the degree of saturation or the amount of cholesterol resulted in a significant decrease in the mean square fluctuation. For comparison, we also show the ensemble averaged fluorescence lifetime in each of these bilayers. An increase in fluorescence lifetime indicates a decrease in average free-area. So, membranes that are packed tightly exhibit lower fluctuations in lifetime (or free-
area). In summary, these results demonstrate that high frequency undulations in planar lipid bilayers and nanoliposomes can be detected by analyzing fluorescence lifetime fluctuations of Dil.

5.4. Conclusions

In summary, we have developed a novel fluorescence lifetime based method to detect thermal undulations of lipid membranes in giant unilamellar vesicles and nanoliposomes. The technique is capable of detecting high frequency undulations up to several hundreds of Hertz. Furthermore, we show that mean-squared fluctuation of fluorescence lifetime could be a very good indicator for measuring dynamic changes in membrane undulations. In future, we would like to develop a theoretical model that relates fluorescence lifetime fluctuations with bilayer bending rigidity modulus. These results will open up several new avenues for understanding the role of membrane undulations in cell membrane processes and the role of membrane bending in cellular mechanosensing.

5.5. References


6.1. Introduction

Lateral compartmentalization of cell membranes is now a well recognized modification of the original Singer-Nicholson membrane model, and has led to a revolutionized view of how the cell membrane regulates cellular signaling. A predominant manifestation of lateral compartmentalization are lipid rafts, 10 to 100 nm dynamic membrane patches enriched in glycosphingolipids (e.g. sphingomyelin) and cholesterol, that are thought to corral signaling proteins such as small and heterotrimeric G proteins, nonreceptor tyrosine kinases, and protein phosphatases for initiation of signaling cascades at the cell surface. Thus, identifying and understanding the nature of lipid-lipid and lipid-protein intermolecular interactions responsible for the raft formation and dynamics in intact cells has now become a fundamental problem in membrane biology.

Through the use of model membranes and biophysical tools, the field is coalescing around the idea that raft existence implies boundaries that arise from the minimization of energy from hydrophobic mismatch and the entropy of mixing. But significant hurdles remain in the characterization of lipid raft organizing principles, and their physiological significance. First, detection of rafts in intact cells under physiological conditions is still very challenging due to their highly dynamic nature and the fact that
their size is below the optical resolution limit of traditional microscopy and second, there are limited tools for controlling raft formation in cells or model membranes that would permit quantitative determination of raft organizing principles or enable study of rafts’ role in signal transduction. Important progress on detection of rafts in cells was recently made using state-of-the-art single-molecule techniques which confirmed the presence of dynamic domains in intact cells under physiological conditions.\(^9,10,11\) The remaining challenge now is to develop the means to control raft formation/disruption in cells that not only permits studying the role of rafts but also facilitate lipid-raft targeted therapies.\(^12,13,14\) Therefore, development of new biochemical and biophysical methods for investigating lipid rafts and their role in membrane control of cell signaling is needed for further progress in this field.

Most experimental tools for controlling raft formation in lipid membranes focus on altering the concentration of cholesterol using methyl-\(\beta\)-cyclodextrin or statins\(^{15,16}\) or by depleting cholesterol through enzymatic degradation with cholesterol oxidase\(^{17}\). Subsequent observations of alterations in signaling cascades or other functions are then interpreted as depending on lipid rafts. There are weaknesses in this approach, however. As pointed out in a review by London,\(^{18}\) association of a membrane process with the concentration of cholesterol is not sufficient to affirm the role of lipid raft domains, as evidenced by the fact that certain processes including cholesterol-dependent cytolysins and virus-induced membrane fusion require high cholesterol concentrations but not necessarily raft domains. In addition, cholesterol plays multiple structural and functional roles in regulating membrane protein activity that are not dependent on localization of proteins to rafts. These include modulating membrane physical properties (e.g. thickness,
fluidity, and diffusion) that affect protein conformation and oligomerization, in addition to modulating lateral organization of the bilayer into raft domains. It is often overlooked that several membrane proteins including G-protein coupled receptors and ligand-gated ion channels possess specific cholesterol binding sites and these specific sterol-protein interactions are thought to be essential for the protein’s activity. Thus, there is a need for compounds that can be used to tune phase separation in membranes but that do not have specific interactions with the lipids or proteins involved in signaling. While membrane protein activity is often correlated to either membrane physical properties or raft stability, these two concepts are highly interrelated. Altering membrane physical properties can promote/disrupt domain formation. For example, lateral membrane tension imposed by osmotic swelling can promote domain formation in cells under physiological conditions.

One strategy that can result in stabilizing or destabilizing rafts while retaining the specific molecular interactions and chemical activity of native raft molecular components (cholesterol and sphingomyelin) is to introduce non-lipid amphiphilic molecules that preferentially interact with and alter the physical properties of non-raft fluid regions. Such a strategy takes advantage of the fact that rafts are thought to exist as liquid-ordered domains while non-raft regions are liquid-disordered. Thus, at low concentrations, non-ionic surfactants that preferentially interact and fluidize the liquid-disordered regions can drive domain formation. Conversely, compounds that rigidify the non raft regions can lead to demixing of lipids and dissolution of rafts. Here we investigated the phase separation behavior of lipid bilayers under the influence of three such amphiphiles: α-tocopherol (Vitamin-E), Triton-X, and benzyl alcohol. Atomistic insights into the
amphiphile interaction with lipids and changes in lipid bilayer physical properties were obtained from molecular dynamic simulations at selected concentrations. Experimentally, phase separation was studied in giant unilamellar vesicles prepared from lipid mixtures that mimic solid-liquid and liquid-liquid phase coexisting regions. These amphiphilic molecules preferentially interact with the fluid-phase lipids and act as a rigidifying or fluidizing agent. Results indicate that each of these amphiphiles alters both membrane thickness and fluidity, but the combined effect is in favor of hydrophobic forces due to thickness mismatch. Thus, the mechanism of amphiphile-induced phase separation or demixing is likely to be through enthalpic interaction energy (hydrophobic mismatch) changes rather than entropic energy changes (i.e. due to conformational flexibility). In summary, we demonstrate that altering the hydrophobic thickness of the non-raft fluid regions by adding certain non-lipid amphiphiles can tune phase separation in model membranes. These molecules, and their molecular quantification provide are potentially useful tools to study rafts while avoiding complicating features such as avoiding specific amphiphile-protein interaction.

6.2. Materials and Methods

6.2.1. Molecular dynamics simulation methodology

Chemical structures of Vitamin-E (VE), Triton-X (TX), and Benzyl alcohol (BA) are shown in figure 6-1. Topologies of these molecules were constructed using DUNDEE PRODRG server. The united atom model was adopted for CH$_n$ groups. Force field
parameters for lipids were identical to Berger et al.\textsuperscript{31} and force field parameters for VE, TX, and BA were adopted from GROMOS 45a4 set.\textsuperscript{32} Partial charges of DPPC were obtained from Chiu et al.,\textsuperscript{33} whereas partial charges for VE, TX, and BA were assigned according to GROMOS force field.\textsuperscript{32} Simple point charge (SPC) model was used for the explicit water molecules.

A simulation box of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) bilayer consisting of 128 DPPC molecules (64 in each leaflet) and 3655 water molecules was obtained from Tieleman et al.\textsuperscript{34} Simulations were carried out on four different systems: pure bilayer (control), 10 mol\% VE, 10 mol\% TX, and 60 mol\% BA. (Note: mol\% is with respect to lipid molecules). VE/TX/BA molecules were placed in the water phase at random locations on both sides of the lipid bilayer, and allowed to freely intercalate into the membrane. Molecular dynamics simulations were carried out using GROMACS simulation software (version 4.0.3).\textsuperscript{35,36} Starting simulation structures were energy

![Chemical structures of cholesterol, vitamin-E, Triton-X 100 (n = 10), and benzyl alcohol.](image-url)

Figure 6-1: Chemical structures of cholesterol, vitamin-E, Triton-X 100 (n = 10), and benzyl alcohol.
minimized using steepest-descent algorithm for at least 1000 steps and further equilibrated under NPT conditions for 1 ns, with temperature and pressure set to 323 K and 1 bar, respectively. Final production runs were carried out under NPT conditions with temperature and pressure were set to 323 K and 1 bar, respectively. Periodic boundary conditions were applied to the simulation box in all three coordinate dimensions (with x,y in the bilayer plane and z normal to the bilayer). Temperature and pressure of the system were controlled using Berendsen’s weak coupling method with the time constants set to 0.1 ps and 1.0 ps respectively. Semi-anisotropic scaling was used for pressure coupling, with 1 bar reference pressure in the xy plane and z-dimension. The LINCS algorithm was used to constrain bond lengths. The Particle-Mesh Ewald (PME) method was used for electrostatic interactions, with a direct-space cutoff of 1 nm, and cubic interpolation for the calculation of long-range interactions in the reciprocal space, with a Fourier transform grid of 0.12 nm maximum. The Lennard-Jones interactions were cutoff at 1.0 nm. A time-step of 2 fs was used with a leap-frog integration algorithm for the equations of motion. Simulations were carried out for a total simulation time of 100 ns. Complete intercalation of the non-lipid amphiphiles was observed by 30 ns and so trajectory analysis was performed on the last 70 ns.

6.2.2. Preparation of giant unilamellar vesicles

1,2-dioleoyl-\textit{sn}-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-\textit{sn}-glycero-3-phosphocholine (DPPC), and cholesterol (CHOL) were purchased from Avanti polar lipids, Inc. Fluorescent probe 1,1’-didodecyl-3,3,3’,3’-tetramethylindocarbocyanine
perchlorate (DiI-C_{12}; \lambda_{ex} = 549 \text{ nm}; \lambda_{em} = 565 \text{ nm}) was purchased from Invitrogen, Inc. Benzyl alcohol and \( \alpha \)-tocopherol were purchased from Sigma-Aldrich, Inc. Triton-X 100 was purchased from MP Biomedicals, Inc. All the chemicals were obtained at their highest purity available and used without further purification.

Lipid mixture solutions were prepared at a concentration of 0.5 mg/ml with dye concentration of 100 nM for fluorescence imaging. VE and TX at the described mole fractions were added to the lipid mixture solution prior to preparation of vesicles. Benzyl alcohol treatment was performed after vesicle sample preparation. Giant unilamellar vesicles (GUVs) were prepared using electroformation method.\(^{39,40}\) A custom-built electroformation chamber consisting of two transparent ITO cover slips was used to apply AC electric fields to the lipid film. The cover slips were separated by a silicone spacer of 1.6 mm thickness. 3 to 5 \( \mu \)l of lipid solution was deposited on the cover slips and dried initially under argon for 5 minutes and then vacuum dried for at least 1 hour. The chamber was filled with deionized water preheated to 50\( ^\circ \)C and moved immediately to a baking oven that was constantly maintained at a temperature of 50\( ^\circ \)C. AC electric fields were then applied across the ITO electrodes using a LabVIEW controlled A/D board (National Instruments, Inc.). Applied voltage at 10 Hz frequency was increased from 0.1 V to 1.6 V at a rate of 30 mV/min, followed by a constant voltage of 1.6 V applied for 3 hours. Frequency was then reduced to 5 Hz and maintained for 1 to 2 hours to detach the vesicles. A single run of electroformation typically resulted in hundreds of GUVs with sizes ranging from 5 to 100 \( \mu \)m. GUVs were allowed to cool down to room temperature for about an hour, before imaging them under an Olympus IX71 inverted microscope equipped with a 60X water-immersion objective.
6.3. Results and Discussion

6.3.1. Effect of non-lipid amphiphiles on membrane thickness and fluidity

To gain an atomistic perspective on how different amphiphiles interact with the lipid bilayer and affect its physical properties, we performed atomistic molecular dynamics simulations of a fluid-phase DPPC bilayer (at 50°C) with different membrane additives at selected concentrations. Simulations were performed on a fluid-phase bilayer, as the additives studied here are thought to preferentially interact with the lower melting temperature lipids.\textsuperscript{41,42,43} It is important to note that MD simulations are faced with some limitations. Molar concentrations used in molecular dynamic simulations are ill-defined and cannot be compared directly to experimental conditions, because of the finite and small system size in simulations compared to giant unilamellar vesicles used experimentally. Due to the use of periodic boundary conditions, the bulk solution phase is not captured, and so partitioning coefficients of the additives between water and bilayer phases cannot be determined. Moreover, simulations were carried out at selected concentrations, as it is computationally prohibitive to study lipid dynamics at many different concentrations. Therefore, we do not expect to capture the concentration-dependent effects that are observed for these additives experimentally and in simulations.\textsuperscript{44,45} In light of these limitations, our focus here is to provide a clear atomistic picture into the location, orientation, and the effect on lipid dynamics, and bilayer structural properties in order to provide qualitative insight into mechanisms of phase separation and demixing observed in experiments.
Snapshots of the equilibrium conformations of the lipid bilayer upon treatment with the additives are shown in figure 6-2 (a-c). Note that, for clarity, only two molecules of the additive are shown in the figures. As hydrophobic forces due to height-mismatch...
between lipids and fluidity together contribute to the mixing free energy, we characterized the changes in membrane thickness and conformational flexibility under the influence of different additives. Bilayer thickness, defined as the peak-to-peak distance between the phosphate groups of the two leaflets was obtained from the mass density profiles that were obtained by calculating the time-averaged mass density of different molecule groups along the membrane normal (figure 6-2 (d)). The thickness of the additive-free bilayer was measured to be 3.68 nm, which is in reasonable agreement with experimental value for DPPC bilayer at 50°C obtained from X-ray studies.46

Unlike the other two amphiphiles (Triton-X and benzyl alcohol), Vitamin-E has a long hydrophobic tail regions, and so adopts a linear conformation along the membrane normal. The terminal methylene groups were located at the bilayer midplane and the chromanol moiety was positioned below the lipid phosphates predominantly hydrogen bonded to the lipid acyl ester oxygen atoms, consistent with neutron scattering and NMR data.47,48 The penetration of the molecule beyond the lipid-water interface indicates that the hydrophobicity of the tail dominates the hydrophilicity of the headgroup hydroxyl group. Intercalation of Vitamin-E in the lipid bilayer induced an increase in bilayer thickness from 3.68 nm to 3.84 nm. This observation is similar to that observed for a structurally similar molecule, cholesterol.49,50

On the other hand, Triton-X and benzyl alcohol have a short hydrophobic tails and Triton-X has a very long hydrophilic polyethylene oxide chain. Bilayer thickness decreased from 3.68 nm to 3.62 nm and 3.52 nm upon addition of Triton-X and benzyl alcohol, respectively. In both these cases, we observed that the headgroup hydroxyl group was located at the lipid headgroup region predominantly hydrogen bonded to the
phosphate or ester oxygen atoms. The ethylene oxide chain of Triton-X adopted a partially extended conformation and is located at the lipid-water interface, while its bulky hydrophobic group penetrated the lipid bilayer core. The decrease in bilayer thickness due to Triton-X is likely due to the reduced surface tension of the lipid-water interface. In the case of benzyl alcohol, the benzyl group was located below the lipid headgroups, which resulted in excess free volume in the bilayer core, thus inducing a decrease in bilayer thickness. Previously, Turner et al. have reported that benzyl alcohol at a molar ratio of 3 to 1 (twice that used in the simulations here) induces a 0.2 nm reduction in bilayer thickness in DMPC bilayers. Decrease in bilayer thickness is also evident from the increased interdigitation of the lipid acyl chains figure 6-2 (d). Increased chain interdigitation in phosphocholine bilayers has been observed experimentally under the influence of benzyl alcohol (10 mM and above) and other alcohols. Interestingly, low concentrations of Triton-X and benzyl alcohol (10 mol%) induced a slight increase in bilayer thickness (data not shown). This concentration-dependent effect is also observed previously for different alcohols and Triton-X. This phenomenon is commonly observed with molecules that interact primarily at the interface, while such effects are not observed with molecules that insert completely into the bilayer core.

It is often observed that an increase in packing or bilayer thickness results in a concomitant decrease in fluidity, and vice versa. Bilayer fluidity was assessed from the molecular trajectories by computing the chain order parameters and rotational relaxation times. While order parameter reports the conformational flexibility of lipid acyl chains, lipid rotational relaxation times reflect the overall bilayer fluidity including the headgroup contributions. Order parameters were computed as an average of both the acyl
chains under different simulation conditions, as described previously\textsuperscript{55} (shown in figure 6-2 (e)). Order parameters of the pure DPPC bilayer are in very good agreement with the previous experimental and simulation values.\textsuperscript{55,56} Incorporation of Vitamin-E in the bilayer caused a significant increase in the order of the acyl chains, uniformly throughout the length of the carbon chain. This is consistent with the linear arrangement of the Vitamin-E molecule along the membrane normal, aligning with the lipid acyl chains.\textsuperscript{57} This is also consistent with electron spin resonance data where the authors observed a slight increase in ordering in the fluid-phase bilayer upon incorporation of vitamin-E.\textsuperscript{58,59} On the other hand, Triton-X and benzyl alcohol do not show a uniform change along the carbon chain. Triton-X induced a significant decrease in chain order near the headgroup region, while the terminal tail region was largely unaffected. This is likely due to the primary interaction of Triton-X with the lipids at the interface. In case of benzyl alcohol, we observed a significant increase near the headgroup region and a significant decrease near the tail region. The close proximity of the benzyl group near the lipid headgroups region orders the lipid acyl chains in this region. The decrease in acyl chain ordering in the tail regions is likely due to the excess free area created in this region that result in overall tilt of the acyl chains.

As the hydrophilic regions of these different membrane additives are located near the lipid headgroup region, these additives could also alter lipid-lipid headgroup interactions. To assess the overall fluidity of the membrane, we characterized the rotational relaxation times of the lipid molecules (figure 6-2 (e)). Rotational relaxation times were determined by fitting the autocorrelation curves to a stretched-exponential function. Rotational relaxation times exhibit a trend that is similar trends to order
parameters. While Vitamin-E caused an increase in rotational relaxation times, both Triton-X and benzyl alcohol lowered the relaxation times. These results are consistent with time-resolved fluorescence anisotropy measurements of fluorescent probes in lipid bilayer.\textsuperscript{54,60,61} Overall, the simulation results indicate that Vitamin-E increases bilayer thickness and lowers acyl chain ordering, whereas both Triton-X and benzyl alcohol decrease bilayer thickness and increase fluidity.

6.3.2. Lateral phase separation in ternary mixtures of DOPC, DPPC, and Chol/VE/TX/BA

Effect of membrane additives on lateral phase separation was determined in ternary and quaternary (next section) mixtures of DOPC, DPPC, Cholesterol, and VE/TX/BA. Microscopic phase separation was determined in giant unilamellar vesicles under wide-field fluorescence microscopy, using DiI-C\textsubscript{12}, a fluorescence probe that has been shown to preferentially partition to the fluid regions.\textsuperscript{62,63} This short chain DiI-C\textsubscript{12} partitions preferentially to the liquid phase in solid-liquid phase coexistence and to the liquid-disordered regions in liquid-liquid phase coexistence.\textsuperscript{63,64} Solid-liquid and liquid-liquid phase coexistence from the fluorescence images were differentiated by the morphology of the domains. For example, liquid-liquid coexisting phases exhibit circular morphology, whereas solid-liquid coexisting phases exhibit irregular domain morphology.\textsuperscript{65} A 1:1 binary mixture of DOPC and DPPC in the absence of any other additives exhibits solid-
Figure 6-3: Solid-liquid coexisting phases in 1:1 DOPC:DPPC lipid mixture (A). Phase separation behavior in 1:1 DOPC:DPPC mixture under the influence of cholesterol (B-E), Vitamin-E (F-I), Triton-X (J-M), and benzyl alcohol (N-Q). The top panel shows the corresponding ternary phase diagrams, grey circle – solid-liquid coexistence, black circle – liquid-liquid coexistence, white circle – no phase separation.
liquid coexisting phase regions, as shown in figure 6-3 (a). Partial ternary phase diagrams of DOPC, DPPC, and VE/TX/BA were constructed by adding different concentrations of the membrane additives (shown in top panel of figure 6-3) to the above mentioned lipid mixture. Vitamin-E and Triton-X were added to the lipid stock solutions prior to the preparation of GUVs at the selected molar concentrations. Because preparation of GUVs using electroformation involves completely drying the solvent (chloroform) before hydration, benzyl alcohol was added to the lipid bilayer after rehydration.

Phase separation in ternary mixture of DOPC, DPPC, and Cholesterol has been studied extensively in the past, and so served here as a positive control. Addition of cholesterol in the range of 10 mol% to 30 mol% resulted in coexisting liquid phases (figures 6-3 (b-d)). Cholesterol partitions preferentially with the gel-phase lipid resulting in liquid-disordered and liquid-ordered coexisting phase domains. Niu et al. have experimentally determined the effect of acyl chain unsaturation on partitioning coefficient of cholesterol in lipid bilayers and showed that cholesterol exhibits high preference for fully saturated lipids. Beyond a certain concentration (40 mol%) of cholesterol, no lateral phase separation was observed (figure 6-3 (e)). Our results with cholesterol are in agreement with the phase diagrams of DOPC/DPPC/Cholesterol in the literature. Despite very similar structure of Vitamin-E compared to Cholesterol (shown in figure 6-1), the phase separation behavior under the influence of Vitamin-E deviated significantly from that of cholesterol. Phase diagrams of DOPC/DPPC/Chol and DOPC/DPPC/VE are shown in the top panel of figure 6-3. First, coexisting liquid-liquid phases were not observed at any concentration of Vitamin-E, up to 30% (figure 6-3 (f-i)). Addition of vitamin-E, even at the lowest concentration studied (10 mol%), resulted in
complete disruption of solid-liquid coexistence. Beyond 30% of Vitamin-E, giant vesicles could not be formed. This is likely due to the formation of mixed micellar phases at high concentrations. While both cholesterol and Vitamin-E both act as rigidifiers when embedded in fluid-phase bilayers, they might interact differently with the gel-phase and fluid-phase lipids in lipid mixtures. Using differential scanning calorimetry and fluorescence polarization, Stillwell et al.\textsuperscript{68} have shown that vitamin-E preferentially interacts with polyunsaturated fatty acids while cholesterol preferentially interacts with saturated fatty acids. According to the “umbrella model”,\textsuperscript{69} lipid polar headgroups cover the bulky hydrophobic tetrameric ring of the cholesterol, and saturated lipids provide better coverage than the unsaturated lipids,\textsuperscript{70} contributing to the preferential interaction of cholesterol with saturated lipids in lipid mixtures. On the other hand, Vitamin-E has a much more flexible and less bulky hydrophobic tail region compared to cholesterol. High conformational flexibility of Vitamin-E might favor its interaction with the unsaturated lipids. Also, these results suggest that Vitamin-E might act as a regulator for lipid raft formation under physiological conditions.

On the other hand, addition of Triton-X or benzyl alcohol – both acting as fluidizers – consistently supported phase coexistence in 1:1 DOPC:DPPC mixture (figure 6-3 (j-q)). The phase diagram for DOPC/DPPC/TX is shown in figure 6-3 (top panel). However, we noticed some differences between these two additives. Domain morphologies in the case of Triton-X were similar to that observed in pure solid-liquid coexistence (figure 6-3 (a)). However, in the case of benzyl alcohol, domain morphologies resembled liquid-liquid coexisting regions. Increase in the concentration of benzyl alcohol showed a gradual increase in the size of the domains (figures 6-3 (n-q)). In
fact, at the highest concentration (100 mM) benzyl alcohol induced large-scale phase separation (figure 6-3 (q)). The persistence of gel-phase domains in the presence of Triton-X indicates that Triton-X shows high preference for the liquid-phase regions, and does not perturb the packing in gel-phase regions. In fact, this forms the basis for extraction of lipid raft domains by selective solubilization of cell membranes using Triton-X 100. Using isothermal titration calorimetry Heerklotz et al. have shown that Triton-X interacts unfavorably with sphingomyelin (high melting temperature lipid) and cholesterol. Induction of liquid-liquid phase coexistence by benzyl alcohol indicates its interaction with both gel and fluid-phase regions. As mentioned earlier, alcohols do favor interaction with fluid-phase lipids, but might not be exclusively. So, liquid-liquid phase coexistence likely resulted from fluidization of both gel and fluid domains. Promotion of large-scale domain formation with increase in concentration also indicates that benzyl alcohol has a stronger effect on the fluid-phase regions.

In summary, all the membrane additives studied here show preferential interaction with the fluid-phase lipid. Vitamin-E acting as a membrane rigidifier promotes raft disruption, while Triton-X and benzyl alcohol acting as fluidizers promote raft formation.

6.3.3. Lateral phase separation in quaternary mixtures of DOPC, DPPC, Cholesterol, and VE/TX/BA

Membrane rafts in intact cells closely resemble liquid-liquid coexisting phase domains exhibited by ternary lipid mixtures (two lipids and cholesterol). In order to understand how these membrane additives influence lipid mixing in the presence of cholesterol, we studied lateral phase separation behavior in two different lipid mixtures:
Figure 6-4: (A) Liquid-liquid phase coexistence in 1:1:0.7 DOPC:DPPC:Chol GUVs. Phase separation behavior of mixture in (A) under the influence of Vitamin-E (B,C), Triton-X 100 (D,E), and benzyl alcohol (F,G). (H) Single-phase GUVs prepared from 1:1:1.2 DOPC:DPPC:Chol. Phase separation behavior of mixture in (H) under the influence of Vitamin-E (I,J), Triton-X 100 (K,L), and benzyl alcohol (M,N).

(Mixture 1) 1:1:0.7 DOPC:DPPC:Chol that forms coexisting liquid-liquid regions (figure 6-4 (a), and (Mixture 2) 1:1:1.2 DOPC:DPPC:Chol that forms randomly mixed liquid
ordered region (figure 6-4 (h)), under the influence of the different additives. These lipid compositions were chosen so that they are close to the boundary (above and below) of the liquid-liquid phase coexistence region. 65

Giant vesicles prepared form Mixture 1 consistently exhibited liquid-liquid phase separation. Addition of Vitamin-E to this mixture resulted in uniformly mixed phase region (figures 6-4 (b&c)), implying that Vitamin-E increases the mixing tendency of the lipids. These results indicate that rigidification of the fluid-phase lipid in mixture 1 induces ideal mixing of the lipids. This is consistent with the observed behavior in binary mixtures. On the other hand, giant vesicles prepared from Mixture 2 exhibit no phase separation due to the high concentration of cholesterol. No change in the phase mixing state was observed with addition of Vitamin-E (figures 6-4 (i&j)), implying that vitamin-E does not induce lipid demixing. That is, further rigidification of the fluid-phase lipid in mixture 2 has no effect on phase separation. This is similar to that observed with cholesterol, where further addition of cholesterol to single-phase liquid-ordered vesicles (above 30 - 40% cholesterol) does not induce lipid phase separation. 65

Both Triton-X or benzyl alcohol promoted or stabilized domain formation. Addition of triton-X to mixture 1 (with preexisting domains) did not alter the coexisting liquid-liquid phases (figures 6-4 (d&e)), whereas addition of benzyl alcohol induced even large-scale phase separation at high concentrations (figures 6-4 (f&g), similar to that observed in binary lipid mixtures. That is, phase coexistence sustained under the influence of these additives. These results together with the results in previous section indicate that benzyl alcohol has a stronger influence of lipid mixing/demixing than Triton-X. In mixture 2, addition of triton-X and benzyl alcohol both promoted the
formation of liquid-liquid coexisting regions (figures 6-4 (k-n)), suggesting that both these amphiphiles drive raft formation, an effect that is exactly opposite to that of Vitamin-E. In fact, we even observed reversible phase separation by adding vitamin-E and Triton-X in a series. That is, addition of Vitamin-E first resulted in uniform mixing of the lipids, and addition of Triton-X to this membrane reversed the effect of Vitamin-E by inducing the formation of domains (figure 6-5).

6.4. Conclusions

Hydrophobic forces due to lipid height-mismatch and the conformational flexibility of lipids play opposing roles in lipid mixing/demixing. As seen in MD simulations, an increase in membrane thickness results in decreased fluidity and vice versa. Increased hydrophobic mismatch between liquid-disordered and liquid-ordered regions favors demixing due to enthalpic interactions. However, the associated increase
in fluidity favors lipid mixing due to the entropic effect. The balance between these two forces determines the phase separation behavior. This is clearly exemplified by the characteristic miscibility temperature of the lipids. Above the miscibility temperature, entropic forces dominate the enthalpic forces resulting in ideal mixing of the lipids, since entropic free energy scales linearly with the temperature. Whereas below the miscibility temperature enthalpic forces dominate the entropic forces resulting in demixing of the lipids.

Each of the three additives investigated in this study showed preferential interaction with the fluid-phase lipid. Vitamin-E caused an increase in thickness of the fluid-phase lipid and lowered the fluidity simultaneously. Increase in thickness drives favorable mixing of the lipids, whereas the associated decrease in fluidity hinders favorable mixing of the lipids. Experimentally, we observe that the combined effect is in the favor of thickness change. Similarly in the case of Triton-X and benzyl alcohol, the decrease in thickness drives favorable demixing of the lipids and the increase in conformational flexibility drives favorable mixing of the lipids. Once again, we observe that the combined effect is in the favor of thickness change. These results are consistent in both ternary and quaternary mixtures of lipids and additives. Estimated thickness changes in lipid bilayer at the studied concentrations are in the order of few angstroms. It is interesting that such a small change (less than the size of a carbon atom) in membrane thickness can influence so strongly the lateral phase separation behavior. In summary, these results suggest that changes in enthalpic interaction dominates the entropic energy changes, indicating that hydrophobic mismatch plays a dominant role compared to the fluidity in determining the mixing behavior. For all practical purposes, hydrophobic
thickness (or mismatch) is a highly relevant structural parameter for qualitatively understanding the non-ideal mixing of lipid mixtures in both model and cell membranes.

### 6.5. References


(34) Tieleman, D. P.; Berendsen, H. J. C. Molecular Dynamics Simulations of a Fully Hydrated Dipalmitoyl Phosphatidylcholine Bilayer With Different Macroscopic


Chapter 7

Conclusions

We described in detail the effect of membrane lateral tension on lipid bilayer structure and dynamics. To quantify the precise relationship between tension, structural properties of the membrane, and the dynamics of lipids and a lipophilic reporter dye (DiI), we performed atomistic molecular dynamics simulations of DiI-labelled dipalmitoylphosphatidyl choline (DPPC) lipid bilayers under physiological tensions ranging from -2.6 mN/m to 15.9 mN/m. Simulations showed that bilayer thickness decreased linearly with tension consistent with volume-incompressibility, and this thinning was facilitated by a significant increase in acyl chain interdigitation at the bilayer midplane and spreading of the acyl chains. Tension caused a significant drop in the bilayer’s peak electrostatic potential, which correlated with the strong reordering of water and lipid dipoles. For the low tension regime, the DPPC lateral diffusion coefficient increased with increasing tension in accordance with free-area theory. For larger tensions, free area theory broke down due to tension-induced changes in molecular shape and friction. Simulated DiI rotational and lateral diffusion coefficients were lower than those of DPPC but increased with tension in a manner similar to DPPC. Direct correlation of membrane order and viscosity near the DiI chromophore, which was just under the DPPC headgroup, indicated that measured DiI fluorescence lifetime, which is reported to decrease with decreasing lipid order, is likely to be a good reporter of tension-induced decreases in lipid headgroup viscosity (discussed in detail in chapters 2 & 3).
Together, these results offer new molecular-level insights into membrane tension-related
mechanostimulation and into the utility of DiI in characterizing tension-induced
changes in lipid packing. These results provide atomistic insights into stress induced
changes in lipid molecular interactions.

We demonstrated the applicability of lateral diffusion coefficient and fluorescence
lifetime of DiI for characterizing microviscosity of lipid bilayers, both qualitatively and
quantitatively. We measured the lateral diffusion coefficient and fluorescence lifetime of
DiI in various lipid bilayer phases in giant unilamellar vesicles: DOPC (fluid-disordered),
1:1 DOPC:Chol (fluid-ordered), 1:1 DPPC:Chol (fluid-ordered), and DPPC (gel).
Microviscosities of these different lipid bilayers were determined from the lateral
diffusion coefficients using the free-area theory. We also observed a strong correlation
between membrane microviscosity and fluorescence lifetime of DiI. So, we assessed
various solvent factors (e.g. viscosity and water content) that influence the photophysical
properties of DiI. A full description of the quantitative model for determining the lipid
packing and packing fluctuations from fluorescence lifetime of DiI was discussed in
chapter 4. Due to selective partitioning ability of different chain-length DiI’s, we also
measured the diffusion coefficient and fluorescence lifetime of DiI-C_{12} (short chain) and
DiI-C_{18} (long chain) in the above-mentioned lipid bilayer phases. We found that DiI
chain length has negligible effect on its diffusion coefficient and fluorescence lifetime
and that these photophysical properties are dictated by the lipid environment of the DiI
molecule. The propensity of these dyes to selectively partition in certain phases was
tested in ternary lipid mixtures with liquid-liquid phase-coexisting regions. Our results
show that DiI-C_{12} preferentially partitions to the liquid-disordered region whereas DiI-C_{18}
preferentially partitions to the liquid-ordered region. However, we also note that DiI-C_{18} partitioning is concentration-dependent, a property not well documented in the literature.

In summary, our results demonstrate the suitability of different chain length DiI’s to selectively label membrane phase domains in model membranes and endothelial cell membranes (previous work by Dr. Tristan Tabouillot), and track the dynamic microviscosity changes occurring in the membrane, under the influence of a chemical or mechanical stimulus.

We derived a quantitative relationship between fluorescence lifetime of DiI and solvent viscosity by measuring the radiative and non-radiative decay rates in solutions of varying viscosities. Additionally, we also derived a quantitative relationship between fluorescence lifetime of DiI and free-area. The method has been extensively tested by measuring area-per-lipid property of nanoliposomes prepared from different chain length lipids and comparing them to the existing X-ray/NMR measurements. Quantitative area-per-lipid measurements obtained here were in very good agreement with published results. For the first time, we also demonstrate the suitability of this fluorescence method for measuring thermo-mechanical properties (thermal expansivity coefficient and free-area compressibility modulus) of lipid bilayers in nanoliposomes. Furthermore, using different chain length dyes (DiI-C_{12} and DiI-C_{18}), we detect nanodomains of lipids in nanoliposomes. While ensemble lifetime measurements report average lipid packing, fluctuations of fluorescence lifetime naturally report the areal fluctuations in the lipid bilayer induced by thermal undulations. We introduce a new technique based on fluorescence lifetime fluctuation autocorrelation to detect membrane undulations with frequencies ranging up to 1KHz. We also demonstrate the detection of such high-
frequency thermal undulations in nanoliposomes. In summary, the fluorescence techniques developed in this thesis provide new means for quantitatively assessing lipid packing and undulations in realistic biological membranes such as cell plasma membrane.

Finally, we studied the lateral phase separation of lipid bilayers in binary and ternary mixture under the influence of various membrane additives (cholesterol, vitamin-E, Triton-X, and benzyl alcohol) that act as rigidifying/fluidizing agents. Lateral phase separation was determined using fluorescence microscopy of DiI-C₁₂ labeled giant vesicles. We found that membrane additives that affect the fluidity of the liquid-disordered region can control lateral phase separation. Specifically, we found that Vitamin-E acts as a rigidifying agent of the liquid-disordered region and addition of vitamin-E results in dissolution of membrane domains in both binary and ternary lipid mixtures. On the other hand, Triton-X and Benzyl alcohol, both acting as fluidizing agents promote domain formation or stability in binary and ternary mixtures. Using computational molecular dynamics simulations of DPPC bilayer with different membrane additives, we assessed the molecular interactions responsible for their roles as rigidifying and fluidizing agents. Finally, our experimental results suggest that enthalpic contributions (hydrophobic mismatch) dominate the entropic contribution (conformational flexibility of the acyl chains) to the free-energy of mixing a gel-phase lipid in fluid-phase. These results strongly support the importance of hydrophobic mismatch as a key determinant of lateral phase separation in lipid bilayers.
VITA

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EDUCATION

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AWARDS/ACHIEVEMENTS

• John C. and Joanne H. Villforth Graduate Scholarship Award, PSU, 2009.
• University Graduate Fellowship, College of Engineering, PSU, 2006.
• Merit Scholarship, National Institute of Technology, India, 2000-2004.
• “Nanoparticles delivering cargo can kill skin and breast cancer cells”, ScienceDaily, Dec 24, 2008.
• “Nontoxic nanoparticle can deliver and track drugs”, ScienceDaily, Nov 26, 2008.
• All-India 33rd Rank, Engineering Common Entrance Test, 2000. (Total entrants: > 100,000)

JOURNAL PUBLICATIONS


CONFERENCE PROCEEDINGS