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THE IMPACT OF MEMBRANE TENSION ON PHASE SEPARATION AND SOLID DOMAIN PROPERTIES IN MODEL MULTICOMPONENT VESICLES

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THE IMPACT OF MEMBRANE TENSION ON PHASE SEPARATION AND SOLID DOMAIN PROPERTIES IN MODEL MULTICOMPONENT VESICLES

A Dissertation Presented

by

DONG CHEN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Department of Physics
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To my family, for their love and support.
ACKNOWLEDGMENTS

The deepest gratitude goes to my advisor, Professor Maria Santore, who has been a teacher and a mentor to guide me through the tough project. She has given me the passion in doing research. She always has amazing ideas and encourages me to pursuit the new frontiers. I have gained great experience in research and motivation in exploring the unknown world.

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ABSTRACT

THE IMPACT OF MEMBRANE TENSION ON PHASE SEPARATION AND SOLID DOMAIN PROPERTIES IN MODEL MULTICOMPONENT VESICLES
SEPTEMBER 2014

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Directed by: Professor Maria M. Santore

Multicomponent phospholipid membranes provide an ideal model to study the complex phase behavior of biological membranes and are directly useful as drug delivery packages. Giant unilamellar vesicles (GUV) formed by mixtures of two or more phospholipids have particular merit as model membranes because of their simplicity, operability, and ease of viewing phase separation and testing membrane mechanics. Until the research in this thesis, biochemistry and biophysical studies of phase separation in phospholipid membranes primarily addressed the influence of membrane composition on the transition temperatures and domain shapes. While the state of understanding in the field had included the extent to which vesicle membranes might or might not be truly equilibrated, other key physics had been neglected, such as the mechanisms of domain formation and factors affecting domain morphology. This thesis focuses on a commonly neglected variable - membrane tension, analogous to pressure in bulk materials, as an important parameter to the phase separation.

By exploring the GUVs formed from mixed 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)
membranes, the study in this thesis comprehensively examines the thermodynamic impact of tension on fluid-solid membrane phase transition and the nature of phase-separated domains. The influence of the tension variable is addressed in terms of its differential thermodynamic effect, analogous to the Clausius Clapeyron equation, and also in terms of its more global thermodynamic effect, such as the phase diagram and domain morphologies.

Quantitative experimental studies of the temperature dependence on the membrane tension and compositions in DOPC/DPPC GUVs were mapped into a 3-dimensional (temperature-tension-composition) phase diagram. Studies employed micropipettes and osmotic pressure to control membrane tension. Depending on the system’s position in this temperature-tension-composition space, giant unilamellar vesicles containing DOPC and DPPC exhibited, in addition to a fluid $L_\alpha$ phase, two different types of solid phases – ripple $P_{\beta'}$ and tilt $L_{\beta'}$, presenting as patchy hexagonal and stripe domains respectively. At low tensions, patch-shaped solid domains formed from the homogeneous fluid phase, transforming into striped-shaped solid domains with further cooling. If the cooling rate was rapid, however, the patchy shaped domains were kinetically trapped and the striped solid was prevented from forming. At high tension, the striped solid domains were observed to form directly from and coexist with the fluid.

To resolve expectations from first principles with the observed large impact of tension to produce domains of dramatically different morphology, a two dimensional analog of the Clausius Clapeyron equation was developed and applied to experimental data. Small shifts in the observed transition temperatures for the formation of patchy and striped domains were shown to be quantitatively consistent with this first-principle
approach. Furthermore, agreement between first principles and the observed data was found only if the patchy domains were assigned the properties of the ripple $P_{\beta^r}$ solid and stripes assigned the properties of the tilt $L_{\beta^r}$ gel. Extending the first principle treatment to several mN/m of tension produced a triple-point like feature in constant-composition sections of the phase space. This feature explained the observed tension-directed production of patchy or striped solid domains on cooling.

Addressing the mechanism of domain formation, the study in this thesis also quantitatively examined the nucleation and domain growth in DOPC/DPPC vesicles. The observed increase of domain density with increases in the cooling rate and DPPC composition was consistent to the classical nucleation theory. Additionally, the domain density was found to be somewhat reduced by increases in membrane tension, consistent with the impact of membrane tension on line tension of the domain perimeter.

Finally, the study in this thesis extends the understanding of the thermodynamic space, including the impact of tension to the hybrid vesicles containing phospholipid and copolymer. Extensive studies employing model vesicles comprised of DPPC and an amphiphilic polydimethylsiloxane-polyethylene oxide graft copolymer revealed striking behavior. The hybrid vesicles exhibited parallel temperature-composition phase space, with only slight reductions in miscibility compared with DOPC/DPPC vesicles. Similarities were found in both the phase transition temperature and the effect of composition and cooling history on the nature and shapes of the solid domains. Further, the hybrid and DOPC/DPPC vesicles exhibited a similar tension-sensitivity for the types of membrane solids and domain shapes formed on cooling. The findings demonstrated that lamella-forming copolymer could be substituted for low melting phospholipid
components in membranes to enhance a membrane’s mechanical properties at the same time retaining key behaviors such as biomimetic multiphase behavior and responsiveness to tension.

The significance of this work spans fundamental membrane biophysics to new materials. The studies in this thesis demonstrated how tension controls the nature of solid domains formed in model biological membranes, relevant to signaling and trafficking, relevant to fundamental understanding of cell surface behavior. At the other extreme, this thesis has demonstrated utility of a copolymer in directing the organization of phospholipids, in response to temperature and tension, potentially useful in delivery applications or tissue scaffolding applications.
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CHAPTER 1
INTRODUCTION

This thesis addresses the role of membrane tension and thermal history in influencing the phase transition, domain morphology transformation and molecular organization in biomimetic model membranes. This work encompasses mixed phospholipid membranes and membranes containing mixtures of phospholipids and copolymers. This study extends the understanding of cell membrane physiology as well as the fundamentals of lipid rafts.

1.1 Phospholipid phases in bilayer membranes.

The membranes of cells, such as the plasma membrane and the membranes of internal organelles are not only functional barriers controlling the transport of substances; these membranes regulate trafficking and signaling through dynamic changes in their organization and shape. The membranes consist mainly of lipid bilayers with embedded functional proteins. The membrane functions depend greatly on the amphiphilic molecular structure of lipids and the bilayer’s lateral organization.

As the major component of membranes, phospholipids consist of a diglyceride or a ceramide, an organic molecule, bridged by a phosphate group. The head group of a phospholipid molecule is hydrophilic, while the alkyl chain backbone tails are hydrophobic. The hydrophilic head contains the phosphate group or other polar groups, while the hydrophobic tails usually consist of long fatty acid hydrocarbon chains. When placed in water, phospholipids self-assemble into lipid bilayers by lining up their
hydrophobic tails against one another, forming a membrane with hydrophilic heads facing the water on both sides.

In order to fully understand the behavior and function of the membrane, physical properties, especially phase transitions of isolated lipid bilayers, have been well studied. Two basic phases commonly exist in phospholipid membranes: the fluid (L\(_{\alpha}\)) phase and the gel phase. The fluid phase exists at elevated temperatures. Here the hydrocarbon chains are randomly arranged, and diffuse freely in the plane of membrane. The gel phase exists below the chain melting temperature. In the gel phase, the hydrocarbon chains are fully extended and close packed into ordered lattices – largely limiting lateral diffusion.

With some phospholipids, more complex phases are observed. When the phospholipid head groups are large enough so that their projected area exceeds that of the hydrocarbon chains in solid phases (PC, PS, PG\(^{-}\), etc.), a variety of solid phases are found below the phase transition temperature, including: P\(_{\beta'}\) (ripple), L\(_{\beta'}\) (tilted or gel) and L\(_{C}\) (subgel)-phases in a sequence of decreasing temperatures\(^1\). The complicated phases are due to the packing of hydrocarbon chains and head groups.

The thermodynamic and structural properties of single component lipid bilayers have been studied by many techniques including calorimetry and X-ray diffraction\(^2,3\). In cases where the bilayer phase exhibits a surface texture, the lipid phases have been examined by freeze fracture EM\(^4,5,6\) as well as other techniques like neutron diffraction\(^7\) and scanning-tunneling microscopy\(^8\). This freeze fracture EM\(^6\) technique gives simultaneous insight into the defect structure of the crystalline phases. Recently AFM has been used to provide a direct view of the surface profile of supported lipid bilayers in various phases\(^9\).
Figure 1.1: Molecular volume (open circles) and heat capacity (solid line) vs. temperature for DPPC bilayers in excess water\textsuperscript{10}.

1.2 Phase transitions and phase diagrams in single-phospholipid membranes and vesicles.

The transitions involving the four membrane phases described above are first-order in hydrated single-phospholipid bilayers and can be clearly seen in NMR measurements\textsuperscript{1}. The phase diagram of pure hydrated DPPC shows the typical phases and phase transitions that are characterized by molecular volume variations as well as heat capacity peaks [Figure 1.1]. The L\textsubscript{α} to P\textsubscript{β'}-transition at 41.5°C\textsuperscript{10} is the primary phase transition from a bilayer fluid to a rippled solid and is accompanied by a substantial molecular volume decrease and heat capacity change.\textsuperscript{10,11} This transition is associated with the transition of the hydrocarbon chains from flexible to a stiff and extended state [Figure 1.2]. Therefore it is called chain melting or main transition. There is an additional
transition at 35.5°C, which is the transition from P$_{\beta'}$ to L$_{\beta'}$ phases.$^{10,11}$ This solid-solid transition is often called a “pretransition”.

The initial recognition of different structures for of P$_{\beta'}$ and L$_{\beta'}$ solid phases increased general interest in solid membrane phases because of their interesting structures. P$_{\beta'}$ is characterized by periodic ripples with a periodicity of around 20 lipid molecules (≈ 130-150 Å) that may be doubled.$^9$ The acyl chains are oriented parallel to the bilayer normal, but tilted to the local surface normal [Figure 1.2]. The periodic arrays of ripples may bend at certain points in directions of 120° or 60°, which most likely indicates the underlying hexagonal packing of lipid acyl chains.$^9$ In 1979, Janiak et al. discovered that the hydrocarbon chain packing of P$_{\beta'}$ phase was a hexagonal array by X-ray diffraction.$^{12}$ They asserted that the ripple phase was a substructure superposed on a two-dimensional hexagonal lattice. The L$_{\beta'}$ phase is characterized by the chains tilted with respect to the bilayer normal. The tilted angles increase greatly from around 20° to 30° with respect to the local bilayer normal.$^{13}$ Because the local angle between the molecules and the surface of ripples is similar in both gel and ripple phases, the two solid packings are difficult to
distinguish by most methods including NMR and FRET and indeed, often a distinction is not made between ripple and gel solid phases.

1.3 The potential impact of tension on the behavior of mixed phospholipid membrane.

In order to model the physiology and phase behavior in complex biological membranes, two or more components of lipids (phospholipids, sphingolipids, and cholesterol, etc.) are mixed to form biomimetic membranes. These membranes are in various forms: supported bilayers, multilamellars, or giant unilamellar vesicles, which are examined by different experimental techniques. Studies in these multicomponent membranes have revealed complex phase behavior and presented various domain shapes. The phase study in vesicles containing multiple lipid mixtures have focused on the phase transition temperatures and the composition dependence. Other important variables, such as membrane tension, have not been considered as a critical parameter in the phase characterization and phase diagrams of lipid membranes. As a mechanical variable, tension can stretch or bend uniform bilayers, affecting the phase transitions, which has been evidenced by micropipette aspiration methods. Relevant to the current focus on the thermodynamic role of tension, tension was shown reduce the liquid-liquid coexistence temperature slightly in cholesterol-containing phospholipid vesicles.

Uline et al. applied Flory’s rotational isomeric states model to single- and three-component planar bilayers to predict the impact of tension on the fluid-solid and fluid-fluid phase transitions, respectively. They anticipate tension will reduce the single component transition temperature on the order of 1.0 K/(mN/m), though their study did
not distinguish ripple and tilt solid phases, which could have an important impact due to substantial differences in their molecular areas. They also predicted that tension would stabilize the liquid disordered phase of the three-component system.

The predictions for the three component system were consistent with the results of Portet, et al. in the vesicles composed of 1:1:1 molar ratio of diphytanoylphosphatidylcholine (DiPhyPC), dimyristoylphosphatidylcholine (DMPC), and cholesterol. In the process of cooling, a reduced liquid-liquid phase separation temperature was observed in vesicles whose tensions were increased using micropipettes. The slope on the tension-temperature phase diagram is $-2.8 \pm 0.7 \text{ K/(mN/m)}$, slightly higher than the value predicted by the model proposed by Uline et al. Such measurement of the impact of tension on the transition temperature comprise a piece of understanding, but may miss interesting features of thermodynamic space that encompasses multiple phases and polymorphs such as the ripple and tilt solid phases. The binary system that allow for the possibility of these complex features may exhibit rich interplay between molecular organization, domain shape and the fundamental variables of temperature and tension. Given the constrained-equilibrium nature of vesicles, additional richness may result from processing history.

1.4 Model giant unilamellar vesicles.

Giant unilamellar vesicles (GUVs) provide a model to examine physical properties, especially the phase behavior of the phospholipids in an unsupported membrane. The simplest systems studied to date have been GUVs formed by single or simple mixtures of diacyl-phospholipids. GUVs are usually prepared by the electroformation technique originally developed by Angelova and Dimitrov. This
method can effectively provide giant unilamellar vesicles, as opposed to multilamellar vesicles.

Scientific questions and properties best addressed using giant unilamellar vesicles rather than monolayer or supported bilayers include: (1) measurements of the phase diagram free from the influence of subphase-related artifacts; (2) the influence of membrane tension on the phase diagram and the phase domains; (3) the influence of curvature on phase composition and structure and (4) dynamic membrane behavior and the influence of a subphase.

While some of these issues could be addressed with smaller systems, for instance, small vesicles via NMR or FRET, GUVs were utilized here for their unique properties and benefits: (1). GUVs are large enough to be directly observed by optical microscopy, providing information about the phase diagram and domain shapes; (2). GUVs have only one bilayer of membrane, the phase behavior is clear; (3). The membrane is fully hydrated and isolated free from a substrate. In order to focus on the phase behavior of one or two phospholipids, a simple two-component system is chosen as the focus of this thesis. Two diacyl-phospholipids - 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) are strategically selected: they have the same head groups (phosphocholine), similar chain length, but they have a great difference in phase transition temperature (-17±1°C for DOPC and 41.5±0.5°C for DPPC). GUVs that contain these two lipid components are fully mixed at elevated temperatures. When GUVs are cooled below phase transition temperature, nearly pure gel domains grow, leaving the rest of the mixture in fluid state. The gel domains grow gradually in size with the temperature decreasing. Such phase behavior is commonly
observed for DPPC-contained vesicles with different secondary components.\textsuperscript{16, 19} The domain size and shape are determined by the molecular structure and other factors, including the choice of the unsaturated phospholipids.

\textbf{1.5 Solid domain nucleation and growth.}

Studies in phospholipid monolayers have revealed the dynamics of solid domain formation and evolution, which are corresponding to either component interaction\textsuperscript{32} or lateral compression\textsuperscript{33}. In supported bilayers, nucleation has been found to be critical in the process from nanoscale to mesoscale domain formation\textsuperscript{9, 34, 35, 36} High resolution scanning via freeze-fracture electron microscopy\textsuperscript{6} and atomic force microscopy\textsuperscript{9} has found the super structure forming from the planar liquid phase, which are corresponding to the L\textsubscript{\alpha} to P\textsubscript{\beta'} phase transition.

There is evidence that cooling DPPC-containing two-component vesicles from the single-phase region produces solid domains by a nucleation and growth mechanism.\textsuperscript{16, 37, 38} Especially, when systems do not fully equilibrate, mechanisms such as nucleation can be critical in determining the ultimate morphology, number density and size of domains. Investigating the nucleation and domain growth in simple binary mixed lamellae provides a foundation for understanding the domain formation in more complex membranes.

\textbf{1.6 Domain shape transformation.}

Varieties of domain shapes has been experimentally observed in phase separated vesicle membranes including round domains in the liquid-ordered systems and flower, hexagonal, or stripe shapes in the solid domains in systems with fluid-solid coexistence. In spite of the diversity of domains, factors dictating these shapes are not well-established.
Schneider et al.\textsuperscript{39} proposed a model to investigate the shapes of crystalline domains on the spherical vesicles. They have examined the stretching energy in several predefined shapes: disk, ring, and ribbon and determined the most stable phase for a given domain ratio and line tension. In the region of small domain area fraction, the shape of “caps” minimized the stretching energy and dominated the vesicle sphere. When the domain area fraction is increased to the order of 0.1, the ribbons are stable and dominant shapes. This theory is based on the stretching energy of various domain shapes in a given crystalline area occupation and line tension, neglecting the dynamic process of domain formation and growth. In the process of phase separation in the vesicles, small domains appear right below the phase transition temperatures, growing larger with further cooling. The shapes are usually determined before the first observation of the theses domains, rooting to the underlying phases and fundamental molecular structures.

Not included in the approach of Schneider et al. is the fundamental connection between molecular ordering and preferred domain shape, even in the limit of a planar membrane. The issue of domain shape therefore is particularly complicated in systems where equilibration involves a preference for one or another molecular organization and these considerations are combined factors such as line tension, curvature, and bending. While this thesis does not address the issue of what domain shapes should be expected for particular molecular orderings or vesicle parameters, it does make specific observations about domain shape and size as a function of thermodynamic and processing considerations. This thesis also makes arguments that map ripple and tilt molecular organization to the domain shape.
1.7 This thesis.

This thesis examines the factors affecting the phase behavior and domain shape in a model phospholipid system, focusing on membrane tension. While extensive work on the phase behavior of multicomponent phospholipid bilayers has been conducted over the past decade, this thesis will distinguish itself from the prior studies by (1) its focus on giant unilamellar vesicles, which enable visualization of the phase domains (2) attention to membrane tension, and (3) systematic variation in the thermal history and composition of the vesicles. The thesis deals not simply with the shifting of a previously established phase diagram with variations in tension and history (for instance described by Clausius Clapeyron), but the ways in which these variables appear to produce entirely different domains.

In particular, this thesis describes the observation of two distinctly different types of solid DPPC-rich membrane domains, “stripes” and “patches”, and provide evidence for differences in their underlying molecular organization. This thesis emphasizes the impact of membrane tension on the phase transition and domain morphology transformation. This work addresses how cooling rates, from the one phase to the two phase region of the phase diagram, affect which phases are preferred. Results are summarized in the form of state diagrams and the relationship of these data to the equilibrium phase diagram is discussed. The study further addresses the size and regularity of the domains, their areal density related to the nucleation rate, also a function of thermal history and tension, and the ultimate area covered by the different phases. DOPC/DPPC two component giant unilamellar vesicles comprise the model system,
chosen to the intricate solid membrane phases that it forms, and because it is historically well-studied.

Chapter 2 describes systematic and highly quantitative study of the thermodynamic impact of membrane tension, over the full range of tensions accessible, on the phase behavior of two-component phosphatidylcholine (PC) lipid vesicles. This chapter examines the specific composition of DOPC/DPPC (30 mol% / 70 mol%) in order to focus on the function of membrane tension.

In Chapter 3, we extend the study of phase behavior of DOPC/DPPC vesicles at the single molar composition (DOPC/DPPC 30:70) to include composition dependence over the full range. The formation of patch-shaped and stripe-shaped domains, rich in DPPC, is further investigated, over the full range of tensions, and from this the 3D phase diagram is mapped out.

Chapter 4 examines the mechanism of domain formation, nucleation and domain growth. Two component DOPC/DPPC giant unilamellar vesicles are continued to be used as a model system. This chapter develops a strategy for determining the nucleation density at different conditions, based on counting the number of domains visible at room temperature. In doing so, arguments are developed for the local equilibrium state of vesicles at room temperature, including an investigation of the liver-arm rule. In studies of nucleation itself, the studies focus on nucleation at low and moderate tensions, accessing the formation of patchy and straight edged hexagonal domains, which are usually compact (but can in other circumstances become dendritic). These compact domains are distinct from striped domains reported by us and others. In addition to
measuring the dependence of the nucleation rate on the cooling rate and composition, the study probes the influence of tension on nucleation.

Chapter 5 extends the new understanding of membranes, developed with the model phospholipid membrane system to hybrid membranes in which a copolymer and DPPC are blended. Critical to the choice of this model, the copolymer employed is a PEG-grafted polydimethylsiloxane copolymer DC5329 that, on its own, forms bilayer membranes and vesicles. The miscibility of phospholipids and copolymers has brought broad interest due to the potential application in bio-inspired materials. By replacing the “fluid solvent” DOPC from Chapter 2-4 with copolymer, the study probes the parallels and underlying chemical physics of DPPC in mixed membranes.

1.8 Reference.


CHAPTER 2

LARGE EFFECT OF MEMBRANE TENSION ON THE FLUID-SOLID PHASE TRANSITIONS OF TWO-COMPONENT PHOSPHATIDYLCHOLINE VESICLES

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2.1 Introduction.

Phospholipid vesicles, capsular lamellar assemblies of phospholipid amphiphiles, are model systems that have advanced our perceptions of material surfaces and thin films, facilitated drug delivery technologies, and anchored the understanding of biological membranes to fundamental physics. Extensive studies of phase transitions in phospholipid bilayers and vesicles have focused almost exclusively on temperature and composition, revealing complex phase behavior1, 2, 3, 4, 5, 6 and beautiful patterns in the domain shapes within vesicle membranes.4, 6, 7 Tension has been mostly neglected as a thermodynamic variable and is unspecified in phase diagrams of vesicle membranes, though in analogous studies of phospholipid monolayers, surface pressure is known to drive transitions between gas-like layers, liquid fluids and ordered crystals, which are sometimes polymorphic.8, 9, 10 Besides its fundamental thermodynamic importance, membrane tension may be biologically important, since stresses on cells can dominate their interactions11, 12, 13, 14 and fates.15 Indeed, tension has been proposed to regulate the
dynamic structure of the cellular surface, for instance through coupling with curvature \(^{16}\) or by clustering proteins in “rafts” \(^{17,18}\).

Employed as a mechanical variable, tension can stretch or bend uniform bilayers. \(^{19,20}\) In multicomponent vesicles containing coexisting fluid domains, coupling of line tension with membrane bending determines vesicle shapes and drives budding transitions. \(^{21,22,23,24}\) Tension has also been hypothesized, but not confirmed, to influence the shapes of solid domains. \(^{25}\) Relevant to the current focus on the thermodynamic role of tension, tension was shown reduce the liquid-liquid coexistence temperature only slightly (a fraction of a Celsius degree for each 0.1 mN/m in tension) in cholesterol-containing phospholipid vesicles. \(^{26,27}\)

In the current work, we systematically examine the broader thermodynamic impact of membrane tension on the phase behavior of two-component phosphatidylcholine (PC) lipid vesicles: Phosphatidylcholine (PC) amphiphiles are an important class of molecules because of their prominence in cell membranes. Important to note, their relatively large hydrated head groups enable PC lipids, in single component membranes, to order into solid bilayers of differing molecular areas (by about 10\(^\%\)) \(^{19}\), such as ripple (P\(_{\beta'}\)) and tilt (“gel” or L\(_{\beta'}\)) phases. \(^{19,28,29,30,31}\)

In order to quantitatively probe the impact of membrane tension on the fluid-solid transition(s) of two-component PC bilayers, giant unilamellar vesicles containing 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), were chosen as model membranes. This work focuses on the impact of tension and temperature at the single membrane composition of 30 mol\% DOPC / 70 mol\% DPPC, taking a constant-composition slice through thermodynamic
space, which qualitatively reflects behaviors at other compositions. Well-hydrated DOPC lamellae melt at $T_m = -17 \pm 1^\circ C$,\textsuperscript{32} while hydrated DPPC bilayers have a higher main transition $T_m = 41.5 \pm 0.5^\circ C$ and a so-called \textit{pretransition} at $T_p = 35.5 \pm 0.5^\circ C$.\textsuperscript{28,33} The ripple solid is found below the main transition temperature while a tilt gel phase is observed below the pretransition temperature; however, many studies of DPPC-containing mixtures cannot distinguish these two solids.\textsuperscript{6,34} Cooling mixed vesicles from the miscible fluid regime at elevated temperatures produces solid-like membrane domains comprised predominantly of DPPC.\textsuperscript{3,34,35} Indeed, cooling from the one phase region is the typical protocol for reliably creating solid domains and measuring transition temperatures.

We report here that in two component phosphatidylcholine membranes, even though the main fluid-solid transition temperature is only mildly reduced with increased tension, consistent with first principles, tension alters the equilibrium and the nature of the solid domains within the fluid membrane. The latter include the molecular ordering and shapes of the domains. Additionally, by shifting a second coexistence line in the opposite direction from the main transition curve, tension produces a triple point-like feature at their intersection. Tension can thus be manipulated to select which solid phase forms on cooling, in turn directing domain morphology.

2.2 \textbf{Results}.

2.2.1 \textbf{Tension selects between different solid phases}.

Figure 2.1 illustrates the dramatic impact of tension, imposed by micropipettes and held constant over the course of an experiment, on the phase behavior of 30/70 mol-
percent DOPC/DPPC vesicle membranes. These vesicles also contain 0.1 mol\% Rh-DPPE fluorescent tracer, \(1,2\text{-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-}
(lissamine rhodamine B sulfonyl) \(\text{ammonium salt}\) ). In Figure 2.1, unilamellar vesicles were aspirated into micropipettes at 43°C (the miscible liquid region) and then cooled at 1-2°C/min at fixed tension into the two phase region. When tension was held well below \(\sim 3\) mN/m, solid-like DPPC-rich \(^3,^{34,35}\) domains that exclude tracer coexist with a fluorescent fluid membrane. In contrast, with fixed membrane tensions of 3 mN/m or greater, no dark solid domains appear on cooling. Instead, phase separation produces finely striped domains that appear brighter than the fluorescent fluid. The solid domains formed at low tensions differ in their shapes and ability to incorporate tracer dye compared with those formed at elevated tension. This suggests different molecular arrangements in two solids.
**Figure 2.1:** Images of vesicles held at fixed tension (indicated) and cooled from 43 °C at 1-2°C / min. Scale bar is 10 μm. Vesicles contain 0.1 mol% Rh-DPPE tracer.
Figure 2.2: Patchy and stripe domains in vesicles formed on cooling from the one-phase region. Parts A and C contain the dye Rh-DPPE; Parts B and D contain the dye Rh-DOPE. A and B have been manipulated to maintain low tension. They contain 0.2 M sucrose inside and 0.25 M sucrose outside. C and D contain DI water both inside and outside. C and D maintain a higher tension averaged at 2 mN/m (further discussed in text). The scale bar is 10 μm.

In addition to precise tension control with micropipettes, tension was manipulated osmotically in a second type of experiment. The separate use of a closed chamber precluded micropipettes, but it additionally facilitated better imaging with a higher numerical aperture objective, more precise temperature control, longer experiments with a broader range of cooling rates, and immediate access to multiple vesicles. Osmotically-conditioned vesicles, cooled from the one-phase fluid region in a sealed chamber,
confirm in Figure 2.2 at room temperature, the same qualitative influence of tension on solid domain formation seen in Figure 2.1. The Rh-DPPE tracer reveals dark patches or imperfect hexagons on vesicles maintained near zero tension. Vesicles subjected to elevated tensions during cooling (in DI water at 1°C/min), exhibit bright striped solid domains at room temperature, in parallel with the pipette studies above 3mN/m in Figure 2.1. In additional cooling experiments employing a different tracer, 0.1 mol% Rh-DOPE, (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) ), vesicles exhibit, in Figure 2.2 at near-zero tensions, the same type of dark patches observed with Rh-DPPE. At elevated tensions, however, Rh-DOPE is excluded from the dark striped domains. The selective incorporation of Rh-DPPE but not Rh-DOPE into the stripes and the exclusion of both dyes from the hexagonal patches, further argues for different molecular ordering in the patches and stripes.

2.2.2 Domain evolution during Cooling.

Monitoring domain formation facilitates an estimate of the transition temperature and provides additional insights. The image sequence in Figure 2.3a, for a freely-suspended vesicle rapidly cooled at 5 °C/min from the single phase region suggests that solid-like domains form via nucleation and growth. Patches clearly visible at 38.4 °C, just below the transition temperature, grow in size but not in number with decreases in temperature. With relatively fast cooling rates in the range 1-5 °C/min, here in the closed chamber but also in the micropipette studies in Figure 2.1, stripes do not appear at low temperatures (and tensions.) The rapidly grown-patches, growing to diameters of several microns, are kinetically trapped though, as we show next, the striped solid phase is preferred at cool temperatures.
**Figure 2.3:** Sequenced images of vesicles phase separating on cooling in the closed chamber. Scale bar is 10 μm. 0.1 mol% Rh-DOPE is used in all vesicles. (A) single vesicle cooled at 5°C/min. (B) Sequence of vesicles, each typical of all the others at a given temperature in a single run, cooled at 0.1°C/min at low tension.

When a similar suspension of vesicles, having substantial excess areas (relative to a sphere of equal volume) and a near-zero membrane tension, is cooled very slowly at 0.1 °C/min, patchy domains first appear in Figure 2.3B near the same transition temperature, Tm, seen for faster cooling in Figure 2.3A. As cooling proceeds very slowly, stripes appear near a temperature of 31.7°C (in this particular run). The stripes always emanate from or intersect the previously-formed patches and, with continued very slow cooling, gradually replace the patches. The appearance of the first stripe and the disappearance of the last patch happen in a sufficiently short time, a minute or two, that we hesitate assert a finite transition window. Such an additional multiphase region may exist, but if it does, it is less than half a degree tall. At temperatures below the pretransition, the vesicles display only stripes. Indeed the observation, for a vesicle composition of 30 mol% DOPC / 70 mol% DPPC, of a main transition at ~38-39°C and a
second at 31-32°C is consistent with the main and pre-transitions for pure DPPC at a few degrees higher.\textsuperscript{36} This further suggests a ripple phase in the patches and a tilt (gel) phase in the stripes. Notably, we have not observed patches turning into stripes in the micropipettes, because micropipette studies cannot be run sufficiently slowly in the open-sided chamber.

### 2.2.3 Phase Diagram.

Experimentally-determined transition temperatures (measured in runs like those in Figures 2.1 and 2.3), are summarized in the Temperature-Tension (T-\(\tau\)) space of Figure 2.4 and reflect the accuracy, within a standard deviation of 0.5°C for 10 vesicles at each datum, of determining the initial appearance of micron-scale domains. Near zero membrane tension, the temperatures recorded for the first appearance of patches, near \(T_m =38.3 \pm 0.5°C\), were consistent between micropipette (hollow points) and free vesicle experiments (solid points for the sealed chamber), with little effect of the cooling rate on the transition temperature. The zero-tension datum (triangle) for the appearance of stripes following the appearance of patches (like Figure 2.3B) near \(T_p =31.6 \pm 0.5°C\), comes exclusively from free vesicle experiments with osmotic conditioning using sucrose solutions in the closed chamber to produce zero tension.
Figure 2.4: Temperature-Tension phase diagram for DOPC/DPPC vesicles with a fixed overall composition of 30/70 mol-percent. Experimental data for the first appearance of patches (squares) on cooling occur at increasingly lower temperatures as tension is increased. Triangles summarize experimental data for the appearance of stripes after the appearance of patches. Hollow points are measured in micropipettes. Solid points are measured in the closed chamber.

In particular, the solid triangular datum, centered at 2 mN/m and ~35°C was obtained in the closed chamber in DI water, as described in the Materials and Methods Section on Elevated Tension in the Closed Chamber. The tension estimate for this data is based on studies described in the “Support information”, Section “Free Vesicles with Elevated Tension” and “Summary of Tension in Free Vesicles”.

Points with error bars are an average based on 10 vesicles with the error bars indicating the full range of temperature data. Hollow circles above $\tau = 3$ mN/m are
individual points for the appearance of stripes directly from the fluid phase, without patches first. Lines with slopes calculated according to the modified Clausius-Clapeyron equation (2.6) are drawn from the observed zero-tension data.

At elevated tensions, micropipettes maintained tension constant within a resolution of 0.02 mN/m during vesicle cooling. For each of at least 10 vesicles per datum (hollow squares), the temperature corresponding to the first appearance of patches from the uniform fluid was noted. Figure 2.4 contains additional individual data (hollow circles) for the appearance of stripes directly from the one-phase fluid at tensions exceeding 3mN/m, similar to Figure 2.1C. The weak tension dependence of the liquid-solid transition temperatures in Figure 2.4 parallels the weak decrease of the liquid-liquid transition temperature with tension reported by Portet et al. for their cholesterol-containing system.26

Figure 2.4 also includes a single datum (solid triangle), averaging 10 vesicles, for the appearance of stripes after patch formation, during slow cooling at 1 °C/min at elevated tension in DI water in the closed chamber. Here, the observed transition temperatures fall within a narrow range (34-36 °C), but the elevated tension at the instant of phase separation carries a large uncertainty, since the tension of free vesicles varies during cooling in deionized water, detailed in the Support information. Despite the large uncertainty on the tension of this particular datum, this point reveals a substantial tension-sensitivity of the temperature for the conversion of patches to stripes.

To demonstrate how the observed tension-mediated switching between phase-separating patches or stripes is consistent with first principles, the lines in Figure 2.4 summarize the tension-sensitivities of the coexistence temperatures, from a model based
on first principles. The treatment, detailed in the Support information, starts with the general differential equilibrium criteria (equality of pressures, temperatures, and chemical potentials) for a two-component mixture of $A$ and $B$ partitioning between phases $\alpha$ and $\beta$:

\[
\left(\frac{\partial P}{\partial T}\right)_{x_A^\alpha(\alpha-\beta)} = \frac{1}{T} \frac{x_A^\alpha (R_A^\alpha - R_A^\beta) + x_B^\beta (R_B^\beta - R_B^\alpha)}{x_A^\alpha (\bar{v}_A^\alpha - \bar{v}_A^\beta) + x_B^\beta (\bar{v}_B^\beta - \bar{v}_B^\alpha)}
\]

(2.1)

Here, $P$ and $T$ are pressure and temperature, $x$ is mole fraction, and $\bar{H}$ and $\bar{V}$ are partial molar enthalpies and volumes. Subscripts identify components while superscripts identify the phase.

For a membrane, a two dimensional analog of this equation replaces pressure $P$ by negative membrane tension $\tau$ and partial molar volume $\bar{V}$ by the partial molar area $\bar{A}$.

For example, for the influence of tension on the temperature for the first appearance of patchy solid hexagons, superscript $\beta$ is assigned to the liquid phase and $\alpha$ to the ripple-solid. Upon cooling a single-phase liquid mixture to the temperature of initial phase separation, the liquid composition is fixed, which gives the subscript, $x_{DPPC}^{liq}$ (70% in our studies) on the left side:

\[
\left(\frac{\partial \tau}{\partial T}\right)_{x_{DPPC}^{liq}(solid-liq)} = -\frac{1}{T} \frac{x_{DPPC}^{solid}(H_{DPPC}^{solid} - H_{DPPC}^{liq}) + x_{DPPC}^{solid}(H_{DOPC}^{solid} - H_{DOPC}^{liq})}{x_{DPPC}^{solid}(\bar{A}_{DPPC}^{solid} - \bar{A}_{DPPC}^{liq}) + x_{DOPC}^{solid}(\bar{A}_{DOPC}^{solid} - \bar{A}_{DOPC}^{liq})}
\]

(2.2)

With solid domains of nearly pure DPPC, we approximate $x_{DPPC}^{solid} = 1$ and $x_{DOPC}^{solid} = 0$. Then, using the latent heat of pure DPPC (between a liquid and ripple phase) and an estimate of the specific area difference between the pure ripple solid and the pure DPPC fluid, the tension sensitivity of the transition temperature, $dT/d\tau = -0.56$ Km/mN, follows. Related property data for the transition from a ripple to a tilted solid in these references anticipates a slope, $dT/d\tau = 1.8$ Km/mN, for the lower
temperature coexistence curve. Lines having these two slopes were drawn in Figure 2.4, starting with the experimentally-measured zero-tension transition temperatures. Another line with the slope \( dT/d\tau = -0.29 \text{ Km/mN} \), for the fluid tilt-solid transition is calculated in a similar fashion and added starting from the intersection of the first two coexistence lines. Calculations in the Support information argue that the exact values of these slopes will not be dramatically influenced by the presence, on the order of 5-10%, of DOPC in the solid phase.

Worth noting, the calculation for the first appearance of patches or striped domains from a completely fluid vesicle is straightforward. The application of this approach to the patch to stripe (ripple to tilt solid) transition (the lower branch of the phase diagram) makes the simplification that this coexistence is dominated by equilibrium between the two types of solids, neglecting the influence of potential changes in fluid composition through the transition. This implicit simplification appears to match the data very well, thus highlighting the dominant physics at play. A possible temperature window, too small to be resolved in our experiments, between the first appearance of stripes and the disappearance of the last patch is neglected by this simple treatment.

Figure 2.4 reveals that the patchy domain (or solid ripple) phase persists in only limited conditions, terminating in a triple point-like feature estimated near a tension of 3mN/m, the exact value being a result of the linear approximation for the coexistence lines. We use the term “triple point” with some caution, as it represents the intersection of coexistence lines and termination of the ripple-solid region. The feature behaves as a triple point in pseudo-one component treatments that consider two pure solid DPPC
phases. The real multicomponent system has fluid-solid coexistence in each “solid” region of the phase diagram (as indicated by the phases seen on the vesicles) and therefore includes coexistence of two solid domains and a fluid along the lower phase boundary (blue).

Most important, the existence of the triple point-like feature is ensured by the opposing slopes of the liquid-ripple solid and ripple-tilt (gel) solid transition curves. This opposition in slopes is unlikely to be eliminated by more refined theory, as long as the areal density of the ripple solid exceeds that of both the fluid and tilt-solid phases over the full range of tensions. Also important, the triple point-like feature is reported here for the specific composition of 30/70 DOPC/DPPC. The locus of such “triple points” for varied DOPC/DPPC content will form a curve in 3-dimensional thermodynamic space.

2.3 Discussion and conclusions.

In this report, we demonstrated how, upon cooling giant unilamellar vesicles from the one phase to the two phase region, membrane tension plays a critical role in determining which of two types of solid-like domains form. The differences in the resulting domains include their formation temperatures, shapes, and ability to incorporate tracers, all suggesting fundamentally different molecular ordering. Thus the two types of solid domains represent thermodynamically different phases, and will occupy different regions of the equilibrium phase diagram.

This study demonstrated that while tension (above or below the “triple point” near 3 mN/m for this overall membrane composition) determines which solid initially forms from the single fluid phase on cooling, other considerations determine the ultimate vesicle morphology. While this can seem complicated, our series of observations are
consistent with the phase diagram mapped in Figure 2.4. For instance, while we have never observed patches at tensions above about 3 mN/m, the stripes (dominant at high tensions,) can also be observed at low tensions. For cooling at tensions below 3 mN/m, however, striped domains are found only after the observation of patches. Stripe formation at low tensions is also influenced by the cooling rate. If, at low tensions, cooling is rapid, the patch-shaped domains become large (and kinetically trapped) then stripes are not seen on experimentally accessible timescales. If cooling is very slow at low tensions, in Figure 2.3B, stripes form while the patches are consumed. We believe the slower cooling history reveals the equilibrium preference for stripes at low tensions and temperatures below a second lower phase transition temperature. The kinetic barriers between the patches and stripes are sufficiently large at room temperature that we find no evidence, based on domain shape, that micropipette manipulation of patchy membranes can shift the domains to tilt-containing stripes.

Additional observations support the fundamental nature of the transition from patches to stripes, particularly at low tensions. If, for instance, vesicles are cooled at low tension from the one phase region and held at fixed temperature above the lower branch of Figure 2.4, stripes do not develop in time. Stripes may ultimately, however, be observed at lower temperatures if cooling is resumed sufficiently slowly. The observation that at low tensions, stripes, if they form at all only form below a distinct tension-dependent (phase transition) temperature, suggests that stripes form when a boundary is crossed in thermodynamic space.

It is significant that we observe two distinct transition temperatures in the 30/70 DOPC/DPPC mixture near zero tensions, because this behavior runs parallel with
literature reports of main and pretransitions for pure DPPC.\textsuperscript{28, 33} For pure DPPC in a hydrated bulk sample where the lamella are, presumably at low tension, the ripple phase is known to occur below the main transition but above the pretransition temperature.\textsuperscript{28} A planar tilt solid occurs below the pretransition temperature. Our mapping of experimental data in the phase diagram of Figure 2.4 does not require us to interpret our stripe versus patchy hexagon data in terms of these molecular structures; however, when calculating estimated phase boundaries, we require physical properties for the solids within the patches and stripes. It is only at this point, in the calculations, where we assign the ripple structure to the patches and the tilt to the solid phase. The qualitative agreement between the calculated boundaries and those observed data supports this interpretation, assigning ripple and tilt molecular structures to the patch and striped domains, respectively.

While it may seem obvious that the tilt phase, with its slightly lower areal density of phospholipid molecules compared with the ripple phase, would be preferred at high tensions, it is also interesting that the tilt phase is associated with stripe-shaped domains and that these stripes tend to run long and straight over vesicle surface. The stripes prefer to intersect each other rather than bend, for instance into ring-like structures. Long thin stripes that wrap the vesicles are observed bend mostly in their long direction, a behavior which may minimize the cost of bending energy and which may also be consistent with a tilt rather than ripple molecular ordering.

With ripple and tilt bilayers, seen for DPPC and characteristic of a broad class of PC lipids,\textsuperscript{31} the phase space in the tension dimension reported in Figure 2.4 is potentially generalizable to other PC lipids. Indeed, because the ripple phase is generally denser than the tilt phase, with the fluid phase being the least dense of the three phases, three-phase
coexistence should occur over a range of overall membrane compositions. Moreover, the phase diagram in Figure 2.4 explains why, for systems like the DOPC/DPPC mixture, the impact of tension generally transcends the small shift in melting temperature anticipated by thermodynamic fundamentals. Still keeping with first principles, the presence of the triple point-like termination of the ripple-solid phase region allows tension to select between the types of solid phases and the domain shapes. Cooling vesicles at elevated tensions directly produces striped domains that are consistent with tilt phase. At lower tension, patchy solid-like domains, likely containing a ripple structure, tend to form and may become kinetically trapped, depending on thermal history. The phase diagram further suggests that, at appropriate temperatures, small changes in tension shift the equilibrium from one type of solid phase to another. The observation of solid phases in bilayers containing as much as 30% cholesterol suggests that this role for tension, shown in Figure 2.4, may be accessible to biological cells as a means to alter the structure of their bilayers and thereby regulate protein interactions and signaling.

2.4 Materials and methods.

2.4.1 Phospholipids and giant unilamellar vesicles.

DOPC and DPPC (1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, respectively) were purchased from Avanti Polar Lipids (catalog numbers 850375C and 850355C) and used as provided. Tracer lipids Rh-DOPE [1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt, catalog number 810150C] and Rh-DPPE [1-2-
dipalmitoy-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt, catalog number 810158C] were also purchased from Avanti.

Giant unilamellar vesicles were produced using established electroformation methods on platinum wires.\textsuperscript{40,41} Phospholipids in the desired molar proportions were dissolved in chloroform at a concentration near 1 mg/ml. When fluorescent tracer was employed, it was used at a concentration of 0.1 mol\% relative to the total phospholipid. (No impact of the tracer concentration, in the range tested from 0.1- 0.5 mol\% was found on the phase separation temperature, or the types of solid domains (stripes or patches) observed). To produce vesicles, a 10 μL quantity of phospholipid solution was placed droplet-wise on the platinum wire electrodes of the electroformation chamber. The chamber was dried under nitrogen then sealed between two glass coverslips and filled with DI (de-ionized) water or sucrose solution that had been preheated to 52°C. (Maintaining the chamber within the 1-phase region at elevated temperatures ensured uniform vesicle compositions.) An alternating current was applied to the electrodes at 3V and10 Hz for 1 hour, while the chamber was maintained at 52°C. After electroforming, vesicles were harvested in a syringe.

2.4.2 Micropipette aspiration.

Micropipettes were pulled on a Kopf Instruments Micropipette Puller and their tips were shaped on a Technical Products International Microforge. Inner diameters were in the range of 3-6 μm, and the tip shape was chosen so that the inner diameter was nearly constant in the range where the vesicle projects into the pipette. Micropipettes were conditioned by adsorbing bovine serum albumin (Sigma catalog number A7511) to prevent vesicle adhesion. Vesicles were chosen in the 15-50 μm diameter range. For
quantitative studies the vesicle diameter was chosen to be at least three times the micropipette diameter to avoid computational error in calculating pressure and area.

2.4.3 Osmotic pressure to maintain zero tension in the closed chamber.

To produce flaccid vesicles with near-zero tensions, vesicles electroformed in 200 mOsm sucrose solution were equilibrated in 250 mOsm sucrose solution. Vesicles become “floppy” only after they equilibrate by water diffusion out of the vesicles across the membrane, a process occurring on the order of several minutes.\textsuperscript{42} The resulting excess area, $A_{xs}$ is defined relative to a sphere of the same volume

$$A_{xs} = \frac{A}{(\frac{36\pi V^2}{\pi})^{\frac{1}{3}}} - 1 \quad (2.3)$$

Here $A$ is the measured vesicle area and $V$ is the measured vesicle volume, both determined from videomicrographs of individual vesicles aspirated into micropipettes at low suction (corresponding to membrane tensions near 0.02 mN/m).

2.4.4 Elevated membrane tension in DI water in the closed chamber.

Vesicles formed and equilibrated in DI water at elevated temperatures did not exhibit excess area at the elevated temperature or upon cooling, for instance at 1°C / min. These vesicles instead sustained membrane tension that was sufficiently high to alter solid domain morphology in vesicles inside the closed temperature control chamber. High membrane tensions develop because of the thermal contraction of the membrane (an effect greater than the contraction of the internal water) on cooling. Opposing this effect and tending to relieve the stress, water diffuses out of the vesicle across the membrane. Fast thermal membrane contractions relative to water diffusion produced membrane
stress that relaxes on timescales of tens of minutes, making its measurement challenging. Detailed studies in the Support information demonstrate that elevated membrane tensions were achieved with sufficient reproducibility to utilize in phase separation studies in the controlled chamber, relevant to the blue triangle point near 2 mN/m in Figure 2.4.

### 2.4.5 Phase separation.

Vesicle phase separation was studied in custom-built temperature-control chambers: Open sided chambers, accommodating micropipette insertion from the side, facilitated tight control and measurement of the membrane tensions of individual vesicles. These chambers were made from coverslips and supported in a gap configuration with a spacing of ~1 mm. The uncertainty on the vesicle tension, near 0.02 mN/m, corresponded to an aspiration pressure on the micropipettes of less than 1 mm head of water and small errors (less than 10%) in measuring pipette and vesicle diameter. Temperature control was accomplished by thermally coupling the ends of the chamber to a heating block whose temperature was regulated by a heating bath.

Use of the open-sided chamber and the micropipettes themselves placed limits on experiments, ultimately necessitating additional studies with free vesicles in a closed chamber. First, micropipettes necessitate long working distance objectives with less light gathering capacity than is usual for fluorescence work, compromising the image quality on the phase separated vesicles. Additionally, evaporation from the open chamber sides limited the experimental duration to about 15 minutes, which subsequently limited the studies to relatively rapid cooling rates. The slowest cooling rates of interest, on the order of 0.1 °C/min, could not be conducted in the open chamber. (Additionally, the convection generated by evaporation made imaging free vesicles in these chambers with
high numerical apertures challenging. While the morphology of the vesicles could be seen by eye, the vesicles were moving too quickly to be imaged clearly at the exposure times appropriate to our fluorescent tracer levels."

A closed chamber design, which did not accommodate micropipettes, enabled better imaging via higher numerical objectives and precise temperature control including a broader range of cooling rates, and avoiding difficulties resulting from evaporation. Here vesicles were placed in a ~125 μm gap between two coverslips that was sealed and heated on all sides. Nominal cooling rates in the range 0.1 - 5 °C / min were possible in addition to rapid cooling and holding at a targeted temperature above room temperature. Additionally, the closed chamber made it consistently possible to observe multiple vesicles for several hours, or as long as they survived. The closed chamber design, while it prohibited use of micropipettes, was appropriate for study of vesicles having osmotically-manipulated membrane tensions.

In studies of phase separation and domain morphology, vesicles were transferred from the heated electroformer to one of the two test chambers where they were heated to one-phase region for at least 5 minutes before cooling was initiated. (The micropipette chamber was held at the elevated temperature for only 5 minutes because of evaporation-limited experimental times, while slightly longer times were achieved in the sealed chamber.) Phase separation and tension studies were then initiated, as needed for a particular test.
2.5 Support information.

2.5.1 Expected influence of membrane tension on phase transition temperatures.

For a single component system, the Clausius-Clapeyron equation describes the influence of pressure on the phase transition temperature. For mixtures, the starting point is still the fundamental equations for equilibrium, written in differential form. As we demonstrate here, the general result for binary mixtures simplifies, for the DPPC/DOPC system, to an expression resembling the Clausius-Clapeyron equation.

As our starting point, the differential representation of the equilibrium criteria (equality of pressures, temperatures, and chemical potentials among phases) leads to the following general expression for a two-component mixture of $A$ and $B$ partitioning between phases $\alpha$ and $\beta$, as described in classical thermodynamics textbooks:

$$
\left(\frac{\partial P}{\partial T}\right)_{x_A^{\beta},(\alpha-\beta)} = \frac{1}{T} \frac{x_A^\alpha (H_A^\alpha - H_B^\beta) + x_B^\beta (H_B^\beta - H_A^\alpha)}{x_A^\alpha (\tilde{V}_A^\alpha - \tilde{V}_B^\beta) + x_B^\beta (\tilde{V}_B^\beta - \tilde{V}_A^\alpha)}
$$  \hspace{1cm} (2.1)

Here, $P$ and $T$ are pressure and temperature, $x$ is mole fraction in the phase of interest, and $H$ and $V$ are partial molar enthalpies and volumes. Subscripts identify components while superscripts identify the phases.

For a membrane, a two dimensional analog of this equation applies, where pressure $P$ is replaced by negative membrane tension and partial molar volume $\tilde{V}$ is replaced by the partial molar area, $\tilde{A}$. Upon cooling a single phase liquid mixture to the temperature of initial phase separation, the liquid composition is fixed, which gives the subscript, $x_{\text{DPPC}}^{\text{liq}}$ on the left side:

$$
\left(\frac{\partial \tau}{\partial T}\right)_{x_{\text{DPPC}}^{\text{liq}},(\text{solid-liq})} = -\frac{1}{T} \frac{x_{\text{DPPC}}^{\text{solid}} (H_{\text{DPPC}}^{\text{solid}} - H_{\text{DPPC}}^{\text{liq}}) + x_{\text{DOPC}}^{\text{solid}} (H_{\text{DOPC}}^{\text{solid}} - H_{\text{DOPC}}^{\text{liq}})}{x_{\text{DPPC}}^{\text{solid}} (A_{\text{DPPC}}^{\text{solid}} - A_{\text{DPPC}}^{\text{liq}}) + x_{\text{DOPC}}^{\text{solid}} (A_{\text{DOPC}}^{\text{solid}} - A_{\text{DOPC}}^{\text{liq}})}
$$  \hspace{1cm} (2.2)
With the solid domains nearly pure in their DPPC content,\textsuperscript{3,34,35} we approximate 
\(x_{DPPC}^{\text{solid}} = 1\) and \(x_{DOPC}^{\text{solid}} = 0\). This gives
\[
\left(\frac{\partial \tau}{\partial T}\right)_{x_{DPPC}^{\text{liq}}(\text{solid-liq})} = -\frac{1}{T} \frac{(H_{DPPC}^{\text{solid}} - H_{DPPC}^{\text{liq}})}{(A_{DPPC}^{\text{solid}} - A_{DPPC}^{\text{liq}})}
\]
(2.4)

Then, treating a liquid mixture as an ideal solution, there is no volume change or heat of mixing, which gives:
\[
\left(\frac{\partial \tau}{\partial T}\right)_{x_{DPPC}^{\text{liq}}(\text{solid-liq})} = -\frac{1}{T} \frac{(H_{DPPC}^{\text{solid}} - H_{DPPC}^{\text{liq}})}{(A_{DPPC}^{\text{solid}} - A_{DPPC}^{\text{liq}})}
\]
(2.5)

OR
\[
\left(\frac{\partial T}{\partial \tau}\right)_{x_{DPPC}^{\text{liq}}(\text{solid-liq})} = -T \frac{\Delta A_{DPPC}^{\text{solid-liq}}}{\Delta H_{DPPC}^{\text{solid-liq}}}
\]
(2.6)

This expression is essentially a two-dimensional form of the Clausius-Clapeyron equation. It was derived from a fundamental form for a two component mixture but, because of the nearly pure nature of the solid phase and an ideal solution approximation in the mixed liquid, the result depends only on the properties of pure DPPC at the transition temperature and tension. At low tensions, this form can be used to describe the fluid-ripple solid transition. The same form applies to the fluid-tilt transition at higher tensions. Likewise for the solid-solid transition from the ripple to the tilt phase, the appropriate heats and area changes at the phase transitions should be employed.

2.5.2 Calculating the tension-sensitivities of transition temperatures.

In equation 2.6, \(\Delta H\) is the enthalpy of the phase change, and \(\Delta A\) is the areal change at the phase transition. An enthalpy of 11.5 cal/g-DPPC is reported for the fluid-solid(ripple P\(_{\beta'}\)) transition,\textsuperscript{38} equivalent to 35,318 J/mol DPPC. An additional 1.5 cal/g-
DPPC is associated with the conversion of the ripple ($P_{\beta'}$) phase to the tilt ($L_{\beta'}$) solid. Also, in the liquid ($L_{\beta'}$) phase DPPC has an areal footprint of 62.9 Å², and upon condensation to a tilt ($L_{\beta'}$) solid phase there is approximately a 10% areal reduction. The area is reduced by an approximate total of 17% from the $L_{\beta'}$ to the rippled $P_{\beta'}$ solid.

Therefore, equation 2.6 predicts the liquid-solid (ripple $P_{\beta'}$) transition is shifted about 1°C for every 2 mN/m of tension applied to the membrane. Table 2.1 summarizes the tension sensitivity of the transition temperatures for all three branches of the phase space of Figure 2.4.

**Table 2.1. Calculated Slopes of Coexistence Curves in Temperature – Tension Space.**

<table>
<thead>
<tr>
<th></th>
<th>$\Delta H_{DPPC}^{\alpha-\beta}$, cal/gDPPC</th>
<th>$\Delta A_{DPPC}^{\alpha-\beta}$, Å²/DPPC</th>
<th>$(\frac{\partial T}{\partial \tau})<em>{x</em>{DPPC}^{\beta}}^{\alpha-\beta}$, K/(mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ripple solid – fluid, 38°C</td>
<td>-11.5</td>
<td>-10.7</td>
<td>-0.56</td>
</tr>
<tr>
<td>Tilt solid – Ripple solid, 31.6°C</td>
<td>-1.5</td>
<td>4.4</td>
<td>1.75</td>
</tr>
<tr>
<td>Tilt solid – fluid, 37°C</td>
<td>-13</td>
<td>-6.3</td>
<td>-0.29</td>
</tr>
</tbody>
</table>

The accuracy of the estimates in Table 2.1 is limited by the precision of the physical property data, with estimates in place for the areal changes. Even with 20% error, however, the signs and magnitudes of the $dT/d\tau$ estimates are unaffected. Also worth noting, the $dT/d\tau$ estimates in Table 2.1 apply in the limit of zero tension. To the extent that $\Delta H$ and $\Delta A$ are sensitive to tension, the coexistence curves between the different phases would be curved rather than straight. As long as the ripple phase is denser than the tilt gel, however, the two co-existence curves will have opposing slopes.
2.5.3 Impact of possible DOPC in the solid on the calculation.

An obvious issue is the extent to which the calculation is affected by the simplification that the solid phases are made of pure DPPC. While some report the presence of other species in solid DPPC phases especially at temperatures well below the onset of phase separation, we do not believe that the DPPC patches or stripes necessarily contain substantial amounts of DOPC, especially at elevated temperatures where they first form. The presence of minimal DOPC in the solid is consistent the observed stark darkness of the patchy domains (ripple solid) that exclude both tracers, one of which (RH-DOPE) has hydrophobe structure similar to DOPC. The stripes selectively incorporate Rh-DPPE, which has a similar tail structure to DPPC, but they reject Rh-DOPE, which is similar to DOPC. These tracer studies suggest the solid phases do not substantially incorporate lipids with unsaturated tails and would therefore reject DOPC. Further, the reduced melting temperature of DPPC with increased amounts of DOPC added to the fluid membrane appears to follow expectations for simple colligative properties, in which the solid phase is pure and the fluid mixtures is treated as an ideal solution. A classic example of this behavior is the use of salt to melt ice, based on the colligative property behavior.

It is well-documented that, in membranes containing a second low-melting phosphatidylcholine lipid in addition to DPPC, the solid domains are DPPC-rich compared to the fluid. In our vesicles, therefore, the solid domains cannot contain more than 30% DOPC and they might realistically, at the point of initial formation, contain less than 5% DOPC. Therefore, using 30% DOPC in the solid as an extreme worst-case scenario, Table 2.2 summarizes calculations from equation 2.2. This
effectiveness of this exercise is limited by the complete lack of physical property data on mixed phospholipid systems that form solid phases. Data are only available for a few pure lipids such as DPPC, in Table 2.2. We therefore take a mathematical approach and examine extremes, for instance if the partial molar latent heat of DOPC in the solid vanished but the area change did not. This approach is completely mathematical and neglects physical restrictions such as adherence to the Gibbs-Duhem theorem. Nonetheless it reports the widest bounds on the slopes of the coexistence lines in Figure 2.4 and Table 2.2. For this reason, and because the impact of DOPC in the solid will roughly follow its amount in the solid, we emphasize these calculations present the most dramatic calculated influence of such solid phase contamination.

Table 2.2 demonstrates that, in the worst possible (unphysical) case, the calculated slopes of the coexistence lines in Table 2.2 would be altered ±50% by the presence of 30% DOPC in the solid domains. This extreme behavior happens when the partial molar properties of the DOPC in the solid are themselves extreme, for instance having no areal or enthalphy change on solidification or taking on twice the value of DOPC. Even with this large uncertainty, the salient features of the phase diagram of Figure 2.4 would be preserved. Conversely, if the partial molar enthalpies and areal changes of DOPC and DPPC are similar, then equation 2.2 demonstrates that the impact of DOPC in the solid phases would be negligible, difficult to discriminate experimentally. For these reasons the simplification of having a pure DPPC solid is a good one in terms of simplifying the calculation so that experimental data can be employed. The key feature of the calculation is the opposing slopes of the fluid-ripple and ripple-tilt
coexistence lines, resulting from the different signs of the area change, going from fluid to ripple solid and then to tilt solid upon cooling (removal of heat).

Table 2.2 Worst Case Examples of the Tension Sensitivity of Transition Temperatures for 30 mol% DOPC in the Solid Phase

<table>
<thead>
<tr>
<th></th>
<th>$\Delta H^{\alpha-\beta}_{\text{DPPC}}$, cal/g</th>
<th>$\Delta \tilde{A}^{\alpha-\beta}_{\text{DPPC}}$, Å$^2$/cal/g</th>
<th>$\Delta H^{\alpha-\beta}_{\text{DOPC}}$, cal/g</th>
<th>$\Delta \tilde{A}^{\alpha-\beta}_{\text{DOPC}}$, Å$^2$/cal/g</th>
<th>$\frac{\partial T}{\partial \tau} \varepsilon_{\text{DPPC}(\alpha-\beta)}$, K/(mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ripple solid-Fluid, 38°C</td>
<td>-11.5  -10.7</td>
<td>-11.5  -10.7</td>
<td>-11.5  -10.7</td>
<td>-10.7  0</td>
<td>-0.56</td>
</tr>
<tr>
<td></td>
<td>-11.5  -10.7</td>
<td>-11.5  -10.7</td>
<td>0</td>
<td>-10.7  -0.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-11.5  -10.7</td>
<td>-23</td>
<td>-10.7  -0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-11.5  -10.7</td>
<td>-11.5  -21.4</td>
<td>-10.7  -0.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tilt solid-Fluid, 37°C</td>
<td>-13</td>
<td>-6.3</td>
<td>0</td>
<td>-6.3  -0.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-13</td>
<td>-6.3</td>
<td>0</td>
<td>-6.3  -0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-13</td>
<td>-6.3</td>
<td>0</td>
<td>-6.3  -0.41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-13</td>
<td>-6.3</td>
<td>-26</td>
<td>-6.3  -0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-13</td>
<td>-6.3</td>
<td>-13</td>
<td>-12.6  -0.38</td>
<td></td>
</tr>
<tr>
<td>Tilt solid-Fluid, 37°C</td>
<td>-1.5</td>
<td>4.4</td>
<td>-1.5</td>
<td>4.4  1.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.5</td>
<td>4.4</td>
<td>-1.5</td>
<td>4.4  1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.5</td>
<td>4.4</td>
<td>0</td>
<td>4.4  2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.5</td>
<td>4.4</td>
<td>-3.0</td>
<td>4.4  1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.5</td>
<td>4.4</td>
<td>-1.5</td>
<td>8.8  2.2</td>
<td></td>
</tr>
</tbody>
</table>
2.5.4 Control of excess area and tension for free vesicles in the closed chamber.

- Zero Tension and Large Excess Area.

To produce flaccid vesicles with near-zero tensions, vesicles electroformed in a 200 mOsm sucrose solution were equilibrated in a 250 mOsm sucrose solution. Vesicles become “floppy” only after they equilibrate by water diffusion out of the vesicles across the membrane, a process occurring on the order of tens of minutes.\(^4\) The resulting excess area, \(A_{xs}\) is defined relative to a sphere of the same volume

\[
A_{xs} = \frac{A}{\left[\frac{36\pi V^2}{I^3}\right]} - 1\tag{2.3}
\]

Here \(A\) is the measured vesicle area and \(V\) is the measured vesicle volume, both determined from videomicrographs of individual vesicles aspirated into micropipettes at low suction (corresponding to membrane tensions near 0.02 mN/m).

Figure 2.5A confirms that the use of sucrose solutions produced vesicles with substantial excess areas upon cooling from an equilibration temperature of 43°C to 35°C (in the range of interest near the onset of stripes) at 1°C/min and transferring to a micropipette chamber for further study. The latter is chosen to be relevant to the phase transitions in the main chapter, especially the appearance of stripes. Greater excess areas, averaging 12%, were found for DOPC (fluid) vesicles. Vesicles containing 30/70 mol% DOPC/DPPC had an average near 8% excess area. With DPPC in a mixed membrane, the excess area was diminished by the formation of solid domains whose areal phospholipid density exceeded that of the fluid phase. Since vesicles contract on cooling as a result of the positive thermal expansion coefficient of the membrane and the increased solid domain area in the two-phase region, the excess areas reported here at 35°C represent a lower limit for the phase separation experiments. This approach
therefore ensured near-zero membrane tension over the course of cooling and phase separation studies, unless tension was directly applied using micropipettes.

Complimentary to the excess areas in Figure 2.5A are measurements of vesicle tension in Figure 2.5B. To determine the tension on a particular vesicle, it was gradually aspirated into a micropipette after cooling from 43°C to 35°C at 1 °C/min and transfer to the micropipette chamber, noting the suction needed to first produce a projection. For vesicles with finite excess areas such as those conditioned in sucrose solutions, this aspiration suction was near zero, corresponding to that needed to produce a gradual membrane curvature (the work of membrane bending) to facilitate aspiration. Vesicles that had no excess areas, however, usually had finite membrane tensions, as described for the case below. These vesicles formed projections inside micropipettes only at elevated suctions necessary to overcome the membrane tension. The LaPlace equation was employed to relate micropipette suction to membrane tension:

$$\tau = \frac{P_s R_p}{(2-\frac{2R_p}{R_v})}$$

(2.7)

Here $P_s$ is the suction pressure, $R_p$ is the micropipette radius, and $R_v$ is the radius of the vesicle external to the micropipette. Figure 2.5B reports near zero membrane tensions for vesicles preconditioned in sucrose solutions (the same ones exhibiting large excess areas).

This study validated the procedure of using sucrose solutions to produce excess area in free vesicles and maintain near-zero tensions during the phase separation of free vesicles in the closed chamber.
Free Vesicles with Elevated Tensions.

Vesicles with elevated tensions at the onset of phase separation were employed in limited studies aiming to best image the striped morphology in Figure 2.2 and also for one data point in Figure 2.4. These vesicles, initially formed in DI water and cooled at 1°C/min from the one phase region near 43°C, were found, in separate studies described here, to exhibit negligible excess areas at room temperature, in Figure 2.5A. Along with this lack of excess area, these vesicles also exhibited membrane tension in Figure 2.5B, as described below. Independent of the exact values of excess area and tension, vesicles cooled in DI water displayed large qualitative differences from those conditioned in sucrose.

The membrane tension for vesicles conditioned in DI water and cooled to room temperature arises because of the membrane’s thermal expansion coefficient, combined with a near-zero or modest excess area at the start of the cooling process. Thermal contractions of the membrane during cooling (about 0.5% strain / °C¹⁹) cause the skin of the vesicle to contract against the more nearly constant volume of water inside the vesicle. (The inner pool of water also contracts with cooling, but not as extensively as the membrane.) The increasing membrane stress on cooling is relaxed, however, by diffusion of water out of the vesicle across the membrane,⁴² a slower process than thermal membrane contraction. Hence the membrane stress at any instant depends on thermal history and, if cooling is too rapid, the membrane ruptures before sufficient water can escape by diffusion, since the lysis tension of these membranes is near 4-6 mN/m (based on our separate micropipette studies). Sometimes ruptured vesicles re-seal
producing vesicles with negligible tension.\textsuperscript{47, 48, 49} Cooling rates, therefore should not be too fast.

Critical to the vesicle morphology on phase separation in the main chapter is the tension just before phase separation initiates, a quantity that is particularly difficult to access because it tends to relax with water-diffusion time. We describe here supporting study in which vesicle suspensions in DI water were equilibrated at elevated temperature nears 43°C and then cooled near 1 °C/min to 35°C, near the general onset of these phase separation processes and the appearance of stripes, and then transferred to a micropipette chamber at 35°C for immediate characterization, giving the data in Figure 2.5.

In Figure 2.5 for 15 vesicles at a 30/70 DOPC/DPPC mol% composition, all vesicles in DI water display a dramatic lack of excess area and substantial elevated tensions, compared with the near zero tensions of the sucrose-conditioned vesicles. Because the tensions relax (due to water diffusion) time in DI water, we expect the measured tension values to represent lower bound on the membrane tensions in phase separation experiments in DI water. An additional point of consistency with our explanation, a comparison to fluid DOPC vesicles demonstrates that in addition to thermal contractions, the formation of solid domains in DPPC-containing vesicles tends to consume excess area and in turn produce elevated stress.

Based on the data in Figure 2.5B, we cautiously estimate a membrane stress value in the range of 2±1 mN/m (with the large error bars representing the full range of measurements with 15 vesicles) for the elevated tensions of vesicles cooled at 1°C/min in DI water and exhibiting the patch-to-stripe transition behavior. This point is represented as the blue triangle with the large error bars in Figure 2.4. While the error bars on the
tension are very large the confidence in the temperature range of the transition is much
tighter.

- Summary of Tension in Free Vesicles.

To summarize, large excess areas and near zero tensions are easily produced by
appropriate choice of the sucrose concentration inside and outside the vesicles. By
contrast, forming vesicles in DI water and cooling from elevated temperatures produces
membrane stresses. Appropriate cooling rates permit water diffusion out of the vesicle at
the same time as membrane contraction and membrane stress build up. In this way, the
stress remains elevated but still below the lysis tension. Supporting studies revealed
elevated tensions in the range 2±1 mN/m as a lower bound on the tension of vesicles in
the main chapter: the high tensioned vesicles in Figure 2.2 and the blue triangular datum
for elevated tensions in Figure 2.4.

Figure 2.5: Excess area (left), and membrane tension (right) of pure DOPC and mixed
30/70 mol% DOPC/DPPC vesicles cooled from 43°C to 35°C at 1°C/min. The vesicles
formed and studied in DI water (gray) develop tension and zero excess area while those
formed in 0.2M sucrose solution and transferred to 0.25 M sucrose sucrose solution (red)
maintain zero tension and substantial excess area. Data represent the average of 15 vesicles each, with the error bars showing the full range of data.

2.6 Reference.


CHAPTER 3
THREE DIMENSIONAL (TEMPERATURE-TENSION-COMPOSITION) PHASE MAP OF MIXED DOPC-DPPC VESICLES: TWO SOLID PHASES AND A FLUID PHASE COEXIST ON THREE INTERSECTING PLANES

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3.1 Introduction.

We have quantitatively probed the impact of tension, by micropipette aspiration of giant unilamellar vesicles and by separate osmotic manipulation, on the formation of DPPC-rich solid domains in mixed DOPC/DPPC membranes. At a fixed composition of 30 mol% DOPC/70 mol% DPPC we found that membrane tension, during controlled cooling, depressed the temperature at which solid domains first appeared. These observations ran parallel to the report of Portet and Keller for the reduction of the liquid miscibility transition temperature. In both cases elevated tension favored the less dense membrane phase, as would be expected based on fundamental arguments.

In single component lamellae, saturated phosphatidylcholines such as DPPC, DLPC, and DMPC form different solid-like bilayer phases because, when hydrated, differences in the sizes of their head and tail groups facilitate molecular tilt within the bilayer. Solid bilayer phases include planar tilt (L_{β'}) also called a “gel”, and a corrugated (P_{β'}) morphology, also called “rippled”. In hydrated lamellar bulk samples, a first order melting peak (near 41.5°C for DPPC) marks the main transition from a pure fluid L_α phase to the P_{β'} rippled solid. A second transition, termed the
“pretransition,” from the rippled solid phase to a tilted $L_\beta'$ solid phase occurs at a slightly lower temperature, in the case of DPPC near 35.5°C. In corrugated lamellae, the molecules are arranged almost perpendicular to the macroscopic membrane surface but at an angle from the local membrane tangent. The reported period of the corrugations for DPPC is ~16 nm though this can vary. In the planar tilt phase the membrane is uniformly flat, and the molecules are at an angle of 29-32° from the membrane surface. Notably, the angle of the molecules with the local surface normal is similar in the two configurations, making distinction of the two phases difficult by some methods. However, there is a clear exotherm on cooling from the corrugated to the tilt solid, and the areal density of the corrugated phase exceeds that of the tilt phase by ~10%. This imparts a potential influence of tension on the phase transition.

In giant unilamellar vesicles of 30 mol% DOPC/ 70 mol% DPPC, we found evidence for two distinctly different types of solid domains, based on shape, dye uptake, and the temperatures at which they first appeared. The occurrence of striped and patchy domains in this system was previously reported and dye selectivity was noted in careful studies by Feingenson. Our assignment of the ripple phase to round or hexagonal domains and planar tilt to the striped phase semi-quantitatively explained, using arguments from first principles, the tension sensitivity (imposed via micropipettes) of the transition temperature between these two solids in the giant unilamellar vesicles.

Extending our prior work at fixed composition, the current study examines the tension sensitivity of the phase separation and the nature of the domains for the DOPC/DPPC system over the full range of compositions, from temperatures near 25°C up to the one phase region, and tensions from ~0 mN/m to that of lysis near 4 mN/m.
Using both micropipette and osmotic manipulation of membrane tension, the 3D thermodynamic phase map is built and describes the coexistence of the Lα fluid, patchy, and striped solid domains. The assignment of the corrugated Pβ′ structure to the patches and tilt Lβ′ structure to the stripes allows a simple fundamental model that qualitatively explains the shape of the phase diagram within the limits of the available physical property data. Reverse assignment of the two solid phases (patchy hexagons – tilt; stripes – corrugated) produces large qualitative discrepancies between the observations and a simple fundamental treatment.

3.2 Experimental description.

3.2.1 Vesicle preparation.

Vesicles were prepared by electroformation, a method established to produce substantial numbers of giant unilamellar vesicles. DOPC and DPPC, in the desired molar proportions, were dissolved in chloroform near a concentration of 1 mg/ml. Rh-DPPE or Rh-DOPE tracers were employed at a concentration of 0.1 mol% relative to the total phospholipid. (Tracer concentrations of 0.1- 0.5 mol% were found not to influence the phase separation temperatures.) A 10 μL quantity of phospholipid solution was placed on the platinum wire electrodes and, after drying under nitrogen, the chamber was sealed. It was then filled with DI (de-ionized) water or sucrose solution that had been preheated to 52°C. An alternating current was applied to the electrodes at 3V and 10 Hz for 1 hour, while the chamber was maintained at 52°C, to maintain the compositional uniformity of the vesicles. The vesicle solution was harvested in a syringe.
3.2.2 Membrane tension measurement and control.

Micropipette manipulation was employed to quantitatively control the tension of single vesicles during cooling and phase separation. Micropipettes, with inner tip diameters of 3-6 μm, were pulled on a Kopf Instruments Micropipette Puller. The tip shapes were refined using a Technical Products International Microforge so that the inner diameters were nearly constant in the region where vesicles projected into the pipettes. Bovine serum albumin (Sigma catalog number A7511) was adsorbed to the pipette tips to prevent vesicle adhesion. Vesicles were chosen in the 15-50 μm diameter range, at least 3 times greater than the pipette diameter, to avoid computational error in calculating tension and area. The tension of aspirated vesicles was controlled by applying suction using a siphon manometer. The uncertainty in the suction pressure was less than 1 mm head of water. Small errors (less than 10%) in measuring pipette and vesicle diameter, contributed to the overall uncertainty on tension of ~0.02 mN/m.

3.2.3 Phase separation characterization.

Vesicle phase separation was studied by fluorescence microscopy in two types of custom-built temperature-control chambers: open and closed. Both fit onto a Nikon Diaphot 300 inverted fluorescence microscope and controlled the rate of cooling. Images were obtained using a CoolSnap HQ CCD camera. Open sided chambers, made of coverslips supported in a gap configuration with spacing of ~ 1mm, allowed micropipette access from the side. The ends of the chamber were coupled to a temperature reservoir that was regulated by a heating bath. The open-sided chamber and the micropipettes themselves placed restrictions on experiments, necessitating additional studies with free vesicles in a closed chamber. First, micropipettes required long working distance
objectives with less light gathering capability than is usual for fluorescence imaging. This compromised the image quality in micropipette studies compared to the closed chamber. Additionally, evaporation from the open chamber limited the experimental duration to about 15 minutes. As a result, the slowest cooling rates, on the order of 0.1 °C/min, could be achieved only in the closed chamber.

Without micropipettes, phase separating vesicles in a closed chamber could be better imaged via higher numerical objectives. Without evaporation in the closed chamber, longer runs, a broader range of cooling rates in the range 0.1 - 5 °C / min, and more precise temperature control was possible. Here a vesicle suspension sealed in a ~125 μm gap between two coverslips.

In studies of phase separation and domain morphology, vesicles were transferred from the heated electroformer to one of the two test chambers where they were heated, placing them within the one-phase region, for at least 5 minutes. A cooling program was then initiated and phase separation and tension studies were then conducted. In these studies, vesicle tension was manipulated directly using micropipettes in the open chamber or, in the closed chamber, osmotic and other conditions were chosen to produce membrane tensions within a targeted range, as described below.

3.2.4 Vesicles with spontaneous elevated tensions in the closed chamber.

In contrast to osmotically-conditioned flaccid vesicles, vesicles formed and equilibrated in DI water at elevated temperatures prior to cooling were observed not to exhibit excess area. These vesicles were instead found (as detailed below) to be tensed at room temperature following controlled cooling at rates of 1°C/min. It is not obvious that
this simple treatment produces elevated tensions. We discovered this to be the case and confirmed this with tension characterization studies, described next.

The hypothesized mechanism for development of membrane stress was critical in the design of our characterization studies and is therefore described here: It involves a starting point at elevated temperatures, for instance near 50°C in our studies, where vesicles exhibit little, if any, excess area, as we confirm below. Subsequent cooling causes both the vesicle membrane and its water contents to contract, as a natural consequence of their thermal expansivities. The membrane, however, contracts more extensively than the aqueous vesicle center, causing development of membrane stress. The membrane stress relaxes as water diffuses out of the vesicles across the membrane, on a timescale of minutes. Thus the actual values of the membrane stress and its variation with time depend on the cooling rate relative to that of water diffusion.

3.2.5 Characterizing stress of free vesicles, like those in the closed chamber.

Characterization of the membrane stresses relevant to the phase transition(s) of vesicles cooling in the closed chamber required attention to the fact that, as a result of continued water diffusion from stressed vesicles, stresses measured at room temperature after vesicle phase separation would likely be lower than the stress occurring during phase separation itself. We therefore designed characterization studies that would probe the membrane tensions of vesicles cooled from 45°C to a temperature of about 35°C, in the range where phase separation had or was about to start. Further, because controlled cooling at different rates could not be accomplished reliably in the open chamber, vesicles were cooled at a programmed rate and then quickly transferred to a constant temperature micropipette (open) chamber for immediate characterization. Measurements
were made within minutes, allowing some tension loss, but minimizing the loss as much as possible.

The stress state of vesicles was characterized by micropipettes by first attempting to aspirate at extremely low suctions, producing membrane stress in the range 0.02-0.04 mN/m, corresponding to areal strains of less than 0.1%. For flaccid vesicles, a projection would appear in the micropipette and excess area could be quantified. Tensed vesicles, however, would be pulled to the mouth of the pipette but not produce a projection. Subsequent increase in suction would produce a projection only once the suction pressure balanced the opposing force from the membrane tension. The suction pressure, $P_s$, corresponding to the first appearance of a projection allowed determination of the membrane tension, $\tau$, of the stressed vesicles according to:

$$\tau = \frac{P_s R_p}{(2 - \frac{2R_p}{R_v})}$$

(2.7)

Here $R_p$ and $R_v$ are the micropipette and vesicle radius (outside the micropipette), respectively.

### 3.3 Results.

#### 3.3.1 Characterizing tension.

While most of the phase diagram was mapped with tension manipulated quantitatively by micropipette aspiration during cooling, the best images of phase separated domains were obtained on free vesicles in the closed chamber. For the relevant cooling histories, Figure 3.1 summarizes separate characterization studies of the membrane tension of free vesicles like those in the images in the next sections, below. Important to note, tension was measured at three different points (in separate studies) in
the cooling history: 1. before cooling, the vesicles are maintained at 45°C, 2. at conditions intended to approach those corresponding to the first instants of phase separation (1°C/min in DI water from 45°C to about 35°C), and 3. later at room temperature.

Figure 3.1: Tension measured for 10-15 vesicles at each condition, using micropipettes. Error bars represent the full range of observed tensions. (When vesicles were flaccid, all vesicles exhibited zero tension and so no error bars are shown.)

Figure 3.1 enforces several important points: (1) Vesicles conditioned in sucrose maintain near-zero tensions before, during cooling, and after cooling. (2) Vesicles cooled at 1°C/min in DI water from 45°C to about 35°C experience substantially elevated tensions. (3) Vesicles cooled at 1°C/min in DI water from 45°C to room temperature and characterized within 15 minutes register a tension loss back to near zero tension. (4) With greater DPPC content in vesicles processed in DI water, slightly higher stresses are
observed on cooling, and (5) faster cooling at 5°C/min produces, for vesicles processed in DI water, lower tensions than cooling at 1°C/min.

The first three points are consistent with the hypothesis that thermal contractions of the membrane, relative to those of the water, produce stress, which tends to relax with time, presumably by water diffusion across the membrane. Additionally, in point 4, the observation of increased tension at 35°C with increased DPPC content may result from greater amounts of solid domains (demonstrated below) in these vesicles. With the solid domains being denser than the fluid domains, their formation causes extra areal reduction compared with the thermal contraction alone.

Finally for vesicles in DI water, the observation of lower finite stress with fast cooling at 5°C/min is consistent with vesicle rupture and resealing (and continued thermal contraction).28, 29, 30 We find vesicles with 30% DOPC/70% DPPC exhibit a lysis strain of ~4 % at room temperature. However, with a reported area-based thermal expansion coefficient of 4.2-6.8 x 10⁻³ K⁻¹ in phosphotidylcholine membranes,23 cooling from 45 to 25°C would produce an area reduction on the order of 10 %. Depending on the original water content of the vesicle, with limited time for water diffusion of the central pool (for fast cooling), there is certainty of vesicle rupture. While we cannot see rupture happening because resealing is relatively rapid, Figure 3.1 establishes the membrane tensions for these different histories, most of which are relevant to the images in Figures 3.2-3.4. Only one history, cooling at 1°C/ in DI water was employed to generate a limited portion of the phase map in Figure 3.6. For the other data in the phase map, phase separation temperatures from measurements in the closed chamber were compared to micropipette studies with carefully controlled tensions.
3.3.2 Qualitative features.

- Appearance at Room Temperature.

The fluorescence micrographs in Figure 3.2 show the influence of the overall DOPC/DPPC ratio on the appearance of solid domains for a series of vesicles processed in DI water and cooled from the 1-phase region above 45°C to room temperature where the images were recorded. These vesicles contain 0.1 mol % Rh-DOPE tracer which, by concentrating in the fluid L_α membrane phase, reveals the solid domains from which tracer is excluded. These images are typical of all vesicles in this size range within the imaging chamber (we did not scrutinize vesicles below 10 μm) and were reproduced for more than 5 electroforming batches each. In this series, tension depends on the cooling rate, 1°C or 5°C/minute, elevated or low but finite respectively, in Figure 3.1.

With low but finite membrane tensions (achieved by cooling at 5°C/min), vesicles exhibited patchy or irregular hexagonal-shaped dark domains if they contained 35 mol% DPPC or greater. Vesicles with lower DPPC content appeared uniform. Besides the exclusion of tracer, further evidence for the solid-like nature of the patchy dark domains includes their fixed shape and lack of coalescence. The increasing dark patch area with increased overall DPPC content argues that the patchy domains are DPPC-rich. These observations are consistent with the long literature on 2-component vesicles in which DPPC comprises the higher melting of the two phospholipid components. Phase separation on cooling produces solid domains composed predominantly of DPPC, suspended within the two-component fluid L_α phase. 31, 32, 33
Figure 3.2: Typical appearance of vesicles at room temperature, for different DOPC/DPPC compositions, and using an Rh-DOPE tracer. The upper series is for cooling at 5°C/min and the lower series is for cooling at 1°C/min, both in DI water. The scale bar is 10 μm.

In Figure 3.2, vesicles experience higher tensions if cooled more slowly at 1°C/minute. The resulting dark domains appear as stripes instead of patches at room temperature, and increase in area as the overall DPPC content is increased. This indicates that the stripes are DPPC-rich. Stripes are not seen when the overall vesicle composition is below ~50% DPPC.

Figure 3.2 raises the question of whether the appearance of stripes versus patches is purely morphological or if there are deeper underlying thermodynamic differences in the solids, for instance in molecular arrangements. Figures 3.3A-D address this question by comparing, at room temperature, the appearance of a vesicles like those in Figure 3.2 at a fixed composition (20/80 mol% DOPC/DPPC), but employing different tracer dyes. (Figure 3.3A-D are like Figure 3.2 in that they are cooled at 5°C/min or 1°C/min in DI water.) While patches exclude both Rh-DOPE and Rh-DPPE tracers, the stripes selectively include Rh-DPPE but exclude Rh-DOPE. This selectivity makes a
compelling argument for different molecular arrangements within the two types of solid domains.

**Figure 3.3:** Influence of processing parameters on the formation of patches or stripes, comparing the uptake or exclusion of 0.1 mol% tracer dyes: Rh-DPPE or Rh-DOPE. Labels in beige boxes indicate conditions for each vesicle. All vesicles are at room temperature with 20/80 mol ratio of DOPC/DPPC. Scale bar is 10 μm.

From Figure 3.2 and just the 4 images in Figures 3.3A-D it is not clear which parameter, membrane stress or cooling rate, fundamentally determines whether a vesicle will form striped or patchy solid domains. Figures 3.3C-F, which show vesicles at a fixed cooling rate at 1°C/min, isolate the impact of tension. In DI water, cooling at 1°C/min produces elevated tensions and striped domains. Conversely, with tension osmotically fixed near 0 mN/m, the same cooling rate produces patch-shaped domains.
In this range of cooling rates, therefore, the tension, not the cooling rate, determines the types of domains.

- Cooling Sequences.

Figure 3.4 shows sequential micrographs for a vesicle with 30/70 mol% DOPC/DPPC during cooling at low tension. The sequence in part A with rapid cooling at 5°C/min shows that, on a single vesicle, similarly-sized small domains first appear and then grow with time. The apparent mechanism of nucleation and growth will be fully explored in chapter 4. Important here is the persistence of the patchy domains at cool temperatures, due to kinetic trapping during rapid cooling.

![Figure 3.4: Domain growth during cooling in DI water for vesicles with a composition of 30/70 mol ratio DOPC/DPPC. (A) Cooling at 5 °C/min (adopted from chapter 1). (B) A series of vesicles during very slow cooling at 0.1 °C/min conditioned in sucrose to maintain tension near zero.](image)

The kinetic trapping that occurs on rapid cooling contrasts with the vesicle appearance and development of domains in slow cooling in Figure 3.4B. Vesicles of the same 30/70 mol% DOPC/DPPC composition in Figure 3.4B, are osmotically conditioned
in sucrose solutions to maintain near zero tension during an extremely slow cooling rate of 0.1°C/min. Figure 3.4B demonstrates the initial appearance of patches, followed by their growth and their subsequent complete conversion to stripes with progressive cooling.

Comparison of Figures 3.4A and B reveal: a) The cooling rate has minimal influence on the temperature corresponding to the first appearance of patches. While we expect some influence of kinetics determining when domains are first visible, this effect here is less than 1°C. b) The cooling rate can, however, dictate whether patches or stripes are ultimately seen at room temperature. We believe that the complete conversion of patches to stripes at low temperatures and very slow cooling indicates that stripes are the preferred (stable equilibrium) phase at lower temperatures. The persistence of large patches after rapid cooling to low temperatures may indicate a kinetic trapping of a robust metastable equilibrium: Conversion of large solid domains from one molecular arrangement to another would involve a large energy barrier and was not observed at room temperature on a timescale of tens of minutes.

Additionally, with regard to Figure 3.4B we observed, with repeat runs with different vesicles, that the temperature for the first appearance of stripes (in the presence of patches) is distinct and is a function of composition (as we will show). The conversion of patches to stripes occurs in less than five minutes at the cooling rate of 0.1°C/min, and therefore the two types of solid domains coexist only over a narrow temperature range (less than 0.5°C). Whether this represents a true equilibrium envelope or a single solid-solid transition point at a fixed temperature for each composition is unclear. Additionally the striped domains grow mainly in length and they intersect each other or they intersect patches. Even when they are first visible the stripes are already long and an increase in
length is not visible. More and wider stripes develop with further cooling once the patches are consumed.

Figure 3.5: Images of vesicles in micropipettes before and after phase separation at the temperatures and tensions indicated. Tension is fixed throughout the cooling process. The cooling rate is 1 - 2°C/min. The molar ratio of DOPC/DPPC is 20/80 and 0.1 mol% Rh-DPPE tracer dye has been added. The scale bar is 10 μm.

- Qualitative Features of Phase Separation by Micropipettes.

While we can osmotically manipulate vesicles to produce excess area and thereby maintain near zero tension during cooling, it is not possible to maintain a fixed known elevated tension during cooling by osmotic conditioning. To achieve quantitative control of elevated membrane tensions, we employed micropipettes, but at the sacrifice of image quality and ability to achieve slow cooling. As an example of micropipette manipulation, the images in Figure 3.5 illustrate, for three different fixed tensions, the appearance of solid phases during the cooling process. While micropipettes quantify membrane tension, facilitating construction of phase maps below, important qualitative features are highlighted here: At higher tensions, above 3mN/m for the composition in Figure 3.5, a striped solid phase was seen to form directly from the fluid. In micropipettes, patches
are not usually seen to convert to stripes because cooling is usually sufficiently rapid to kinetically trap the patchy solid domains, consistent with Figure 3.4.

- **Summary of Qualitative Findings.**

  Even without precise quantification of tension or temperature it is evident that domains of thermodynamically different solids can be observed, depending on vesicle composition and tension and, to a lesser extent in certain cases, cooling history. Striped domains are seen in two situations: 1) they form, at the highest fixed tensions, directly from the one-phase fluid during moderate cooling (1-2°C/min, no other cooling rates could be tested) in micropipettes and 2) at lower tensions and with sufficiently slow cooling (0.1 °C/minute), stripes can be produced by conversion of previously formed hexagonal patchy domains. The stripes observed DI water in Figure 3.2, with the elevated membrane tension, could fit into either of these categories depending on the vesicle tension at the instant of phase separation, as discussed below. Patchy hexagons, on the other hand, form directly from the one-phase fluid mixture at low and moderate tensions, fairly independent of cooling rate. However, with time and especially for slow cooling they tend to convert to stripes. The patches are stabilized however, by rapid cooling that produces large domains. The patchy domains are distinctly different in their molecular arrangement from the stripes, evidence by selective incorporation of tracer dyes. Patchy solid domains have never been observed at substantially elevated tensions, for instance 3mN/m.

  These observations suggest that tension is a fundamentally important thermodynamic variable influencing more than domain size and therefore visibility. The
next section further explores the phase diagram of DOPC/DPPC in temperature-tension-composition space.

**Figure 3.6A:** Three dimensional phase space of DOPC/DPPC membranes, (temperature, tension, total DPPC content) from an angle that looks into the region where patches (ripple phase) forms. From other angles the region containing patch-fluid equilibrium pinches closed.
Figure 3.6B:  **B i.** Temperatures for the first appearance of patches and for their conversion to stripes in zero-tension vesicles. Near-zero tension was maintained by osmotically conditioning in sucrose solutions.  **B ii.** Controlled-tension sections through DOPC-DPPC phase space, indicating first appearance of patches or stripes as a function of temperature, with solid curves guiding the eye. The conversion of patches to stripes at constant overall DPPC content is indicated with dashed lines. Hollow data points are obtained in micropipettes with tensions as indicated. Solid points were obtained in the closed chamber. Here the zero tension data is precisely controlled through choice of sucrose solutions, while the elevated tension data carries large uncertainty for cooling in DI water at 1°C/min as indicated in Figure 3.1.
Figure 3.6C: Temperature-tension sections of DOPC/DPPC phase space, with slices for different overall compositions. Error bar on lower pink triangle datum is typical only of all the pink triangle data (for conversion of patches to stripes in chamber in DI water). The uncertainty in tension for the other data is 0.02mN/m.

3.3.3 Phase maps.

Figure 3.6 shows different representations of the three dimensional phase map of the DOPC/DPPC mixture, measured by noting the temperature and shape of the first appearance of solid domains when vesicles were cooled from the one phase region. Here temperature, composition, and tension are the three independent variables defining a 3-dimensional thermodynamic space in Figure 3.6A. While it is difficult to see the details, there are three “branches” or curved planes intersecting in this three dimensional space. These represent fluid-patch solid coexistence, fluid-stripe coexistence, and patch-stripe coexistence. Because of the complexity of the experimental results in Figure 3.6A, we additionally present slices through this space, at constant composition (Figure 3.6B) or
constant tension (Figure 3.6C). The particular features of the phase diagram are discussed in the context of these constant composition and tension sections, which are easier to see.

With a few exceptions, noted below, micropipettes were employed to control tension and elucidate the solid domain formation, typically patches at low tension and stripes at high tension (hollow points). Probing the tension dependence of the patch-to-stripe transition required our best imaging and slow cooling, neither possible in micropipettes. Therefore, the patch to stripe transition was tracked in the closed chamber (solid points).

Figure 3.6B presents temperature –composition plots at a series of fixed tensions. Figure 3.6Bi shows, for clarity, data collected exclusively at zero tension. Then in Figure 3.6Bii additional curves for data at elevated tensions are included. At zero tension in Figure 3.6Bi the upper data set shows the temperatures at which solid domains were first observed on cooling. All of these initially-formed solid domains turned out to be patches. The data approach the reported melting transition of pure DPPC (41.5°C5) as the proportion of DPPC is increased in the mixture. As the DPPC is increasingly diluted with DOPC, the appearance of patches occurs at progressively lower temperatures, as expected for the classical case of melting point depression.

The lower curve in Figure 3.6Bi summarizes the temperatures for the patch-to-stripe transition measured with the same vesicles, still at $\tau \sim 0$, employed to determine the upper curve. It is interesting that this lower curve extrapolates, in the limit of high DPPC concentrations, to the so-called “pretransition” reported for the conversion between a
ripple solid and tilted solid phase in pure DPPC (35.5°C). The significance of this will be discussed in the following section.

Figure 3.6Bii expands the content Figure 3.6Bi with additional temperature-composition curves (solid lines) measured in micropipettes, each at a different fixed tension. Each curve constitutes a different slice through the 3-dimensional map of Figure 3.6A. On each of these fixed-tension solid curves, patches first appear from the cooling fluid membrane and the temperature of their first appearance shifts downward slightly with increases in tension. It is gratifying that the temperatures measured near zero tension (0.02 mN/m) in micropipettes for patch appearance agree well with the temperatures recorded for vesicles osmotically conditioned with sucrose to near zero tension in the closed chamber. At tensions exceeding ~3mN/m, the first solid phases to form from the fluid were stripes, not patches. The points for the appearance of stripes directly from the fluid on cooling in micropipettes, are also included but will be shown to fall on a separate branch of the phase diagram.

In addition to the zero-tension dashed curve for the conversion of patches to stripes (from Figure 3.6Bi) Figure 3.6Bii also includes a data set (also a dashed line) at elevated tensions for the conversion of patches to stripes. Here the elevated tension was achieved in the closed chamber in DI water by cooling at 1°C/min, and thus carries a large uncertainty. Additionally, for this data set, the tension at the moment of phase separation varies slightly (consistent with Figure 3.1) with composition. Nonetheless, it is clear that the patch-stripe conversion temperature increases with imposed membrane tension.
It is worth emphasizing that, while temperature-composition maps for DPPC mixed with other phospholipids have been reported many times previously,\textsuperscript{32, 34, 35} the data in Figure 3.6B differ in their quantification of tension and its assurance of a constant value for each curve (excepting the pink dashes where tension varies is as described in Figure 3.1.)

Figure 3.6C, which presents temperature-tension curves at different fixed overall compositions, explicitly shows the impact of tension on the observed transitions. These data are the same as those in Figure 3.6A and 3.6Bii, now on different axes. The lines represent a model, described below.

In the temperature-tension perspective of Figure 3.6C, the three-branch nature of the thermodynamic space is evident. The upper branch at tensions less than $\sim 3\ \text{mN/m}$ describes the first appearance, with progressive cooling, of patchy solid domains in the fluid membrane. At tensions exceeding $\sim 3\text{mN/m}$ (depending on composition) the striped solid domains are the first and only phase to appear on cooling, constituting a separate branch. Finally at low tensions and temperatures a third branch describes the transition of patchy to striped domains in the presence of the fluid membrane. Importantly, the upper two branches, for the first appearance of solid or striped domains in the fluid, both have slightly negative slopes. The lower branch for the patch to striped transition has a positive slope and intersects the upper branches creating a triple-point-like feature at each composition.

A few points are worth making. First near zero tension (on the left axis) temperatures measured for the first appearance of patches in the closed chamber (at the ultraslow cooling rate) show excellent agreement with those measured in micropipettes
and cooled at a rate of 1-2°C/min. Further, at zero tension, the data for the conversion of patches to stripes are obtained by osmotically conditioning vesicles with sucrose solutions and cooling at 0.1°C/min. The patch to stripe transition was inconsistently seen in micropipettes because micropipettes necessitate a faster cooling rate, which tends to kinetically trap large patchy domains, demonstrated in Figure 3.4.

For clarification, we mention that all the data on the upper two branches of Figure 3.6C were measured using micropipettes for tension control, except at zero tension where data points from both micropipettes and osmotic manipulation agree well. Below tensions of about 3 mN/m, data points are averages based on 10 vesicles. At tensions above 3 mN/m, rupture limited the numbers of vesicles that could be studied so data are plotted for individual vesicles.

Finally worth highlighting are data for the patch-to-stripe transition at elevated tensions near 2 mN/m, the pink triangles in Figure 3.6C. (These are the same pink triangles on the pink dashed line in Figure 3.6Bii.) These data are the only stand-alone data on the phase diagram obtained in the closed chamber. The points each represent an average of temperatures measured at the patch-to-stripe transition temperatures for 10-15 vesicles at each composition. The data carries a large uncertainty in tension, but they fit well on each of the three calculated lines (model to be presented below), suggesting a stronger tension-sensitivity of the patch to stripe transition than the other two branches.

A key feature of the phase diagram is the triple point-like feature that is most evident in Figure 3.6C for each composition when transition temperature was mapped as a function of tension. The triple points are not clear in the familiar temperature-composition representation (Figure 3.6B) but, in the 3D space of Figure 3.6A, they
appear as a line (a locus of triple points) where the three curved surfaces intersect. The “triple point locus” marks, for a particular composition, the tension above which the patchy phase is not seen. As a result, for tensions above the triple point locus, cooling vesicles into the one phase region always produces striped domains irrespective of the cooling rate. In this way, tension regulates the choice of solid formed and the related domain morphology, somewhat independent of cooling rate.

3.3.4 A model for the effect of tension on the transition temperature.

A simple first-principles treatment provides an interpretation of the phase diagram and, in particular the shapes of the temperature-tension curves in Figure 3.6C. The treatment starts with the general fundamental expression for chemical equilibrium expressed in differential form as found in textbooks. Treating the negative membrane tension as the two dimensional (2-D) analog of pressure, and considering area rather than volume, a 2-D membrane version of the general expression results, written here for a fluid-solid transition of a binary mixture:

\[
\left( \frac{\partial \tau}{\partial T} \right)_{x_{\text{DPPC}}^{\text{liq}}(\text{solid-liq})} = -\frac{1}{T} \left( \begin{array}{c}
\frac{x_{\text{DPPC}}^{\text{solid}} (H_{\text{DPPC}}^{\text{solid}} - H_{\text{DPPC}}^{\text{liq}})}{x_{\text{DPPC}}^{\text{solid}} A_{\text{DPPC}}^{\text{solid}} - A_{\text{DPPC}}^{\text{liq}}} + \frac{x_{\text{DOPC}}^{\text{solid}} (H_{\text{DOPC}}^{\text{solid}} - H_{\text{DOPC}}^{\text{liq}})}{x_{\text{DOPC}}^{\text{solid}} A_{\text{DOPC}}^{\text{solid}} - A_{\text{DOPC}}^{\text{liq}}} \end{array} \right)
\]

(2.1)

This expression relates temperature and tension along the 2-phase coexistence curve at constant composition \((x_{\text{DPPC}}^{\text{liq}})\). It can apply separately to the equilibrium between the patchy solid and the fluid or between the striped solid and the fluid. \(x_a^\alpha\) refers to the mole fraction of component \(a\) (DOPC or DPPC) in phase \(\alpha\) (solid or liquid). \(H\) denotes enthalpy and \(A\) denotes area, and the overbars refer to partial molar properties. A version of the expression for equilibrium between the patchy solid and striped solid would look the same, substituting a second solid superscript for the liquid superscript.
Simplifying assumptions were made only at this point: The fluid \( L_\alpha \) phase was treated as an ideal solution while the two solid phases were treated as being pure DPPC. (Now the partial molar properties become equal to the pure solution properties on a molar basis. Additionally \( x_{solid}^{DPPC} = 1 \) while \( x_{solid}^{DOPC} = 0 \).) This led to:

\[
\left( \frac{\partial T}{\partial \tau} \right)_{\text{solid-liquid}} = -T \frac{\Delta A_{\text{DPPC-solid-liquid}}}{\Delta H_{\text{DPPC}}} \tag{2.6}
\]

In equation 2.6, the slope of the coexistence curve depends only on the temperature and the area and enthalpy changes at the transition.

The simplifications of a pure solid and an ideal solution are practical because of the absence of physical property data on partial molar areas and enthalpies, but they also turn out to be good estimates from the physical perspective. Over the temperature range of interest, the binary DOPC/DPPC fluid is reasonably approximated by an ideal solution: The temperature-composition curves of Figure 3.6Bi, at zero tension, are of the general shape described by ideal solution behavior. Additionally consistent with ideal solution behavior, very similar curves for the first appearance of solid at low tensions are found for DPPC mixed with DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine)\(^{35}\) or POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine).\(^{34}\) The choice of second component does not matter for an ideal solution. Second, a moderate amount of a second species (up to 30\% for instance) in the solid phase, turns out not to change the calculated slopes by more than a factor of two, even if relatively extreme physical property data values are arbitrarily substituted for the partial molar properties of the DOPC.\(^1\)

From Equation 2.6, the slopes of lines estimating the tension sensitivity of phase transition temperatures were calculated. Table 2.1 summarizes the calculations, using physical property data from the literature. Important in making this calculation, the
physical properties of the corrugated DPPC phase are assigned to the patches and the planar tilt phase to the stripes. Lines having the calculated slopes were drawn in Figure 3.6C, starting with the phase transition temperatures measured at low tension.

The calculation reasonably approximates the data in Figure 3.6C, but is not expected to be fully quantitative. Worth noting, the composition dependence of $dT/dτ$ vanishes in Equation 2.6, so nearly the same of slopes are calculated for the transition lines at the different compositions in Figure 3.6C. Because composition influences the transition temperature (per Figure 3.6B), and because $dT/dτ$ is proportional to the temperature in Equation 2.6, there turns out to be a very slight effect (less than 1% in the range of interest) of composition on the calculated slopes. Also, without physical property data at different elevated tensions, the zero tension physical property data are applied over the full range of Figure 3.6C. In reality, some influence of tension on the physical properties would impart real curvature to the lines that have been drawn as straight. This would shift the positions of the “triple points” in ways difficult to anticipate. The presence of the triple points would, however, be preserved as long as the areal densities of the different phases retain the ranking that occurs at low tensions, which is almost sure to be the case.

We wish to emphasize a single important point by comparing the data and the calculation: Assigning the patchy phase as the ripple $P_{β'}$ phase and the stripes as the tilt $L_{β'}$ phase is necessary for qualitative agreement. Both solids are denser than the fluid $L_{α}$ phase. As a result the membrane contracts upon cooling through the transition from the fluid to either solid. This imparts negative slopes, in Figure 3.6C, to the two upper branches at low and high tension respectively. The corrugated phase is, however, more
dense than tilt phase and thus, upon cooling from the patch to the striped phase, the membrane expands. This produces a positive slope for the impact of tension on the patch-to-stripe transition. The positive slope on the lower branch is also responsible for the appearance of the “triple point” like feature for a fixed composition. Different assignment of the patches and stripes would have given slopes of the wrong sign and the triple point like feature would not occur. While the model is extremely basic, it captures the key experimental observations because of the correct assignment of the phases and because a ball park estimation of the physical property data is “good enough.”

While others report different signs on $dT/dt$,37,38 our first principles treatment argues that our phase diagrams in Figure 3.6 are correct. Worth mentioning, our observed trend that tension favors the less dense phase agrees with the report of Portet in a different system.2 Our work and Portet’s quantify tension with micropipettes in contrast to the dissenting investigations where tension is manipulated osmotically. Those dissenting reports did not quantitatively confirm the membrane tensions.

3.4 Interpretation in terms of equilibrium.

Overall we believe the phase diagram in Figure 3.6 to be a reasonable approximation (within limits of measuring boundaries by the visual appearance of dark domains) of the equilibrium phase boundaries: With tension controlled, there were minimal effects of cooling rate on the temperature of first appearance of solid phase, patches or stripes from the $L_\alpha$ fluid. Studies at zero tension but with different cooling rates confirmed the (usually minor) influence of cooling rate. The only instance where we found rate to be important was in the patch-to-stripe transition. Here rapid cooling rates favored retention of the originally formed solid patchy domains: Stabilization of patches
by rapid cooling into a region of the phase diagram where stripes were preferred likely produced large energy barriers to the conversion of the less-preferred patches to the more preferred stripes. However, even when the preferred stripe phase was kinetically disallowed, the proportions of patches (greater areal coverage with increased overall DPPC content) was consistent with equilibrium and a mass balance (“the inverse lever-arm rule”). This suggests that the patchy domains formed and trapped during rapid cooling represent a metastable or restricted equilibrium, rather than, for instance a glass. It further suggests that the boundaries for the first appearance of patches or stripes from the one-phase fluid represent, over the full range of compositions, the equilibrium surface. The same curve for the first appearance of patches, for instance, is the locus of the ends of tie lines.

3.5 Conclusions.

This study reports the complex three-dimensional phase map for mixed DOPC/DPPC membranes and it related the different solid domains observed in different regions of thermodynamic space previously reported molecular packings. While the composition dependence of the fluid-solid phase separation temperature follows expectations from previous reports, the tension-dimension of the phase diagram is rich because of the presence of two distinctly different solid phases in different regions. Ultimately three different coexistence branches (curved planes) were discovered: One for the equilibrium of the ripple Pβ' (at low tension) solid with the Lα fluid, one for the equilibrium of the tilt gel Lβ' solid (at high tension) with the Lα fluid, and a third for the coexistence of the two solids in the presence of the Lα fluid. While the Lα fluid persists at high temperatures over a full range of tensions, and the Lβ' solid is preferred at cool
(room) temperatures over a wide range of tensions. At intermediate temperatures, on the order of 30-38°C, the choice of solid is tension-sensitive: the ripple $P_{\beta'}$ phase occurs at low tensions and the $L_{\beta'}$ phase at higher tensions. The ripple $P_{\beta'}$ phase was found to always produce irregular patchy or hexagonal domains that excluded the tracer dyes considered, while the $L_{\beta'}$ solid appeared as stripes that could selectively incorporate some tracers.

3.6 Reference


CHAPTER 4
DPPC–RICH DOMAIN FORMATION IN BINARY PHOSPHOLIPID VESICLE MEMBRANES: TWO DIMENSIONAL NUCLEATION AND GROWTH

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4.1 Introduction.

Nucleation phenomena in bilayer membranes and Langmuir monolayers are important two-dimensional manifestations of ubiquitous nucleation processes that can proceed differently, depending on system specifics. Particularly in membranes, nucleation comprises the first step in one of several potential mechanistic pathways leading to phase separation and the formation of membrane domains.\(^1\) Especially when systems do not fully equilibrate, mechanisms such as nucleation can be critical in determining the ultimate type and shapes of domains, not to mention the conditions susceptible to domain formation. Relevant to biology, the concentration of active species inside small membrane domains, similar to those nucleating in model systems, may facilitate processes such as trafficking and signaling.\(^2,3\) Likewise, some enzymatic reactions are concentrated at domain boundaries.\(^4\) From the materials physics perspective, the fundamental aspects of nucleation in bilayers teach us about nucleation in real two dimensional systems where thermodynamic variables couple in intricate ways with other membrane features such as tension and bending.\(^5,6,7,8\) In addition to their role in the formation of solid membrane domains within multicomponent fluid membranes, nucleation has been implicated in the formation of voids that influence membrane
permeability, and in the interaction of pharmaceuticals and proteins with biomembranes. Related nucleation phenomena, for instance in monolayers compressed to a supersaturated state at fixed temperature, determine the formation of creases and 3D collapsed states related to lung surfactant function. Nucleation has also been shown to be a critical step in the crystallization of fatty acids in a monolayer.

Among model membrane systems potentially exhibiting nucleation and growth are binary mixtures of saturated phosphatidylcholines with lower-melting unsaturated phospholipids. Understanding phase separation in such simple binary mixed lamellae builds a foundation for the understanding of more complex mixtures containing the combined binary mixtures. Saturated phosphatidylcholines are also particularly interesting because, along with phosphatidylglycerol and sphingomyelin, they comprise a class of phospholipids commonly found in natural membranes in which the solid membrane phases exhibit a diversity of orders. The pure one-component solids include a planar tilt gel \( \text{L}_{\beta'} \), a sub-gel, and a variety of symmetric and asymmetric ripple or nano-corrugated phases \( \text{P}_{\beta'} \). Just as fascinating as these molecular orderings are the shapes of the domains seen in vesicles and supported bilayers, ranging from flowers and dendrites to uniform hexagons and intersecting stripes. It is thought that the phase behavior of the ordered-phase domains is dominated by the high-melting lipid component. We therefore expect a correlation between the nanostructure of the pure solids in single phospholipid systems and the nearly pure solids that form, usually at depressed transitions temperatures, in multicomponent bilayers.

Studies in supported bilayers have begun to connect the nanoscale morphologies to the mesoscale domain shapes with nucleation being a key step in domain formation.
Scrutinizing supported phospholipid bilayers at high magnification via electron microscopy and, more recently, AFM has sometimes revealed fascinating membrane textures, including the corrugations of the $P_{\beta'}$ ripple solid, and other features of tiny membrane domains as small as 0.5 μm. In the case of supported bilayers, in addition to the formation of solid or condensed phases on cooling, phospholipid lamella also exhibit nucleated voids that may not heal on cooling and likely arise from thermal membrane contraction. Additional evidence suggests interactions between supported phospholipid bilayers and the underlying solid supports can affect phase separation, for instance to depress the apparent phase separation temperature or to sustain membrane stresses and strains. The solid support has been shown to influence the morphology and type of phase separation, and nucleation itself.

While studies with supported bilayers bear directly on their use for sensors and templates, important additional factors, including curvature and membrane stress, motivate direct studies of vesicles to address the relationship of domain morphology to the thermodynamic phase diagram. To this end we report here on the nucleation processes of DPPC-rich domains from model mixed vesicles containing different proportions of DOPC and DPPC (1,2-dioleoyl-$sn$-glycero-3-phosphocholine and 1,2-dipalmitoyl-$sn$-glycero-3-phosphocholine, respectively). This binary mixture, one of the simplest and most studied, still exhibits great complexity in terms of the variety of domain shapes (flowers, dendrites, patches, hexagons, and stripes and underlying solid molecular ordering (symmetric and asymmetric corrugations, and planar tilt). The connection between the nano-scopic and meso-scale features is debated. Worth noting for this system, DOPC is a fluid above room temperature (with a
melting temperature of $-17\,^\circ C$\textsuperscript{39} while pure DPPC exhibits a main melting transition at $T_m = 41.5 \pm 0.5\,^\circ C$.\textsuperscript{20, 40} Below $T_m$ a corrugated ripple ($P_{\beta'}$) phase persists down to the “pre-transition” temperature ($T_{pre} = 35.5 \pm 0.5\,^\circ C$ for pure DPPC.)\textsuperscript{20, 40} Below the pre-transition temperature, a tilt “gel” $L_{\beta'}$ is the preferred equilibrium bilayer structure.

Our prior work with two component DOPC/DPPC giant unilamellar vesicles revealed a substantial tension sensitivity to the domains that formed, along with a tension sensitivity of the transition temperatures and the molecular ordering (the latter evidenced by tracer incorporation into different domain types.)\textsuperscript{35, 36} With different solids forming in different regions of thermodynamic phase space, and with the potential for dependence on thermal history, the current work focuses on nucleation at low and moderate tensions, accessing the formation of patchy and straight edged hexagonal domains that are usually compact (but can in other circumstances become dendritic). These compact domains are distinct from striped domains reported by us\textsuperscript{35, 36} and others.\textsuperscript{25, 26, 27, 41} At slow cooling rates the initially formed patchy phases can convert to stripes.\textsuperscript{35, 36} This chapter focuses exclusively on domains that nucleate and grow, and avoids the issues of the subsequent conversion of the initially formed domains.

The present work is unique in its attention to membrane tension, which is poorly controlled in supported bilayers and most studies of vesicles. In the current work, nucleation of compact irregular and straight edged hexagonal phases is put in the context of a phase or state diagram (including composition and tension) that distinguishes the first appearance of these compact domains from the first appearance of other domains types. Vesicles are osmotically conditioned to maintain zero membrane tensions and the rate of cooling from the one phase region varied to produce different nucleation densities.
(Carefully maintaining near-zero tension is key to accessing a large range of cooling rates and nucleation densities without interference from other types of phases.) These results are then compared with nucleation in vesicles at moderately elevated tensions. Care is taken to avoid extreme tensions where stripes were observed to form more directly, and potentially by different mechanisms. Importantly, while membrane tensions are controlled osmotically, the tensions themselves are measured with micropipettes. Simply calculating or estimating membrane tensions based on osmotic pressure misses thermal membrane contraction, diffusion of water across the membrane, and the impact of transient membrane tears and pores. Finally, the influence of membrane composition on nucleation is examined.

4.2 Experimental description.

4.2.1 Phase separation Studies.

Vesicles were studied in a closed chamber, sealed between two coverslips with a gap of approximately 125 μm. The chamber was coupled to a custom temperature-control block through which temperature control fluid from a heating bath was pumped. Slow cooling was accomplished through adjustments in the bath temperature. More rapid quenches required a switch to a second temperature control bath through quick-connect fittings. Examples of thermal histories, measured within the liquid of the vesicle chamber are shown in Figure 3.1. Several different examples are included, for instance cooling to room temperature or quenching and holding at a targeted temperature. Of note, for rapid (5°C/min) quench and hold protocols, the target temperature is approached within a minute or two; however, there is a slower final approach of a few minutes to the
intermediate target temperature, an unavoidable feature of thermal manipulation and approach to a fixed intermediate temperature.

In phase separation studies, vesicles were imaged before, during, and/or after cooling at different rates. Vesicles were examined on a Nikon Diaphot 300 TS inverted fluorescence microscope and images captured using a CoolSnap HQ CCD camera. Following previous procedures,\textsuperscript{35} the temperature for the first appearance of solid domains, appearing dark as a result of excluding the tracer dye, was recorded in the generation of the phase diagram.

4.2.2 Tension Control.

To maintain tension at zero (within an error of 0.02 mN/m), vesicles were electroformed in 200 mOsm sucrose solution and transferred to a 250 mOsm sucrose solution prior to study. In the tens of minutes following the transfer, water diffusion out of the vesicles caused them to become flaccid, with near zero tension. This zero-tension state was confirmed using micropipettes before (at elevated temperatures near 43 °C) and after cooling to room temperature.\textsuperscript{35, 36} The zero tension state was additionally confirmed via micropipette aspiration, in cooling runs to ~35°C, a temperature in the neighborhood of phase separation.\textsuperscript{35, 36}

Separately, to produce vesicles with elevated tensions, vesicles were electroformed and studied in DI water. Here the cooling history was chosen to reproducibly produce a membrane tension, near 1mN/m near during the onset of phase separation for the case studied below with a membrane composition of 30/70 DOPC/DPPC.\textsuperscript{35} The elevated membrane tension results from thermal contraction of the membrane during cooling and, in addition, a reduction in area when the fluid phase
solidifies. (The reduction in the area of the membrane exceeds the effect of the shrinking water volume within the interior.) The tendency to increase stress is balanced by water diffusion from the core of the vesicle and, at elevated stress, temporary pores or tears that leak the vesicle contents and then reseal.\textsuperscript{42, 43, 44} As a result, the actual vesicle tension is strongly history dependent but well characterized in our prior work.\textsuperscript{35, 36}

The area of solid phase in certain vesicles was determined by manually measuring the areas of dark domains. Where possible, the exact domain shape was included and, where this was less than clear, domains were approximated by circles. (This was shown to be reasonable approximation within a few \% error, when circles were imposed on hexagonal domains and comparisons made.) To account for the influence of the spherical vesicle surface, the radial dimension of each domain was renormalized by the circumferential domain dimension.
4.3 Results.

4.3.1 The relationship of quenching to boundaries within the phase diagram.

The state diagram constitutes a starting point, and a map, for our understanding of the kinetics and mechanisms of domain formation. The temperature-composition plot, in Figure 4.2 contains the locus of points corresponding to the first appearance of solid domains when vesicles are cooled from the one phase fluid region at elevated temperatures. The solid circle points summarize the first appearance of patchy or
compact solid domains for vesicles at maintained at zero tension through osmotic control. (These data were independently confirmed in a separate publication using micropipettes.\textsuperscript{35, 36}) Increased membrane tensions tend to slightly shift the boundary for the first appearance of patchy solid domains to cooler temperatures. An example is shown for a limited range of compositions, for a fixed membrane tension of 1 mN/m, imposed using micropipettes.\textsuperscript{35, 36} With tensions exceeding \( \sim 3\) mN/m during cooling, striped domains are the first solid domains seen.\textsuperscript{35} With their different molecular organization\textsuperscript{35} and potentially different mechanism of formation compared with the patchy solid domains studied here, the formation of striped phases is not included in the current work, though an example is shown in the “Additional discussions” section.

While we found little impact of the cooling rate on the first appearance of solid domains in Figure 4.2, the cooling rate was found to have a strong influence on domain stability. For vesicles cooled more rapidly than about 1°C/min, the solid domains grew in size with progressive cooling down to room temperature. Typical examples of the resulting phase-separated vesicles are shown at the bottom of Figure 4.2, imaged at room temperature and centered on the amount of DPPC corresponding to their overall composition. In these images, the solid domains appear as dark areas, sometimes patchy and sometimes hexagonal. The solid membrane domains are visible because they exclude the fluorescent tracer lipid, which remains in the fluid membrane phase. For vesicles cooled more slowly, on the order of 0.1°C /min, the patchy solid domains that formed when the upper phase boundary was crossed are completely consumed at intermediate temperatures by conversion to stripes. The temperature for the patch-to-stripe conversion at zero membrane tension is indicated by the solid triangles and is unique to each vesicle.
composition. (The conversion process is in itself fascinating and outside the scope of the current work.) Cooling histories that allow quantification of nucleation without complication by conversion to stripes are the focus of this chapter.

**Figure 4.2:** State diagram of DOPC/DPPC binary membranes of giant unilamellar vesicles, with example thermal histories and vesicles appearances (at room temperature) superposed. The red data mark the first appearance of solid domains upon cooling. The solid red points are for zero tension and the hollow red squares show a slight depression as a result of a tension increase to ~1mN/m. The lower green triangles mark the first appearance of a striped solid phase upon slow cooling (absent with the faster cooling histories in this chapter.) The blue arrows show example thermal quenches, shallow and deep. The vesicle images, taken at 25°C, are centered above the DPPC composition relevant to each. A tie line at room temperature is shown in gray, with the right-most end point determined based on measured solid areas. The solid composition at room temperature connects to the melting point of the pure solid.
A final point about Figure 4.2 is worth noting: The data formally represent the temperatures for the first appearance of patchy domains in fluid membranes containing differing amounts of DPPC. However, we show next that the data also approach the equilibrium envelope, across which horizontal (constant temperature) tie lines can be drawn. This interpretation is best illustrated at room temperature from the appearance of the vesicles and their increasing total area of dark solid domains with increased overall DPPC content. The images in Figure 4.2 are characteristic of all vesicles at each composition, based on at least 5 different electroformed vesicle batches for each composition. Different solid (dark) area fractions were measured for over 10 vesicles at each composition and are reported in Table 4.1.

Table 4.1 also summarizes a mass balance (detailed in the “Additional discussions”) that demonstrates how vesicles, like those in Figure 4.2, represent different points (total vesicle compositions) on the same 25°C tie line. The relationship of the observed total solid area with the mass balance involving the compositions at the ends a tie line is known as the inverse-lever arm rule. While the fluid composition at one end of the tie line is inferred from the cooling experiment, the composition of the solid is not directly accessible in our experiments, but is inferred from this exercise. Phase separation in DOPC/DPPC mixtures is, however, known to produce solid domains that are nearly pure in DPPC.29,45,46 High purity of the solid DPPC domains is consistent with our observation that tracer lipids, having saturated or unsaturated tails, were excluded from the solid domains.35 Table 4.1 therefore, compares calculations that assume pure DPPC in the solid phase with calculations for a solid phase containing 95% DPPC.
Figure 4.3: Appearance of domains on a vesicle containing a molar ratio of 35/65 DOPC/DPPC, following a quench at 5°C/min to 34.5 °C, and holding at that temperature. The vesicle contains 0.01 mol % Rh-DOPE tracer and the scale bar is 10 μm. Time zero is chosen arbitrarily, based on our ability to focus quickly after the temperature drop.

Table 4.1  Observed and Expected Solid Domain Areas at 25°C for Different Overall Amounts of DPPC

<table>
<thead>
<tr>
<th>Overall mole fraction DPPC</th>
<th>Observed Dark Area Fraction</th>
<th>Calculated Solid Area Fraction assuming pure DPPC in solid</th>
<th>Calculated Solid Area Fraction assuming 95% DPPC in solid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.17 ± 0.05</td>
<td>0.19 ± 0.03</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>0.6</td>
<td>0.28 ± 0.06</td>
<td>0.33 ± 0.03</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>0.7</td>
<td>0.42 ± 0.06</td>
<td>0.48 ± 0.03</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>0.8</td>
<td>0.56 ± 0.07</td>
<td>0.64 ± 0.03</td>
<td>0.70 ± 0.03</td>
</tr>
</tbody>
</table>

The calculated values in Table 4.1 result from a mass balance. The calculation employs an estimated molar area difference between the fluid and solid phases of ~20%, consistent with the literature on ripple-fluid and gel-fluid transitions. The tolerances reported on the calculations represent uncertainty in this parameter, allowing it
to vary between a 10-30% change in areal density. The precision on the liquid phase composition on the left side of the tie line has a similar influence on the calculated solid areas: 5% uncertainty in the liquid DPPC molar content translates approximately to a ~5% uncertainty in the expected solid domain area fraction, depending on the overall composition with a lesser effect for systems with greater amounts of DPPC. While the reported error on the observed dark area fractions represents statistical variation from vesicle to vesicle, additional error may result from inaccuracies in resolving the edges of the domains, an issue that becomes increasingly pronounced with decreasing domain size. Even with the uncertainty, the observed solid domain areas fall within expectations for the equilibrium amounts of relatively pure DPPC (including only a few percent DOPC) solid phases.

![Sequenced images of a vesicle phase separating during cooling at 5°C/min to room temperature. The vesicle contains a mole ratio of 30/70 DOPC/DPPC and 0.01 mol % Rh-DOPE tracer has been added. Scale bar is 10 μm. Images reproduced from reference 35.](image)

**Figure 4.4:** Sequenced images of a vesicle phase separating during cooling at 5°C/min to room temperature. The vesicle contains a mole ratio of 30/70 DOPC/DPPC and 0.01 mol % Rh-DOPE tracer has been added. Scale bar is 10 μm. Images reproduced from reference 35.

The calculation in Table 4.1 demonstrates that, for a broad range of initial fluid membrane compositions from 50-80 mol% DPPC, the solid DPPC phase that is retained at room temperature following a rapid thermal quench has a nearly fixed DPPC composition between 95 and 100 mol%. This DPPC composition represents the solid-side end point on an equilibrium tie line, drawn in Figure 4.2. The observation that the
same solidus datum is approached from different compositional starting points argues for the equilibrium nature of this solid point. While we understand that striped shaped domains are preferred near zero tension and at these low temperatures, when the system is kinetically constrained, the patchy solid domains (with their molecular arrangements that differ from those of the stripes) are the preferred state. One can think of this solid point as a metastable equilibrium point, a distinct local minimum in thermodynamic space. In Figure 4.2, this datum is connected to the pure solid at its 41.5°C melting temperature.

4.3.2 Evidence for Nucleation and Growth of Domains.

The image sequence in Figure 4.3 shows the typical response of a vesicle (here with a molar ratio of 35/65 DOPC/DPPC) during a thermal quench that ends inside the two phase region, illustrated in Figure 4.2. The final temperature, 34.5 °C, falls 2.5 Celcius degrees inside the phase boundary near 37°C, giving the quench depth of $\Delta T_q = 2.5 \, ^\circ\text{C}$. For simplicity, the final temperature was chosen to miss the temperature at which patchy domains convert to stripes. This composition produces only a handful of domains, making them easy to track for purposes of illustration. In this run rapid cooling, with an average rate of $5\, ^\circ\text{C}/\text{min}$, occurred within the first few seconds of the study. Then, as explained in the Experimental Description section, the final few degrees of approach to the targeted temperature occurred more slowly, over a period of $\sim$1 min. This slow final approach to the final temperature could not be avoided. The final temperature was, however, constant, within an error of $\pm 0.2^\circ\text{C}$. 
As this vesicle was cooled, solid domains, about 1 μm in diameter were immediately visible. The domains then increased more in size than in number as the final temperature was approached. The change in numbers was seen, when viewed in real time by eye, to result from translation of the domains over the surface and into/out of view. Watching the series by eye also made clear that the domains were not dissolving, re-developing, or coalescing. (The lack of coalescence is consistent with the solid-like nature of the domains along with a relatively stable fluid region between the domains, potentially stabilized by vesicle curvature.) Also in Figure 4.3, the domains are relatively uniform in size. Important observations from Figure 4.3 include the rapid initial appearance of domains, their fixed numbers after the initial quench and, once the temperature was fixed, a constant size.

Figure 4.4 shows a related series of images obtained when a vesicle having a molar ratio of 30/70 DOPC/DPPC was quenched to room temperature at a rate of 5°C/min. Solid domains appeared at short times and grew in size with continued cooling down to room temperature. While our separate studies have established the conversion of patchy solid domains to stripes upon very slow cooling to room temperatures, the patchy and hexagonal solid domains grown with faster thermal histories like those in Figure 4.4 are relatively stable (over the period of observation) at room temperature. An interesting observation in Figure 4.4, when two small domains are in close proximity, sometimes appearing aggregated, progressive growth from all sizes moves their centers of mass further apart. The “aggregated” domains were not, in this case, in true contact and their proximity did not interfere with their growth.
Taken together, Figures 4.3 and 4.4 support the mechanism of nucleation and growth. Specifically, Figures 4.3 and 4.4 establish a narrow time window for the first appearance of patchy domains, and a broader subsequent time and temperature window for domain growth upon further cooling. The effectively narrow window for nucleation can be exploited to produce uniformly sized domains.

While, in the next section we probe factors affecting the nucleation density, here we note that the rate of domain growth appears not to be limited by the diffusion of DPPC to the edges of the domains. During cooling, the domains grow with the temperature change itself, tracking the equilibrium amount of solid phase. There is no visible growth once the temperature is fixed. Since the fluid phase contains a substantial amount of DPPC, the diffusion length necessary to bring DPPC to the domain edge is small and the characteristic diffusion time fast. This behavior is consistent with the observed compact domain shapes rather than dendrites or flower-shapes.

4.3.3 Effect of Cooling Rate on Nucleation.

The nuclei-producing the domains shown in Figures 4.3 and 4.4 are too small to be seen. Only once nuclei grow to micron-sized domains and larger are they clearly discernable in our microscope. Three factors (the ultimate stability of the micron-sized domains, their growth only with continued decreases in temperature, and their lack of coalescence or ripening) all suggest that the number of visible domains is an indicator of the number of stable nuclei per unit area forming during nucleation. The observation that the domain numbers do not change with cooling to room temperature enables us to count the final domain numbers as an indicator of the density of stable nuclei. The nucleation
density is, in turn roughly proportional to the nucleation rate of classical theory, discussed below.

Figure 4.5A, B, and C illustrate the impact of cooling rate on the numbers and sizes of domains found at room temperature, for vesicles with a molar ratio of 30/70 DOPC/DPPC. Here the thermal histories, indicated in Figure 4.1, started with annealing at 43°C in the one phase region for 5 minutes followed by cooling past the transition temperature of ~38°C to room temperature. For each cooling rate three vesicles from different batches are shown to demonstrate the reproducibility of the morphology. All these vesicles have been conditioned in sucrose to maintain zero membrane tension. Clear in Figure 4.5A-C is that the number of domains increases dramatically with the cooling rate. Equally stunning is the size uniformity of the domains, reinforcing the previous discussion of a sharp nucleation period prior to the growth step. Notably, when histories produce more nuclei, the final state at room temperature necessarily comprises smaller domains, since the total amount of solid phase at room temperature at equilibrium is fixed.

Part D of Figure 4.5 shows additional vesicles subject to a 5°C/min cooling history in DI water. This procedure was established to produce membrane tensions near 1 mN/m as the temperature passes in the neighborhood of 35°C, near the moments of initial phase separation.36 (Also established was a minor influence of tension on the apparent equilibrium phase separation temperature, included in Figure 4.2.) Careful examination of Figure 4.5D reveals a slightly smaller number of domains in the tensed vesicles compared with zero tensioned vesicles also cooled at 5°C/min, suggesting that membrane tension slightly reduces the nucleation density.
Figure 4.6 summaries the nucleation number densities for different cooling rates and for the effect of tension. Each datum averages 10 vesicles like those in Figure 4.5. The number of nucleated sites was obtained by counting the domain numbers on each vesicle, per unit area. The assumption that the final domain number is equal to the number of nucleated sites is supported by the observations, in Figures 4.3 and 4.4, concerning domain evolution (with a lack of domain dissolving or coalescence) and subsequent stability.

The data in Figure 4.6 at zero membrane tension reveal a greater than linear dependence of the nucleation density on the cooling rate where the latter is typically thought to be proportional to effective quench depth. Additionally it is found that tension has a modest, but statistically significant, effect to reduce the nucleation rate.

Figure 4.7 illustrates the impact of membrane composition on the domain formation. The series of images, in Figure 4.7A, show the sizes and domain shapes typical of vesicles at each composition, for vesicles quenched at 5°C/min from the one phase region to room temperature. Figure 4.7B summarizes the nucleation densities as a function of membrane composition, with each datum averaging the domain numbers over 10 vesicles. The numbers of domains on each vesicle becomes small, just one or two, as the overall molar ratio of 65/35 DOPC/DPPC is approached. This is the composition where the phase boundary of Figure 4.2 approaches room temperature. At compositions with less DPPC, the vesicle will remain a single-phase fluid membrane at room temperature. As a result of the small domain number in this limit, the statistical error on the nucleation densities grows large. Figure 4.7 illustrates that, as expected, the domain number increases with composition. Also evident in Figure 4.7A is an increased
irregularity in the domain shape at low compositions. This may be a result of the lowered diffusive driving force and smaller diffusion rate for growth as the fluid phase is made increasingly less concentrated in DPPC.

4.4 Discussion.

This study examined the impact of cooling rate, tension, and composition on the formation of compact DPPC-rich solid domains in giant DOPC/DPPC unilamellar vesicles. Tracking the domain evolution, starting with the initial appearance of solid domains to a final temperature near 25°C, revealed a lack of coalescence or domain dissolving as the domains grew with progressive cooling. With this, it was possible to interpret the final domain number as the nucleation density. Important in the interpretation of the nucleation study, the envelope of temperatures and compositions where the first solid domains become visible from the single-phase fluid was shown to be consistent with an equilibrium phase boundary. The current study further analyzed the dark areas of the patchy solid domains and found their amounts to be consistent with a lever arm rule with a solidus point corresponding to DPPC concentrations near 95 mol% or more. The amounts of solid patchy phase were consistent with the same endpoint of the tie line, regardless of the vesicle starting composition, arguing that the kinetically-trapped state resulting from these thermal histories was a local equilibrium. Our previous work presented qualitative and quantitative arguments that the patch-shaped solid domains are comprised of a corrugated or ripple $P_{β'}$ solid phase.$^{35}$
Figure 4.5: Vesicle appearance, emphasizing domain density for different vesicles all having a molar ratio of 30/70 DOPC/DPPC. The scale bar is 10 μm.

In nucleation studies, rapid cooling from the single-phase region to the unstable two-phase region is associated with deep quenches, ΔTq, (because little time is spent at temperatures slightly inside the phase boundary, and because the thermal history accesses regions deep into the unstable two phase region.) The expected influence of quench depth or other properties such as a surface tension on the nucleation rate is classically...
thought to depend on the assumed nucleus shape. For instance, in the textbook example for three-dimensional systems in which a solid phase grows from a spherical nucleus, the height of the energy barrier to form a critical nucleus scales as the quench depth, $\Delta T_q$, squared, as reviewed in the “Additional discussions”. This is a result of the driving force to form the nucleus, which increases as the heat of the fusion and the depth of quench, balanced against the expense (surface tension) to produce the surface of the nucleus.

**Figure 4.6:** Dependence of nuclei density on the cooling rate and tension for vesicles having a molar ratio of 30/70 DOPC/DPPC. At least 10 vesicles were measured for each datum and the error bars show the full range of the data. Solid square are for zero tension while the red point is for $\tau \sim 1$mN/m.

In a membrane, the nuclei are likely to be two-dimensional. The energy barrier opposing their formation contains a driving force (the heat of fusion times the depth of the quench, similar to the 3D case) balanced against a term containing the line tension at
the nucleus perimeter. The result depends on the shape of the nucleus, as described in the “Additional discussions”. In the two dimensional geometry, if nuclei are assumed isotropic circular discs, the energy barrier to form a critical nucleus, $\Delta G^*$, scales as the inverse of the quench depth, $\Delta T_q$:

$$\Delta G^* = -\frac{\pi}{4} \left[ \frac{\mu_L^2 T_{eq}}{4 \Delta H_{fus}} \right] \frac{1}{\Delta T_q} \quad (4.1)$$

Here $\mu_L$ is the line tension, $T_{eq}$ is the equilibrium temperature for phase separation at the composition of interest, and $\Delta H_{fus}$ is the molar heat of fusion. Additionally, the size of the critical membrane nucleus, $r_c$, is

$$r_c = \frac{-\mu_L T_{eq}}{4 \Delta H_{fus} \Delta T_q} \quad (4.2)$$

The inverse scaling on quench depth is therefore expected to be markedly different for membranes than in classical three-dimensional systems. Proving that this is the case from experimental data is, however, difficult: the nucleation rate scales as the exponent of the energy barrier:

$$\text{Nucleation rate} = \text{attempt frequency} \, e^{\Delta G^*/kT} \quad (4.3)$$

A convincing determination of the experimental scaling with quench depth would require several decades in cooling rate. Additionally, because of the tendency of DPPC to assemble into lamellae, there is no obvious three-dimensional system for comparison. This said, we note a greater than linear dependence of the nucleation density of Figure 4.5 with cooling rate. Worth noting, the decreased nucleation density with membrane tension is consistent with an increased line tension around the nuclei as the membrane tension is manipulated and increased.

An important take-home point from the current work is the significance of the observed range of nucleation densities, from 0.01 - 0.1 / $\mu m^2$ in Figures 4.5 and 4.7 over
the full range of cooling rates and compositions accessible. An interesting point of
comparison is the nucleation densities reported by Nam et al.\textsuperscript{1} For DPPC mixed with a
polymer that is more viscous than fluid DOPC, these authors report a similar dependence
of the DPPC nucleation rate the cooling rate, but a strong dependence of the final solid
area fraction on cooling rate. Conversely, we report no significant dependence of the
final solid area fraction on cooling rate, more consistent with approach to local
equilibrium in our system.
Figure 4.7: (A) Appearance of vesicles of different compositions after cooling to room temperature at 5°C/min. The molar compositions are indicated and the scale bar is 10 μm. (B) Summary of the influence of vesicle composition on the nucleation density for vesicles cooled at 5°C/min in DI water.
Another interesting point of reference is provided by a study of nucleation in single immobilized DOPC/DPPC bilayers on mica. For a narrower window of cooling rates, still overlapping those in our work, that study reports nucleation densities of 0.01 - 0.1 / μm². Their exact values for the nucleation densities for a given cooling rate and composition are slightly greater than a factor of two times our observed values for the same conditions. That prior work reports, however, roughly half of the domains formed repeatedly at the same surface positions upon melting and re-cooling. Thus slightly more than half the domains in the supported bilayers were attributed to interactions with the mica substrate. The remaining domains were thought to have nucleated from physical processes within the membrane. To first order, therefore, the previous chapter on immobilized DOPC/DPPC membranes is in agreement with our reported nucleation densities.

An important distinction between the domains, nucleated and grown in vesicles, compared with analogous domains in supported bilayers is also worth pointing out: The domains in our study are compact and in some cases reflecting a hexagonal shape with distinct edges and vertices. This contrasts the dendritic structure of domains nucleated and grown in DOPC/DPPC bilayers on mica. It is interesting to speculate that the differences in domain growth patterns result from a lower mobility of domains on the mica and the more fluid environment of the vesicles. While the DPPC diffusion may be faster in free membranes, the steps of DPPC incorporation at the edge of each domain would also appear to proceed more easily toward equilibrium in the free membranes, producing the compact domains in our vesicles.
4.5 Conclusions.

This work examined the nucleation and growth of solid domains that formed upon cooling of giant unilamellar vesicles containing different proportions of DOPC and DPPC. The nucleation study was put in the context of the state diagram for mixed DOPC/DPPC vesicles at zero tension, where application of the lever arm rule to the solid area fraction of the vesicles argued that the final state of the nucleation study was a local equilibrium. The domains were first visible, by fluorescence microscopy, when they attained a diameter of about a micron. Because the domain numbers did not change considerably with cooling, and because the identity of individual domains was easy to track during cooling, we associated the numbers of domains present on vesicles with the nucleation density. The nucleation densities were in the range of 0.01 to 0.10/μm² for cooling rates of 1-10 °C/min. The highest nucleation densities were found with the most rapid cooling, consistent with an increase in nucleation rate with depth of quench, as expected from classical theory. We found a greater than linear dependence of the nucleation density or rate on the cooling rate, in support of classical nucleation theory. We also observed a slight decrease in nucleation density with an increase in membrane tension. The nucleation densities were compared with nucleation reported previously with a similar membrane in a supported configuration. The prior study, done at a smaller range of cooling rates, reported additional nuclei as a result of interactions with the substrate, but the reported nucleation densities originated from the membrane agree with our findings. Also interesting, the solid domains formed in the vesicles were compact and even hexagonal in shape. These shapes were in sharp contrast to the dendritic shapes reported for supported membrane analogs. This difference suggests that a membrane
support can interfere with domain formation, and that domain formation may more nearly approach an equilibrium structure in giant unilamellar vesicles.

4.6 Additional discussion.

4.6.1 Lever arm rule for solid area in a two component membrane

![Schematic of two component phase diagram](image)

**Figure 4.8:** Schematic of two component phase diagram, like that for DPPC/DOPC. Species A solidifies on cooling, with a pure component melting temperature where the equilibrium curves intersect the right-side y-axis.

A vesicle having overall mole fraction DPPC, $z_A$, initially in the fluid phase is cooled into the two phase region. The resulting fluid and solid phases have DPPC mol fractions $x_A$, and $y_A$, respectively. As illustrated in Figure 4.8, the mole fractions of A in the fluid and solid lie on the ends of a tie line and the solid is predominantly DPPC. Also based on 1 mole of total lipid, the moles (or mole fractions) in the fluid and solid phases are $L$ and $S$, respectively.
A mass balance on the DPPC, stating that the amount of DPPC in the fluid and solid phases must sum to the overall DPPC in the mixture is written:

\[ x_A L + y_A S = z_A \]  \hspace{1cm} (4.1)

Rearrangement for the mole fraction of lipid (DPPC and DOPC together) in solid phase gives:

\[ S = \frac{z_A - x_A}{y_A - x_A} \]  \hspace{1cm} (4.2)

The fraction of moles that are found in the solid domains is related to the area covered by those domains by the ratio, \( R \), of the molar area of lipid in the solid phase, \( A_s \), to that in the fluid phase, \( A_l \). That is,

\[ R \equiv \frac{A_s}{A_l} \]  \hspace{1cm} (4.3)

\( R \) is simply a property of the types of solid and fluid and is temperature dependent. On a phase separated vesicle, the observable fluid and solid areas are \( A_L \) and \( A_S \). (These are not usually per mol but are extensive.) As a result the fraction, \( F_s \), of the vesicle surface covered by solid domains, at equilibrium, is

\[ F_s = \left( 1 + \frac{A_L}{A_S} \right)^{-1} = \left( 1 + \frac{L}{S R} \right)^{-1} \]  \hspace{1cm} (4.4)

This gives

\[ F_s = \left( 1 + \frac{1}{R \frac{z_A - y_A}{x_A - z_A}} \right)^{-1} \]  \hspace{1cm} (4.5)

Therefore, with the overall vesicle composition, \( z_A \), set at the time of vesicle fabrication, and \( x_A \) and \( y_A \) determined from the equilibrium curves, (and \( R \) determined from the literature), the fraction of vesicle surface covered by the solid can be calculated and compared with observations.
4.6.2 Nucleation theory.

The rate at which stable nuclei are formed (and have the opportunity to grow) depends on the free energy of their formation.

\[ \Delta G(r) = \Delta G_{\text{bulk}}(r) + \Delta G_{\text{surf}}(r) \]  

(4.6)

\( \Delta G(r) \) is the free energy to create a nucleus of size \( r \) and is negative when nuclei formation is favorable. \( \Delta G(r) \) is made up of bulk and surface terms, an assumption that requires that nuclei have well-defined surfaces that can be described in terms of accessible physical properties. In classical theory, the surface term opposes nucleation (the surface term is positive) because it costs energy to form a surface. The bulk term favors nucleation (is negative) because the system has been quenched (usually thermally) from the one-phase region to a state where global equilibrium favors phase separation (condensation or crystallization). In classical theory, the two terms combine to produce an energy barrier against nucleation at a maximum in \( \Delta G(r) \), denoted \( \Delta G^* \).

The energy barrier, \( \Delta G^* \) is associated with a critical nucleus of radius \( r_c \), and hence the nucleation rate depends on the height of the energy barrier, \( \Delta G^* = \Delta G(r_c) \).

\[
\text{Nucleation rate } \propto e^{-\frac{\Delta G^*}{kT}}
\]

(4.7)

In classical theory the nucleation rate is described on a per-volume basis.

*Classical Theory for Three-Dimensional Systems.* The surface term is the surface tension, \( \mu \), times the area of a spherical nucleus:

\[ \Delta G_{\text{surf}}(r) = 4\pi r^2 \mu \]  

(4.8)

Here surface tension \( \mu \) is treated to be independent of quench depth.
Classically, the bulk term is nucleus volume times the is the free energy density (corresponding to the bulk phase), $\Delta G$ ($\Delta G$ is independent of $r$.) For a spherical nucleus this is:

$$\Delta G_{\text{bulk}}(r) = \frac{4\pi r^3}{3} \Delta G$$ (4.9)

Classical nucleation theory predicts that the energy to form a nucleus experiences a maximum (at critical nucleus size, $r_c$, where the surface and bulk terms are balanced.) Practically this means that small nuclei are unstable and disperse but large nuclei can grow.

In order to address how the nucleation rate should depend on the depth of quench, we must evaluate the height of the energy barrier as a function of the quench depth. This requires first identifying $r_c$, the size of the critical nucleus and then evaluating $\Delta G(r_c)$.

For a classical 3-dimensional system

$$\Delta G(r) = \frac{4\pi r^3}{3} \Delta G + 4\pi r^2 \mu$$ (4.10)

Taking the derivative of $\Delta G(r)$ with respect to $r$, gives

$$\frac{\partial \Delta G(r)}{\partial r} = 4\pi r^2 + 8\pi r \mu$$ (4.11)

Setting this to zero gives the size of the critical nucleus

$$r_c = \frac{-2\mu}{\Delta G}$$ (4.12)

We note here that the opposition of surface tension to nucleation means that $\mu$ contributes a positive (repulsive) contribution to $\Delta G(r)$. The bulk term $\Delta G$ favors nucleation and is therefore negative, so that $r_c$ is a positive number.

For classical three dimensional systems $\Delta G$ is proportional to the depth of quench, $\Delta T_q = |T - T_{eq}|$. (Because of the absolute values, the quench depth is defined to be
positive.) $T_{eq}$ is the temperature of the phase transition and $T$ is the final temperature of the quench-and-hold experiment.

$$\Delta G = \Delta H_{trans} \frac{\Delta T_q}{T_{eq}}$$  (4.13)

Here, $\Delta H_{trans}$ is the heat of the phase transition at the equilibrium temperature, for instance condensation of a liquid or the heat of fusion, in the case of crystallization, and is negative.

So the critical radius depends on the depth of quench:

$$r_c = \frac{-2 \mu T_{eq}}{\Delta H_{trans} \Delta T_q}$$  (4.14)

And thus the depth of quench, $\Delta T_q$, influences the energy barrier for nucleation in a complicated way:

$$\Delta G^* = \frac{4 \pi}{3} \left( \frac{-2 \mu T_{eq}}{\Delta H_{trans}} \right)^3 (\Delta T_q)^{-3} \Delta H_{trans} \frac{\Delta T_q}{T_{eq}} + 4 \pi \mu \left( \frac{-2 \mu T_{eq}}{\Delta H_{trans}} \right)^2 (\Delta T_q)^{-2}$$  (4.15)

$$= \frac{16 \pi}{3} \left( \frac{\mu^3 T_{eq}}{\Delta H_{trans}} \right)^2 \frac{1}{(\Delta T_q)^2}$$

The energy barrier ultimately is seen to scale with the inverse square of the quench depth and also as the cube of the surface tension. The latter is assumed, in classical theory, not to depend on the depth of quench. This result, in addition to making classical assumptions, has been developed for a spherical nucleus forming in three-dimensional space.

Here we examine the predictions of classical theory for the influence of quench depth on the nucleation rate in a two-dimensional system such as a membrane. The assumptions and development are the same as before, but now the dimensionality of the system is reduced to two. The free energy to create a nucleus of radius $r$ now contains a
two-dimensional bulk term which is favorable and an interfacial term, associated with the perimeter of the nucleus, which opposes nucleation.

The energy at the nucleus perimeter, involves the line tension, $\mu_L$: (Here treated as isotropic, since we have no specific information otherwise and since the domains are round or hexagonal, rather than elongated.)

$$\Delta G_{\text{perimeter}}(r) = 2\pi r \mu_L$$  \hspace{1cm} (4.16)

The line tension, $\mu_L$, is treated to be independent of quench depth, as was the surface tension in the three-dimensional treatment.

The bulk energy of the two dimensional nucleus includes an energy density, now per unit area $\Delta \bar{G}$:

$$\Delta \bar{G} = \frac{\Delta H_{\text{trans}}}{T_{\text{eq}}} \frac{\Delta T_q}{T_{\text{eq}}}$$  \hspace{1cm} (4.17)

which is still proportional to the same quench depth, along with the heat of formation of the membrane solid from the membrane fluid. Combining edge and area contributions to the nucleus, the free energy to form a nucleus of radius $r$ becomes:

$$\Delta G(r) = 4\pi r^2 \Delta \bar{G} + 2\pi r \mu_L$$  \hspace{1cm} (4.18)

Setting the derivative to zero and substituting for the bulk free energy gives the size of the critical membrane nucleus:

$$r_c = \frac{-\mu_L T_{\text{eq}}}{4 \Delta H_{\text{trans}} \Delta T_q}$$  \hspace{1cm} (4.19)

The height of the energy barrier follows:

$$\Delta G^* = 4\pi \left(\frac{-\mu_L T_{\text{eq}}}{4 \Delta H_{\text{trans}}}\right)^2 \left(\Delta T_q\right)^{-2} \frac{\Delta H_{\text{trans}}}{T_{\text{eq}}} \frac{\Delta T_q}{T_{\text{eq}}} + 2\pi \mu_L \left(\frac{-\mu_L T_{\text{eq}}}{4 \Delta H_{\text{trans}}}\right) \left(\Delta T_q\right)^{-1}$$  \hspace{1cm} (4.20)

$$= \frac{-\pi}{4} \left[\frac{\mu_L^2 T_{\text{eq}}}{\Delta H_{\text{trans}}}\right] \frac{1}{\Delta T_q}$$
This result is interesting in that the height of the energy barrier for the two-dimensional system scales as the inverse of the quench depth whereas before, in three dimensions the energy barrier scaled as the inverse quench depth squared. Hence there is a weaker dependence in membranes of the nucleation rate on the quench depth than in the equivalent three-dimensional system. Worth noting, the minus sign here combines with the negative sign on the enthalpy change to give positive energy barriers (as was the case in three dimensions.)

4.7 Reference.


CHAPTER 5

HYBRID COPOLYMER-PHOSPHOLIPID VESICLES: PHASE SEPARATION
RESEMBLING MIXED PHOSPHOLIPID LAMELLAE BUT WITH
MECHANICAL STABILITY AND CONTROL

5.1 Introduction.

Multicomponent phospholipid vesicles have long held scientific interest because of the insight they provide into biological membranes and their utility as drug delivery agents. Giant vesicles made of copolymers, called “polymersomes” by some,\textsuperscript{1,2} are a relatively newer construct. Polymer vesicles have attracted focus for the past two decades because of their exceptional mechanical properties by comparison with phospholipids,\textsuperscript{1,2} including tunable viscoelasticity, membrane diffusion,\textsuperscript{3} and bending mechanics;\textsuperscript{4} large lysis strains and tough behavior;\textsuperscript{1} and resistance to surfactants.\textsuperscript{5} While parallels have been drawn between the polymer and phospholipid vesicles, relatively little work has focused on hybrid membranes containing both phospholipids and copolymers. (Here we distinguish hybrid vesicles as those whose primary components form lamellae on their own, different from the addition of non-lamellae-forming surfactants and nanoparticles to polymeric or phospholipid bilayers.) Factors working against the formation of hybrid vesicles include fundamental incompatibilities between the two types of lamella such as differing thicknesses, and chemical incompatibility between the components themselves (which would lead to high line tensions and lack of cohesion in phase separated hybrid membranes). Indeed, some descriptions of hybrid vesicle systems mention broad compositional ranges where vesicles (or at least hybrid vesicles) do not form.\textsuperscript{6,7,8}
Recent studies demonstrate that the formation of hybrid vesicles is facilitated by careful choice of the copolymers, with appropriate chemistry, architecture, and molecular weight. While some studies have focused on hybrid liposomes of submicron diameter, giant unilamellar vesicles are particularly appealing because membrane mechanics can be probed and interesting membrane physics visualized. The list of combinations forming giant hybrid unilamellar vesicles includes a copolymer of polyethylene oxide (PEO)-co-polybutadiene mixed with DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine, or DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) with and without cholesterol; a PEO-co-polyisobutylene copolymer mixed with DPPC; a polyoxozaline-PDMS-polyoxozaline ABA triblock mixed with phosphatidylethanolamine or DPPC, and a graft PDMS-PEO mixed with DPPC and POPC. It is interesting to ask how the overall membrane features are dominated by one or the other compound and how the mixed vesicles compare with other systems, such as well-studied model phospholipid mixtures. In particular, one might wonder how well biomimetic features (for instance the phase separation and responsiveness of phospholipids) can be achieved, still maintaining the mechanical robustness of polymer vesicles. The prospects look good, as hybrid vesicles have been shown to have improved delivery properties and exhibit key biomimetic functionality, such as adhesion-triggered phase separation.

The current work develops a qualitative and quantitative account of the physical features that underlie the biomimicry and robust mechanics in a potentially powerful hybrid system: poly(dimethyl siloxane)-co-poly(ethylene oxide) [PDMS-co-PEO] graft copolymer, mixed with DPPC, over the full compositional range. The silicone graft
copolymer, Xiameter OFX-5329, previously sold under the name Dow Corning 5329, is established to form lamellae and giant unilamellar vesicles.\textsuperscript{15, 16} It has been well studied by us\textsuperscript{17, 18} and others.\textsuperscript{15, 16} Important to the current work, the graft copolymer, while polydisperse, has an overall molecular weight of 3250 g/mol with 2-3 PEO side arms per each PDMS backbone, and each PEO arm containing 12 EO units. When hydrated, it spontaneously forms lamellae and the membrane thickness, including the hydrated PEG coronas, is 7.7 nm.\textsuperscript{15}

DPPC, a solid at room temperature, is a particularly interesting phospholipid because of its prominence in cell membranes and because its molecular shape imparts complexity to its lamellar organization. DPPC’s relatively large phosphatidyl head group compared with its smaller hydrophobic tails facilitate a molecular tilt within the solid phase, a feature also found in other saturated phosphatidylcholine,\textsuperscript{19, 20} PS, and PG lamellae. Cholesterol-containing membranes comprised of DPPC and low melting phospholipids display a variety of phases including a mixed fluid (L\textsubscript{$\alpha$}), liquid condensed, and “gel” or solid-like phases, depending on composition.\textsuperscript{21, 22, 23} Even without cholesterol, membranes containing DPPC mixed with low melting phospholipids exhibit a variety of solid domains in a stunning array of patterns including flowers, blobs, stripes, and hexagons.\textsuperscript{24, 25, 26} Feigenson Contributing to observed domain morphologies are the mechanisms and the conditions during DPPC solidification, the molecular arrangement of the DPPC, and kinetic trapping of the system in a local equilibrium compared with access to a more global equilibrium.

Important in determining domain morphology in mixed vesicles are the properties of DPPC itself. Pure (but hydrated) DPPC melts at 41.5°C and mixed membranes
containing DPPC are usually single L_α phase above this temperature. Below 41.5°C but above the pretransition temperature of 35.5°C, DPPC lamellae exist in a “ripple” or “corrugated” structure where the molecules are aligned at 20 degrees from the local membrane surface normal but their overall projection is roughly perpendicular to the macroscopic membrane. At temperatures below the pretransition, DPPC forms a “gel” phase in which the membrane is relatively flat and smooth, and the molecules are tilted approximately 30 degrees from the surface normal. The gel and ripple solids are difficult to distinguish by NMR and FRET because of their similar short-range molecular orderings; however, other methods (XRay and DSC) clearly identify different polymorphs having different areal densities in their lamellae.

Ultimately, the control of a membrane’s molecular organization and the domain shape is important because the solid type and the domain shape determine ability of different membrane phases to connectedly span large distances, affecting transport along the membrane. Additionally, curvature at the domain edges may influence biochemical reactions at these locations.

The current work benchmarks hybrid copolymer/DPPC membranes against a well-studied model membrane system, DOPC/DPPC. DOPC/DPPC membranes are, in their own right, complicated by the different DPPC solid polymorphs and the sensitivity of the membrane to tension. The current study compares the domain morphology in the two systems over a broad range of composition. This study addresses membrane mechanics, the phase transition temperatures, and the utility of membrane tension in selecting the solid DPPC molecular organization and domain shape.
5.2 Experimental description.

5.2.1 Lipids and copolymer.

1,2-dioleoyl-\textit{sn}-glycero-3-phosphocholine and 1,2-dipalmitoyl-\textit{sn}-glycero-3-phosphocholine (DOPC and DPPC respectively) from Avanti Polar Lipids (catalog numbers 850375C and 850355C) were used as received. 1,2-dioleoyl-\textit{sn}-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt, [Rh-DOPE, catalog number 810150C] and 1-2-dipalmitoyl-\textit{sn}-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt, [Rh-DPPE, catalog number 810158C] from Avanti were employed as tracer lipids.

A graft copolymer having a PDMS (polydimethylsiloxane) backbone with PEO (polyethylene oxide) graft side chains was a gift from Xiameter. This compound is the same as previously sold under the name Dow Corning 5329, on which there is a large literature documenting its lamellar structure and tendency to form giant unilamellar vesicles.

5.2.2 Lipid and hybrid vesicle formation.

Giant unilamellar vesicles were prepared by electroformation, as previously described.\textsuperscript{32} Copolymer and DPPC, or DOPC and DPPC, in the desired molar proportions, were dissolved in chloroform near a concentration of 1 mg/ml. Rh-DPPE or Rh-DOPE tracers were employed at a concentration of 0.1 mol%. (Tracer concentrations of 0.1-0.5 mol% were found not to influence the results reported here.) A 10 μL quantity of solution was placed on the platinum wire electrodes and, after drying under nitrogen, the chamber was filled with DI (de-ionized) water or sucrose solution that had been
preheated to 52°C. An alternating current was applied to the electrodes at 3V and 10 Hz for 1 hour, while the chamber was maintained at 52°C, to ensure the compositional uniformity of the vesicles. The vesicle solution was harvested in a syringe.

5.2.3 Characterizing Vesicle Stress and Strain.

Some studies focused on vesicles whose membrane tensions were manipulated osmotically to near zero (within instrumental error of 0.02 mN/m). To produce flaccid vesicles with near-zero tensions over a range of temperatures, vesicles were first electroformed in a 200 mOsm sucrose solution, and then equilibrated in 250 mOsm sucrose solution. Over a few tens of minutes, water diffusion out of the vesicles made them flaccid. The extent of “flaccidness” has been previously quantified in terms of an excess area parameter.30 For the hybrid vesicles prepared in this fashion, zero-tension was confirmed using micropipettes, with data included in Figure 4A of the Results section.

In contrast to osmotically-conditioned flaccid vesicles, most of the vesicles in the current program were formed and equilibrated in DI water at elevated temperatures prior to cooling. Phospholipid and hybrid vesicles conditioned in this way were observed not to exhibit excess area and were, instead, discovered to be tensed after controlled cooling to intermediate and room temperatures. It is not obvious that this simple treatment should produce elevated membrane tensions; however, characterization studies described previously29,30 and applied below to hybrid vesicles demonstrated that this was indeed the case, as detailed in the Results section.

The hypothesized mechanism for development of membrane stress involves a starting point at elevated temperatures, for instance near 50°C in our studies, where
vesicles exhibit little, if any, excess area, as was confirmed. Subsequent cooling causes both the vesicle membrane and its water contents to contract, as a natural consequence of their thermal expansivities. The membrane, however, contracts more extensively than the aqueous vesicle center, causing development of membrane stress. The membrane stress relaxes as water diffuses out of the vesicles across the membrane, on a timescale of minutes. Thus the actual values of the membrane stress and its variation with time depend on the cooling rate relative to that of water diffusion. If cooling is extremely fast, the membrane ruptures and reseals, so that it can be difficult to anticipate whether fast or slow cooling will produce higher tensions. Extremely slow cooling, however, always results in low tensions throughout the phase separation process.

Micropipette aspiration was employed to probe two different features of the stressed state of vesicles. The first was the traditional stress-strain curve, including the area expansion modulus, $K_a$, and the second was the membrane strain inherent to free vesicles manipulated in DI water versus sucrose solutions. Both types of micropipette studies employed open sided micropipette-accessible chambers, made of coverslips supported in a gap configuration with a spacing of ~1mm. The ends of the chamber were coupled to a temperature reservoir that was regulated by a heating bath to maintain a constant temperature.

Stress-strain curves were measured following established procedures. A test vesicle was aspirated at low suction (1-2 cm water) and then stretched to a great extent, to incorporate any tethers back into the membrane and ensure good lubrication with the micropipette. After the tension was set close to zero, the suction was increased stepwise and held at each step for about 10 seconds. Vesicle images and suction pressure were
recorded on video during the process. If the vesicle did not break, the tensions could be stepped back down and then back up to confirm reversibility. Mixed phospholipid vesicles, however, tended to lyse easily.

Video images of vesicles were analyzed to determine the membrane area and therefore the strain at each step. The membrane tension, \( \tau \), was calculated from the LaPlace Equation (2.7):

\[
\tau = \frac{P_s R_p}{(2 - \frac{2R_p}{R_v})} \quad (2.7)
\]

Here \( R_p \) and \( R_v \) are the micropipette and vesicle radius (outside the micropipette), respectively. \( P_s \) is the suction pressure applied to the micropipette from the manometer.

Apart from the characterization of the membrane mechanics, separate studies quantified the combined impact of cooling history and osmotic conditioning on the hybrid membrane stress, targeting the condition of the membrane at the initial instants of phase separation. Following the procedure previously developed for DOPC/DPPC vesicles, hybrid vesicles were cooled at the rate of interest to 35°C, a temperature within range of the phase transition, and the vesicle were transferred from the closed chamber to the micropipette chamber. (Precision cooling in the micropipette chamber was confounded by its open architecture, necessary for micropipette access.)

Measurements were made within minutes of transfer to the micropipette chamber, allowing some tension loss, but minimizing the loss as much as possible. The stress state of vesicles was characterized by micropipettes by first attempting to aspirate at extremely low suctions, producing membrane stress in the range 0.02-0.04 mN/m, corresponding to areal strains of less than 0.1%. For flaccid vesicles, a projection would appear in the micropipette and excess area could be quantified. Tensed vesicles, however, would be
pulled to the mouth of the pipette but not produce a projection. Subsequent increase in suction would produce a projection only once the suction pressure balanced the opposing force from the membrane tension. The suction pressure corresponding to the first appearance of a projection allowed determination of the membrane tension of the stressed vesicles according to the LaPlace equation, above.

![Figure 5.1: Domain morphologies of hybrid copolymer/DPPC vesicles comparing to DOPC/DPPC vesicles at broad range of compositions. Images were photographed at room temperature after cooling from one phase temperature (43°C) at 5°C/min or 1°C/min as indicated. The scale bar is 10 μm.](image-url)
5.3 Results.

5.3.1 Vesicle morphology.

Figure 5.1 contains images of hybrid copolymer/DPPC vesicles photographed at room temperature after cooling at different rates from the single-phase region at 43°C. These data demonstrate how the resulting vesicle morphology depends on composition and the thermal history during the approach to room temperature. Figure 5.1 also presents remarkable parallels between copolymer/DPPC and DOPC/DPPC vesicles suggesting that copolymer and DOPC play similar roles as the lower melting fluid membrane component. (The images of the DOPC/DPPC vesicles are reproduced from a paper focusing on DOPC/DPPC phase separation for pairwise comparison with hybrid vesicles). The similarities between copolymer/DPPC and DOPC/DPPC vesicles go beyond the classes of domain morphologies formed: Qualitative similarities include the shapes of the domains and the temperatures, compositions, and histories where they are observed.

In Figure 5.1, single-phase fluid membranes are consistently found, for both hybrid and DOPC/DPPC vesicles at low overall DPPC content. This single-phase hybrid membranes are also observed at temperatures above 41.5°C (the melting point of DPPC), in parallel with the well-established single phase behavior of mixed DOPC/DPPC vesicles in the same elevated temperature range. (Images of single-phase fluid vesicles at elevated temperatures are not included because they are identical to those having low DPPC content.) In addition to fluorescence uniformity, single-phase fluid vesicles appear round and relatively smooth, with an absence of rigid facets that are sometimes associated with solid domains.
With increased amounts of DPPC in vesicles containing either copolymer or DOPC, dark domains, which exclude the tracer dye Rh-DOPE, become visible in Figure 5.1. These dark domains occupy an increasing membrane area fraction at higher overall mole fractions of DPPC. This is consistent with domain compositions that are predominantly DPPC. Vesicles electroformed and cooled in DI water to room temperature at a rate of 5°C/min from about 43°C display patchy solid domains which, upon close examination, often have hexagonal facets. Vesicles cooled more slowly, for instance at 1°C/min, can exhibit stripes or patches, depending on composition. Interesting in both hybrid and phospholipid systems, there are distinct ranges of compositions where cooling history, not the composition itself, determines whether striped or patchy solid domains persist. Also worth noting, for relatively slow cooling of 1°C/min, the particular compositions, which produce stripes (for a given cooling rate) are well-defined and highly reproducible. The patches are of similar size and number (to first order) in hybrid and phospholipid vesicles and likewise, the stripes have similar widths, for a given composition and thermal history.

Subtle quantitative differences distinguish the phase separation in the hybrid vesicles from the DOPC/DPPC bilayers. Most notably, at the cooling rate of 1°C/min, in the phospholipid system there is a broader composition window, above about 45 mol% DPPC, where stripes are the solid morphology at room temperature. In the case of the copolymer/DPPC vesicles, the compositional window for the striped solid morphology is narrower, starting around 70 mol% DPPC. Indeed it is interesting that at 70 mol% DPPC, a solid patch is seen among the solid stripes. The composition of 70 mol% DPPC in the hybrid vesicles apparently lies close to the boundary between the two solid morphologies.
5.3.2 Molecular organization in striped and patchy solid domains.

Differences in the interaction of tracer lipid with the striped and patchy solid domains provide convincing evidence for differences in the molecular organization within the two domain types. This is the case whether the stripes and patches appear in hybrid or in purely phospholipid vesicles. Figure 5.2 compares the appearance of copolymer/DPPC and DOPC/DPPC vesicles containing either Rh-DPPE or Rh-DOPE tracer. (The latter was employed in all the vesicles of Figure 5.1.). In Figure 5.2, the patchy domains always exclude the two tracers, in both hybrid and phospholipid vesicles. The striped domains exclude Rh-DOPE but take up Rh-DPPE at higher density than in the fluid phase, in either hybrid or mixed phospholipid vesicles. Thus, depending on the tracer but not on the vesicle system (hybrid or strictly phospholipid) the stripes can appear light or dark. This makes a strong case for different molecular organization in the two types of solid domains. It also suggests that the patchy domains in the hybrid vesicles are nearly the same, in terms of molecular organization and composition, as those in phospholipid vesicles, with the same being true for the striped solid domains.

5.3.3 Membrane mechanics.

An important factor in vesicle design is membrane mechanics. The presentation of membrane mechanics data at this point enables the phase-separated morphologies in Figure 5.1 and 5.2 to be understood in the context of the mechanical state of the membrane.

Figure 5.3 compares example stress-strain curves for copolymer, hybrid, and phospholipid vesicles aspirated in micropipettes. The mixed membranes contain 70 mol% DPPC. Data were obtained at room temperature and were generally reversible. The
mixed vesicles were in the two-phase region at room temperature, with prior thermal histories to produce patches rather than stripes. Because entry of solid domains (especially stripes) into the micropipette can sometimes interrupt the progress of aspiration and membrane stretching, we chose data sets for the phase-separated vesicles where domains did not happen to hang up upon entry into the micropipette. We do not wish to place too much emphasis on the quantitative aspects of stress-strain curves of the phase-separated vesicles (because of possible artifacts introduced by phase separation), but include the data here because it appears reasonable, at least qualitatively. We also included the single component vesicles as an important point of reference.
Figure 5.2: Patch and stripe domains in copolymer/DPPC (30 mol% / 70 mol%) and DOPC/DPPC (30 mol% / 70 mol%) vesicles formed under low and high tension respectively. Stripes contain more tracers of Rh-DPPE, while excluding Rh-DOPE. Patches exclude both tracers. The scale bar is 10 μm.
Figure 5.3: Tension – strain measurement on giant unilamellar vesicles formed by DOPC/DPPC (3:7), DOPC, DC5329/DPPC (3:7) and DC5329. Membrane tension is measured by micropipette aspiration.

Figure 5.3 makes two important distinctions between phospholipid and copolymer-containing membranes. First, the lysis stress and strain for the copolymer and hybrid vesicles far exceeds that of the pure phospholipid and especially the phase separated phospholipid, which is relatively fragile. Lysis strains for the copolymer-containing vesicles exceed 8-10%, compared with 2-4% for the mixed phospholipid vesicles. These values are consistent with the literature for both polymeric and phospholipid vesicles. A second take-home point from the mechanical experiments is the difference in moduli between the hybrid and phospholipid membranes. Area expansion
moduli in the neighborhood of 200 mN/m, observed for our phospholipid vesicles, are
typical of these liposomes. The copolymer-containing vesicles have area expansion
moduli a factor of two lower. Additionally, the copolymer seems to dominate the
mechanics of mixed vesicles: Hybrid vesicles containing 70% DPPC have an area
expansion modulus only slightly greater than that of the pure copolymer.

Taken together these results suggest that for a given strain, the copolymer and
hybrid vesicles will experience lower stress than the pure phospholipid system.
Additionally, hybrid membranes, even when phase separated, exhibit a smaller tendency
to rupture compared with entirely phospholipid membranes.

5.3.4 Tension and mechanism of morphological fate.

Figure 5.1 illustrates how, in both systems, cooling rate appear to dictate the
morphological vesicle fate of vesicles over a range of membrane compositions. Indeed
cooling rate turns out to be among the most experimentally-accessible means of tuning
morphology. For DOPC/DPPC vesicles, however, it was demonstrated that membrane
tension, imposed osmotically or using micropipettes, was the underlying factor governing
the morphology. If the tension of DOPC/DPPC membranes was controlled
independently, the influence of cooling rate on the initial appearance of stripes or patchy
solid domains was removed. The exception was that large domains either type could be
kinetically trapped at low temperatures, and were mostly unresponsive to subsequent
changes in temperature.
Figure 5.4: Membrane tension measurement (A) and domain morphology variation under different conditions (B) as indicated.

Figure 5.4 suggests that tension has a similar influence on hybrid vesicles, and that tension, not cooling rate, is the dominant underlying variable in establishing the type of solid and therefore the type of the domains formed on cooling, in Figures 5.1 and 5.2. Figure 5.4A first presents data demonstrating that higher membrane tensions are sustained at the cooling rate of 1°C/min compared with lower near-zero tensions at 5°C/min. In the experiments of Figure 5.4A vesicles, initially electroformed and annealed in DI water at 43°C were cooled at one or the other of the two rates to 35°C, in the neighborhood of the initial phase separation temperature (corresponding to vesicle compositions of 70% DPPC). The vesicles were rapidly transferred to the micropipette aspiration chamber also maintained at 35°C and the membrane tension measured. The higher tensions seen at the slower cooling rate are statistically significant within 99% certainty. Additionally the differences in tension between the hybrid and copolymer vesicles are significant within 99% certainty.
Membrane tension develops and increases during cooling because of thermal contractions of the membrane. This shrinkage tightens the “skin” around the water pool inside the vesicle. The water itself also contracts on cooling, but to a lesser extent, permitting the membrane contraction to dominate and increase the tension. The effect can be substantial for phospholipids, a 5% area decrease is expected for each 10°C of cooling. The areal strain from thermal contraction of phospholipid membranes exceeds the lysis conditions of Figure 5.3. Therefore, in the absence of water diffusion across the membrane, tears in the bilayer must break and reseal after some loss of internal water, reducing the tension. Indeed, rupture and resealing has been observed by others. If cooling is sufficiently slow, however, tension can be reduced by water diffusion through the membrane, a process that equilibrates on the order of tens of minutes for phospholipid bilayers. While it is difficult to anticipate the relative contributions of thermal
contraction, water diffusion, membrane stretching, and lysis and resealing, Figure 5.4A establishes a lower bound for the range of tensions experienced by vesicles in the temperature range corresponding to the onset of phase separation. Over time, membrane tension continues to decrease to zero. This explains why vesicles maintained at room temperature for some time no longer exhibit the higher stress observed in Figure 5.4A at temperatures corresponding to phase separation.

Once solid domains form, their morphology is retained for hours, on the order of vesicle lifetime. A large energy barrier opposes the transformation of one solid domain type to another, even when stress is adjusted at room temperature to favor one or another morphology. In obtaining the mechanical data in Figure 5.3, we observed no qualitative alteration of the dark solid domains in either class of mixed membranes, even with tensions as high as that needed for lysis.

Figure 5.4B illustrates, for hybrid copolymer vesicles osmotically manipulated to maintain zero membrane tension, solid patches are observed independent of cooling rate. A similar preference for patch-shaped solid domain formation was previously reported in mixed DOPC/DPPC vesicles maintained near zero tension. These observations for a similar role of tension in dictating solid domain formation suggest more broadly parallel underlying thermodynamics (in the tension dimension) for hybrid and purely phospholipid vesicles. The results also demonstrate that while cooling rate and tension are correlated, it is the tension that fundamentally controls whether the solid morphology is patchy or striped. The cooling rate is a convenient processing variable through which tension, and therefore the solid morphology can be tuned.
5.3.5 Composition-temperature space.

The previous section revealed a similar thermodynamic influence of tension on solid domain formation in hybrid and purely phospholipid vesicles. Figure 5.5 illustrates the additional thermodynamic parallel between the two classes of vesicles in terms of the temperature-composition space for fluid-solid phase separation. Figure 5.5 plots the temperatures for the initial appearance of solid domains in vesicles of varying composition that were heated to about 43°C in the one phase fluid region and then cooled sufficiently quickly to produce patches. The domains start as small nuclei that become clearly visible when they reach a size of about a micron. The temperature of first appearance is not significantly dependent on the cooling rate as long as the conditions are chosen to produce tensions sufficiently low for patchy domain formation.

Figure 5.5A demonstrates that for hybrid and phospholipid vesicles the temperature-composition state diagram appears similar, with only modest differences between the temperatures for the onset of solid domain formation in the two vesicle types. Phase separation occurs at slightly elevated temperatures in the hybrid system over the full range of compositions. Comparing to the data measured at mass composition [Figure 5.5 B], Figure 5.5A indicates the similar molecular orientation of the copolymer and the phospholipids, and similar molecular area occupation respect to the vesicle bilayer.

Drawn on Figure 5.5A (black squares) is the melting temperature of the patchy domains in the phospholipid vesicles, obtained by application of the inverse lever arm rule to the solid domain area fraction in DOPC/DPPC vesicles at room temperature. Together these points sketch out the solidus curve describing the solid membrane potentially in equilibrium with the mixed phospholipid fluid membrane. The nearly pure
solid phase motivates the calculation of the standard colligative property behavior for melting point depression in an ideal solution fluid mixture with a pure solid, also shown. It is interesting to note that both real membrane systems exhibit solid domain formation at temperatures only modestly greater than predicted. The purely phospholipid system has a fluid phase slightly less favorable for mixing than a strictly ideal solution. The copolymer-DPPC interaction is slightly less favorable than the DOPC/DPPC interaction so that DPPC solidifies sooner on cooling. These deviations from ideality suggest that the phospholipid mixture is a slightly poorer solution than ideal and that the copolymer-DPPC mixed fluid is somewhat less ideal; however, neither system is sufficiently non-ideal to produce fluid-fluid demixing.

5.4 Discussion.

The behavior of hybrid copolymer phospholipid vesicles is remarkable in several regards.

First, given the sentiment in much of the literature that polymers forming bilayer phases are fundamentally incompatible with phospholipids at least at some compositions, our ability to create single phase vesicles at room temperature containing up to 20 mol% DPPC content or at elevated temperatures over a full range of compositions may come as a surprise to some: A combination of chemical mismatch along with mismatch in lamellar thickness argues against single phase hybrid vesicles, and against phase separated hybrid vesicles having mechanical strength. Our hybrid lamellae are almost as compatible as mixed phospholipid membranes.

Phase separation in membranes such as ours, where DPPC is mixed with a low melting lamella former, results from the fundamental tendency for DPPC to crystallize
below 41.5°C, rather than from a fundamental incompatibility between the two components. The region of thermodynamic space in which DPPC crystalizes in hybrid vesicles is only slightly larger than the two-phase region for DOPC/DPPC mixtures, arguing for substantial miscibility of both systems. Miscibility is favored by similar bilayer thicknesses in of DOPC and copolymer, 5.5 and 7.7 nm respectively. Additionally in the case of the copolymer, the PDMS chains likely assume a random coil or brush conformation where conformational freedom renders nanometer-scale changes in lamellar thickness entropically inexpensive. In this way, local thinning or thickening of the hydrophobic membrane core could accommodate “objects” such as solid DPPC domains.

Our findings concerning thermodynamic compatibility must be put in the context of error associated with the membrane composition. Copolymer polydispersity introduces uncertainty in the molecular weight and therefore uncertainty in the mole fractions of the mixed hybrid membranes. Polymer blend compositions are often prescribed as mass or volume fractions. If this approach is applied to the membrane blends in Figure 5.4A, the state diagram appears as Figure 5.4B. In Figure 5.4B, the hybrid membrane phase diagram shows further apparent deviations from ideal solution behavior, but the underlying parallel behavior is preserved.

Domains in DPPC-containing membranes must assume at least one of the distinct solid polymorphs exhibited by DPPC. These include a family of corrugated ripple (P\(_{\beta'}\)) morphologies that persist, for pure (but hydrated DPPC) between 35.5 and 41.5°C, and a planar tilt gel (L\(_{\beta'}\)) phase below 35.5°C down to room temperature. The assignment of these different bilayer solids to patches and stripes within mixed DPPC-containing
lamellae has been debated. Striped regions with corrugated morphology found by AFM within DOPC/DPPC membranes immobilized on mica suggest a similar relationship in vesicles. Our more recent data, with mixed DOPC/DPPC vesicles, argue differently: We document the formation of stripe solid DPPC domains at elevated tensions above a triple point and patchy solid domains at lower tensions. The observed tension-dependence of the phase transition temperatures of DOPC/DPPC vesicles was found in quantitative agreement with fundamental thermodynamic arguments (somewhat similar to Clausius Clapeyron) when the patchy solid domains were assigned the properties of the DPPC ripple phase. This strongly suggests the ripple P_{\beta'} solid phase within the patchy DPPC domains and a gel L_{\beta'} phase in the stripes.

The most articulate argument, however, for the ripple P_{\beta'} phase (with its greater areal density of DPPC) within the patchy solid is the tendency of the membrane to favor patches at low tension and stripes at high tension, for example in Figures 5.1, 5.2 and 5.4. Simply put, the less dense membrane phase L_{\beta'} persists at higher tensions. The parallels in Figure 5.1 therefore suggest that the patches and stripes in the hybrid vesicles are ripple and gel DPPC solid domains, and that the patchy DPPC solid domains have similar DPPC content in both case. In this sense, manipulation of the tension of a polymer membrane, in our hands, directs the crystallization and morphology of a phospholipid.

Beyond thermodynamic parallels between the two systems (temperature, tension, and composition), other observed parallels between the vesicle morphology in the hybrid and phospholipid systems include similar domains sizes and numbers, and a similar influence of cooling history. This suggests similar membrane thermodynamics and similar mechanisms for domain formation. Patchy DPPC domains in phospholipid
membranes are found to result from nucleation and growth, and the same mechanism is likely to produce DPPC domains during cooling of hybrid membranes. To the extent that the domains sizes and numbers are similar for a given compositions and cooling rate, the nucleation rate, with its dependence on the enthalpy of domain formation and the line tension around nucleated domains, suggests that line tensions in the hybrid system are not unusual. Indeed the mechanical strength of phase separated hybrid vesicles suggests that line tension is not high.

An important difference between hybrid and phospholipid membranes, evident in Figure 5.1, is the greater tendency for patch formation and smaller compositional window for stripe formation in hybrid vesicles. With the correlation between low tension and stripe formation established for both systems, this suggests lower tensions in the formative instants of phase separation of hybrid vesicles. This hypothesis is affirmed, at least in part by the mechanical data of Figure 5.4A. For a given membrane strain (such as a thermal contraction), the softer copolymer-containing vesicles experience a lower stress and a greater tendency to produces patches rather than stripes. We expect that separate manipulation of the hybrid vesicles to sustain large membrane tensions will produce stripes over a larger compositional range.

### 5.5 Conclusions.

This study examined multicomponent membranes that contained copolymer and phospholipid mixtures to probe how well a synthetic or hybrid system could approximate the behavior of mixed membranes containing only phospholipid components while retaining the mechanical advantages of the polymeric system. Comparison of giant unilamellar hybrid vesicles to an analogous phospholipid membrane system containing
DPPC, examining the full compositional range, revealed fundamental physical and thermodynamic similarities. First, the general appearance of the vesicles was similar into the two systems, with fully-mixed membranes at temperatures above the melting point of DPPC or at low DPPC content.

Phase separation in both systems resulted from the tendency for DPPC to solidify in the membrane to form nearly pure DPPC domains with complicated large and nanoscale morphologies. The DPPC domains appeared patchy or stripe-shaped, depending on composition, tension, and thermal history, with striking similarity between the two types of membranes. The temperatures observed for the onset of phase separation was, to first order the same in the two systems. Small differences included slightly higher solidification temperature in copolymer-containing membranes, indicative of slightly less ideal mixing in the hybrid compared with the phospholipid system, with both classes of mixed membranes exhibiting small deviations from ideal solution behavior. The mixing non-idealities were not so great as to produce fluid-fluid demixing.

Tension was shown to play a dominant role, compared with cooling history, in determining the types of membrane domains to form. Based on findings with DOPC/DPPC mixed membranes, parallel behavior of the hybrid membranes suggested that the striped and patchy domains, with their different tracer dye incorporation, were a result of differences in their molecular organization. A ripple DPPC polymorph in the patchy domains along with a tilt gel structure in the stripes was the best explanation for the differences in the domains. The current study demonstrated how cooling rate was a natural variable for tuning the membrane morphology switching between stripes with long-range solid connectivity at high tensions to patches with long range fluid
connectivity at low tensions. However, with osmotic manipulation of the vesicles to isolate tension, the impact of cooling rate on the domain type could be removed to facilitate processing on desired timescales.

5.6 Reference.


6.1 Conclusions.

This thesis has described an investigation of the equilibrium phase behavior and thermodynamics of binary lipid and hybrid unilamellar vesicles. This work focused on the model membrane system comprise of DOPC and DPPC phospholipids, which are both biologically significant and physically complex in their thermodynamic space and ability to form a variety of membrane morphologies. The observed transformation between domain morphologies in the mixed DOPC/DPPC vesicles is novel and suggests an underlying change in the molecular arrangement within solid DPPC membrane domains. Further, the discovery that tension shifts phase transition temperatures and directs the phase transition from fluid to one or another type of solid provides a third dimension of thermodynamic space. Prior work in the relatively mature field of membrane biophysics focused exclusively on membrane temperature and composition.

The first step toward a more comprehensive understanding of model phospholipid membrane behavior was, working with a fixed overall membrane composition (30 mol% DOPC/70 mol% DPPC), demonstrating the role of membrane tension in directing to direct the phase transition from a uniform phase at elevated temperatures to one of two different solid DPPC phases having dramatically different features at the microscopic and nanoscale length scales. The two solid DPPC phases (P$_{\beta'}$ and L$_{\beta'}$) differ in their formation temperatures, formation tension, domain morphology, and molecular area. They have different molecular structures as evidenced by selective incorporation of fluorescent dye. Our recognition of the two distinctly different solid phases in vesicle
membranes not only clarified the controversial viewpoint regarding the domain shapes and underlying structures, but also enabled us to apply a simple Clausius-Clapeyron-like treatment to anticipate the impact of tension on the temperatures for different phase transitions.

Both theoretical calculations and experimental measurements indicated the existence of a triple point when the overall membrane composition was fixed. In three dimensional thermodynamic space that included composition variations, the locus of triple points was represented by a slightly curved line. Beyond the triple point, the fluid phase was shown to coexist with the tilt solid phase (L\(\beta\')), and solidification of the tilt gel solid occurred directly without passing the region of space corresponding to a rippled solid-phase. The resulting temperature-tension phase diagram provides a tool to interpret the phase transition and domain morphology transformation in the binary lamella. In particular, the elevated stripe formation temperatures in the vesicles that contain large amount of DPPC can be explained by the induced tension during main transition.

The approach to studying the influence of tension demonstrated first for a single overall membrane composition of 30 mol% DOPC / 70 mol% DPPC was next extended to DOPC/DPPC mixtures of broad compositional range. The same qualitative behavior reported initially for vesicles containing 70% DPPC was shown to broadly apply across all compositions in the qualitative sense, but with downward shifting of phase transition temperatures. The reduction in the phase transition temperatures with increased DOPC membrane content was qualitatively consistent with the expected influence of mixing entropy on the formation of a solid from a mixed fluid. The main transition temperature decreases as the DOPC content of the vesicles was increased, close to predictions from
ideal solution theory. A similar trend of transition temperature reduction with increased DOPC content was observed for the pretransition temperature between the two solid DPPC phases. In the vesicles that contained lower amounts of DPPC, the stripe formation temperature was decreased sufficiently so that stripe formation was not accessible at room temperature (when the pretransition temperature fell below room temperature). This was observed in the vesicles contained 50 mol% or less of DPPC. With addition of the composition dependence to the temperature-tension space, the three dimensional phase diagram (temperature-tension-composition) of DOPC/DPPC binary system was mapped out. This representation presented a complete picture in the binary phospholipid system. This first description of the three dimensional thermodynamic space of a two-lipid membrane including the impact of tension is the first of its kind and is a unique contribution of this thesis to both the understanding the complex biological membranes, and to the physics of mixtures with additional dimensional parameters.

Next, a study of the nucleation and subsequent domain growth in DOPC/DPPC vesicles revealed the mechanism of solid domain formation from a mixed fluid membrane. It was found that number of domains does not change with temperature and time after the domains were first observed, though the areal domain number density varies with cooling rate. Specifically, the domain density increased from 0.01/μm² to 0.1/μm² when the cooling rate changed from 1 to 10 °C/min. A slight decrease of domain density was also discovered as the membrane tension was increased. These findings are consistent with an increase in nucleation density with depth of quench and with a reduction in tension, as expected from the classical nucleation theory.
This thesis also documents an inaugural investigation of hybrid vesicles formed by DPPC lipid and copolymer mixtures. The work documents their miscibility at elevated temperatures and modest DPPC content. The hybrid vesicles formed by DPPC and DC5329 copolymer presented similar phase behavior and domain morphology as the lipid mixtures of DOPC/DPPC, with some important exceptions. Most obvious was a slightly elevated temperature for the solidification of DPPC from the mixed fluid. The slight elevation in solidification temperature suggests slight non-idealities in the polymer-DPPC mixed membrane, but the mixture is not so non-ideal as to produce fluid-fluid phase separation. Importantly, the copolymer renders the hybrid membranes soft and highly extensible, so that the hybrid membranes are mechanically robust. As result of the hybrid membranes lower area expansion modulus (compared to purely phospholipid membranes), tension accumulated during cooling and the phase separations is lower than the lipid mixtures, which favors patch formation over stripes. The high lysis tension and strain of the hybrid membranes suggests, however, that should extreme conditions favor high tensions that drive stripe formation, membranes would be stable and sustain their integrity. These are favorable properties that can be used to design stable drug delivery materials.

6.2 Future directions.

The current study contains findings sufficiently important to influence future research in several different directions, both in membrane biophysics and in new materials systems.

One direction is the investigation of ternary phase behavior of DPPC, DC5329 and cholesterol. Many studies have revealed the fluid-fluid phase separation in the
vesicles containing multi-component phospholipids together with cholesterol. The fluid-ordered and fluid-disordered separation is particularly relevant to the physiology in the biological membranes, especially the proposed functional regions called “lipid rafts”. The experiments in this thesis have suggested the miscibility and phase separation in the DC5329/DPPC/Cholesterol ternary system. The incorporation of amphiphilic copolymer in the biomembranes would be a significant advance in the hybrid vesicle research and development, which would accelerate the development of stable and biocompatible drug delivery carriers.

Another important future direction is to study the water and drug permeability in the lipid and hybrid vesicle membrane. The drug storage and targeted release is an active topic. It is observed that there is a great surface area change during the phase transition. These findings suggested the methods of controlling membrane permeability by utilizing the phase behavior of the binary system. Specifically, by manipulating osmotic pressure and cooling rate, a program can be designed to quantitatively release substance carried by the vesicles.


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