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Environmental Perturbations of Soft Matter Assemblies and their Mechanical Response

By

ARCHAN P. VYAS
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Chemical Engineering

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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Committee in Charge

2026

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COPYRIGHT ACKNOWLEDGMENTS

Several chapters of this dissertation are largely based on publications, manuscripts in review, and/or work in progress. The copyrighted content is also adapted to fit the formatting guidelines of this thesis.

- Chapter 2 is loosely based on a publication that appeared in ACS Nano (2025)¹. The dissertation author is listed as a co-author and the figures used are from the publication with proper credit given.
- Chapter 3 is largely based on a publication that appeared in Soft Matter (2026)². This dissertation author is listed as a co-author and the figures are used from the publication with proper credit.
- Chapter 4 is work currently in progress as an extensive collaboration with the labs of Prof. Eliza Morris and Prof. Mikkel Herholdt Jensen from Sacramento State University.

DISSERTATION ABSTRACT

Soft matter assemblies, such as lipid bilayer membranes, liquid crystalline multilamellar phases, and cytoskeletal protein networks, are stabilized by collective interactions near the $k_B T$ scale, rendering them sensitive to environmental perturbations. This dissertation provides experimental evidence that structural reorganization of such assemblies is not governed by the strength of the environmental perturbation but by the physical mechanism of the stressor. Across two broad categories of environmental stressors, chemical and physical, three geometrically distinct model systems are investigated to assess this claim and capture evidence.

In the first system, this dissertation reviews the theoretical framework connecting molecular packing geometry to membrane morphology before developing the pH-responsive bolaamphiphile GC18:1 as an experimental case study. When GC18:1 is inserted into DOPC giant unilamellar vesicles, it converts a chemically inert host membrane into a pH-responsive material. These synthetic liposomes bud inward at mildly acidic conditions and outward at basic ones, a direct consequence of the molecule's geometry shifting with its chemical state. This establishes a theoretical and experimental foundation for this dissertation in which the character of environmental stressors, here the molecular geometry, drives membrane remodeling.

In the second system, concentric cylindrical multilamellar assemblies treated as lyotropic smectic-A liquid crystals, known as myelin figures, are subjected to osmotic stresses from solutes spanning molecular weights of 92 g mol^{-1} to $10\,000 \text{ g mol}^{-1}$, with the osmotic pressure matched across solutes by adjusting concentrations. Small-molecule osmolytes produce no surface instability at any pressure tested, while larger macromolecular osmolytes drive periodic axial corrugations consistent with the Helfrich-Hurault elastic instability within seconds of exposure.

This result demonstrates that the determining variable is the entropic, excluded-volume character of the macromolecular stressor: depletion forces generated at inter-myelin interfaces by solute-excluded coronas couple to the smectic's elastic instability mode, driving symmetry-breaking buckling that colligatively equivalent small-molecule solutes cannot produce.

Ongoing work in the third system examines whether this excluded volume mechanism generalizes beyond lipid membranes to cytoskeletal protein assemblies. TActin, one of the most abundant proteins in the eukaryotic cell, is examined within dextran-rich aqueous two-phase system droplets stabilized by small unilamellar vesicles as Pickering agents, approximating cytoplasmic macromolecular volume fractions of 20–40%. Preliminary results reveal that actin preferentially partitions into these crowded droplets and that the dextran-rich environment promotes its assembly into a filamentous state, even in the absence of conventional polymerizing buffer conditions. These results thus far suggests a novel approach for synthetic cell engineering and biophysical design.

Taken all together, the completed and ongoing work supports a unifying framework in which stressor character is the primary determinant of soft matter structural response, independent of stressor magnitude. Across multiple geometries and soft matter assemblies, these findings advance a physical basis for understanding and engineering environmentally responsive soft matter systems.

Chapter 1

Introduction

1.1 The Responsive Boundary

Boundaries define systems. In biology, materials science, engineering, and other fields, the ability to demarcate and compartmentalize is what allows complex functions to emerge from simple components. Yet many of these important structures across science, whether they are biological or synthetic, are not held together by strong rigid bonds. Rather, they are held together by collective weak interactions, rendering them responsive, dynamic, and tunable, therefore retaining interest for engineering and biomedical purposes. It is precisely this soft responsiveness which makes them functionally essential and scientifically fascinating.

These structures held together by collective non-covalent interactions are often generally called “soft matter” and remain a highly engaging field of study. Consisting of (but not limited to) liquids, colloids, polymers, and biological tissues, these assemblies are mesoscopic (larger than atoms but smaller than the overall material) and the characteristic energy scale is on the order of thermal energy ($\sim k_b T$)^{3,4}. These types of materials behave radically differently compared to metals or covalent solids. Such soft matter assemblies can rotate, twist, deflate, inflate, divide, and behave in ways unlike their more rigid counterparts. This type of dynamic environmental response allows them to adapt to their circumstances and enables scientists to leverage their

response for engineered purposes.

A remarkably simple and instructive example is a eukaryotic cell's response to osmotic stress. In steady-state, the cell exists in an isotonic state with osmotic pressures balanced, the membrane at an equilibrium, and cellular function operates normally. Now, with the smallest perturbations, osmotic stress, the cell must accommodate the osmotic pressure differential (Figure 1.1). There are two ways the water can move: either into the cell or exit the cell. If external concentration drops (hypotonic), water enters and the cell swells; if it rises (hypertonic), water exits and the cell shrinks. In either case, physics remains the same: the boundary responds to an environmental stressor to regulate proper function. This simple example illustrates a key general principle that soft matter assemblies adapt to environmental stress through a structural reorganization across multiple length scales.

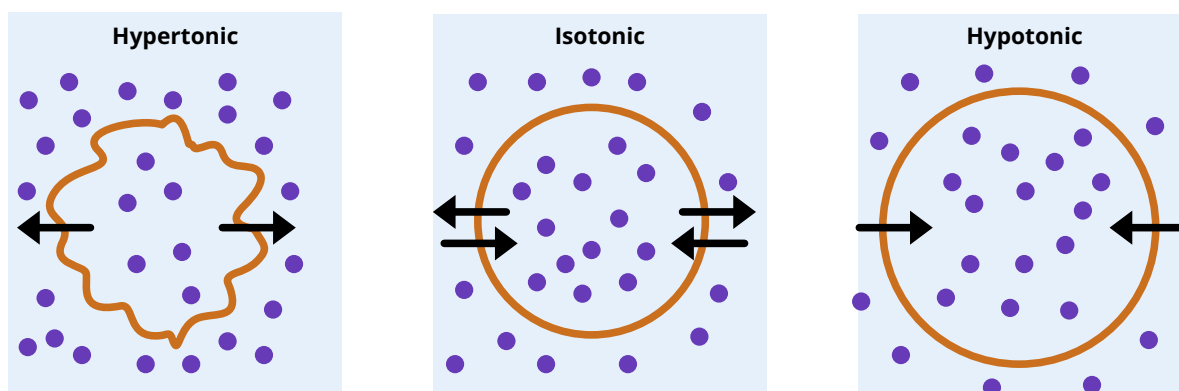


Figure 1.1: (Left) Under hypertonic stress, excess solutes on the exterior than the interior induces a net efflux of water. This volumetric decrease forces the membrane to change by wrinkling and shrinking to release elastic compressive energy. (Center) In an isotonic steady-state system, the solute concentrations are balanced, resulting in zero net water flux and an unstressed equilibrium volume. (Right) Under hypotonic conditions, more solutes on the inside than the outside drives a net influx of water, causing the soft matter system to swell and placing the boundary under lateral tension.

1.2 Phospholipids: The Building Blocks

To understand how environmental stressors drive structural reorganization in the soft matter systems studied in this thesis, it is important to first establish the physical and chemical properties

of their primary structural components. All the soft matter systems studied here are fundamentally organized around or in the presence of phospholipid bilayer membranes. Phospholipids are therefore a critical molecular foundation on which the rest of this thesis is built, and a working understanding of their structure, self-assembly behavior, and mechanical properties is prerequisite to understanding everything that follows.

1.2.1 Chemistry of Phospholipids

Phospholipids are a class of amphiphilic molecules that have both hydrophilic and hydrophobic regions, constituting the primary structural component of biological membranes. In fact, alongside cholesterol and glycolipids, phospholipids are the primary structural component of all cellular membranes, numbering $\sim 3 \times 10^6$ lipids per square micron of a cell's membrane⁵. The most abundant form of phospholipids are glycerophospholipids, all of which share a common molecular structure. Structurally, these molecules feature a glycerol backbone esterified to two hydrophobic fatty acid chains ("tails"), while a phosphate group is attached via a phosphodiester linkage to form the hydrophilic region ("head"). This tail and head structure divides the molecule into a well-defined hydrophobic region (the tails) and a hydrophilic region (the head), giving these typical phospholipids their characteristic amphiphilic character (see Figure 1.2). This amphiphilic nature is a key property for the maintenance of cellular life and an integral component of soft matter systems⁶.

The fatty acid tails in the hydrophobic region are typically 14-22 carbon atoms in length and can be saturated (all single carbon-carbon bonds, fully extended) or unsaturated (containing one or more carbon-carbon double bonds, introducing kinks into the chain). The degree of unsaturation in these tails profoundly affects the physical properties of phospholipid assemblies. Saturated chains will pack more tightly and order into more crystal-like arrangements since they are straight. Unsaturated chains with their kinks cannot pack as tightly, forming more fluid membranes at lower temperatures. For example, one of the common lipids used in this thesis, POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), contains one saturated chain and one

unsaturated chain, rendering it more fluid at room temperature⁷.

While the non-polar chain properties heavily influence membrane properties such as the packing and fluidity of these soft matter systems, the membrane properties are equally heavily influenced by the polar head groups. These polar headgroups additionally help dictate properties such as the electrostatics, shell thickness, and curvature, among others. In eukaryotic cells, the most common phospholipid head group is phosphatidylcholine (PC), an amphiphilic and zwitterionic molecule which contains phosphate and choline groups. Other common head groups include phosphatidylethanolamine (PE; zwitterionic), phosphatidylinositol (PI; polar), and phosphatidylserine (PS; negative). The identity of the polar headgroup helps determine its electrostatic interactions with the environment, surface interactions, and how curved the membrane structure might be. Together with the influence of the polar headgroup, these lipid characteristics are critical for membrane function and environmental responsiveness, with the latter a key theme of this thesis.

1.2.2 Self-Assembly into Bilayers

Having discussed the fundamental properties of typical phospholipids, it is now imperative to examine their interaction with the most fundamental fluid in nature: water. Given their amphiphilic nature, the lipid molecules need to thermodynamically orient themselves to satisfy both the hydrophilic head and hydrophobic tails. These molecules cannot exist independently in aqueous environments and prefer to form structures which shield the tails and expose the heads. To achieve this, the system minimizes its free energy by forming either micelles or bilayers, the former being favored by fatty acids and detergents and the latter being favored by phospholipids.

The reason for this preference is because of how these molecules are shaped and the ratio between the head and tail substructures. The specific geometry is dictated and formalized by Israelachvili's packing parameter⁸ (see Figure 1.2b):

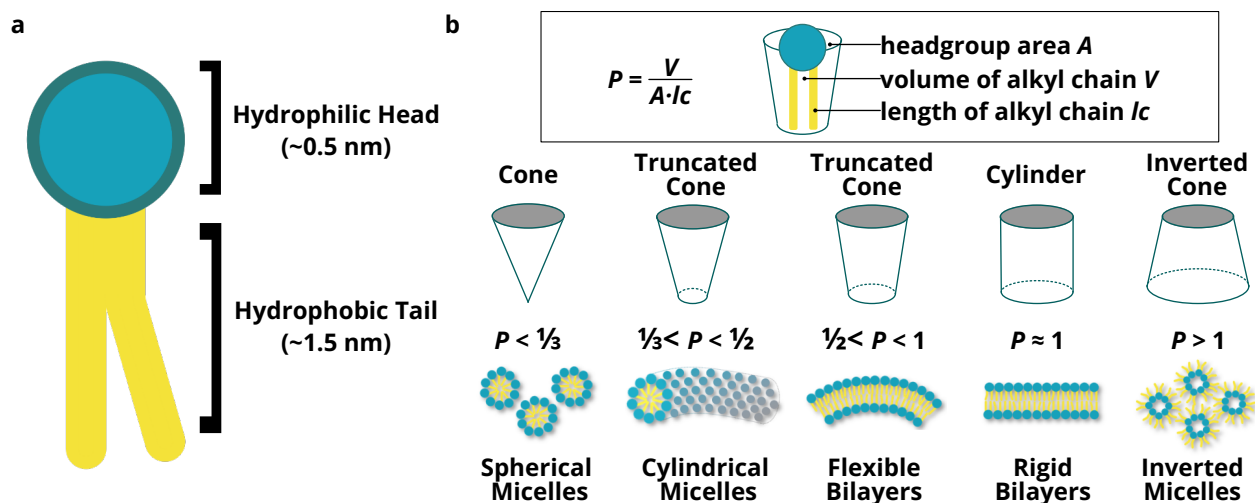


Figure 1.2: [a] illustration of the molecular structure for a typical phospholipid showing its hydrophilic head (~ 0.5 nm) and hydrophobic tail (~ 1.5 nm) regions. [b] Israelachvili's packing parameter $P = V / (A \cdot l_c)$ and the associated assembled structures⁹ corresponding to ranges of the packing parameter.

For detergents and single tailed fatty acids, there is a considerably larger headgroup area relative to the volume occupied by their tails, creating either a truncated cone structure with a $P < 1/3$ or a worm-like micelle structure with $1/3 < P < 1/2$. This geometry forces the molecules to bury their hydrophobic tails and expose their hydrophilic headgroups. As the molecule characteristics change (and so does the packing parameter), the geometry which minimizes the system free energy also changes.

For more typical phospholipids, such as those used in this thesis here, as discussed earlier they possess two bulky hydrocarbon tails. The packing parameter here is much closer to 1, which gives these molecules a flexible bilayer shape ($1/2 < P < 1$) or rigid bilayer shape ($P \approx 1$). Cylindrical lipid molecules pack best side-by-side so for typical phospholipid systems, a more planar structure is achieved. To further minimize the free energy and to satisfy the hydrophobicity requirements, two planar sheets will typically come together to hide the tails and expose the heads, creating the more standard phospholipid bilayer. This bilayer architecture efficiently shields the hydrophobic regions (the hydrocarbon tails) and exposes the hydrophilic regions (the polar head groups), creating a $\sim 4\text{--}5$ nm¹⁰ thick bilayer with a hydrophobic core flanked by two hydrophilic surfaces.

For typical phospholipids, while the flat bilayer is much more thermodynamically favorable

than a micelle, a flat sheet by itself is unstable. At the edges of this sheet, the hydrophobic core would be exposed to the aqueous environment, creating an unfavorable line tension¹¹. To eliminate this, the system performs a simple geometric adjustment and bends itself into a stable enclosed sphere known as a vesicle¹². This spontaneous geometric closure forms one of the most fundamental structural boundaries in biology and has countless uses.

1.2.3 Key Properties of Bilayers

1.2.3.1 Bending Rigidity:

The resistance to curvature deformation of a typical phospholipid bilayer is quantified by its bending modulus κ . For a typical bilayer, the value of κ is in the range^{13,14} of $8-25 k_B T$. Because the κ value is on the order of $10 k_B T$, unlike the much larger values characteristic of rigid materials, low $k_B T$ deformations in these structures are much more probable and accessible. These include budding, tubulation, poration, and other geometric transformations which may happen due to environmental stressors such as those discussed further in this thesis. This mechanical accessibility is the physical reason that lipid bilayers are such sensitive environmental transducers. This is discussed further in Chapter 2 and Chapter 3.

1.2.3.2 Spontaneous Curvature:

The spontaneous curvature C_0 of a membrane is the average curvature that a membrane would take in the absence of external geometric or topological restrictions.¹⁵ For a typical symmetric bilayer of a single lipid, $C_0 = 0$. Nonzero curvature values arise from asymmetric lipid composition, the extramembrane environment, or wedge-shaped molecules in either leaflet.^{15,16} In this thesis, changes in the curvature of the membrane can be seen via modification by chemical stressors in Chapter 2 and via environmental physical stressors in Chapter 3.

1.2.3.3 Permeability:

Considering the role that bilayers play as boundaries and environmental interfaces, it is evident that these bilayers have selective permeability. As simple bilayers, they are impermeable to ions, large macromolecules, and proteins without the presence of pumps and channels. Water moves much more readily through these bilayers and its movement is a key regulatory process. It is this selective permeability that allows osmotic pressure gradients to be established and maintained across the bilayer and is fundamental to Chapter 2 and Chapter 3.

1.2.3.4 Thermal Undulations:

Since the bending rigidity of these fluid bilayers are reasonably low ($\sim 10k_B T$), these membranes are susceptible to shape deformations due to thermal fluctuations. In single bilayer systems such as GUVs, these occur with varying undulation amplitudes and are mostly unrestricted. However, for multilamellar systems, due to the physical barrier of the neighboring layers, these undulations become much more repressed and this repulsive force between the bilayers is called the Helfrich repulsion¹⁷. This force can stabilize the equilibrium interlayer spacing of multilamellar stacks and resists compression. This is discussed further in Chapter 3.

1.3 Environmental Stressors

Having established the molecular architecture and mechanical properties of phospholipid bilayers, we now consider the environmental inputs that drive their structural reorganization. The word “stressor” or some form of it is used throughout this thesis to signify any environmental conditions which perturbs a soft matter system from its equilibrium. The core idea of this thesis is that the character of the stressor plays a more noticeable role in the structural reorganization rather than the magnitude alone. For developing this idea in this thesis, it is important to consider two distinctly important classes of environmental stressors: chemical and physical.

1.3.1 Chemical Stressors

Considering the complexity of biology, it is no surprise that an abundance of various molecules causes chemical perturbations which alter a soft matter systems properties directly. Examples include a change in pH altering membrane stiffness¹⁸, a change in the ion concentration can modify the electrostatic interactions and change membrane curvature¹⁹, and compositional changes directly affect stiffness and bending^{20,21}. The consequences of these typically affect the packing parameter by altering lipid headgroup area or some other change in molecular shape. A small molecular change thereby cascades into a larger morphological change in the membrane. A much further discussion happens in Chapter 2 where this thesis attempts a brief review of chemical stressors on lipid bilayer structures and a case study¹ involving bolaamphiphile that this author was involved in.

1.3.2 Physical Stressors

Physical stressors do not alter the membrane components' molecular properties like chemical stressors do. Instead, they rely on a thermodynamic approach by applying constraints on possible configurations without altering the membrane's compositional properties. The most common physical stress imposed on such systems is osmotic pressure²². For a simple, ideal, dilute solute unable to cross the membrane, the osmotic pressure is described by the ideal van't Hoff relation²³: $\Pi = cRT$. In this expression, c is the molar concentration, R is the universal gas constant, and T is the absolute temperature. This equation is colligative which means that the osmotic pressure depends only on the number of solutes present and not their size, shape, or chemical identity. For non-ideal systems, such as in the presence of large macromolecules, the van't Hoff equation can be adapted to account for the non-ideal interactions by a virial expansion²⁴:

$$\Pi = RT \left(\frac{c}{M_w} + A_2 c^2 + \dots \right)$$

where M_w is the solute molecular weight and A_2 is the second virial coefficient, which grows with molecular size. At equivalent mass concentrations, a larger macromolecule will generate a higher osmotic pressure than a smaller one, a direct consequence of their excluded-volume interactions.²⁵

Aside from a larger osmotic pressure in non-ideal systems (which much of biology exists as), these larger macromolecules introduce a new physically distinct effect known as the depletion interaction.^{26,27} When macromolecules with radius R_p are present near a surface, their finite size prevents their centers of mass from approaching the surface closer than R_p , creating an excluded-volume region around the surface. When two surfaces approach to within $2R_p$, their coronas overlap, restoring volume to the bulk solution and increasing the translational entropy of the depletant, thereby minimizing the free energy of the system.

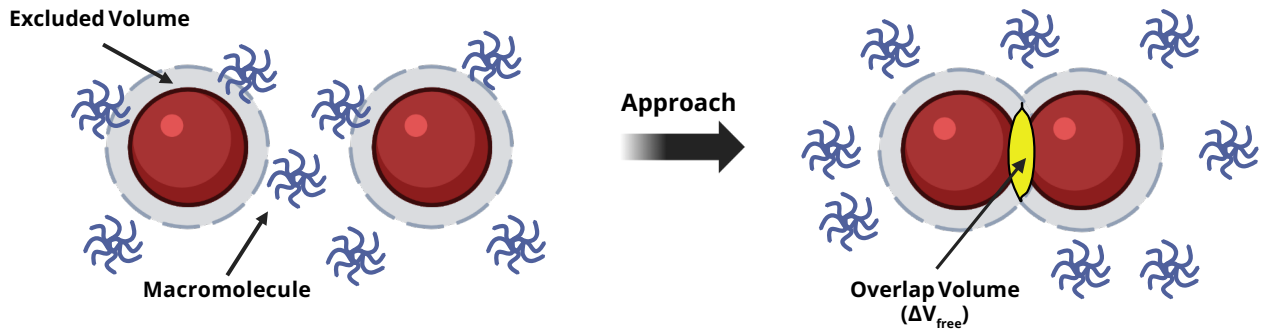


Figure 1.3: Schematic illustration of the depletion interaction between two surfaces in the presence of macromolecular depletants. When surfaces are separated (left), depletant molecules are excluded from a corona of radius close to the surface (R_p). As the surfaces approach due to Brownian motion, the excluded-volume coronas overlap, returning the overlap volume ΔV to the bulk and increasing the translational entropy of the depletant macromolecule. The resulting entropic free energy gain drives an effective attraction between the surfaces known as the depletion force.

Mathematically, it is given by: $\Delta F_{\text{depletion}} \approx -nk_B T \Delta V$ where n is the depletant number density and ΔV is the overlap volume. The depletion force grows with depletant size because larger depletants have thicker coronas and generate greater ΔV upon surface-surface approach. Considering that 20-40% of the cellular environment is filled with macromolecules, the depletion effect is a key biological result of these excluded-volume interactions based solely on geometric constraints. Examples include thylakoid membrane stacking in chloroplasts²⁸ or bacterial colony

aggregation²⁹.

1.4 Thesis Road-map and Overarching Theme

This thesis investigates the structural response of soft matter systems across three distinct model systems at differing scales of complexity and biological relevance. Within each of these models, there is an overarching idea that the character of the stressor plays a much deeper role than its magnitude in driving structural response across differing material types and geometric arrangements. The first system explores chemical stressors as a method of modulating soft matter system boundaries, with a case study using giant unilamellar vesicles (GUVs) interacting with a pH-responsive bolaamphiphile (G-C18:1). GUVs comprised of DOPC provide a clean membrane interface where the response to the pH-dependent molecular geometry of G-C18:1 can be isolated and studied. The second system uses a multilamellar cylindrical liquid crystalline smectic system of myelin figures to test a physical stressor framework. By matching osmotic pressures across small-molecule and macromolecular solutes and observing whether surface instabilities develop, Chapter 3 demonstrates that depletion-force character, not osmotic magnitude, is the determinant of structural response in a cylindrical multilamellar geometry. The third system uses aqueous phase-separated droplets in which actin can be sequestered, to further explore the physical stressors that soft matter systems may experience, in this case within a protein-based material. This chapter focuses on how macromolecular crowding drives actin polymerization in an environment which would otherwise not support it, specifically inside dynamic droplets. Across all three systems, the unifying theme is the same: the character of the stressor, rather than its magnitude alone, determines how soft matter systems structurally reorganize.

Chapter 2

Chemical Stressors as Geometric Perturbations in Lipid Membranes: A Review and Case Study

2.1 Preface

The work presented in this chapter is loosely based on a publication¹ in *ACS Nano*: "Driving a Stimuli-Responsive Wedge in the Packing of Phospholipid Membranes Using Bolaamphiphile Intercalants" by Niki Baccile, **Archan Vyas**, Ramanujam Ramanujam, Daniel Hermida-Merino, Ingo Hoffmann, Lionel Porcar, and Atul N. Parikh (*ACS Nano*, 2025,).

2.2 Introduction

Soft matter assemblies, like those briefly discussed in Chapter 1, are often exposed to their environmental surroundings and in fact rely on them for a dynamic adaptation. When compared to traditional crystalline structures where the assembly is held together by more strong, directional interactions, soft matter assemblies rely on an adaptive assortment of collective weak interactions on the order of the $k_B T$ scale. As discussed in Chapter 1, this collective arrangement allows for

soft matter assemblies to be dynamic and environmentally responsive as their mechanics are not as rigid nor are they fixed properties. For example, the chemical character of the environment is an extremely relevant source of perturbing agents for the soft matter assemblies. Changes in pH¹⁸, ionic composition³⁰ and electric charge, solvent chemistry³¹, and the molecular composition^{5,32} are just some of the chemical properties which impact how the soft matter assemblies respond. Some of the properties of the soft matter assemblies which are affected include the lipid packing parameter (Figure 1.2), interfacial electrostatics³³, hydration state, and the membrane effective geometry. Any changes in the properties mentioned prior mean that the soft matter assembly must respond accordingly to redistribute its internal stress. The extent of response, whether subtle or dramatic, is highly dependent on the chemical stressor.

The central premise of this chapter does not aim to provide an exhaustive review on chemical perturbations on soft matter assemblies, but rather to build sufficient context. Instead, what this chapter will do is to motivate the idea that these diverse chemical stressors, no matter their differences, will all affect the geometry of the soft matter assemblies' molecular building blocks. In return, these assemblies will respond to this change in stress by modulating their morphology, making it more compatible with the changed constituents. More typically, these transformations are well studied for lipid membranes where the framework of the lipid packing parameter^{34,35} and curvature elasticity theory³⁶ provide clear explanations as to how local, molecular perturbations can propagate to system-wide changes. While much of this chapter will focus on these membrane-based structures, the same principles can be applied to the other structures such as wormlike micelles³⁷, polymer vesicles (polymersomes), and coacervates to name a few. All these systems respond to the chemical environment by re-balancing their internal stresses based on their respective geometry and governing mechanics.

This chapter will first revisit the physical and theoretical frameworks discussed in Chapter 1 and frame them for a more in-depth interpretation for how the soft matter systems respond. Next, this chapter discusses how major chemical stressors affect the template lipid membrane soft matter assemblies with some small discussion on other broader materials. The latter part of this

chapter distills the main ideas into interpreting a specific case study¹, discussing bolaamphiphiles in phospholipid bilayer membranes, to help elucidate further the importance of understanding how chemical stressors can impact soft matter assemblies.

2.3 Extending the Physical Framework

As alluded earlier in Chapter 1, there are a couple of important concepts which help understand how the lipid bilayer assemblies respond under environmental stressors: the critical packing parameter, which connects the molecular geometry to the preferred assembly state; the Helfrich elasticity framework, which connects the bending modulus (κ) and the spontaneous curvature (C_0), to describe the cost of membrane deformation. This section develops these ideas further to better understand the morphological transformations occurring in soft matter assemblies under chemical stressors.

2.3.1 The Packing Parameter Extended as a Perturbation Response

As introduced in Chapter 1, the lipid critical packing parameter characterizes the geometric shape of an amphiphile and predicts what self-assembled structure the molecule prefers and minimizes the free energy of the system. Chapter 1 presented this framework as a predictor of which structure may form based on this parameter. This section discusses how this value changes when it encounters perturbations that may alter the geometry. Consider a bilayer made of PC lipids where the $P \cong 1$. This means it exists in a lamellar state with a near-zero spontaneous curvature. Those with $P < 1$ are inverted-cone-shaped and favor positively curved structures; those with $P > 1$ are cone-shaped and favor negatively curved structures.

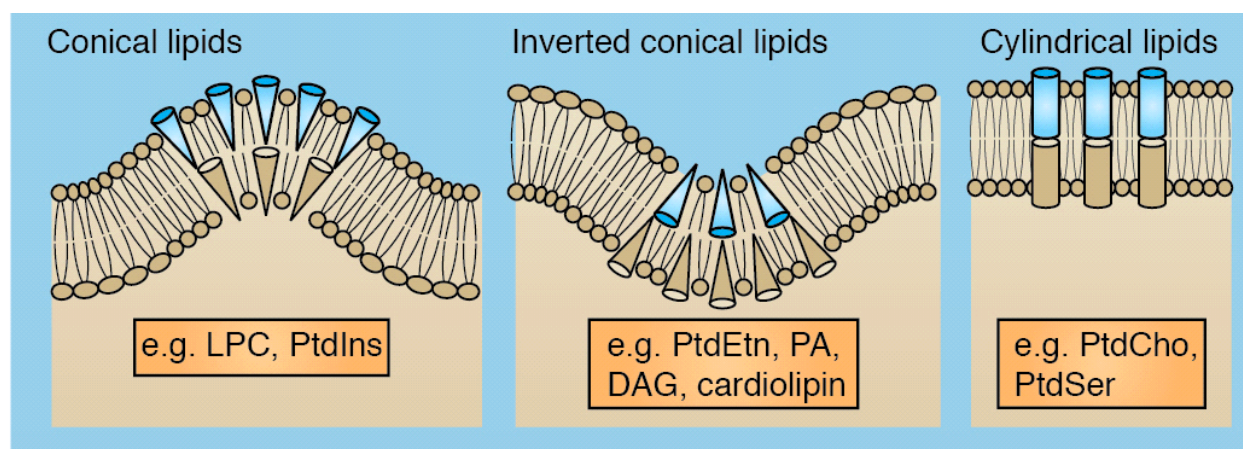


Figure 2.1: Lipid molecular geometry determines the direction of membrane curvature. Conical lipids ($P > 1$) generate negative curvatures; cylindrical lipids ($P \approx 1$) favor flat bilayers; inverted-conical lipids ($P < 1$) generate positive curvature. Adapted from McMahon and Boucrot(2015)³⁸.

In Chapter 1, the focus was on the types of structures which are made based on the predictive nature of the lipid packing parameter when placed in water. In this chapter, we aim to focus on what happens when these made structures are impacted by external environmental chemical perturbations. When a chemically mismatched molecule enters this system, subsequent changes in the membrane structure and curvature are possible since now the established geometry needs to account for this new stressor. The packing parameter captures, for this type of perturbation, changes in the membrane head group and structure. For example, how would a primarily lamellar straight membrane respond if an inverted-cone lipid was somehow introduced? Or if the chemical change alters the parameters within the lipid packing parameter formula (ex. pH to protonate or deprotonate a headgroup, electrostatic changes, salt conditions)? In any of these circumstances, the changes alter the expression for the packing parameter.

However, changes in the packing parameter are not sufficient to explain the mechanical response of the assembled membrane structures. The packing parameter does not contain information about bending modulus, elastic energies, or when certain P values cause things like budding or poration. For those questions, the Helfrich framework discussed below is more appropriate. The packing parameter can characterize the molecular geometry of the lipid structure used and predicts the direction of the favored spontaneous curvature. One important distinction to make

is that this packing parameter is for a single-component, equilibrium layer. When applied to a multi-component membrane, it can only predict the curvature shift generally for a specific chemical perturbation. Regardless, the packing parameter remains critical in predicting the curvature changes for a chemical perturbation to membranes.

2.3.2 The Helfrich Elasticity Theory: Curvature and Bending Responses

When these perturbations happen, the membrane has to accommodate the stress somehow. Typically, they respond by bending in some form but this morphological response is not free. There is an energy penalty that is paid. This penalty is governed by the Helfrich curvature elastic free energy framework³⁶ which helps define the total bending free energy stored across a defined surface area of a fluid membrane. The equation's two key terms can be broken into terms which discuss the **Mean Curvature** and the **Gaussian Curvature** terms:

$$\int \left[\frac{\kappa}{2}(2H - C_0)^2 + \bar{\kappa}K \right] dA$$

The **Mean Curvature** term, $\frac{\kappa}{2}(2H - C_0)^2$ accounts for the energy penalty associated with variations in mean curvature. Here, $H = (c_1 + c_2)/2$ defines the local mean curvature based on the principal local curvature values c_1 and c_2 . The spontaneous curvature is denoted by C_0 and the bending modulus of the bilayer is represented by the κ . Since this expression squares the difference between the local geometry and the spontaneous curvature, it acts like a penalty rule. This term therefore controls the large-scale, non-disruptive membrane shape transformations such as tubulation.

The second term, $\bar{\kappa}K$, accounts for **Gaussian Curvature** changes. Within this expression, $K = c_1c_2$ represents the Gaussian curvature, and $\bar{\kappa}$ defines the Gaussian (saddle-splay) modulus. According to the Gauss-Bonnet theorem, the surface integral $\int K dA$ remains a strict topological invariant for any closed surface under continuous deformation. As a result, this term yields a

constant energetic value during standard bending or stretching and does not influence continuous path fluctuations. It contributes to the overall energy landscape exclusively during topological transitions, becoming physically relevant only when a localized bud pinches off into a distinct daughter vesicle or when a pore opens and closes in the membrane.

Of the above terms, the key state variable that connects the molecular-level perturbations to membrane response is the spontaneous curvature C_0 . For a symmetric bilayer composed of a single lipid with $P \cong 1$, curvature should be 0 by symmetry. If this symmetry is broken in a single leaflet, say by insertion of a molecule or a chemical change to the headgroup, the preferred curvature of that leaflet becomes non-zero, and the whole bilayer acquires nonzero curvature. This is where the packing parameter and the Helfrich framework come together, since the sign of the curvature is based on the molecular parameters, but the magnitude of the resultant elastic stress response is governed by the Helfrich energy formula.

As noted earlier, the more typical fluid PC bilayers^{3,4} have a κ in the range of $8 - 25 k_B T$. What this also means is that since κ is on the order of $10 k_B T$, membrane deformations of a similar order are thermally and chemically accessible. Even a few mole percent of a geometric mismatch in the membranes can cause noticeable shifts in the spontaneous curvature, generating elastic stresses which propagate over the entire membrane quite readily. This stress propagation can be relieved by global shape change, such as budding, tubulation, or poration. This amplification from a small molecular perturbation to large scale changes in the membrane morphology is a clear basis for the sensitivity of membrane structures to chemical perturbations. Together, the packing parameter and the Helfrich framework provide a multi-level approach for interpreting every morphological membrane transition in this chapter. The packing parameter informs which way the perturbation pushes, while the Helfrich framework determines what the membrane does about it.

2.3.3 Membranes as the Main Template Example

Lipid membranes, typically unilamellar structures such as vesicles, provide the clearest template for case studies which study the impact of chemical perturbations. There is a plethora of research into this field for two main reasons. The first being that the molecular architecture of the typical phospholipid bilayers is well-characterized and any environmental chemical changes can be traced to specific aspects of the molecular composition with great precision. The second main reason is that the various types of membrane transformations observed, such as budding, tubulation, pearling, and poration, are readily visible via optical microscopy. With these two factors, it becomes possible to treat these unilamellar membrane structures as an ideal template to understand environmental chemical perturbations.

2.3.3.1 pH and Amphiphile Geometry: Molecular Shape Changes

Of the possible chemical perturbations, pH is one of the most direct chemical stressors which impacts the membrane structures. Changes in the pH can alter the headgroup charge, through protonation or deprotonation, which changes the electrostatic repulsion of neighboring headgroups. Connecting this back to the packing parameter, this in turn changes the effective area per molecule which changes the packing parameter and curvature. This is a straightforward cascade from molecular pH changes to large scale geometric transformations within these lipid bilayer structures.

The most direct route for pH to alter the membrane properties is via the amphiphile itself, whose geometry can be dependent on the surrounding pH. Consider when such amphiphiles are incorporated into a bilayer as pH-dependent geometric wedges. The packing geometry of the membrane changes based on the ionization state of the molecule. Amphiphiles that are inverted cone ($P < 1$) generate a positive spontaneous curvature contribution which drives outward budding and possible vesiculation. Those that are cone shapes ($P > 1$) generate a negative spontaneous contribution contribution which drives invagination, inner tubulation, or something similar.

This type of wedge insertion logic has been demonstrated across various classes of amphiphiles, such as lysophospholipids³⁹ where lysolipid insertion into the outer leaflet of GUVs generated asymmetric stress and caused outward budding, or in another complementary example⁴⁰ where GM1 concentration asymmetry drove clear shape remodeling.

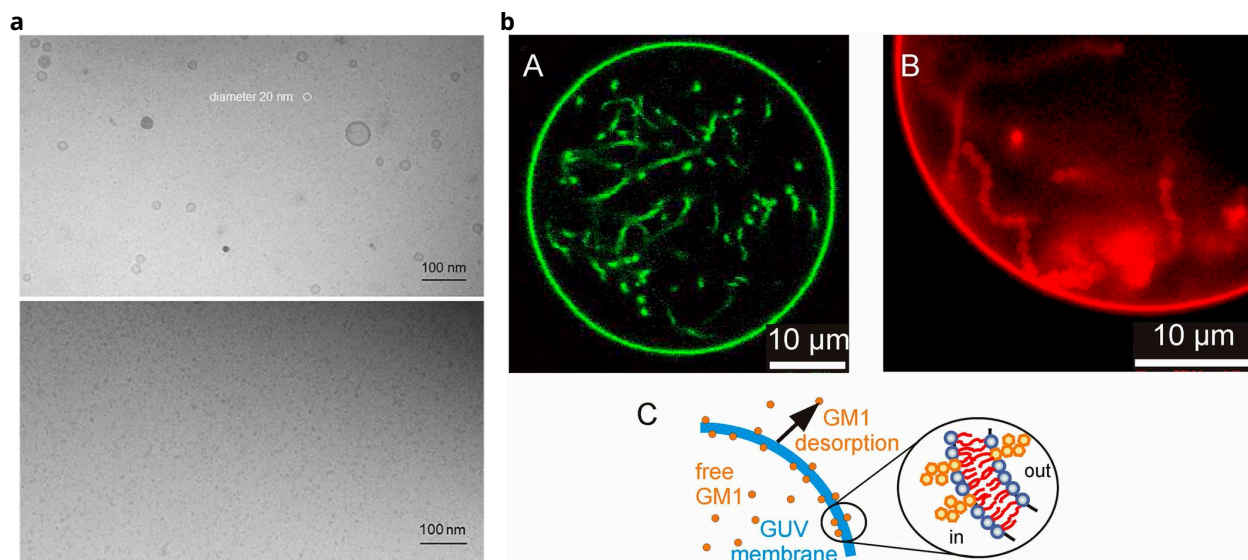


Figure 2.2: [a] Cryo-TEM images of POPC vesicles exposed to lysolipid (C12LPC). Outer leaflet lysolipid produces small daughter vesicles (20 nm, white circle, top). When lysolipid redistributes to the inner leaflet, vesicles break down into mixed micelles (bottom). Adapted from Hua et al.³⁹ [b] Confocal fluorescence images of GUVs containing GM1 showing extensive inner tubulation driven by asymmetric GM1 distribution between leaflets (A, B). The schematic (C) illustrates how reduced outer leaflet GM1 generates the curvature imbalance responsible for the observed tubes. Adapted from Dasgupta et al.⁴⁰

Beyond just the insertion of a different shape amphiphile in the bilayer, this type of curvature based geometry changes can be directly modulated by controlling the pH without the insertion of an extra molecule. Both theoretical^{41,42} and experimental approaches^{18,43} give clear evidence to cases where the protonation or deprotonation of the amphiphiles alters the curvature and causes subsequent morphological changes due to stress propagation.

Khalifat et al (2008)⁴³ provides particularly clear visual evidence of this pH effect on membrane structure. The authors successfully showed that applying local pH gradients to GUVs produced optically visible membrane tubulation and invagination-like morphological responses. These collected responses mapped directly to the curvature changes that can be predicted by the packing

parameter. This result is important since it visually confirms that pH alone is sufficient to drive large scale membrane morphology changes without any compositional change to the membrane. Furthermore, a complementary study by Zhou and Raphael¹⁸ shows the mechanical measurement side of this observed phenomenon. They successfully measured that the bending rigidity changes with pH by increasing both the acidic and basic extrema relative to the neutral conditions. This is in line with the idea that interfacial electrostatics modify the effective head group geometry, which can further drive the morphological changes discussed. When these pH-based curvature perturbations are large enough, the membrane response can go beyond shape transitions within the bilayer topology and instead changes can occur in the phase structure themselves.⁴⁴

What is clear is that pH changes can cascade system wide and show large-scale morphological changes which can be from local properties, surface topological changes, and into the extreme of lamellar phase changes. It is reasonably clear now that membrane based structures containing amphiphiles can have a large propensity to alter based on local pH changes; these changes can propagate through the system to accommodate the stress.

2.3.3.2 Ions and Electrostatics

While pH changes can affect the head group size and the molecular geometry, the effect of ions on lipid membranes is most frequently described as simple electrostatic screening. The ions are unable to cross the lipid bilayer naturally (without the explicit channels or pores) and remain on whichever side of the bilayer they were initially present. There is a large amount of literature which discusses how ions can bind to the lipid head groups, preferential accumulation at interface, and can generate morphological response through its asymmetric distribution.

Consider the studies by Petrache^{30,45}. They were able to show that monovalent salt ions can swell neutral PC bilayers by weakening the attractive van der Waals forces that hold membranes together. These results were supported by X-ray scattering data and osmotic stress measurements. They then built on this work to ask how different ions affect the membranes differently. What they found is that there are two complementary effects happening. Firstly the salts do weaken

the van der Waals attraction in membranes. Secondly, some ions such as bromide can adsorb to the membrane interface more than chloride. So while most salts can impact the structure by weakening the van der Waals attraction, certain salts are more likely to adsorb to the membrane to create larger electrostatic effect.

Some of the most striking ionic effects arise from concentration asymmetry on either side of the membrane. When there are two different ionic concentrations on two sides of the bilayer, the differing effect on each leaflet imposes differing curvature effects without more complex methods. Consider this study by Karimi et al.¹⁹ in which asymmetric salt and sugar solutions were on either side of the POPC GUVs. They were able to optically observe nanotubes on the interior or small buds on the exterior, depending on the salt concentration difference and where lithium ions were particularly effective at driving inner tube formation. In a different study⁴⁶, asymmetric calcium concentrations induce membrane shape remodeling as they favor negative spontaneous curvature.

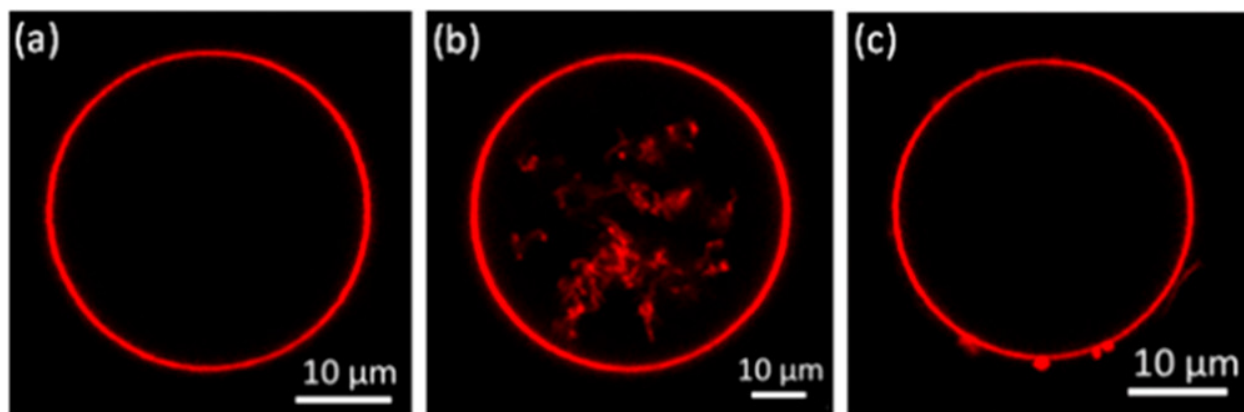


Figure 2.3: Confocal fluorescence microscopy of POPC GUVs with different ionic solutions on each side of the membrane, showing three possible morphological outcomes: no structural change (a), inner nanotube formation (b), or outward buds, tubes, and folds (c). Higher salt concentration asymmetry generally increases the frequency of structural remodeling, and the ion identity matters. Low LiCl drives 69% internal structures while low NaCl produces structures in fewer than 10% of vesicles. Adapted from Karimi et al.¹⁹

Charge concentrations also have an effect after some unrelated structural effect has taken place. In this study⁴⁷, it was shown that charged membranes become highly unstable once a poration event occurs since pore edge tension falls considerably at high surface charge density. Calcium ions were successful in stabilizing the system due to charge screening. Taken together,

it is clear that the ionic environment of the membrane affects local van der Waals forces, causes ion-specific curvature preferences, and can even destabilize membranes in the event of other phenomena.

2.3.3.3 Osmotic Forces: Chemical Potential Differences to Drive Shape Change

The final simple, yet powerful chemical stressor discussed in this chapter is that of osmotic stress. While discussed briefly in chapter 1 and later in chapter 3, osmotic stress is discussed here in the context of chemical osmotic potential. Osmotic stress can convert the chemical potential gradient of water into membrane shape transformations. Because bilayers resist changes in area, the volume change from osmotic flux must be accommodated by a corresponding change in shape. Changes in the encapsulated volume's water volume and the subsequent stress must be accounted for by membrane shape changes. There is a simple path from solution chemistry through water potential to membrane response.

Consider this experimental study⁴⁸ in which the authors successfully demonstrated that osmotic gradients across the membrane can make the simple lipid vesicles undergo cell-like shape changes. In GUVs, by imposing solute asymmetry, the GUVs showed inward budding, tubulation, pearling, and drastic remodeling. These results showed that a simple water and solute imbalance alone can push membranes far from their relaxed preferred state into states where the osmotic stress must be addressed. Basic membranes, without complex architecture or proteins, can already undergo complex remodeling due only to the difference in water's chemical potential. While the authors did caution that the response can vary from vesicle to vesicle, they did show the high impact that water potential can have on membrane remodeling. Another study⁴⁹ showed another vesicle based system in which the giant phospholipid vesicles underwent pearling as a transient geometric response. The osmotic stress alone was again sufficient to push the lipid vesicles past their initial stressed shape and remodel them by developing pearling instabilities, before relaxing into daughter vesicles. This sequence can be considered a Rayleigh-Plateau-like instability driven by the imbalance between osmotic compression and bending rigidity. The paper, like the other,

frames osmotic stress and the chemical potential of water as an active regulator of membrane geometry. Simple changes in the chemical potential of water clearly drive large scale morphological change.

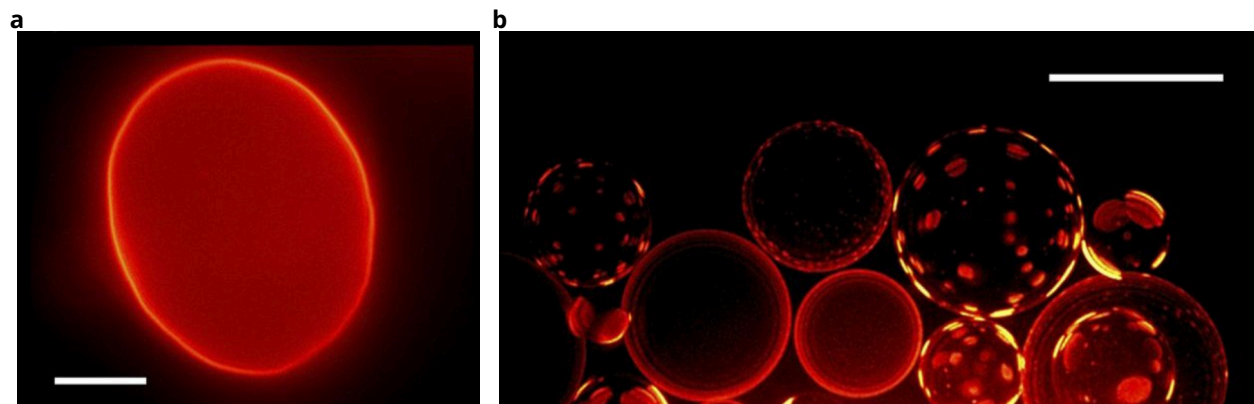


Figure 2.4: Fluorescence microscopy of vesicles in isotonic conditions [a] and under an osmotic differential [b]. The initially smooth, uniform vesicle boundary is replaced by a complex pattern of co-existing domains, daughter vesicles, and membrane structures upon osmotic imbalance. Images were captured with fluorescence microscopy. Scale bar = 15 μm . Adapted from Oglecka et al.⁴⁸

The variety of osmotic responses became apparent in the recent work⁵⁰ by a former lab member where the author pushed this osmotic stress as chemical perturbations much further. The authors successfully demonstrated that a sequential cycle of hypertonic and hypotonic stresses generates inwards buds and daughter vesicles respectively. The hypertonic step first drives the inward budding and stores the excess membrane area in invaginations. The subsequent hypotonic step either reincorporates the membrane back to normal or can convert some into budding daughter vesicles. This cyclic water stressing cycle frames osmotic gradients as active chemical stressors that clearly reshape membrane geometry and allow for these structures to accommodate stress in many forms.

It is worth noting here that osmotic stress acting on bilayers through area/volume imbalance, as described above, is distinct from the question addressed in Chapter 3, where osmotic pressure is applied to a multilamellar system using solutes of very different molecular sizes at matched pressures. That experiment asks whether the *character* of the osmolyte, such as its molecular size

and associated excluded-volume depletion forces, determines the structural response independent of pressure magnitude. The finding that macromolecular osmolytes drive surface instabilities while small-molecule osmolytes at identical pressures do not motivates the distinct entropic stressor framework developed there.

2.3.4 Shared Rules

In compiling the literature above, there are several principles that occur consistently across the stressor types and length scales. Note that these are not universal laws and exceptions do exist. The most consistent is that the strongest morphological responses tend to occur in systems that already exist in boundaries between states or at the edge, such as bilayer-to-bud threshold or phase transitions. In these cases, a small chemical perturbation shows up as a dramatic structural response. The second core principle is that asymmetry matters more than uniform exposure such as concentration gradients or compositional differences in the leaflets. Such asymmetry is what drives the curvature to change via the Helfrich framework.

Another recurring observations is that chemical stressors can act via various pathways. For example, osmotic cycling pairs shape changes to the membrane area regulation and solute transport. These paired responses mean that the most informative experiments are those which can combine structural, mechanical, and dynamic measurements rather than looking at only one observable. The systems most worth studying are those that respond to environmental stress by changing morphology in a controlled, reversible way without losing their structural identity — which is precisely what the case study in the next section demonstrates.

2.3.5 A pH-Responsive Geometric Wedge: G-C18:1 Intercalation in DOPC Membranes

2.3.5.1 Preface

The confocal fluorescence microscopy results below represent the author's direct experimental contribution to this published work and are discussed in more detail. All scattering data (SAXS, SANS, NSE) are findings of the broader study and are discussed briefly credited to the co-authors on the paper.¹ The author does not claim expertise in those measurements and has included them here to serve as a complementary dataset to the confocal microscopy images.

2.3.5.2 Introduction to the G-C18:1 System

The earlier sections of this chapter establish that chemical stressors act on membranes through molecular geometry: pH shifts the headgroup charge, ions create asymmetry, and compositional changes affect curvature and the average packing parameter. This case study examines all of these mechanisms operating simultaneously in a single representative system: a single amphiphilic molecule that has a pH sensitive geometry is inserted into a initially pH inert membrane. The resultant membrane is now environmentally sensitive and can respond accordingly.

The representative bilayer initially has DOPC, a fluid-phase PC lipid with a $P \approx 1$ and $C_0 = 0$, and this is true for the experimental pH range. As confirmed by control experiments, DOPC GUVs are completely insensitive to the pH range tested and shown no noticeable changes in their shape or morphology when pH is changed in the absence of any additive. Because they are inert, this makes them an ideal experimental platform to study a mixed system since any changes can be attributed to the newer molecule.

This newer molecule is an intercalant named G-C18:1, a linear glucolipid bolaamphiphile, a single-glucose derivative of oleic acid⁵¹, with a $MW = 460 \text{ g mol}^{-1}$. What makes G-C18:1 useful here is that its molecular shape is directly pH tunable^{52,53} through the protonation state of the head group. Above pH 7, full deprotonation enlarges the effective headgroup through electrostatic

repulsion, shifting with a $P < 1$ (inverted-conical). Between pH 4–6.2, the molecule is partially protonated, near-cylindrical to slightly cone-like ($P \cong 1$). Below pH 4, full protonation removes all electrostatic repulsion and sugar-sugar interactions dominate, driving aggregation. This three-state pH behavior provides a direct, controllable test of how molecular geometry shifts drive membrane morphological response.

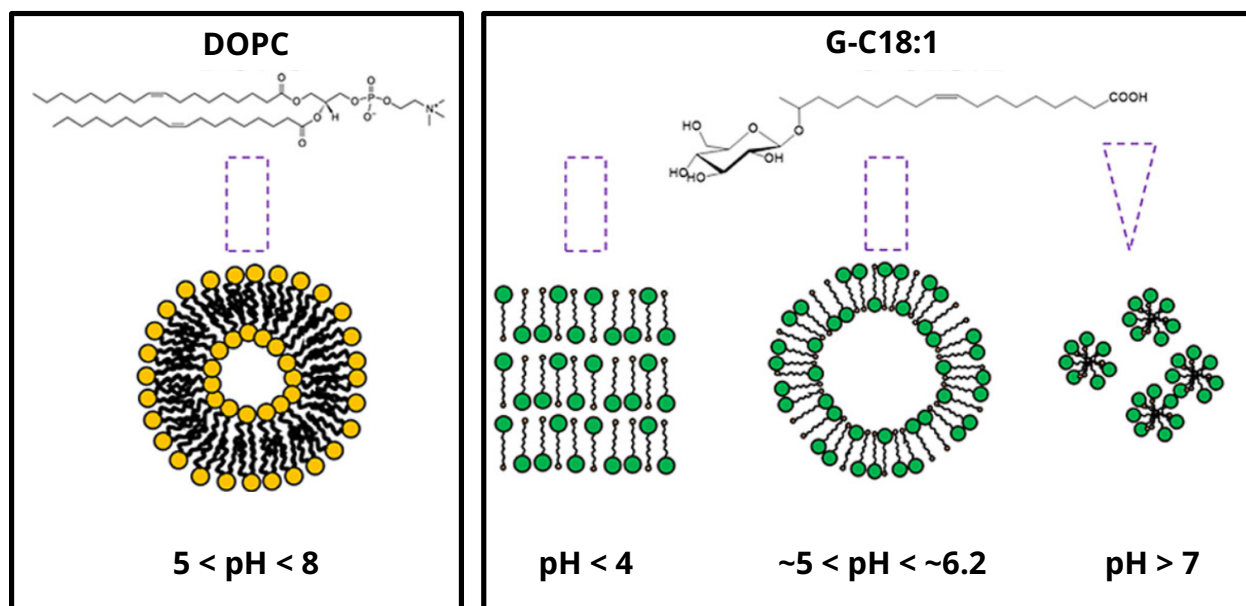


Figure 2.5: Molecular structures of DOPC and G-C18:1 with their known pH-dependent self-assembled geometries in water. G-C18:1 adopts a cylindrical shape under acidic and near-neutral conditions and shifts to an inverted-cone geometry upon deprotonation at basic pH. Adapted from Baccile et al.¹

2.3.5.3 Experimental Setup

To prepare the DOPC GUVs, we used the standard electroformation⁵⁴ technique and labeled the GUVs with a rhodamine-based lipid. For the G-C18:1 prepared vesicles (SUVs) vesicles of 100 nm – 1 μ m were prepared after dispersion of the dry powder in water, adjusting the pH to 5.5 using NaOH, and sonicating in bath until the solution was highly turbid. The vesicles were labeled with perylene, which partitions in the G-C18:1 membrane so that they can be tracked independently from the DOPC GUVs in a multi-channel experiment.

To assemble the samples, 96 μ L of 0.1 M glucose (pH 5.5) was pipetted into individual wells of

a 96 well-plate glass bottom plate. Then, 1 μL of electroformed DOPC GUVs in 0.1 M sucrose were added, with the density difference between the sucrose and glucose letting the GUVs sediment to the bottom of the plate for accessible imaging and the osmotic balance maintained. To start the interaction with the G-C18:1, 1 μL of the G-C18:1 vesicles at either 0.5 mg mL^{-1} or 5 mg mL^{-1} were added and imaging started right after. When pH adjustment was needed, 1 μL of 0.1 M of NaOH raised the pH to approximately 9 or 2 μL of 1 M HCl lowered the pH to approximately 3.5. The images were acquired using a Marianas 3i spinning disk confocal microscope using a 60 $\times$ oil immersion objective (NA 1.4) and an EMCCD camera at 50 $\times$ gain, at an acquisition rate of 20 Hz (one frame every 200 ms). All experiments ran at 23 $^{\circ}\text{C}$, and we repeated each condition at least three times independently ($n = 3$).

2.3.5.4 Fluorescent Microscopy Results over the pH Range

The following sections explain the chemical stressor's state (i.e. the pH of the system), how the G-C18:1 molecule chemically alters its geometry, and the corresponding microscopy result.

2.3.5.4.1 pH 5.5 = Near Cylindrical G-C18:1 At pH 5.5, just below the pK_a (meaning the environment is more acidic and the molecule exists in its protonated state), the G-C18:1 SUVs readily interacted with the DOPC GUVs, producing large-scale morphological changes within seconds of mixing. The microscopy tracked the transfer of the perylene dye from the blue bath of the G-C18:1 SUVs, providing the evidence that the interaction can proceed through vesicle fusion and coalescence rather than simple exchange. Within 15-25 seconds of introduction, there were some unusual vesicular structures: small inner vesicles enclosed within the larger DOPC GUV boundary. With longer incubation times, the features become inner tubulation dominated, with noticeable membrane tubes filling the GUV interior ($n=3$). This inward direction of the deformation is consistent with G-C18:1 near its pK_a , adopting a cone-like geometry ($P > 1$). The insertion into the DOPC GUVs shifts the curvature of the bilayer towards negative values and the Helfrich framework energy then drives the membrane towards concave geometry, bending

inward and forcing it to generate large amount of tubules.

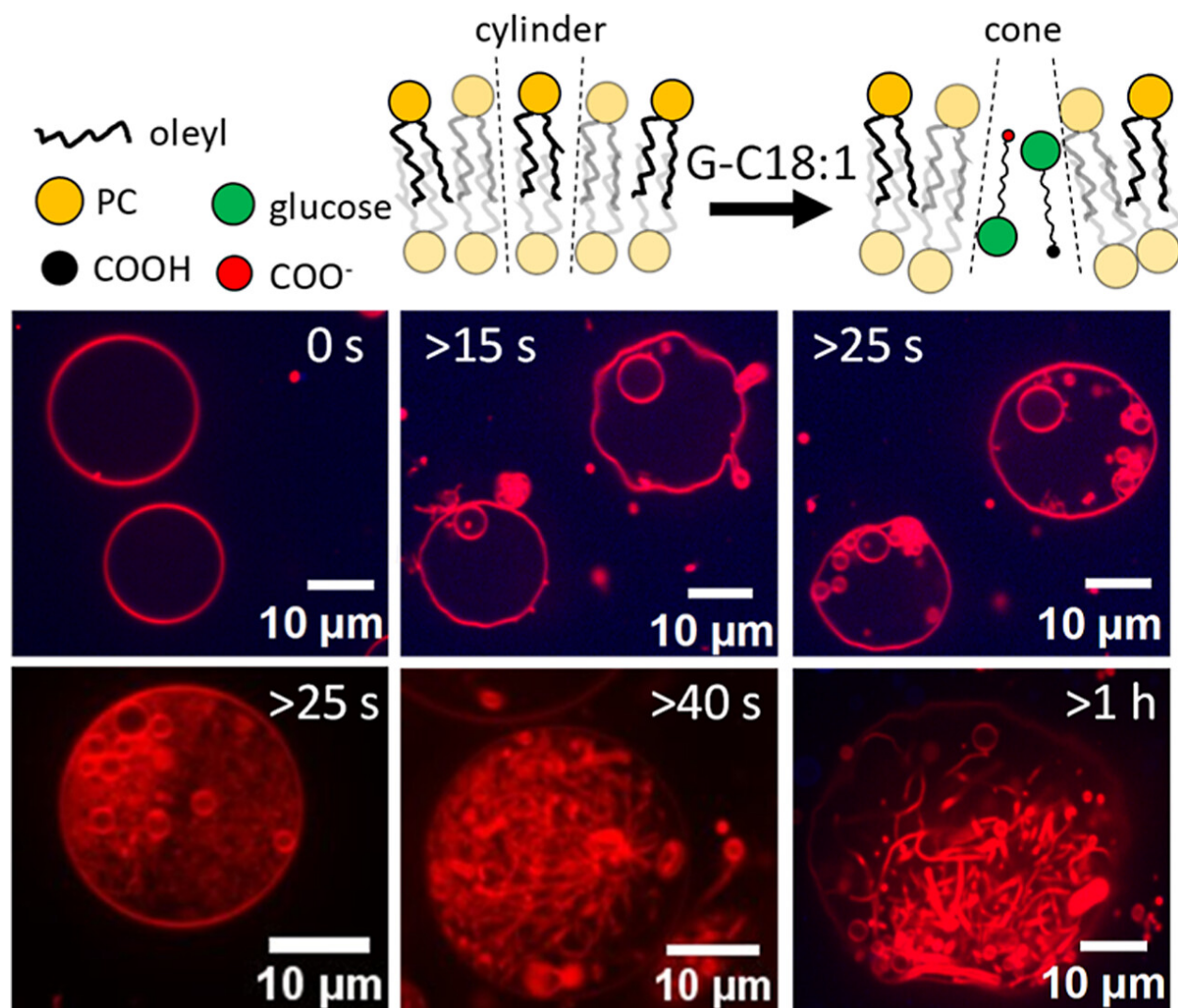


Figure 2.6: Real-time time lapse confocal fluorescence microscopy montage of DOPC GUVs after addition of G-C18:1 vesicles at pH 5.5. Within 15 s-25 s, vesicle-in-vesicle structures appear at the membrane boundary ($C = 0.5 \text{ mg mL}^{-1}$). At longer times (>40 s and >1 h), inner tubulation becomes the dominant morphological feature ($C = 5 \text{ mg mL}^{-1}$). The cone-like geometry of G-C18:1 in the cartoon is deduced from the events observed after 15 s. Scale bars=10 μm . Adapted from Baccile et al.¹

2.3.5.4.2 pH 9.0 = Inverted Conical G-C18:1 Adding NaOH to the samples that had equilibrated at pH 5.5 for 1 hour, for a final pH of 9, produced an immediate and optically different response. Within seconds of the pH increase, multiple distinct events were occurring in the vesicle suspension. Firstly, there was internal membrane fusion at the inner membrane

boundary, there was pore formation at the outer GUV surface, and there was expulsion of inner vesicular structures and content outward through the aforementioned pores. Over the course of the transformation, the structures generated through the pH 5.5 equilibration disappeared and stable, uniform well-defined GUVs remained.

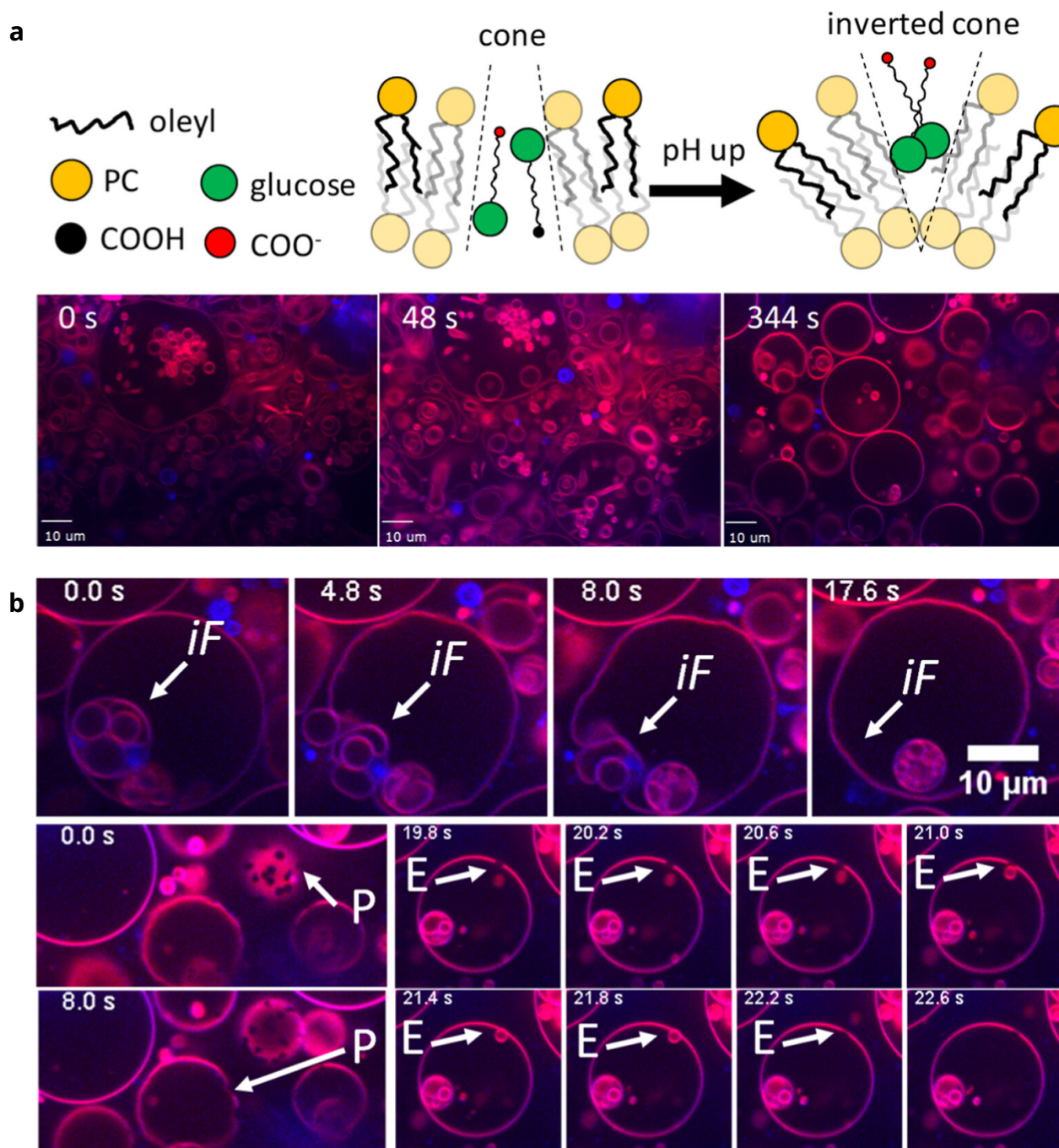


Figure 2.7: [a] Time-resolved overview showing progressive simplification of the GUV population after NaOH addition. [b] Close-up panels illustrating the three mechanisms of membrane modification: internal fusion (*iF*), poration (*P*), and expulsion of inner content (*E*). Scale bars=10 μm. Adapted from Baccile et al.¹

This apparent complete reversal of the deformations in the interior is direct evidence of the geometric stressor mechanism in this system. The only thing that changed was the protonation

state of the G-C18:1, a singular chemical event that shifted the molecule from its initially cone like structure to the inverted cone structure, reversing the curvature perturbation from negative to positive. The resultant membrane morphological changes are a direct consequence of this chemical stressor propagating via the Helfrich framework, changing from favoring inward budding to outward deformation. These changes were immediate and visible.

2.3.5.4.3 pH < 4 = Cylindrical Aggregated G-C18:1 When this system stabilizes at pH 3.5 (from a pH 5.5) by adding HCl, there was a third distinct geometric response. Over the first 100 s, the inner vesicular and tubular structures migrated towards the membrane edge and aggregated at the GUV boundary. Over the next few minutes, visible phase separation appeared within the membrane itself as there was clear, optically resolved groups of increased fluorescence intensity, indicating more denser membrane domains. Vesicles also seemed to aggregate with each other over this timescale. With full protonation of the G-C18:1 molecule, the electrostatic repulsion between neighboring molecules is removed and van der Waals attraction between the headgroups starts to dominate. At strongly acidic conditions, the curvature-based morphological transformation breaks down since the attractive forces overwhelm the packing geometry and the system collapses into lumped aggregates.

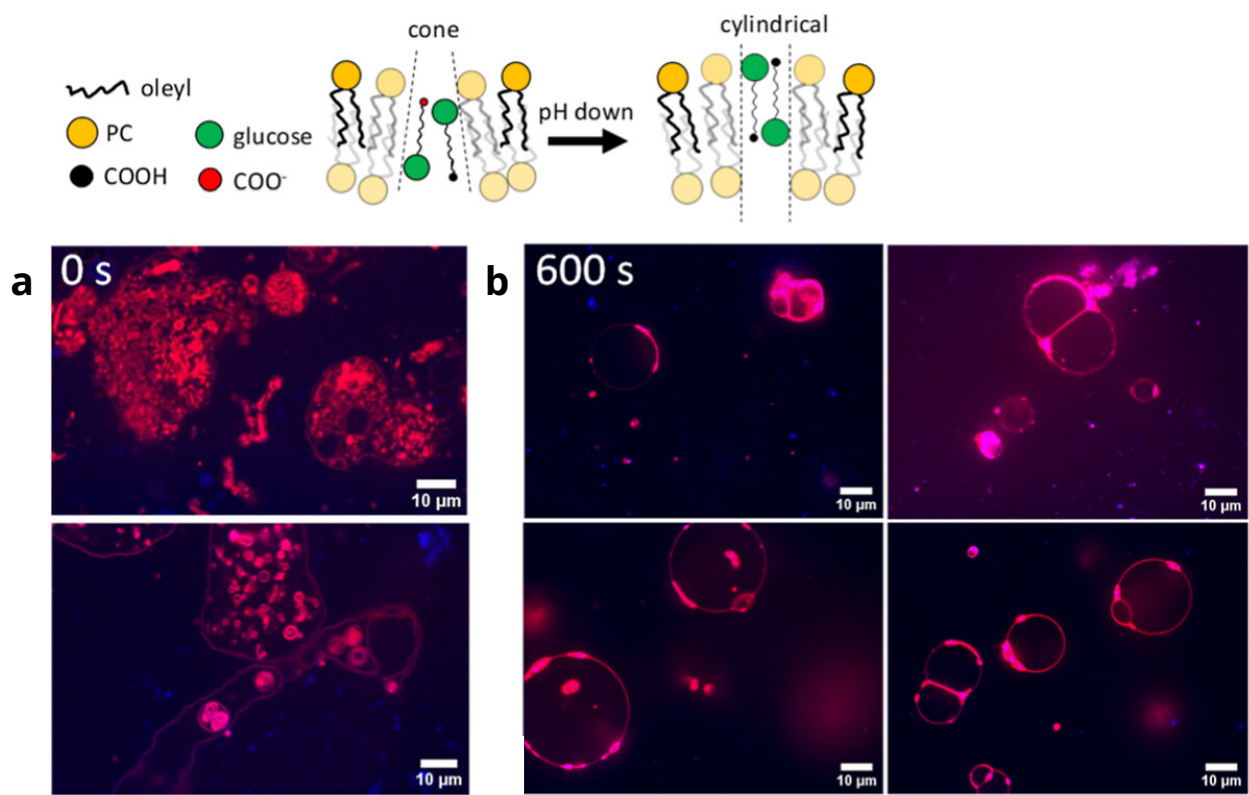


Figure 2.8: [a] Equilibrated sample showing inner vesicular complexity before adding acid. [b] After HCl addition, images recorded at 600 s show membrane phase separation and vesicle aggregation as G-C18:1 becomes fully protonated. Scale bars=10 μm. Adapted from Baccile et al.¹

2.3.6 Complementary Scattering Studies

In this publication, as a part of the broader collaborative study, the other co-authors performed extensive complementary small-angle x-ray scattering (SAXS), small-angle neutron scattering (SANS), and neutron spin echo (NSE) measurements. These appear as additional published findings and provide molecular-scale and mechanical evidence which supports the confocal observations above.

SAXS measurements confirmed that G-C18:1 physically incorporates into the DOPC GUVs, with a measured 17% reduction in membrane thickness at pH 5.5, from 4.8 nm for pure DOPC to 4.0 nm for the DOPC-G-C18:1 mixed system. A time resolved SAXS measurement further showed that this structural change has a two stage process, consistent with the fast fusion and slower equilibrium that was shown in the confocal fluorescence images.

NSE measurements quantified the bending rigidity of the membrane across the various pH regimes. Pure DOPC showed $\frac{\kappa}{k_B T} = 10.2 \pm 0.5$, consistent with established literature values.^{55,56} At basic pH the $\frac{\kappa}{k_B T}$ dropped to 0.4 ± 0.1 , which explains the poration and expulsion visible by confocal microscopy. At the acidic pH, $\frac{\kappa}{k_B T}$ increased to 96.0 ± 31.0 , which is consistent with the membrane aggregation. When taken all together, these measurements give more deliberate, quantified evidence of the morphological transformations which were captured via confocal fluorescence microscopy.

2.3.7 Summary

This G-C18:1-DOPC system gives clear, experimental evidence about the importance of chemical stressors on membrane-based systems and supports the claims about the central argument of this chapter. At each pH, due to the protonation state of the G-C18:1 molecule, the packing parameter changes and predictions of the favored curvature also change. Inward deformations when the G-C18:1 is cone shaped, outward poration and budding when it is inverted cone like, and aggregation when the attractive forces overwhelm the molecular geometry. Further complementary SAXS, SANS, and NSE give quantitative evidence to support the observations made by confocal fluorescence microscopy.

Crucially, the bilayer structure survives across all three pH regimes and supports the claims made in this thesis about the character of the stressor. The membrane does not fail. It remodels, adapts, and responds. G-C18:1 gives the membrane an environmental sensitivity it did not possess before, without destroying the structural identity that makes it a membrane. This is what a chemical stressor acting through the geometric mechanism looks like: a single molecule, a single pH-tunable geometry switch, one inert host membrane, three distinct and predictable morphological outcomes. The review sections of this chapter built the framework to make exactly this result interpretable and motivate why it is so powerful.

Chapter 3

Surface Wrinkling of Liquid

Crystalline Myelin Figures in Aqueous Solutions of Polymers

3.1 Preface

The work presented in this chapter is based on a publication² in *Soft Matter*: "Entropic depletion of macromolecular solutes induces symmetry-breaking surface wrinkling in myelin figures" by Archan Vyas, Pallavi D. Sambre, Leren Wang, Prianka Deshmuck, and Atul Parikh (*Soft Matter*, 2026, DOI: [10.1039/d6sm00044d](https://doi.org/10.1039/d6sm00044d)).

3.2 Introduction and Motivation

Chapter 2 established that chemical stressors, such as changes in pH, ionic strength, or molecular composition can drive membrane morphological transformations by modifying the molecular packing parameter. The membrane must respond via changes in the curvature elasticity. This chapter turns to a more qualitatively different class of environmental stressors: physical stressors which impose thermodynamic constraints on a soft matter system without altering its molecular

composition. Here, the main question is whether the magnitude of such a stressor (osmotic pressure) causes structural reorganization, or whether it is the character of the stressors that matters. In this chapter, we use a different soft matter model system, myelin figures. These are well-defined, cylindrical multilamellar smectic assemblies that serve as a tractable model for compression-driven mechanical instabilities in lipid systems.

3.2.1 Smectic Liquid Crystals and the Helfrich-Hurault Instability

Compress a soft, thin-walled tubule radially and it lengthens.⁵⁷ This familiar behavior – arising from volume conservation and the Poisson effect – is universal for a broad class of soft, essentially incompressible, tubular materials, including tubular lipid bilayers, hydrogels, and elastomeric tubes.^{58–60} Now compress a thick-walled, multilamellar smectic-A liquid-crystalline tube, and it responds very differently.⁶¹ This is because, in multilamellar, cylindrical smectics—three-dimensional samples with one-dimensional inter-layer periodicity—mechanics are governed by a complex interplay of competing interactions. Forces resisting radial compression originate from the bending rigidity of individual layers, the interlayer compression modulus, and in-plane area elasticity. Opposing axial deformation, however, is the end-cap energy, which penalizes changes in tube length.⁶²

Because these energetic contributions cannot all be simultaneously minimized, the tube is unable to relax its interaction energy fully: The inter-layer separation (a) remains larger than that expected for the compressed myelin at the new equilibrium (a_o). The stack is therefore effectively hyper-swollen – a seemingly counterintuitive outcome since the tube is under mechanical compression.⁶² This situation is phenomenologically comparable to a swollen stack of planar, lamellar smectic liquid crystals, in which mechanically stressed lamellar stacks — including those obtained through compression — relieve the compression energy by undulating and buckling to maintain their preferred interlamellar spacing^{63–65} through the Helfrich-Hurault instability.^{36,66,67}

For lamellar smectics, this phenomenology of mechanical instability is well described by continuum elasticity theory.⁶⁸ The same framework extends naturally to cylindrical geometry.^{61,68} In

this description, the layer displacement, δR , can be decomposed into two contributions: (i) a radially uniform shrinkage, $\delta \bar{R}$, which is constant along the cylinder axis, and (ii) a sinusoidally varying component, $\delta R'(r, z) \propto \cos(k, r) \cos(q, z)$, where k and q are the radial and axial wave vectors of the undulation modes, respectively.

The energy density (energy per unit volume) corresponding to such a deformation takes the form:

$$f = \frac{B_A}{2h} \left(\frac{\delta \bar{R}}{R} + \frac{1}{2} |\nabla \delta R'| \right)^2 + \frac{B}{2} \left(\frac{\partial \delta \bar{R}}{\partial r} \right)^2 + \frac{K}{2} (\nabla^2 \delta R')^2$$

In this expression, B_A represents the in-plane membrane area compression modulus (energy per membrane area), B is the smectic inter-layer compression modulus (energy per unit volume), K (energy per unit length) captures the bulk bending modulus of the multilayer tube, and h is the repeat distance of the layer prior to the osmotic or dehydration perturbation.

Chen and co-authors⁶⁸ demonstrated that for a trial deformation $\delta R = \bar{a}(r) + a'(r) \cos(qz)$ which penetrates to a finite depth d_o from the outer surface of the myelin, the minima of the total free energy per unit length yield an optimal wave-number of $q_*^2 = \frac{3B_A \bar{a}}{8hKR_0}$. At the onset of the instability, where the minimized free energy vanishes at the critical radial deformation of $\bar{a}_c = \frac{8hR_0 \sqrt{KB}}{\sqrt{3}d_o B_A}$, the corresponding wavelength of the axially propagating instability is $\lambda = \frac{2\pi}{q_*} \cong 5\sqrt{(\xi d_0)}$ where $\xi (= \frac{K}{B})$ is the standard smectic penetration depth.⁶⁹ For a typical phospholipid bilayer, $\xi (= \frac{K}{B})$ is on the order of 10 nm. Thus, the wavelength of undulations λ is on the order of micrometers if d_0 is several micrometers deep.^{68,69}

A variety of real-life condensed-phase systems that exhibit smectic-like lamellar morphologies and cylindrical organization display comparable surface instabilities.⁵⁸ In a range of scenarios involving externally applied or internally generated stresses, these liquid-crystalline systems experience geometric frustration that impedes complete relaxation.⁶⁵ Consider, for example, the tubular organs of animals such as the esophagus and pulmonary airways.^{70,71} Here, the outer layer (the muscle) is much stiffer than the inner layer (the mucosa). This stiffness mismatch creates a geometric incompatibility. During tissue growth, the stress generated is unable to relax, resulting in an instability that produces wrinkling, creasing, and folding of the inner surface of the

mucosal layer.⁵⁸ Other biological examples include the lipid layers of stratum corneum (skin)⁶³ and cortical folding in the human brain.⁷² In all of these cases, differential growth or external strains can give rise to instabilities through geometric frustrations. Synthetic soft matter systems, including smectic liquid crystals, elastomers in the smectic liquid-crystalline phase, and diblock copolymers^{73,74} are susceptible to similar instabilities.

3.2.2 Myelin Figures as Cylindrical Smectics

In the work reported here, we consider a class of smectic-A cylindrical liquid crystals known as myelin figures. First reported by Virchow^{75,76}, now more than 150 years ago, myelin figures form during the hydration and/or swelling of many common amphiphiles, including surfactants⁷⁷, lipids⁷⁸, and amphiphilic block polymers.^{79,80} They form at the amphiphile-water boundary as ensembles of finger-like tubular protrusions, tens of micrometers wide, and hundreds of micrometers long, when a dry mass of nearly insoluble ($10^{-10}M$) amphiphiles encounters water.

Molecular organization of myelin figures resembles that of a lyotropic smectic liquid crystals in cylindrical geometry^{78,81–83}: concentric arrays of discrete tubules consisting of thousands of cylindrically stacked alternating lamellae of lipid bilayers and aqueous channels wrapping a central ($\sim 100 - 200\text{ nm}$) aqueous core (Figure 3.1). Individual lamellae, comprising single fluid bilayers, allow for in-plane lateral diffusion, but the spatial confinement due to neighboring layers suppresses the thermally excited out-of-plane undulation fluctuations. The corresponding entropy loss gives rise to the inter-lamellar repulsive interactions (Helfrich repulsion), which stabilize the one-dimensional smectic order in myelins¹⁷ and inhibit molecular exchanges between individual lamellae.

As soft multilamellar smectic tubes, are myelins susceptible to compression-induced Helfrich-Hurault type interfacial instability? To our knowledge, there is one previous study⁶⁸, which explores this question. In that study, Chen, Schmidt, Olmsted, and Mackintosh subjected isolated myelin figures to macroscopic dehydration.⁶⁸ The accompanying compressive stress, they find, transforms the smooth surface of single, isolated myelin figures into an undulatory one displaying

periodic “bumps.” The bumps, they observe, are spatially periodic and appear to grow into longer “arms” before the myelins lose their cylindrical structure and become an amorphous mosaic. The authors argue that the observed initial instability arises from hypertonic osmotic stress induced by dehydration. They propose that as the bulk water evaporates, the concentration of impurities in the water rises, which, in turn, produces hypertonic stress on the myelins. This then triggers the flow of water from the outer layers of myelin figures to the impurity-laden bath, compressing the myelin. Adapting the formulation of Helfrich-Hurault type mechanical instability of smectics^{66,67,84}, the authors provide a theoretical description of how the relief of accumulated intra- and interlayer compression energies through the bending of lamellae can lead to an instability of surface undulations beyond a threshold compression. However, the presumed correspondence between dehydration and osmotic stress, and the causal link between the two that drives surface instability at the myelin surface, has not been experimentally verified.

3.2.3 Scope of this Chapter

In the work reported here, we monitor the real-time deformation of myelin figures under osmotic stress. We find that exposing pre-formed myelin figures to hypertonic aqueous solutions containing low-molecular-weight, colligatively ideal, osmotically active solutes (e.g. glycerol, 92 g mol^{-1} ; sucrose, 342 g mol^{-1}) does not induce undulatory instability. Remarkably, however, myelin figures exposed to larger macromolecular osmolytes (i.e., polyethylene glycol (PEG) and dextran (DEX), molecular weights 2000-10,000 g mol^{-1}) exhibit striking undulatory instability in bundles of myelin figures. Individual myelin figures undergo spontaneous chiral symmetry breaking and stabilize an axial pattern of interlocking topographic corrugations or wrinkles, which extend over the entire length of the myelin. The corrugations are almost exclusively localized at the surfaces where neighboring myelin figures (or loops of single myelins) are in direct juxtaposition. Our results suggest that this qualitatively significant difference, dependent solely on the size of the osmolytes, originates from the colligatively non-ideal, differential contribution of depletion interactions²⁶ to the osmotic pressure in macromolecular solutions.^{85,86} More generally, these ob-

servations suggest a heretofore underappreciated role of solute entropy in inducing morphological transitions in soft and cylindrical smectic tubules, pervasive in man-made and biological systems.

3.3 Formation & Characterization of Myelin Figures

The preparation of myelin figures under standard isotonic conditions is accomplished by hydrating a dried mass of fluid-phase phospholipids with deionized water. Adapting previous protocols^{81,87–89}, we first prepare a dry plaque of lipid (e.g., 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC)) with some fluorescent label (e.g., 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss-Rho-PE)) if needed. This is achieved by drying a droplet ($4\text{ }\mu\text{L}$, $\approx 100\text{ }\mu\text{g}$) of a chloroform solution containing desired lipids at high concentrations ($(\geq 25\text{ mg mL}^{-1})$) onto a planar cover glass (Supplementary Information). Subsequent hydration with deionized water roughens the edges of the lipid mass, producing a dense tangle of rapidly growing tubular protrusions, or myelin figures.

Monitoring these formation dynamics in real time using brightfield optical and fluorescence microscopy (epi- and confocal) (Supplementary Videos 1, 2) confirms the established features of myelin figures. A selection of still micrographs extracted from the video during the first 180 s is shown in Figure 3.1. The images reveal that the tubes grow sinuously, branch occasionally into secondary myelins, and juxtapose almost invariably into closely spaced myelin bundles. After the rapid growth during the first 100 s, myelins reach their quasi-steady state lengths, and the rafts of myelin exhibit slow, cooperative movements.

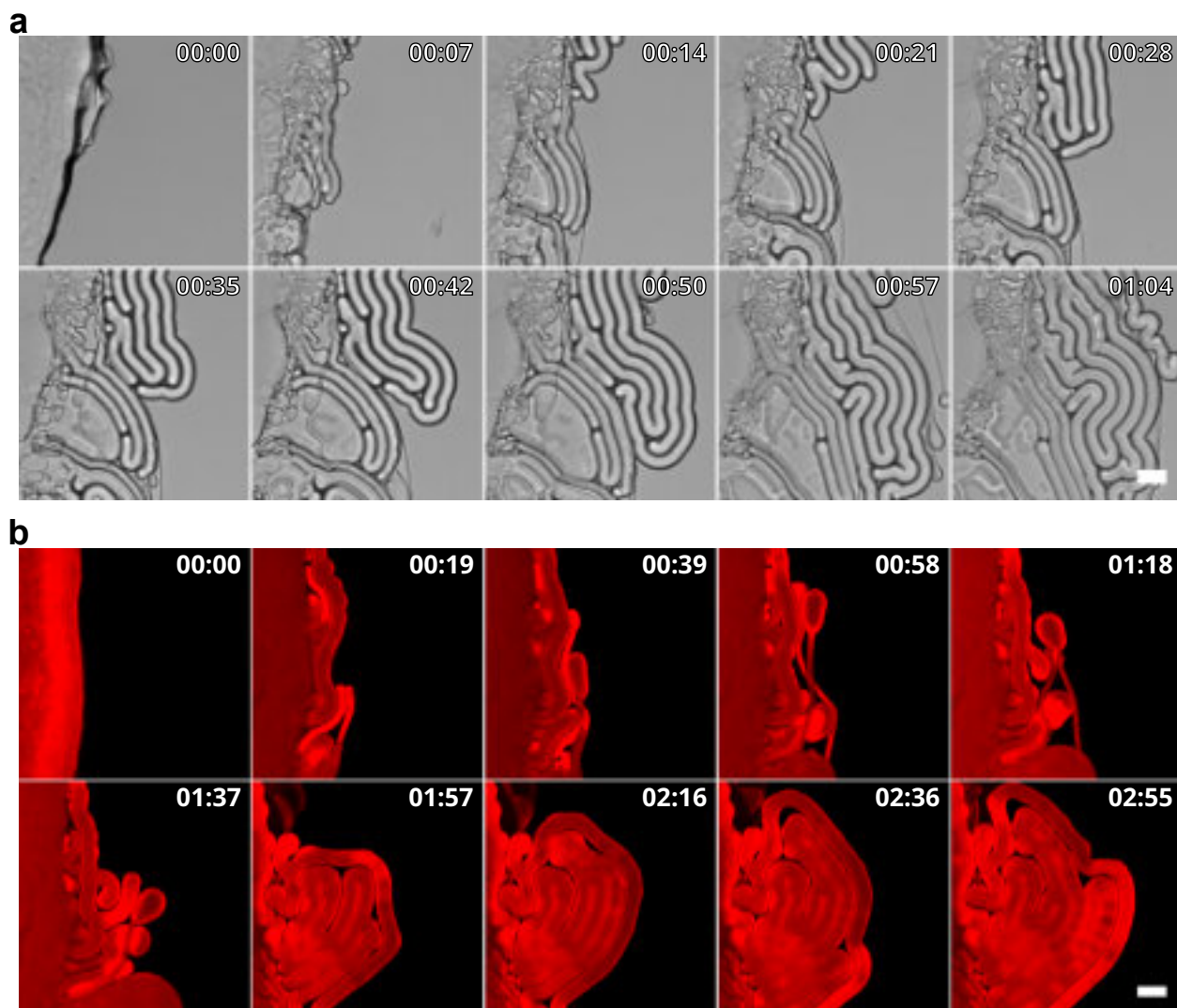


Figure 3.1: [a, b] A montage of a selection time-lapse images from movies (Supplementary Videos 1 and 2) showing the formation of myelin figures upon hydration. The sample compositions: [a] 100% POPC lipids, brightfield microscopy and [b] 99.9% POPC and 0.1% Rhodamine-PE, confocal microscopy. All timestamps are shown in mm:ss. (Scale Bar = 25 μm)

Individual myelin figures in single experiments are essentially monodisperse. Measuring the sizes of several tubules in multiple different experiments reveals a strikingly uniform average size. Analyzing 32 myelin tubes in a single representative experiment (the same experiment seen in Figure 3.2), we estimate a mean myelin diameter of $10.69 \pm 0.62 \mu\text{m}$. Assuming that a single POPC bilayer is roughly 4 nm¹⁰, an average inter-layer separation of $\sim 1 - 2 \text{ nm}$ ^{5,90,91}, and the central aqueous core of 100 nm^{78,82,83}, we estimate that each myelin is made of roughly one thousand (~ 940) bilayers. These preliminary findings are in excellent agreement with those

reported previously in the literature.⁸⁷ While Figure 3.2 illustrates the mean diameter for a representative trial $10.69 \pm 0.62 \mu\text{m}$, similar narrow distributions were consistently observed across various independent experiments. This indicates that while the absolute mean may vary between experiments, the formation of these myelin figures remains consistently monodisperse.

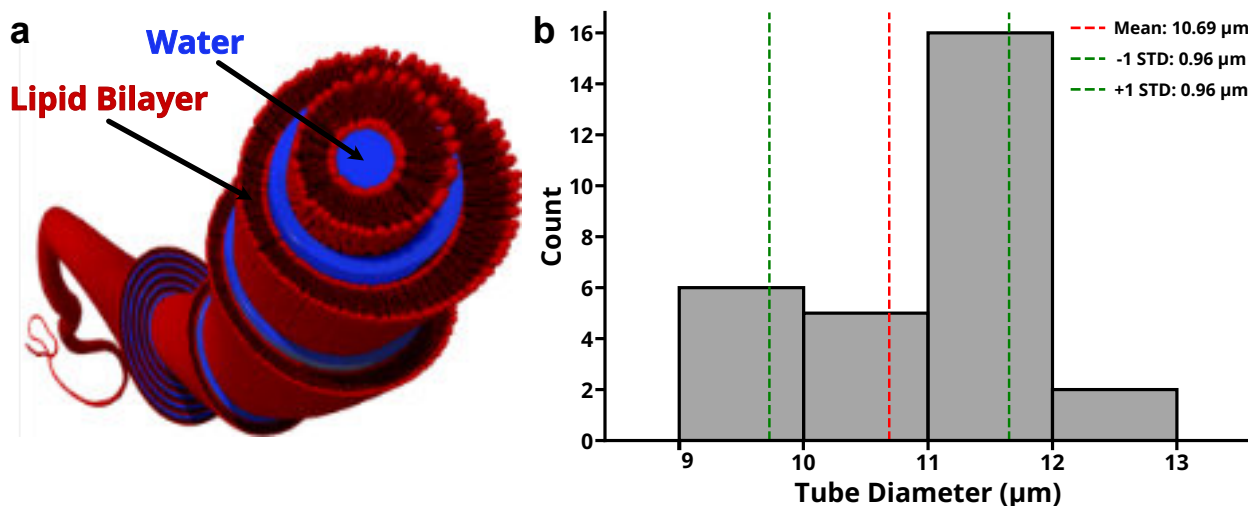


Figure 3.2: [a] Schematic illustration of a single myelin figure highlighting the multilamellar molecular organization in the cylindrical smectic-A phase (Adapted with permission from Ho et al.⁸¹). [b] Histogram depicting the distribution of myelin diameters for a representative experiment (same experiment as Figure 3.1) where the average diameter is $10.69 \pm 0.62 \mu\text{m}$ ($n = 32$ tubes). Further details on how measurements were made are found in the Methods section.

3.4 Dehydration-induced Instability in Myelin Figures

Next, we subjected the well-formed myelin populations to a bulk dehydration.⁶⁸ Images shown in Figure 3.3 reveal striking remodeling of the myelinic surface, characterized by interdigitated corrugations, similar to those reported previously.⁶⁸ Unlike the previous findings, which reported corrugations of isolated single myelins, we find that the bundles of myelins corrugate asymmetrically: The surface undulations are most pronounced at the juxtaposed myelin-myelin interface; those at the free myelin surfaces are far less prominent, bulge-like protrusions.

The time-lapse movies of brightfield and fluorescence microscopy data shown in Figure 3.3, respectively, capture the formation of the corrugations in real-time. These observations reveal that the undulations initiate stochastically, couple across the inter-myelin surface, subsequently

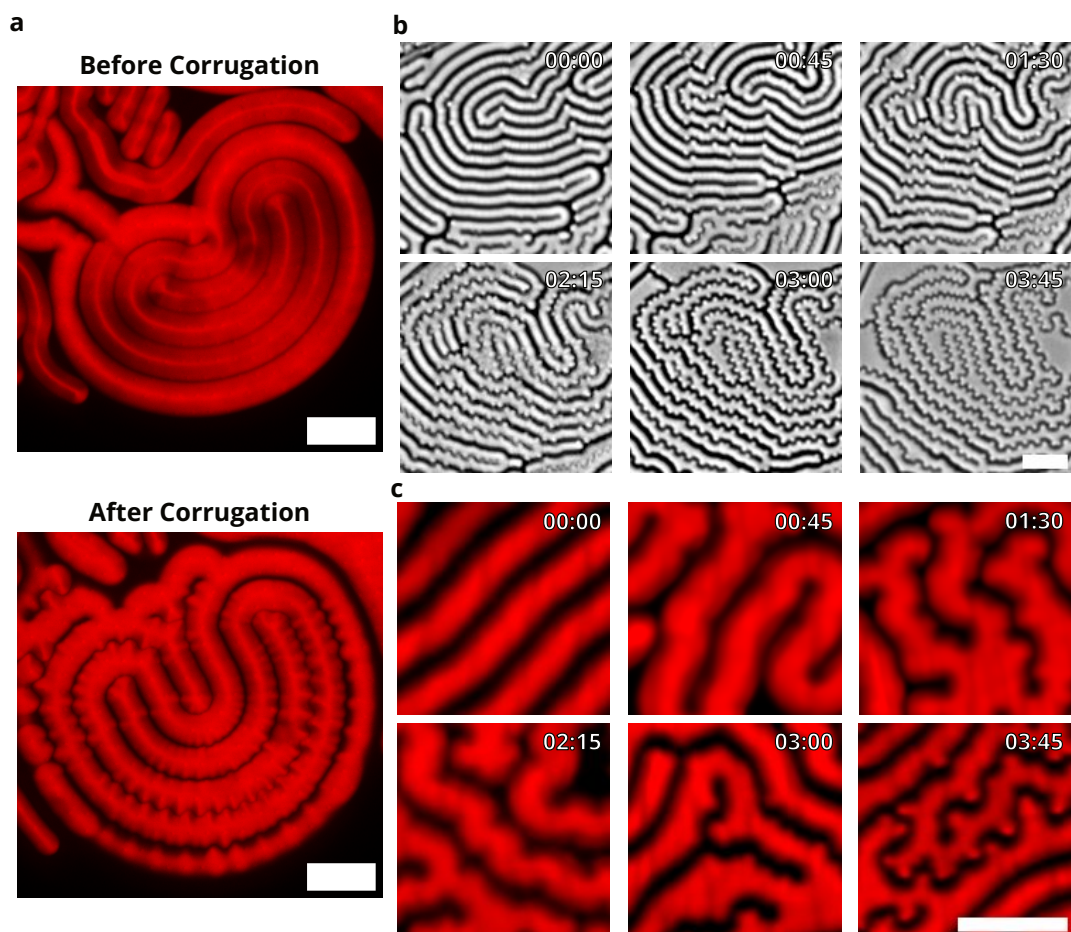


Figure 3.3: [a] A representative confocal fluorescence microscopy image of a well-formed myelin bundle in the fully hydrated state (top) and upon ambient dehydration (bottom). The quasi-periodic undulations are most pronounced at the inter-myelin boundaries with less conspicuous bulging at the free surfaces. Composition: 99.9% POPC doped with 0.1 mol% of Liss-Rho-PE (scalebar = 25 μm). [b and c] A selection of frames from a video of time-lapse brightfield microscopy images for 100% POPC myelins [b] and confocal fluorescence microscopy images for myelins consisting of 99.9% POPC and 0.1 mol% of Liss-Rho-PE [c]. (scalebar = 25 μm). (Supplementary Videos 3 and 4). The images reveal the localized appearance followed by a rapid axial propagation of the surface undulations along the lengths of the myelin figures.

propagating along the length of the myelins, effectively transforming the entire bundle into a remarkable pattern characterized by a strikingly corrugated surface. The previous study by Chen et al.⁶⁸, which reported observations of the appearance of periodic bumps with the wavelengths on the order of a 0.9 μm but did not examine the formation dynamics, proposed that dehydration-induced wrinkling arises from hypertonic stresses generated by increased impurity concentrations in the aqueous bulk surrounding the myelins. This hypothesis, however, has not been experimentally tested and warranted further study. Specifically, by systematically looking at the size and pressure exerted due to macro-molecules, the corresponding stress on these myelin figures can be calculated and any subsequent topological transformations can be captured via microscopy.

3.5 Osmotic Stress Induced Instability in Myelin Figures

The prior dehydrative observations raise a fundamental question: Does bulk dehydration produce a separate effect that is not accounted for by the increased concentration of small solutes and the subsequent change in osmotic pressure in the bulk ? It was clear from Chen et al.⁶⁸ that a dehydrative stress of some kind induced large macroscopic topological change within the Myelin figures. But their explanation and subsequent datasets did not provide a clear experimental basis to further develop the claim of bulk impurities driving such a transformation. Therefore, a clear experimental process for exploring this osmotic stress on myelin figures was warranted. However, to be able to properly control this, it is important to first discuss how the osmotic stress can change based on solute properties and their subsequent deviations from ideal behavior.

3.5.1 The van't Hoff Equation and Deviations from Colligative Non-Ideality

As established earlier in Chapter 1, the strength of the applied osmotic stress is determined by the ideal van't Hoff equation⁹²:

$$\Pi = cRT$$

where Π is the osmotic pressure in [Pa], R is the universal gas constant of $8.314 \text{ m}^3 \text{ Pa mol}^{-1} \text{ K}^{-1}$, T is temperature in [K], and c is the osmolyte concentration in $[\text{mol m}^{-3}]$. This expression treats the solution as thermodynamically ideal: solute molecules are assumed to be point-like, non-interacting, and do not interact any differently with themselves than they do with the solvent. Under these conditions, osmotic pressure is a purely colligative property — determined entirely by the number density of solute particles, independent of their size, shape, or chemical identity. By contrast, it is well known that colloidal and macromolecular impurities, whose larger sizes exclude volume, produce colligatively non-ideal contributions⁹² to osmotic stresses during dehydration. Recall that, unlike colligatively ideal solutions containing typically low-molecular-weight solutes, large macromolecules and/or colloidal solutes generate non-ideal aqueous solutions which have non-negligible solute-solute interactions, causing deviation from the behaviour predicted by the ideal solutions.^{24,93} Therefore, for aqueous solutions containing larger macromolecular solutes, osmotic pressure varies non-linearly with concentration:

$$\frac{\Pi}{RT} = \frac{c}{M_w} + A_2 c^2$$

In this modified van't Hoff expression, M_w is the molecular weight of the solute, R is the gas constant, c is the mass concentration of the polymer solution and $A_2 \left[\frac{\text{mol mL}}{\text{g}^2} \right]$ represents the second virial coefficient^{94,95} (see subsection 3.9.4). For low-molecular-weight solutes, deviations from ideal osmotic behavior are minimal at millimolar concentrations⁹⁶ and the standard van't Hoff expression is valid where the osmotic pressure is primarily a function of concentration. In contrast, for moderate- to high-molecular-weight solutes, the nonlinear contribution involving the second virial coefficient A_2 becomes substantial and the colligative non-ideality due to the solute-solute and solute-solvent interactions cannot be ignored. As molecular weight increases, so does the solute's effective size, amplifying excluded-volume interactions that restrict the configurational degrees of freedom available to each solute. This reduction in entropy increases the osmotic pressure non-linearly with concentration, increasing far above that predicted by the van't Hoff

equation. For further details, see subsection 3.9.4.

3.5.2 Small Molecule Osmolytes: A Negative Result

To address this gap and to determine whether the undulatory profiles observed upon dehydration can be attributed solely to osmotic stress, we subjected pre-formed myelin figures to externally imposed osmotic stresses under controlled conditions by using a secondary hydration step in which we add an osmolyte-laden solution to the preformed myelin figures. Specifically, we replaced the aqueous bath surrounding the freshly formed myelins with a hypertonic aqueous solution containing several pre-determined concentrations of bilayer-impermeable sucrose (M.W. 342 g mol^{-1}) as well as glycerol (M.W. 92 g mol^{-1}) (see Appendix). Strikingly, we find little or no surface undulations even after extended incubation (~ 25 minutes). One possible explanation for the absence of myelin responsiveness to the introduction of solutes in the external bath is that small-molecule solutes permeate into the myelin structure, thereby eliminating any osmotic gradient across the lamellae. However, this possibility can be safely ruled out. Experiments using labeled sucrose confirm that no detectable exchange of solute occurs between the interior of the myelin and the surrounding bath. These observations suggest that the osmotic stresses generated by colligatively ideal solutions of low-molecular-weight solutes are generally insufficient to drive the surface instabilities observed during dehydration, even to upwards of 2.5 MPa (see Appendix).

3.5.3 Large Macromolecule Osmolytes: Positive Results

The prior section establishes that small molecules are unable to replicate the instability that is induced by dehydration. To test whether excluded-volume effects contribute to undulatory instability, we next subject pre-formed myelins to aqueous solutions containing macromolecular solutes, namely poly(ethylene glycol) (PEG) (M.W., $8,000 \text{ g mol}^{-1}$, hydrodynamic radius, $R_H = 2.45 \text{ nm}$ ⁹⁷). The ideal van't Hoff equation is no longer valid, as the macromolecule is large enough that solute-solute interactions play a role. (see Appendix). In the representative experiments shown in Figure 3.4, we exposed pre-formed myelins to an aqueous solution containing PEG8K

(12.7 mM, osmotic pressure 0.26 MPa). Within seconds of exposure, the myelin figures undergo a striking morphological remodeling (Appendix and Figure 3.4). The most pronounced change is the emergence of a mesoscopic undulatory surface profile, most pronounced at the interfaces between myelins. In the case highlighted in Figure 3a, wrinkling begins at a bend in a folded myelin. The initial local wrinkle propagates (over ~ 30 s) down the length of the myelin, producing a global undulatory myelin-myelin interface with a quasi-sinusoidal profile. Although less conspicuous, the outer free surface of the myelin figure also exhibits the appearance of long-wavelength, low-amplitude bulges, like that observed under global dehydration (see Figure 3.3) and which reflects the expected area conservation across tubular topology.

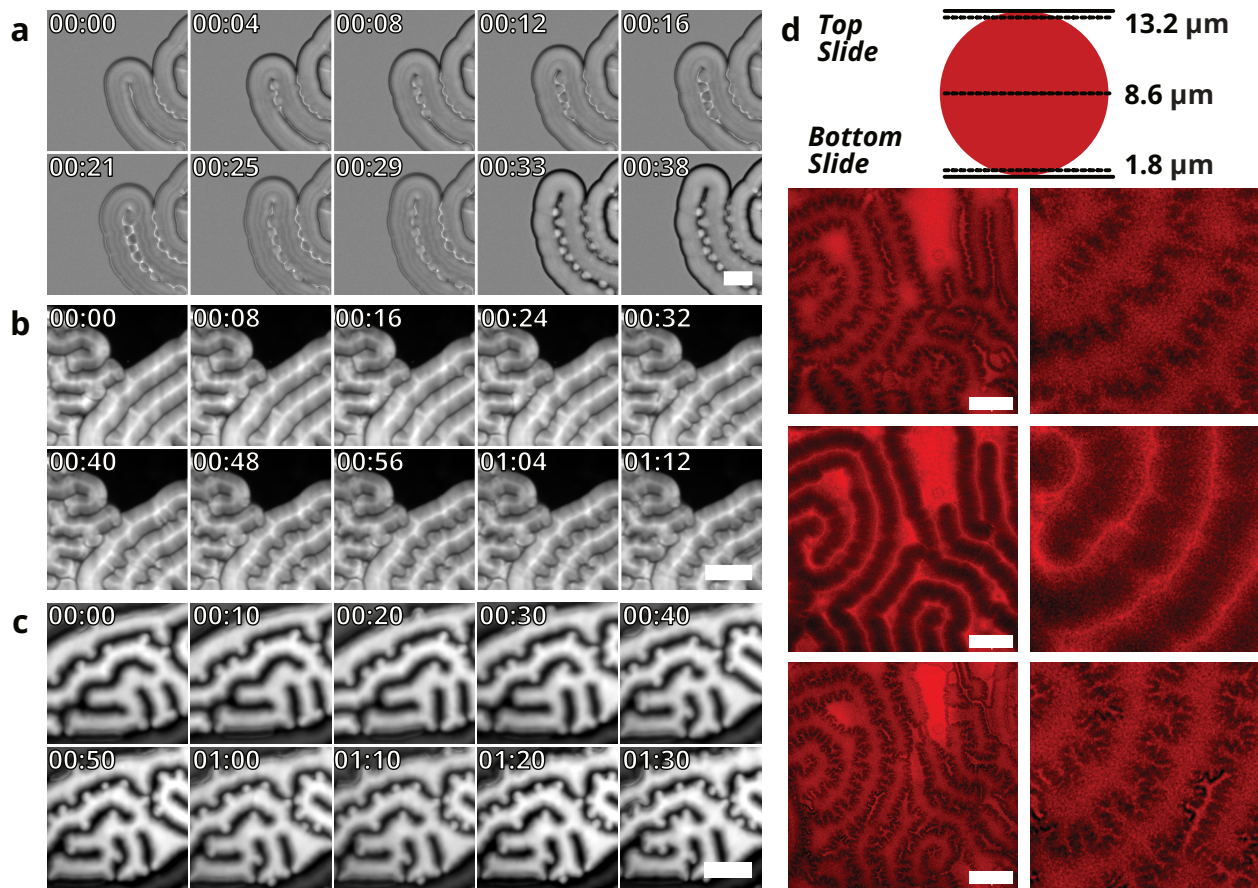


Figure 3.4: [a] A montage [mm:ss] of time-lapse brightfield microscopy images revealing the initial seed and the propagation of the undulatory surface profile. Sample: Pre-formed POPC myelin figures upon exposure to an aqueous solution containing 12.7 mM PEG 8,000 (scalebar = 25 μm) [b, c] Montages [mm:ss] of time-lapse confocal fluorescence microscopy images revealing undulatory surface profiles when pre-formed myelins were exposed to [b] 37.5 mM PEG 8,000 and [c] 55.6 mM DEX10,000 (scalebar = 25 μm) [d] A Z-stack slice montage of the experiment in [a] (scalebar = 10 μm).

Analyzing the undulations at the inter-myelin junctions, we estimate the average wavelength of the quasi-sinusoidal, wrinkled myelin to be $\sim 12.2(23) \mu\text{m}$ with the average amplitude of $\sim 3.8(7) \mu\text{m}$ ($n = 2$ measurements, $m = 28$ inter-myelin junctions) where all values represent mean $\pm$ SD. The long-wavelength undulations at the free surfaces have a much longer apparent average wavelength of ($\sim 21 \mu\text{m}$). Qualitative features of the appearance of undulatory surface profiles and their growth trajectories are fully reproducible for several different myelin compositions tested (i.e., POPC and POPC/Cholesterol mixtures). (For details, see Appendix)

To test whether the observed instabilities are unique to PEG, we compared the myelin response

to perturbations induced by another common macromolecular solute, distinctly different from PEG. We used high-molecular-weight (M.W. 10,000 g mol⁻¹) dextran, a branched polysaccharide composed of glucose monomers linked together by glycosidic bonds. For our study, 37.5 mM (~30 weight%) PEG 8000 and 55.6 mM DEX 10,000 were the concentrations chosen to generate comparable osmotic stress (2.15 MPa or 21.2 atm in the present case).^{22,98} Images shown in Figure 3.4, with the corresponding time-lapse movies (Appendix), establish unambiguously that PEG and dextran produce comparable patterns of surface wrinkling, thus suggesting that the response is independent of the chemical identity of the macromolecular osmolytes. Even if the chemical identity does not have a dependent response on the surface wrinkling patterns, PEG 8,000 and DEX 10,000 have a size based difference reflected in their hydrodynamic radii R_h , which are 2.45 nm⁹⁷ for PEG 8,000 and 1.85 nm-3.24 nm⁹⁹ for DEX 10,000 respectively. Considering that sucrose has a $R_h = 0.52$ nm¹⁰⁰ and no surface undulations were observed after extended incubation, the effect of osmolyte size on surface wrinkling patterns is experimentally tested.

3.6 Size Dependence Effects at Large Osmotic Pressures

To explore the effect of molecular sizes of the polymeric osmolytes, we next used a self-consistent series of polyethylene glycol solutions (molecular weights [g/mol], 600, 2000, and 8000) under comparable osmotic pressures. This is achieved by subjecting freshly formed myelins to polymer solutions containing 400 mM for PEG 600, 182 mM for PEG 2000, and 37.5 mM for PEG 8000, all of which have an identical osmotic pressure ($\Pi=2.15$ MPa). The results summarized in Figure 3.5 confirm the size dependence. The larger osmolyte (PEG 8000) produced the most well-defined corrugations with weaker, bulge-like corrugations produced by the shortest PEG 600 osmolyte. These observations are consistent with our proposition that the non-ideal contribution to osmotic pressure from large macromolecular osmolytes is critical for the surface instabilities in myelin figures.

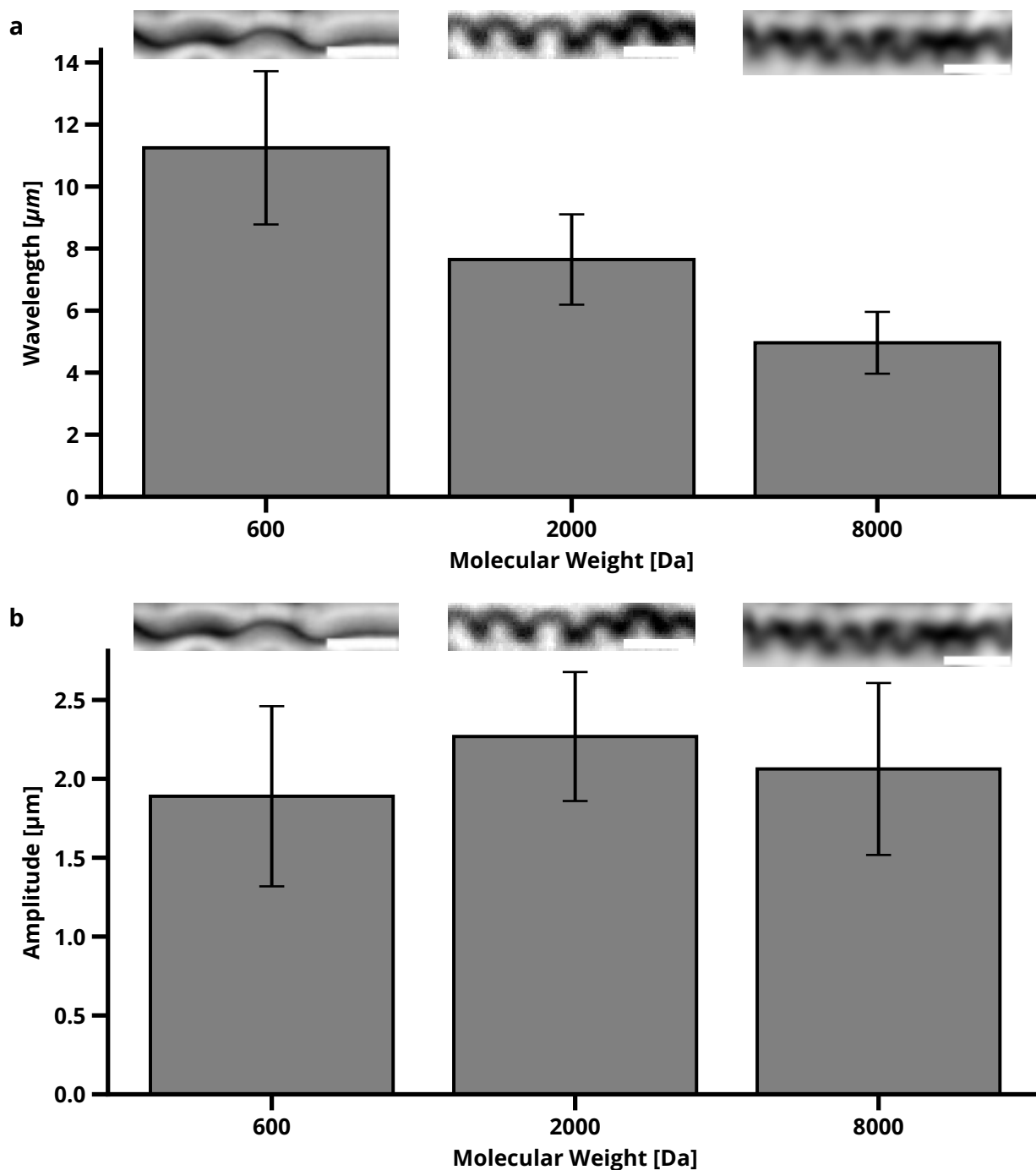


Figure 3.5: The average wavelengths and amplitudes of the surface undulations produced in myelin figures upon exposure to aqueous solutions containing three different PEG osmolytes (PEG 600, PEG 2,000, and PEG 8,000) at nominally identical osmotic pressures (See text for details). Statistics: PEG 600 ($n=76$), PEG 2000 ($n=81$), and PEG 8000 ($n=137$). (Scale Bar = 10 μm)

3.7 Direct Evidence for Depletion Based Mechanism

The corrugated myelins are in close juxtaposition. Are macromolecular osmolytes present in the narrow space between the roughened myelins? To address this question, we exposed preformed myelin figures to a 55.6 mM dextran mixture containing a small concentration (0.1 mol%) of fluorescently labeled dextran of comparable molecular weight ($\sim 10,000$ Da). Confocal fluorescence microscopy images shown in Figure 3.6 documents the experiment. The images shown in Figure 3.6 (top panel) correspond to the formation of myelin figures, which follow the expected trajectory (See Figure 3.1). Upon exposure to the exterior bath containing fluorescently labeled dextran, the pre-formed myelin figures exhibit secondary shape changes. A selection of these images is shown in the bottom panel of Figure 3.6. Here, $t = 0$ is arbitrary, chosen to reflect the time point at which labeled dextran becomes visible in the field of view. The images reveal three noteworthy features. First, the myelins in dextran-laden exterior are bundled closer together. This bundling is not unexpected because of the depletion forces arising from macromolecular crowding¹⁰¹ in the solution surrounding the myelin figures. Second, the sequence of images reveals the appearance and propagation of the surface wrinkling, consistent with the experiments reported in Figure 3.4 and Figure 3.5.

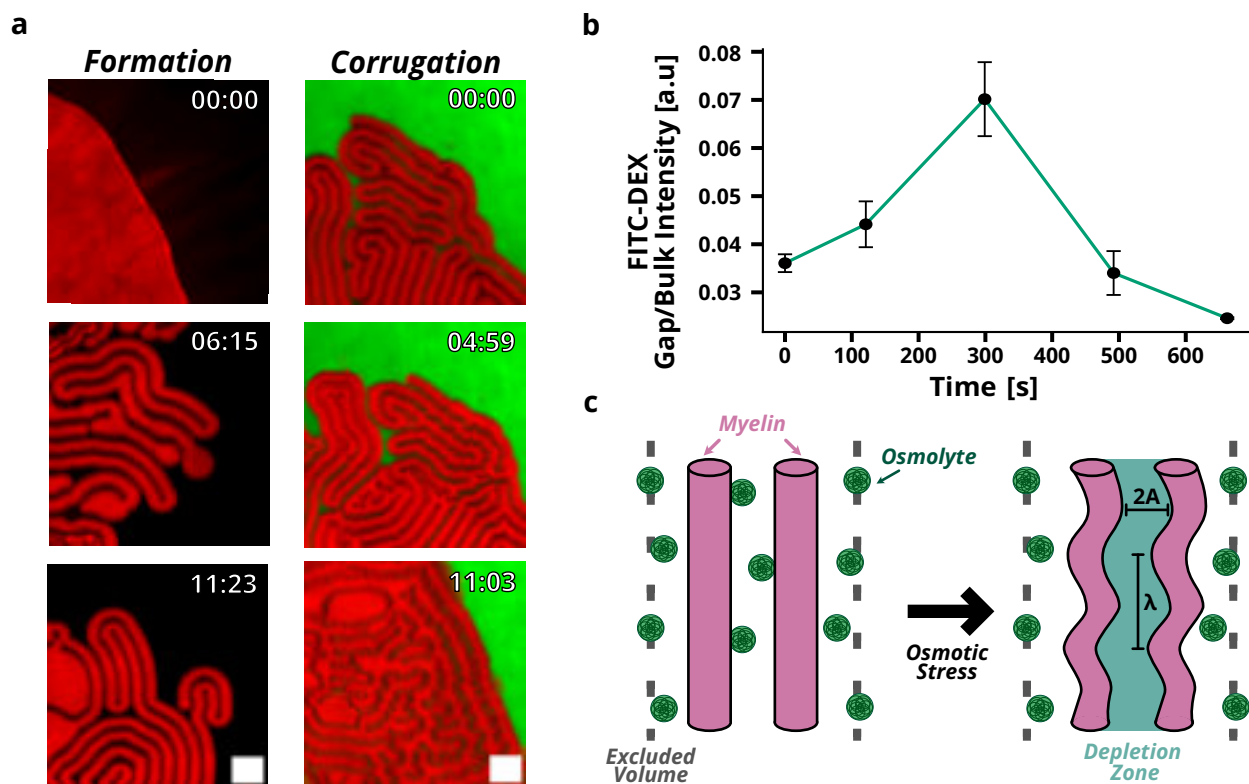


Figure 3.6: [a] Monitoring the formation of POPC myelin figures upon primary hydration with 30 μ L deionized water (a, first column), followed by secondary hydration with 30 μ L Dextran 10,000 Da containing 0.1% fluorescently labeled dextran (55.6 mM; 1000:1 unlabeled:FITC-labeled dextran) (a, second column). (Scale Bar = 25 μ m). [b] Time-dependent changes in the gap-to-bulk FITC-dextran fluorescence intensity ratio during the corrugation process. [c] Schematic depiction of the depletion forces exerted by exclusion of macromolecular osmolytes on neighboring myelin figures, stabilizing surface undulations. λ denotes the corrugation wavelength and $2A$ denotes double the transverse inter myelin distance, where A is the plotted amplitude. (Supplementary Videos 17 and 18).

Third, by monitoring the fluorescence intensity due to FITC-dextran in the bulk relative to that between the myelin we can assess how dextran dissolved in the bath is distributed relative to the bundled myelins. This is shown as gap-to-bulk intensity fluorescence ratio for the FITC-Dextran 10K as a function of time in Figure 3.6. Here, the gap refers to the narrow aqueous space between adjacent myelin figures, and the bulk refers to the surrounding aqueous free dextran solution far from the myelin interface. This ratio is therefore a direct measure of the relative dextran concentration in the confined inter-myelin space compared to the surrounding solution. (A ratio of 1.0 would indicate uniform dextran permeation in the gap; a ratio $\ll$ 1.0 confirms

depletion of the macromolecular osmolyte within the inter-myelin gap.) The measured values fall between 0.03–0.07, well below 1.0, indicating that dextran is excluded from the inter-myelin space.

3.8 Discussion and Conclusion

Taken together, the results presented here establish that the concentration imbalance due to low-molecular-weight osmolytes (e.g., glycerol $\sim 92 \text{ g mol}^{-1}$, sucrose, $\sim 342 \text{ g mol}^{-1}$) – those that produce colligatively ideal osmotic pressure difference—is insufficient to induce the surface wrinkling in myelin figures. By contrast, when myelins are exposed to solutions containing macromolecular osmolytes (i.e., polyethylene glycol and dextran, molecular weights 2.000 g mol^{-1} – $10.000 \text{ g mol}^{-1}$), they break symmetry, generate chirality, and stabilize an axial pattern of interlocking topographic corrugations or wrinkles, which extend over the entire length of the myelin. Furthermore, it is notable that the corrugations are almost exclusively localized at the surfaces where neighboring myelin figures (or loops of single myelins) are in direct apposition, and they produce near-perfect interdigitation at the inter-myelin junctions.

We propose that the extended surface wrinkling we observe in myelins exposed to macromolecular solutes can be understood in terms of synergistic effects from three factors. These include: (1) elevated osmotic stress due to the colligative non-ideality of the macromolecular solutes; (2) macromolecular crowding; and (3) depletion interactions arising from the excluded volume effects – a consequence of the translational entropy of polymeric osmolytes (Figure 3.6). First, the osmotic stress due to the macromolecular solutes (i.e., PEG or dextran) in the bath expels water from the myelins, prompting Helfrich-Hurault type instability, as described previously by Chen et al.⁶⁸ Second, the macromolecular crowding from the bath bundles myelins closer together. This then sets the stage for short-range depletion interactions to stabilize the wrinkled topography of myelin figures, as depicted in Figure 3.6.

Consider large macromolecular osmolytes as particles of radius R_p . The center-of-mass of

smaller osmolytes cannot approach the myelin figures beyond R_p , developing a corona of the solute-inaccessible excluded-volume that surrounds the myelin figures. Thus, when closely spaced myelins roughen and interdigitate, their excluded-volume coronas overlap, effectively increasing the total space accessible to the center-of-mass of the depletant particle. Consequently, the entropy of the polymeric depletants increases, and the overall free energy of the system decreases. The magnitude of the free energy change can be approximated as $\Delta F_{\text{depletion}} \approx -nk_B T \Delta V$, where n is the number density of the polymeric osmolytes, ΔV represents the overlap volume, k_B is the Boltzmann constant, and T is the temperature. The net result then is an osmotic pressure imbalance ($\Delta \Pi = nk_B T$) arising from the local difference in the concentration of polymeric depletants between the interdigitating myelins and the surrounding bulk. This then acts as a depletion force stabilizing the surface instability in juxtaposed myelin figures. This scenario then suggests a heretofore underappreciated role of solute entropy in inducing large-scale morphological transitions in soft and cylindrical smectics, which are pervasive in man-made and biological systems.

It is important to recall here that depletion interactions are intrinsically short ranged. In the presence of macromolecular solutes, myelin figures (like other filamentous assemblies) can experience crowding-induced bundling, which brings myelins closer together.¹⁰¹ Moreover, myelins are highly dynamic structures. These properties allow segments of large, bundled myelins to spatiotemporally localize in close juxtaposition. These effects can give rise to transient regions of significantly reduced separation, within which macromolecular exclusion becomes appreciable. Under such conditions, depletion interactions can couple with osmotically induced Helfrich-Hurault instability thereby stabilizing wrinkled surface topographies. A quantitative assessment of this mechanism will require further investigation and is beyond the scope of the present study.

More broadly, our finding suggests that myelin wrinkling in macromolecular solutions arises from the cooperative yet distinct roles of Helfrich–Hurault–type elastic instability and depletion-mediated interactions. In multilamellar cylindrical smectics, radial compression frustrates the preferred interlamellar spacing, rendering uniform relaxation energetically unfavorable and predis-

posing the system toward undulatory deformation modes characteristic of the Helfrich–Hurault instability. However, in contrast to planar lamellar systems subjected to uniform compression, the instability in myelin figures emerges in a spatially heterogeneous environment shaped by bundle geometry and interfacial confinement. Macromolecular osmolytes introduce a non-uniform osmotic pressure landscape through excluded-volume effects: depletion of polymeric solutes from narrow inter-myelin gaps generates a local osmotic pressure imbalance that stabilizes corrugated interfaces by increasing the configurational entropy of the depletants. In this framework, Helfrich–Hurault elasticity defines the accessible deformation modes and characteristic length scales, while depletion forces selectively stabilize interfacial, long-wavelength undulations and lock in their axial propagation and handedness. The resulting morphology thus reflects an interplay between elastic frustration intrinsic to cylindrical smectics and entropic forces arising from macromolecular crowding, providing a general mechanism for symmetry breaking and large-scale structural remodeling in soft multilamellar assemblies.

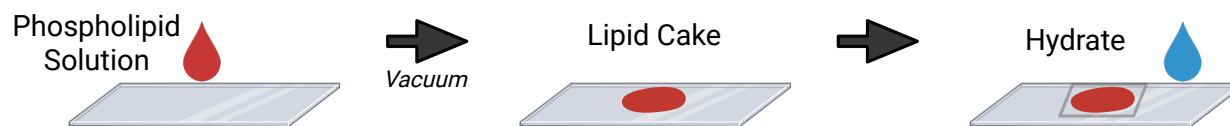
3.9 Materials and Methods

3.9.1 Materials

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), cholesterol (Ch), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss-Rho-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Sucrose, Poly(ethylene glycol) (BioUltra, Molecular weights 300, 600, 2000, 8,000), Dextran from *Leuconostoc mesenteroides* (Average molecular weight 9,000–11,000), and chloroform were purchased from Sigma Aldrich (St. Louis, MO). Slides (22×50×0.5mm and 22×50×1 mm) were purchased from Corning Incorporated. All the depletant solutions were prepared in deionized water (DI) (Millipore, Sigma, St. Louis, MO) for experimentation.

3.9.2 Formation of Myelin Figures

Lipid solutions were prepared with a desired concentration of 25 mg mL^{-1} in chloroform, using either 99.9 % of lipid stock with 0.1 mol% Liss-Rho-DOPE or using 100 mol% lipid. From the prepared solutions, $4 \mu\text{L}$ was placed onto clean slide ($22 \times 50 \times 0.5 \text{ mm}$) which was desiccated for at least 15 minutes under vacuum prior to usage. This slide was then sandwiched with a clean base slide ($22 \times 50 \times 1 \text{ mm}$) and introduced to the microscope where deionized water is introduced at the edge of the cover slip. Capillary action pulls the water into the sandwich from the air-glass interface and flows toward the dried lipid cluster to produce metastable myelin figures.



3.9.3 Secondary Hydration of Myelin Figures with Depletants

$30 \mu\text{L}$ of depletant-rich solution at the appropriate concentration was introduced into the existing pool of deionized water at the edge of the sandwich. It interacted with myelin figures via diffusion-mediated uptake and subsequent capillary action into the sandwich. The corresponding interactions of the new solution to those of the myelin colonies were captured by optical microscopy.

3.9.4 Osmotic Pressure Calculations

The physical presence of macromolecules in solution gives rise to nonideal, colloidal osmotic pressure¹⁰² Π which is well appreciated in polymer physics. When dealing with ideal polymer solutions, van't Hoff's law ($\Pi = cRT$) is valid, where $R = 8.314 \frac{\text{J}}{\text{mol} \cdot \text{K}}$ is the universal gas constant, T = temperature in Kelvin, and c = concentration in $\frac{\text{mol}}{\text{m}^3}$. However, when dealing with non-ideal solutions as in this case, the expression must be extended to include non-colligative

effects. A virial expansion of the osmotic pressure²⁴ accounts for these non-colligative effects and is written as:

$$\frac{\Pi}{RT} = \frac{c}{M_w} + A_2 c^2$$

In the above expression, $R = 8.314 \frac{J}{mol \cdot K}$, T is the temperature in Kelvin, c is concentration in $\frac{g}{mL}$, M_w is the molecular weight in $\frac{g}{mol}$, and A_2 is the second virial coefficient with units of $\frac{mol \cdot mL}{g^2}$. The second virial coefficient accounts for the colligative non-ideality in the solution due to solute-solute and solute-solvent interactions. Higher order terms in the virial expansion, such as $A_3, A_4, \dots$ can be neglected as we are primarily interested in the pairwise solute-solute interaction understanding and not three molecules at a time. It is also experimentally difficult to determine the higher order terms in the expansion.

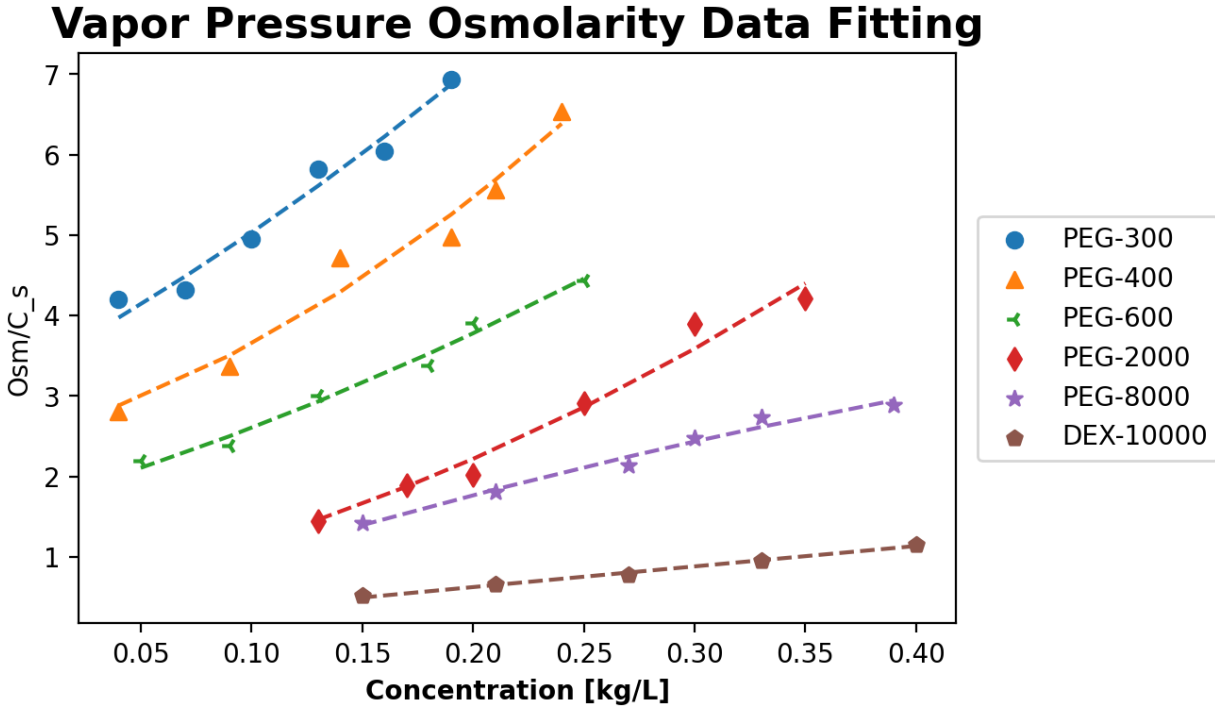
To be able to calculate the osmotic pressure for the chosen osmolytes, the chosen osmolytes second virial coefficient needs to be determined. This was achieved by utilizing a technique known as vapor pressure osmometry.¹⁰³ Vapor pressure osmometry measurements, using an osmometer, of these larger macromolecules can be determined by plotting mass concentration-normalized osmolality $\frac{Osm}{c_s}$ against a mass concentration c_s of the solute.^{95,98,104} When the ideal van't Hoff law is expanded to fit the modified van't Hoff, we get the earlier expansion with the second virial coefficient.

Vapor pressure osmometry measures the osmolality of a solution as $Osm = \phi m_s = -m_w^* \ln(a_w)$ where ϕ is the molal osmotic coefficient of the solute, m_s is the molality of solute, m_w^* is the molality of water ($55.51 \frac{mol}{kg}$), and a_w is water activity. Furthermore, the osmotic pressure is directly proportional to the water activity via $\Pi V_w = -RT \ln(a_w)$. If the prior 3 expressions from above are algebraically combined, it yields an expression which relates the output of a vapor pressure osmometer to the concentration, molar mass, and second virial coefficient:

$$\frac{Osm}{c_s} = V_w m_w^* \left(\frac{1}{M_s} + A_2 c_s + A_3 c_s^2 + \dots \right)$$

To determine the second virial coefficient of the depletant molecules, we generated a plot of the

concentration normalized osmolality $\left(\frac{Osm}{c_s}\right)$ versus the mass concentration. The osmolality is measured directly from the vapor pressure osmometer. Given that these experiments are done in ambient conditions (25 °C 1 atm), the molality of water $\left(55.51 \frac{mol}{kg}\right)$, the molar volume of water $V_w = 18.01 \frac{mL}{mol}$, and the M_s is the molecular weight of each depletant measured. The results are shown in the figures and table below:



We would like to thank the Gray Lab in the Neuroscience department for letting us use their vapor pressure osmometer. For the above plot with the vapor pressure osmolality fitting, we get the following results for the second virial coefficient for PEG at various molecular weights and for the DEX 10K:

- PEG 300 g mol^{-1} , $A_2 = 0.014960 \text{ mol} \cdot \text{mL/g}^2$, $R^2 = 0.971$
- PEG 400 g mol^{-1} , $A_2 = 0.007974 \text{ mol} \cdot \text{mL/g}^2$, $R^2 = 0.967$
- PEG 600 g mol^{-1} , $A_2 = 0.008088 \text{ mol} \cdot \text{mL/g}^2$, $R^2 = 0.982$
- PEG 2.000 g mol^{-1} , $A_2 = 0.005168 \text{ mol} \cdot \text{mL/g}^2$, $R^2 = 0.973$

- PEG 8.000 g mol⁻¹, $A_2 = 0.009\,215\text{ mol} \cdot \text{mL/g}^2$, $R^2 = 0.979$
- DEX 10.000 g mol⁻¹, $A_2 = 0.002\,659\text{ mol} \cdot \text{mL/g}^2$, $R^2 = 0.993$

Based on the above process to experimentally determine the second virial coefficient, the following are sample calculations for showing how the osmotic pressure was calculated for various macro molecular samples for the same osmotic pressure.

PEG 600 g mol⁻¹, 400 mM

$$\begin{aligned}\frac{\Pi}{RT} &= \frac{c}{M_w} + A_2 c^2 \\ \frac{\Pi}{RT} &= \frac{400\text{ mM}}{600\text{ g mol}^{-1}} + (0.008\,088\text{ mol} \cdot \text{mL/g}^2) (400\text{ mM})^2 \\ \frac{\Pi}{RT} &= \frac{0.24\text{ g mL}^{-1}}{600\text{ g mol}^{-1}} + (0.008\,088\text{ mol} \cdot \text{mL/g}^2) (0.24\text{ g mL}^{-1})^2 \\ \Pi &= (8.658 \times 10^{-4}\text{ mol mL}^{-1}) (RT) \\ \Pi &= (8.658 \times 10^{-4}\text{ mol mL}^{-1}) (298\text{ K}) (8.314\text{ J mol}^{-1}\text{ K}) \\ \Pi &= 2.146\text{ J mL}^{-1} \\ \Pi &\cong 2.15\text{ MPa}\end{aligned}$$

PEG 2.000 g mol⁻¹, 182 mM

$$\begin{aligned}\frac{\Pi}{RT} &= \frac{c}{M_w} + A_2 c^2 \\ \frac{\Pi}{RT} &= \frac{182\text{ mM}}{2.000\text{ g mol}^{-1}} + (0.005\,168\text{ mol} \cdot \text{mL/g}^2) (182\text{ mM})^2 \\ \frac{\Pi}{RT} &= \frac{0.364\text{ g mL}^{-1}}{2.000\text{ g mol}^{-1}} + (0.005\,168\text{ mol} \cdot \text{mL/g}^2) (0.364\text{ g mL}^{-1})^2 \\ \Pi &= (8.667 \times 10^{-4}\text{ mol mL}^{-1}) (RT) \\ \Pi &= (8.667 \times 10^{-4}\text{ mol mL}^{-1}) (298\text{ K}) (8.314\text{ J mol}^{-1}\text{ K}) \\ \Pi &= 2.147\text{ J mL}^{-1} \\ \Pi &\cong 2.15\text{ MPa}\end{aligned}$$

PEG 8.000 g mol⁻¹, 37.5 mM

$$\begin{aligned}\frac{\Pi}{RT} &= \frac{c}{M_w} + A_2 c^2 \\ \frac{\Pi}{RT} &= \frac{37.5 \text{ mM}}{8.000 \text{ g mol}^{-1}} + (0.009 215 \text{ mol} \cdot \text{mL/g}^2) (37.5 \text{ mM})^2 \\ \frac{\Pi}{RT} &= \frac{0.3 \text{ g mL}^{-1}}{8.000 \text{ g mol}^{-1}} + (0.009 215 \text{ mol} \cdot \text{mL/g}^2) (0.3 \text{ g mL}^{-1})^2 \\ \Pi &= (8.668 \times 10^{-4} \text{ mol mL}^{-1}) (RT) \\ \Pi &= (8.668 \times 10^{-4} \text{ mol mL}^{-1}) (298 \text{ K}) (8.314 \text{ J mol}^{-1} \text{ K}) \\ \Pi &= 2.147 \text{ J mL}^{-1} \\ \Pi &\cong 2.15 \text{ MPa}\end{aligned}$$

3.9.5 Sample Imaging

Myelin figures were monitored in real time using a brightfield microscope and a fluorescence microscope equipped with a spinning disk confocal configuration using a Nikon Ti2 microscope. Real time image sequences were recorded in confocal mode, with a Yokogawa X1 spinning disc and a Hamamatsu Flash 4 SCMOS camera operated through Nikon Elements V 4.0. For visual inspections, long working distance dry objectives were used, for the confocal image acquisition either a 20x NA 0.75 Plan Apo or 60x NA 1.4 Plan Apo objective was used. Intensities of the Toptica iChrome MLE 4 color laser engine were set to 10% for the 488 nm and 561 laser lines, with exposure times to reduce photobleaching. Rho-DOPE (Ex/Em; 560/583) was exposed with a 50 mW 561 laser line and NBD-PE (Ex/Em; 460/535) was exposed with a 50 mW 488 laser line. Images were acquired by Hamamatsu ORCA-Fusion Digital CMOS camera (model #C14440-20UP) that collected images (2304 × 2304 pixels) and was controlled using Nikon NIS-Elements Advanced Research software.

3.9.6 Image Processing

Microscopy images were collected using NIS Elements software (Nikon Instruments). They were further processed using Fiji^{105,106} and various Python modules^{107–109} to quantify surface topographies of myelin figures. Myelin figure diameters were measured from line intensity profiles drawn manually in Fiji across individual tubes. The resulting gray value versus distance data were exported and processed in Python. The raw intensity profiles were smoothed using a Savitzky Golay filter (second-order polynomial, 7-point window) to reduce the high frequency noise while greatly preserving the shape of the intensity curve. The tube edges appear as local minima in fluorescent intensity and a peak-finding algorithm will detect the troughs. Tube diameter was calculated as distance between adjacent detected troughs and pooled across all line profiles for a single experiment. Measurements falling outside ± 2 standard deviations from the mean were excluded. Final diameter distributions were reported as mean $\pm$ standard deviation.

Observed wrinkled surface profiles had quasi-sinusoidal patterns and corresponding wavelengths and amplitudes were measured using a manual approach in FIJI. Frames of interest were isolated and individual tubes displaying surface corrugations were straightened using Fiji's straightening tool. For each wrinkled tube, noise was reduced, contrast enhanced, and the straightened fluorescent image was binarized to facilitate wrinkle detection. The wavelengths and amplitude were then manually measured on this processed image. Due to the morphological complexity and variability of the wrinkled structures, a manual approach was favored over automated analysis as it provided more consistent and reliable results without the extensive parameter tuning required by algorithmic methods. On average, 10 edges ($n=10$) were selected at each time point, with each experiment conducted in triplicate.

Chapter 4

Aqueous Transient Phase Separated Model Systems and their Interplay with Actin

4.1 Preface

This work is a collaborative project with researchers both at University of California, Davis and at Sacramento State University. This project is in preparation for publication and I would like to acknowledge the following: Chris Carnahan, Michael Pham, Roanna Pham, Changyang Zhang, Victor Hernandez, Jasmine Doyle, Riya Kaur Sandhu, Abhijaya Singh, Lynne Usanase, Prof. Eliza Morris, Prof. Mikkel Herholdt Jensen. The confocal microscopy, FRAP, and phalloidin experiments represent the author's direct contribution; the bulk rheology was performed in collaboration with the Morris and Jensen labs at Sacramento State.

4.2 Introduction

Eukaryotic life requires structure but not rigidity. Without a cell's ability to modulate its boundary and structure, proper function would cease and thus the cytoskeleton has evolved within

eukaryotic cells. At the core of this natural architecture is a single cytoskeletal protein, actin, a highly ubiquitous protein that is one of the most abundant macromolecules in the eukaryotic cytoplasm¹¹⁰. Actin is critically essential for a wide array of physiological processes, including but not limited to the maintenance of cellular morphology¹¹¹, complex mechanical movements in cell motility¹¹² and division¹¹³, and intracellular transport¹¹⁴.

At the core of this functional versatility of actin is its remarkable structural adaptability in transitioning between a monomeric globular form (G-actin) and a helical filamentous form (F-actin)¹¹⁵. This assembly of actin from monomers into filaments is one of the most consequential biochemical reactions in biology. This G-to F actin transition is a well-regulated non-equilibrium process which involves the nucleation of protein monomers into intermediate dimers, followed by an exponential elongation into the filamentous form¹¹⁶. Often, this polymerization equilibrium is triggered via biochemical markers, such as salt concentration, ATP, and pH fluctuations. Simultaneously, actin is also responsive to its physical environment, where factors such as temperature and, crucially, the physical space available for individual monomers play an important role in the polymerization equilibrium.

The physical space for actin monomers is directly a result of an underappreciated feature of the cellular interior, which is its extreme macromolecular crowding¹¹⁷. The eukaryotic cytoplasm is densely packed with proteins, organelles, and other macromolecules to occupy ~20-40% of the available volume.¹¹⁸ The crowded milieu has a pronounced thermodynamic effect through exclude volume effects since the macromolecules have less space to occupy and their effective concentration increases. For cytoskeletal proteins such as actin, this effect is not trivial.

Given its importance, actin behavior has extensively been characterized¹¹⁰ in chemically defined buffer conditions and concentrations, with emphasis placed on structural properties, protein-protein interactions, and polymerization kinetics under well-controlled salt conditions. Less work, however, has been done with actin in cell-free systems or cell mimetic systems. While membrane enclosed giant unilamellar vesicles (GUVs) have been used to study actin networks^{119,120}, a significant fraction of cellular organization occurs within membrane-less compartments or more fluid

boundaries. One of the key methodologies for exploring membrane-less organelles is the idea of liquid-liquid phase separation¹²¹ (LLPS), a thermodynamically driven process where two or more different domains in solution can spontaneously de-mix to form compositionally distinct phases. These dynamic condensates¹²² can selectively concentrate macromolecules, create local chemical microenvironments, and help regulate biochemical reactions without a distinct barrier, making LLPS systems a compelling framework to study actin dynamics.

To utilize this framework, we utilize a specific form of LLPS system known as an aqueous two-phase system (ATPS)¹²³ using two well-characterized polymers: polyethylene glycol (PEG, 8.000 g mol⁻¹) and dextran (DEX, 10.000 g mol⁻¹) at concentrations where they prefer to form their own phases, specifically 10wt% each. These concentrations were chosen such that they phase separated naturally just barely¹²⁴ (see Appendix)) By using such a system, we can generate cell-sized droplets which can be a phenomenal biomimetic model. Building on this, we generated further stabilized protocols by adding small unilamellar vesicles (SUVs) of 100nm to act as Pickering agents, letting us create discrete DEX10K rich droplets in a PEG8K continuous phase¹²⁵. This system provides us with a unique platform to study actin behavior within these Pickering stabilized protocells and investigate how the phase-separated microenvironment influences their structural state.

In this work, we investigate how the successful preferential partitioning of actin into the DEX-rich phase modulates its polymerization in the absence of conventional buffer conditions. We can successfully generate cell-sized (20 μ m-60 μ m) Pickering stabilized DEX10K droplets which initially contained monomeric G-actin. We believe the heightened crowded state in the droplet interior drives spontaneous actin assembly and polymerization, even in the absence of more traditional high salt buffers. Using a combination of confocal fluorescence microscopy, rheological measurements, and fluorescence recovery after photobleaching (FRAP), we demonstrate that the actin inside the droplets alters the state of the actin, leading to the formation of a viscoelastic network within. These results suggest that ATPS is not merely a passive organizational strategy but also an active physical regulator that influences cellular organization within the eukaryotic

cytoskeleton and biomimetic droplets.

4.3 Results

The central premise of this work is that the physical microenvironments of these cell-mimetic droplets are a sufficient trigger to drive actin polymerization. To establish this, we must first define the system compositionally and demonstrate that actin does not seem to distribute uniformly within these droplets. By showing that actin is sequestered within the DEX10K-rich phase, we establish the baseline of what these droplets look like, where the actin lives, and what form it may be in. This can then build the ground for interpretation of more complex dynamics.

4.3.1 System Characterization and Actin Partitioning

To investigate how a crowded, phase-separated microenvironment influences actin assembly (Figure 4.1), we constructed Pickering-stabilized ATPS droplets¹²⁵ composed of a dextran-rich (DEX 10K) dispersed phase suspended in a PEG-rich (PEG 8K) continuous phase, with the droplet interface stabilized by PEGylated SUVs of approximately 100 nm diameter (Figure 1B). Actin monomers (G-actin) labeled with rhodamine were initially incorporated into the system prior to droplet formation, allowing their spatial distribution to be tracked by multi-channel confocal fluorescence microscopy.

Actin distributed non-uniformly between the two phases. Quantification of fluorescence intensities inside and outside droplets yielded a partition coefficient of $K = 2.82 \pm 0.45$, confirming preferential sequestration of actin into the crowded dextran-rich interior at the concentration tested (0.2 mg mL^{-1}) (Figure 4.1). This preference of actin into the DEX-rich phase is consistent with the well-established tendency of other macromolecules and proteins to prefer the DEX rich phase, particularly F-actin¹²⁶.

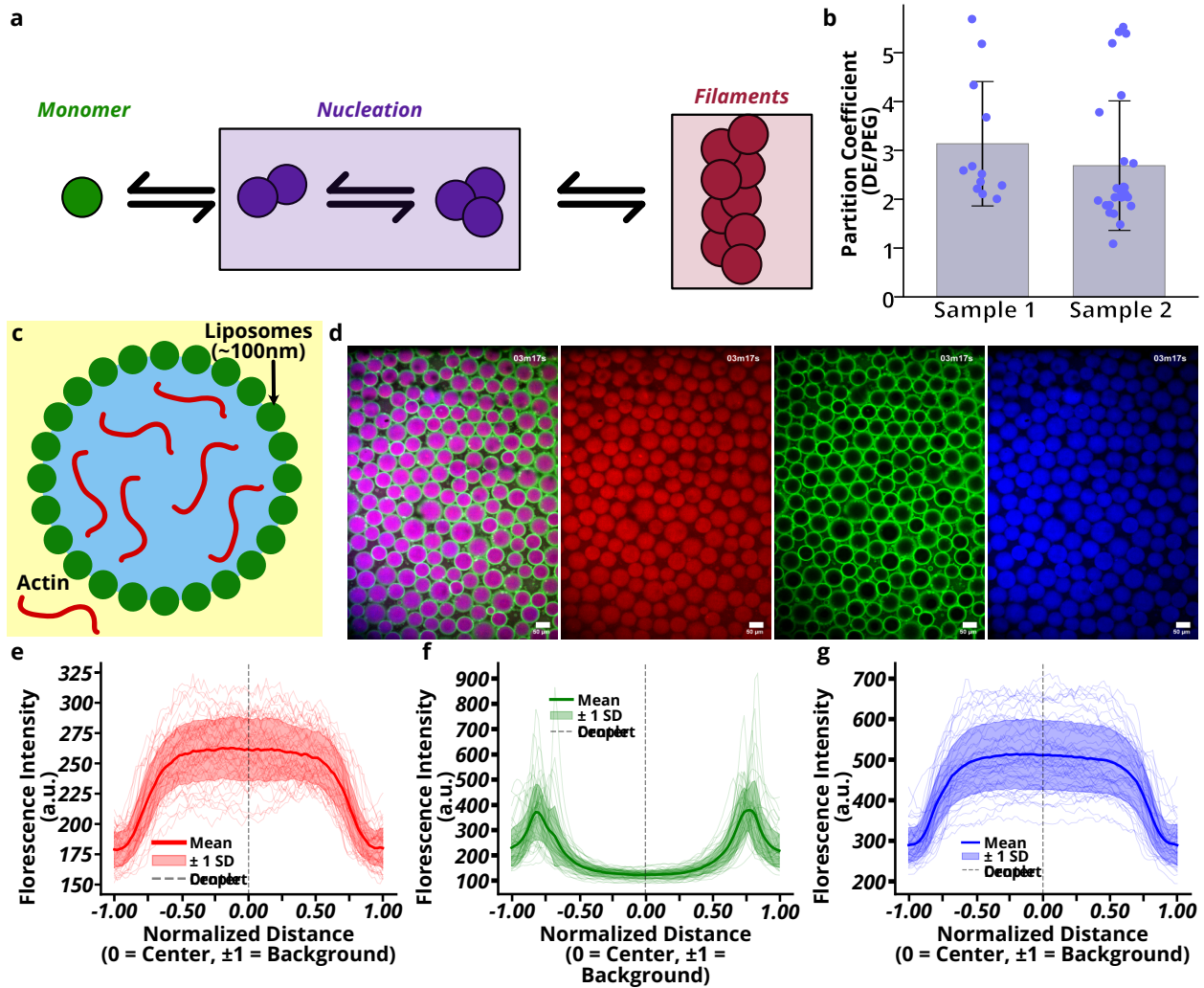


Figure 4.1: (A) Schematic of actin polymerization states. G-actin monomers (green) nucleate into short oligomeric intermediates (purple) that elongate into filamentous F-actin (red); equilibrium arrows indicate the reversible nature of each transition. (B) Partition coefficients ($K = \text{DEX/PEG}$) for rhodamine-labeled actin at 0.2 mg mL^{-1} , quantified from mean fluorescence intensities inside and outside DEX-rich droplets. $K_{\text{avg}} = 2.82 \pm 0.45 > 1$ indicates preferential sequestration of actin into the dextran-rich phase at the concentration tested. Error bars represent standard deviation ($n = 21$ droplets across 2 independent preparations). (C) Schematic cross-section of a Pickering-stabilized ATPS droplet showing the DEX 10K-rich interior (blue), PEG 8K-rich continuous phase (yellow), PEGylated SUVs localized at the droplet interface (green), and encapsulated rhodamine-actin (red). (D) Representative confocal fluorescence images of a single droplet shown as a three-channel composite (leftmost) and individual channel images for rhodamine-actin (red), NBD-PE SUVs (green), and Alexa Fluor 647-dextran (blue). Actin signal is concentrated in the droplet interior, SUV signal is localized to the droplet boundary, and dextran signal confirms the identity of the DEX-rich dispersed phase. Scale bar = $50 \mu\text{m}$. (E) Normalized fluorescence intensity profiles along a diametric cross-section of the droplet shown in (D) for all three channels, confirming the spatial colocalization of actin with the dextran-rich interior and the interfacial localization of SUVs. ($n=50$ droplets)

In measuring these droplets, we looked at a representative sample, shown in Figure 4.1. 50 droplets were measured where intensity profiles across representative droplets confirmed that actin fluorescence was concentrated in the droplet interior, that the SUV membrane signal was localized to the droplet boundary, and the dextran remains sequestered in the droplet interior.

4.3.2 Bulk Rheology of Actin inside Macromolecule-laden Solutions

From the prior section, the preferential partitioning of actin establishes that the actin locally concentrates into the DEX-rich droplets, but this enhanced localized concentration is not enough to claim that these droplets contain polymerized actin or that the formation of these droplets causes polymerization. To address this directly, it is necessary to characterize the local mechanical state of the actin within each phase environment. With the help of my collaborators at Sacramento State University, we ran complementary bulk rheological measurements to probe the actin state. We performed rheological measurements on solutions prepared to match the bulk polymer concentrations in both PEG and DEX rich phases, conducting oscillatory frequency sweeps and flow ramp experiments. Oscillatory frequency sweeps report the storage modulus G' (the elastic, solid-like component of the mechanical response) and the loss modulus G'' (the viscous, liquid-like component) as functions of angular frequency. Flow ramp experiments characterize the steady-state viscous response under increasing shear rate. Taken together, these two measurements aim to distinguish between the freely-flowing monomer state, a loose structured intermediate assembly, and a more entangled filament network. Each measurement was performed on three sample conditions within each phase: polymer alone (no actin), polymer with G-actin, and polymer with pre-polymerized F-actin as a positive control, at 0.2 mg mL^{-1} actin. The corresponding results for PEG and DEX are discussed below.

4.3.2.1 Actin inside the PEG-Rich Phase: A Newtonian Baseline

Across all the actin conditions measured in the PEG-rich phase, the rheological measurements indicated a similar result, that the actin behaved as a purely-Newtonian fluid (Figure 4.2). In

the oscillatory tests, as shown in Figure 4.2, the storage modulus was negligible across the tested frequencies, and the loss modulus was always dominant with no crossover between these two. Furthermore, in the flow ramp tests, all the sample conditions tested demonstrated a linear stress–shear-rate relationship and no noticeable deviations were observed. The lack of any non-Newtonian behavior, even when the sample contained pre-polymerized F-actin, suggests that the PEG at this 10wt% concentration is insufficient to drive deviations in actin behavior and is seemingly inert with respect to filament formation.

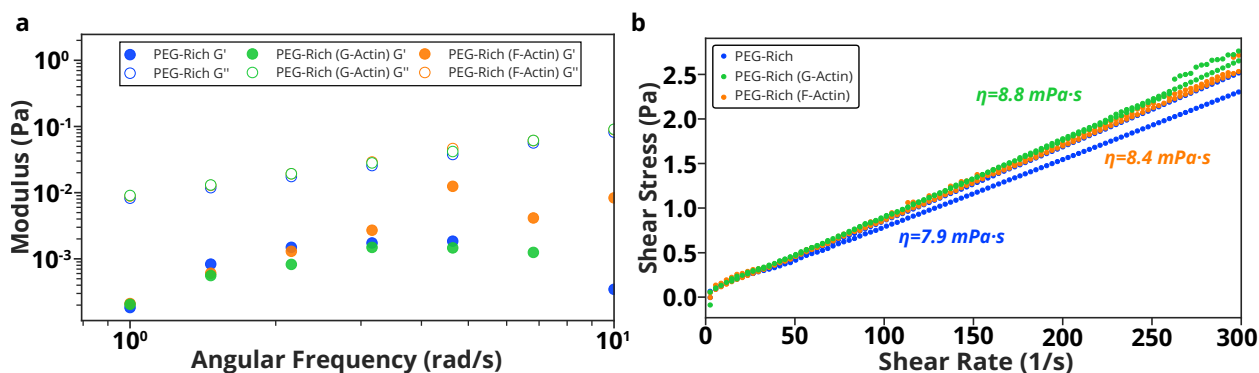


Figure 4.2: [a] Oscillation test of PEG8K-rich regions without actin, with G-Actin, and with F-actin. Storage and loss moduli are plotted as function of angular frequency and show negligible storage modulus due to Newtonian behavior. [b] Flow ramp test of PEG8K-rich regions without actin, with G-Actin, and with F-actin. All samples behaved as standard Newtonian fluids

4.3.2.2 Actin inside the DEX-Rich Phase: A Viscoelastic Transition and Non-Newtonian Flow

Compared to the PEG-rich phase, the DEX-rich phase produced qualitatively different results across both measurement types (Figure 4.3). Of note are the following observations:

Firstly, the DEX-rich G-actin samples demonstrated a non-zero storage modulus at higher frequencies, something that was absent in the controls without actin. This appearance of a slight elastic character only at higher frequencies, rather than the whole range, is consistent with the appearance of a partially assembled filamentous actin state. Unlike the PEG, it seems that the DEX crowding environment is sufficient enough to cause the actin to start some degree of polymerization in low salt conditions, albeit retaining more liquid like structure at longer time

lengths.

When looking at the flow ramp data for the DEX-rich G-actin, they showed some shear thickening behavior where this apparent viscosity increases with shear rate. If the solution contained only G-actin monomers, the increase in shear rate would have shown a linear stress vs rate, a classic Newtonian response. Since this result is inconsistent with this expected behavior, this suggests the presence of a loosely organized actin assembly that stiffens under mechanical perturbation. The most striking and intriguing rheology result is the behavior of DEX-rich F-actin. In the flow ramp tests, this phase behaved as a viscoelastic Bingham fluid with a measurable yield stress of $\sigma_0 = 0.61$ Pa. This type of fluid requires a finite stress to be applied before it begins to flow, which is a characteristic mechanical attribute of loosely formed network with some degree of inter-connectivity. The defined yield stress of this DEX-rich F-actin phase is consistent with that of other semi-dilute entangled actin networks at comparable concentrations, supporting the idea that the dextran crowding environment supports the crowding based formation of an actin filament network.

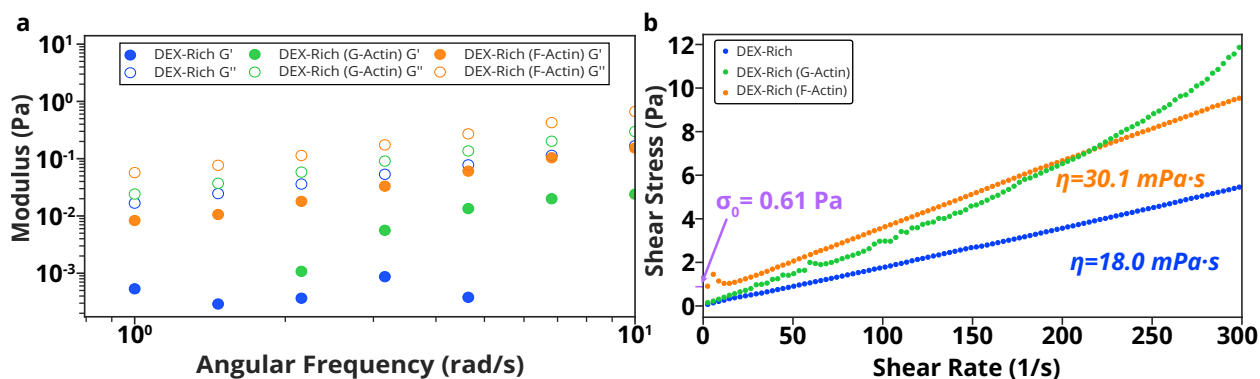


Figure 4.3: [a] Oscillation tests of DEX10K-rich regions without actin, with G-Actin, and with F-actin. Oscillatory test results from DEX rich phase. Samples with actin showed some viscoelastic behavior. [b] Flow ramp tests of DEX10K-rich regions without actin, with G-Actin, and with F-actin. The G-actin sample showed shear-thickening behavior while the F-actin behaved like a Bingham plastic fluid with a measured yield stress of $\sigma_0 = 0.61$ Pa.

When taken together, these results bring to life this idea that a crowding environment, in the absence of typical salt concentrations, is sufficient to drive spontaneous polymerization. The no-actin DEX control behaves as a viscous Newtonian fluid, whereas both the G-actin and F-

actin samples deviate from this baseline. The DEX-rich G-actin shows some non-Newtonian behavior and shear thickening consistent with partial actin network formation and the DEX-rich F-actin forms a noticeable developed viscoelastic fluid behavior with a clear yield stress of $\sigma_0 = 0.61 \text{ Pa}$. This actin-dependent emergence of elasticity, absent in the crowded polymer environment alone, develop the argument that macromolecular crowding, specifically dextran, can drive actin network formation in the absence of the typical salt environment. There exists a purely physical mechanism, rather than a biochemical, which helps promote this development.

4.3.3 FRAP and Phalloidin Staining of Actin Within Individual Droplets

The bulk rheological measurements from the section prior establish that macromolecular crowding, specifically DEX10k-rich phases, can produce non-Newtonian responses consistent with a polymerized actin network. However, the rheology described above characterizes the bulk phase and cannot confirm the assembly state of actin within. To directly probe the polymerized state of actin within the droplets, we performed two different complementary techniques to address this: fluorescence recovery after photobleaching (FRAP) and phalloidin staining. Of these, phalloidin staining provides the most direct evidence for filament formation, since phalloidin binds F-actin specifically; the FRAP and rheology results corroborate this by showing restricted mobility and actin-dependent mechanics.

FRAP is an optical microscopy technique that measures the mobility and dynamic behavior of fluorescently labeled molecules inside both living cells and cell-mimetic structures like the droplets here. By photobleaching a small area and monitoring how quickly unbleached molecules move into the previously bleached region, we can quantify the dynamics of the system. By using FRAP in droplets which were thought to contain monomeric actin and those containing filamentous actin for sure, we can experimentally determine whether the initially monomeric actin has polymerized or not within our droplets. G-actin monomers should undergo rapid, complete fluorescence recovery which would reflect unobstructed diffusion and unpolymerized networks. By contrast, actin filament networks remain largely immobile in FRAP measurements since the actin

is polymerized and cannot move through the entangled network¹²⁷, producing a slow incomplete recovery. FRAP therefore provides a direct, spatially resolved readout of actin assembly state that operates independently of bulk mechanical measurements.

We performed FRAP measurements on droplets containing 0.2 mg mL^{-1} of 7:1 unlabeled to rhodamine-labeled actin under both the intended monomeric 1xG conditions and the 1xF polymerized conditions. The montage in Figure 4.4 shows a representative photobleaching timelapse over the course of 10mins for the 1xG condition. We quantified the recovery rate across multiple droplets for both the 1xG ($n=11$) and 1xF conditions ($n=15$), shown in Figure 4.4, normalizing to the initial intensity inside the droplet for comparison. Importantly, the samples were prepared under 1xG conditions because we expected to keep actin monomeric; the incomplete recovery therefore occurred despite a nominally non-polymerizing buffer. Across both results, over the course of the 10 min experiment, the intensity never fully recovered to 1.0 and instead plateaued between 0.6-0.7 under both 1xG and 1xF conditions. This incomplete recovery for both conditions indicates that actin within the crowded droplet interior occupies a network-constrained¹²⁸, largely immobile state regardless of buffer condition, even under the nominally monomeric (1xG) condition. The almost-identical recovery profiles between 1xG and 1xF conditions further suggest that the crowded droplet DEX microenvironment drives actin into an assembled state mechanically comparable to that achieved by typical high-salt polymerization conditions.

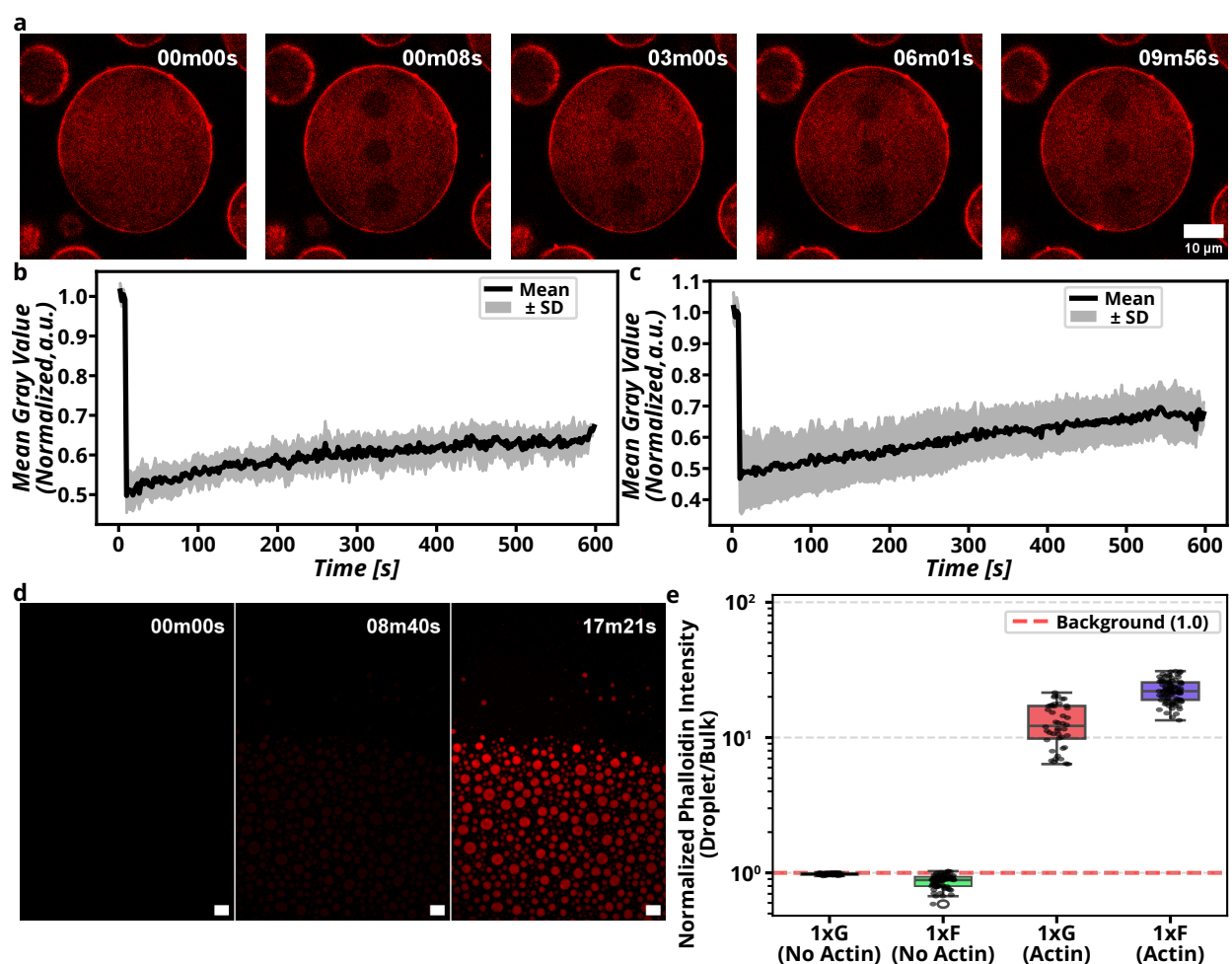


Figure 4.4: [a] Representative FRAP timelapse montage of a DEX-rich droplet containing 0.2 mg mL^{-1} actin (7:1 unlabeled to labeled ratio) under 1xG buffer conditions. Five frames are shown at $t=0\text{m}00\text{s}$ (pre-bleach), $t=0\text{m}08\text{s}$ (immediately post-bleach), $t=3\text{m}00\text{s}$, $t=6\text{m}01\text{s}$, and $t=9\text{m}56\text{s}$, illustrating the photobleaching event and subsequent partial fluorescence recovery over approximately 10 minutes. Scale bar = $10 \text{ }\mu\text{m}$. [b] Normalized mean gray value as a function of time for actin inside DEX droplets under 1xG conditions ($n = 11$). Intensity is normalized to the pre-bleach value, plateauing between 0.6–0.7 over the acquisition window. [c] Normalized mean gray value as a function of time for actin inside DEX droplets under 1xF conditions ($n = 15$). Recovery plateaus at a comparable level to the 1xG condition, indicating equivalent immobility regardless of buffer condition. [d] Representative phalloidin staining timelapse montage of DEX-rich droplets containing 0.2 mg mL^{-1} unlabeled actin under 1xF conditions, following addition of 100 mol% rhodamine phalloidin. Three frames are shown at $t = 0\text{m}00\text{s}$, $t = 8\text{m}40\text{s}$, and $t = 17\text{m}21\text{s}$, demonstrating time-dependent accumulation of phalloidin signal inside the droplets as phalloidin penetrates the droplet and binds to F-actin. Scale bar = $50 \text{ }\mu\text{m}$. [e] Normalized phalloidin intensity (droplet interior to bulk PEG phase ratio) shown on a logarithmic scale for four conditions: no-actin controls under 1xG ($n = 58$) and 1xF ($n = 69$) buffer conditions, and actin-containing droplets under 1xG ($n = 44$) and 1xF ($n = 88$) buffer conditions, all at 0.2 mg mL^{-1} actin and 100 mol% phalloidin. Actin-containing droplets show median enrichment ratios of 12.98 and 22.29 for 1xG and 1xF conditions respectively ($P < 0.0001$, statistically significant), reflecting specific phalloidin binding to filamentous actin concentrated within the crowded droplet interior. Box plots show median, interquartile range, and individual data points. See Appendix for 1xG montages for FRAP and phalloidin probing. 70

To further probe the state of the actin within these droplets, we stained the droplets using phalloidin, a well-known^{129,130} chemical stain to probe filamentous actin properties and kinetics (Figure 4.4). Phalloidin is a bicyclic peptide toxin that binds specifically and stoichiometrically to F-actin with high affinity, with no detectable binding to G-actin monomers, making it a great tool to optically confirm if the actin inside these droplets is polymerized or not. To eliminate cross-channel contamination, we used entirely unlabeled actin at 0.2 mg mL^{-1} within these droplets, ensuring that any red channel fluorescence arises solely from the phalloidin and no background actin fluorescence. The montage shown in Figure 4.4 is a representative time-dependent accumulation of phalloidin signal inside the droplets containing actin at 0.2 mg mL^{-1} in the 1xF polymerized condition. Given phalloidin's propensity to bind to filamentous actin alone, the signal expectedly accumulates inside the droplets where filamentous actin should be present. Signal is absent at $t = 0$, negligible at $t = 8$ minutes 40 seconds, and strongly concentrated inside droplets by $t = 17$ minutes 21 seconds, reflecting the equilibration time required for phalloidin to penetrate the droplet interior and bind available filaments. To assess whether crowding-driven assembly in the 1xG condition similarly produces phalloidin-accessible filaments, we performed the same staining assay on droplets containing actin under 1xG conditions alongside the 1xF positive control and droplets under both 1xG and 1xF conditions containing no actin.

Of these, phalloidin staining provides the most direct evidence for filament formation, since phalloidin binds F-actin specifically; the FRAP and rheology results corroborate this by showing restricted mobility and actin-dependent mechanics. We quantified normalized phalloidin intensity (droplet interior to the bulk PEG phase ratio) across multiple droplets for multiple conditions, presenting the results in Figure 4.4. We measured after the phalloidin concentration dispersed within the droplet system and had equilibrated, around 20 mins. No actin controls under both the 1xG ($n = 58$ droplets) and 1xF ($n = 69$) produced ratios indistinguishable from the background ratio, consistent with the fact that phalloidin is not known to have a phase preference for either the PEG or DEX phases. In contrast to the phalloidin signal contained in the actin containing droplets (0.2 mg mL^{-1}) under both the 1xG ($n = 44$ droplets) and 1xF ($n = 88$ droplets), the

elevated normalized intensities of 12.98 and 22.29 respectively reflect specific F-actin binding rather than nonspecific accumulation of phalloidin within the system. The higher signal enrichment in the 1xF relative to 1xG is consistent with a greater degree of polymerization under the typical high-salt conditions. The substantial enrichment in 1xG confirms that crowding-driven assembly produces genuine F-actin within the droplet interior even in the absence of conventional polymerization triggers.

When the results in Figure 4.4 are taken together, we provide complementary spatially resolved evidence that actin inside these Pickering-stabilized droplets exists in a filamentous, network-constrained state, even when conventional salt conditions are not present. The FRAP results establish that the actin mobility and recovery inside these droplets is severely restricted relative to the freely-moving diffused monomers initially introduced. The phalloidin assay further confirms the presence of assembled filamentous actin within the droplets through F-actin preferential staining. Given that both the 1xG monomeric buffer and the polymerizing 1xF buffer were tested, the only variable distinguishing the interior droplet state is the crowded Dex-rich microenvironment, establishing that macromolecular crowding within these cell-mimetic Pickering emulsions is physically sufficient to trigger actin filament assembly and can act independently to do so.

4.4 Conclusion

This chapter investigates how the physical environment of cell-mimetic compartments influences actin, using SUVs as Pickering agents to stabilize aqueous two-phase system droplets as an experimental platform. The main finding of this work thus far is that macromolecular crowding within the dextran-rich droplets is enough to drive actin polymerization in the absence of conventional salt conditions, establishing crowding as a physically sufficient and independent trigger for filament formation.

Using confocal fluorescence microscopy, we can establish that actin preferentially concentrates within the DEX-rich droplet and that the phase separated, cell-mimetic droplets are formed.

Bulk rheological experiments demonstrate that actin in DEX-crowding conditions develops non-Newtonian mechanical behavior, including a measurable yield stress in the F-actin condition and shear-thickening behavior even for nominally monomeric G-actin. FRAP and phalloidin staining provide further confirmation that some degree of a polymerized network is localized within individual droplets, with FRAP curves showing minimal recovery over 10 mins and with considerable phalloidin signal enrichment above background for 1xG and 1xF conditions.

Taken together, these results reveal that the molecular architecture and crowding character of the phase-separated environment can determine whether actin polymerizes. The PEG-rich phase, despite presenting comparable polymer concentration and crowding effects, leaves actin mostly unperturbed and seemingly unpolymerized. The DEX-rich phase, with its branched macromolecular architecture and resulting excluded-volume interactions, promotes the formation of F-actin-containing structures. This distinction between crowding character and crowding magnitude parallels the broader argument developed in this thesis regarding the role of stressor character in determining soft matter structural responses.

4.5 Materials and Methods

4.5.1 Materials

POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine), DOPE-mPEG-2K (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)), Rhodamine-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)(ammonium salt)), NBD-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)) were all sourced from Avanti Polar Lipids (Alabaster, AL) and were stored in chloroform solution at -20°C. Dextran (Alexa Fluor™ 647; 10,000 MW, Anionic, Fixable) was sourced from ThermoFisher (Waltham, MA). PEG (Poly(ethylene glycol)(BioUltra, Molecular weight 8,000 g/mol)), Dextran (from *Leuconostoc mesenteroides* (Sigma Aldrich) (Average molecular weight 9,000-11,000 g/mol)) was sourced from SigmaAldrich (St. Louis, MO). Actin

protein (>99% pure, rabbit skeletal muscle), Actin protein (rhodamine, rabbit skeletal muscle), and rhodamine phalloidin were all sourced from Cytoskeleton (Denver, CO).

4.5.2 Fabrication of Small Unilamellar Vesicles (SUVs)

Small unilamellar vesicles (SUVs) of 100 nm were made using extrusion¹³¹. To prepare the PEGylated SUVs, 94.5 mol% POPC, 5 mol% DOPE-mPEG-2K, and 0.5 mol% NBD-PE at 4 mg mL⁻¹ was dried under low nitrogen flow. The vial was then desiccated for > 3 hours to remove residual solvent. Once fully dried, it was rehydrated with PEG8K 10wt% (dissolved in 1xG buffer) at 4 mg mL⁻¹, then was sonicated at elevated temperature 45 °C to create a solution of multilamellar vesicles. This solution was then extruded using 100 nm filters to create a homogenous solution of PEGylated SUVs. The sample sizing was then checked using dynamic light scattering.

4.5.3 Sample Preparation

The formation of the droplets is highly based on a previously established protocol¹²⁵ to make water-in-water droplets. For this project's protocol, we required stock polymer solutions, actin solutions, the liposome solutions, and buffers. Stock solutions of PEG8K 40w/w% and DEX10K 40w/w% are first formed in buffers (0.2 mM CaCl₂, 2 mM Tris-HCl, pH 8.0). Stock lyophilized unlabeled actin is hydrated the night prior with 50 µL of water and stored on ice for a final concentration of 5 mg mL⁻¹. Stock labeled rhodamine actin was hydrated the night prior with 10 µL of water and stored on ice for a final concentration of 2 mg mL⁻¹. The stock actin solutions were used within the week. The liposome solution was formed according to the section prior. The two types of actin buffers, 1xG (0.2 mM CaCl₂, 0.2 mM ATP, 0.2 mM dithiothreitol (DTT), 2 mM Tris-HCl, pH 8.0) and 10xF (2 mM CaCl₂, 20 mM MgCl₂, 500 mM KCl, 5 mM ATP, 2 mM dithiothreitol (DTT), 20 mM Tris-HCl, pH 7.5) were prepared in advanced and stored in the freezer till usage. Fresh buffers were thawed when needed and no freeze-thaw cycles occurred. the 10xF buffer was diluted to a working stock of 1xF when being used in experiments.

Then, a single aliquot of 200 μL was prepared which had final concentrations of PEG8k 10wt% (1xG), DEX10K 10wt% (1xG) with 1500:1 unlabeled-to-labeled Alexa Fluor 647 DEX10K, 0.4 mg mL^{-1} of SUVs, actin (7:1 unlabeled-to-labeled ratio) at various concentrations, and remainder 1xG buffer. These samples were vortexed for 30s to form a single homogenous cloudy mixture. These mixtures were then allowed to preferentially phase separate for $t > 60$ min after which 10 μL aliquots were taken from the newly formed PEG-rich region for imaging and further experimentation.

4.5.4 Sample Imaging

There were two main optical configurations used within this project, one for the confocal imaging and the other for the fluorescence recovery after photobleaching experiments. Both are expanded on below.

Droplet samples were monitored in real time using a brightfield microscope and a fluorescence microscope equipped with a spinning disk confocal configuration using a Nikon Ti2 microscope. Real time image sequences were recorded in confocal mode, with a Yokogawa X1 spinning disc and a Hamamatsu Flash 4 SCMOS camera operated through Nikon Elements V 4.0. For visual inspections, long working distance dry objectives were used, for the confocal image acquisition either a 20x NA 0.75 Plan Apo or 60x NA 1.4 Plan Apo objective was used. Intensities of the Toptica iChrome MLE 4 color laser engine were set to 10% for the 488 nm and 561 laser lines, with exposure times to reduce photobleaching. Rho-DOPE (Ex/Em; 560/583) was exposed with a 50 mW 561 laser line and NBD-PE (Ex/Em; 460/535) was exposed with a 50 mW 488 laser line. Images were acquired by Hamamatsu ORCA-Fusion Digital CMOS camera (model #C14440-20UP) that collected images and was controlled using Nikon NIS-Elements Advanced Research software. Rho-DOPE (Ex/Em; 560/583) was exposed with a 100 mW 561 laser line and NBD-PE (Ex/Em; 460/535) was exposed with a 100 mW 488 laser line. Alexa Fluor 647 (Ex/Em; 650/668) was exposed to a 100 mW 640 laser line.

Fluorescence recovery after photobleaching (FRAP) experiments were performed on an in-

verted Zeiss LSM 980 with Airyscan 2 using a 20x 0.8 NA dry objective. Images were acquired using the Zen Blue software platform from ZEISS Microscopy. Image acquisition for the three channel image, was set up with a dedicated Roh6g track and a combined NBD-PE Alexa Fluor 647 track. NBD-PE was excited at 445 nm with ca. 1% laser power, Alexa Fluor 647 was excited at 633nm with ca. 0.3% laser power, Roh6g was excited at 514 nm at ca. 1% laser power. Pre-bleach images were collected for 5 frames at imaging laser powers to establish baseline fluorescence intensity. A defined region of interest (ROI) was photobleached using the 514 laser nm for Roh6g at 100% laser power for 5 iterations, resulting in approximately 50–80% loss of fluorescence within the selected region. Following bleaching, fluorescence recovery was monitored at initial low laser intensities, to minimize additional photobleaching, with images acquired at intervals of 1 second, for 600 seconds reflecting the recovery kinetics. Fluorescence intensities were quantified using Zen Blue and further analyzed in Origin. Recovery curves were normalized to pre-bleach intensity values.

Chapter 5

Conclusions and Future Directions

5.1 Summary

This dissertation examined multiple soft matter systems to investigate how environmental perturbations can drive structural reorganization in membrane-based structures. Using widefield and confocal fluorescence microscopy as the main experimental tools, we collected a substantial dataset on how environmental stressors, whether they are chemical or physical, and how they alter the membrane environment. Each chapter built a case that the character of the stressor is as significant as, if not more significant than, its magnitude in how structural organization occurs. This chapter briefly summarizes the central findings of the three body chapters and closes with a discussion of future directions.

5.1.1 Chapter 2: G-C18:1

In Chapter 2, we discussed further how chemical stressors can alter soft matter assemblies. This chapter opened with a review of how stressors such as pH, the ionic conditions, and osmotic chemical potential alter properties at the molecular scale, showing in each case that the perturbation ultimately produces a change in the packing parameter and/or the spontaneous curvature. These changes propagate through the system via the Helfrich elasticity framework, transducing

them as a global morphological response. The chapter used this review to frame the GC18:1 case study, a publication this author contributed to, in which a pH-responsive bolaamphiphile inserted into DOPC GUVs demonstrated the mechanism in clean experimental form. In this system, the molecule's protonation sets the packing geometry of the individual molecule, which in turn dictates whether the membrane is going to have inner tubulation, outward budding, or aggregation across a biologically relevant pH range. Taken together, the brief review and case study combined to explain a single point: that chemical stressor character is geometric in nature and that the change in the molecular shape, not the perturbation itself, is what drives membrane reorganization.

5.1.2 Chapter 3: Osmotically Stressed Myelin

Chapter 3 tested whether the character of the osmotic stressor, specifically its molecular size and excluded volume properties, determines how structural responses change in myelin figures. These are cylindrical smectic-A liquid crystals in which small molecule osmolytes, such as sucrose and glycerol, even at substantial osmotic pressures, left the myelin figures largely smooth and unperturbed. However, larger macromolecular osmolytes such as PEG and DEX, at matched or lower osmotic pressures, triggered pronounced, long wavelength, quasi-sinusoidal surface corrugations that propagated axially and localized preferentially at the inter-myelin interfaces. Quantitative analysis showed that the corrugation wavelength depended on osmolyte size rather than osmotic pressure and fluorescently labeled molecules were excluded from the corrugated interfacial regions. These results continue to develop the idea that the stressor character, in this case the physical size, drives the membrane remodeling due to the entropic depletion force acting on these systems to cause an elastic instability.

5.1.3 Chapter 4: Actin Interplay with ATPS Droplets

Chapter 4 investigated how the physical microenvironment of a phase separated, cell-mimetic droplet compartment acts as an environmental stressor on actin assembly. Using SUVs as Picker-

ing stabilizers to make aqueous two-phase separated droplets (discrete DEX phases dispersed in a bath of PEG), this chapter characterized how macromolecular crowding and buffer conditions affect the actin assembly state, specifically in the absence of conventional biochemical triggers. Confocal fluorescence microscopy established that actin preferentially partitions into the DEX-rich droplet interior, raising the effective actin concentration. Bulk rheology showed that actin in the DEX-rich phase developed a viscoelastic response absent from polymer-only controls, indicating that the mechanical response is actin-dependent rather than arising from the crowded polymer environment alone. FRAP and phalloidin staining further confirmed that the response of actin to this structural stressor, in the absence of typical salts, was to polymerize. The FRAP recovery curves were similar across conditions, and both conditions showed significant enrichment in the phalloidin assay. Stressor magnitude alone, such as the total polymer volume fraction, is insufficient to predict the structural response. It is the character of the crowding agent, specifically the DEX10K, that determines whether actin assembly is triggered, extending the central thesis argument from lipid membrane systems to cytoskeletal protein networks.

5.1.4 Final Thoughts

Throughout this thesis, the goal has been to further expand the work done on stressors in soft matter systems. The three experimental chapters of this thesis work to support a single main principle: that the structural response of various soft matter assemblies to environmental stressors is not just based on the magnitude of the stressor alone, but on its intrinsic character. Chapter 2 establishes that chemical stressors, regardless of their specific chemistry, act by changing the geometry of the molecular constituents of the soft matter system. These changes are interpreted by the soft matter assembly as changes in its spontaneous curvature and global morphology. Chapter 3 shows a multi-lamellar system where the physical stressor has its own distinct character such as excluded-volume, which drives large scale morphological changes and not the osmotic pressure they exert. The resulting elastic instability manifests as large-scale morphological change in the cylindrical smectic. Chapter 4 shows that the same entropic mechanism can operate within

an entirely different soft matter assembly, focusing on a cytoskeletal protein network within cell-mimetic droplets. All three systems across vesicles, cylindrical multilamellar tubes, and crowded protein networks show the impact of character over magnitude.

This idea has much practical consequence in designing novel soft matter systems and for understanding the physical biology of the cell interior. From the perspective of materials design, the character of the stimulus provided is just as consequential as the magnitude it exerts. For cell biology, these findings reaffirm the importance of crowding in shaping how membranes and cytoskeletal networks respond to their environment. Whether these cells exploit this deliberately or passively remains a crucial question and consequential for the future. The central goal of this thesis is simple but consequential: to understand how soft matter responds to its environment, it is not enough to ask how much stress is applied. One must ask what kind.

Appendices

Appendix A

Osmotically Stressed Myelin

Supplementary Images

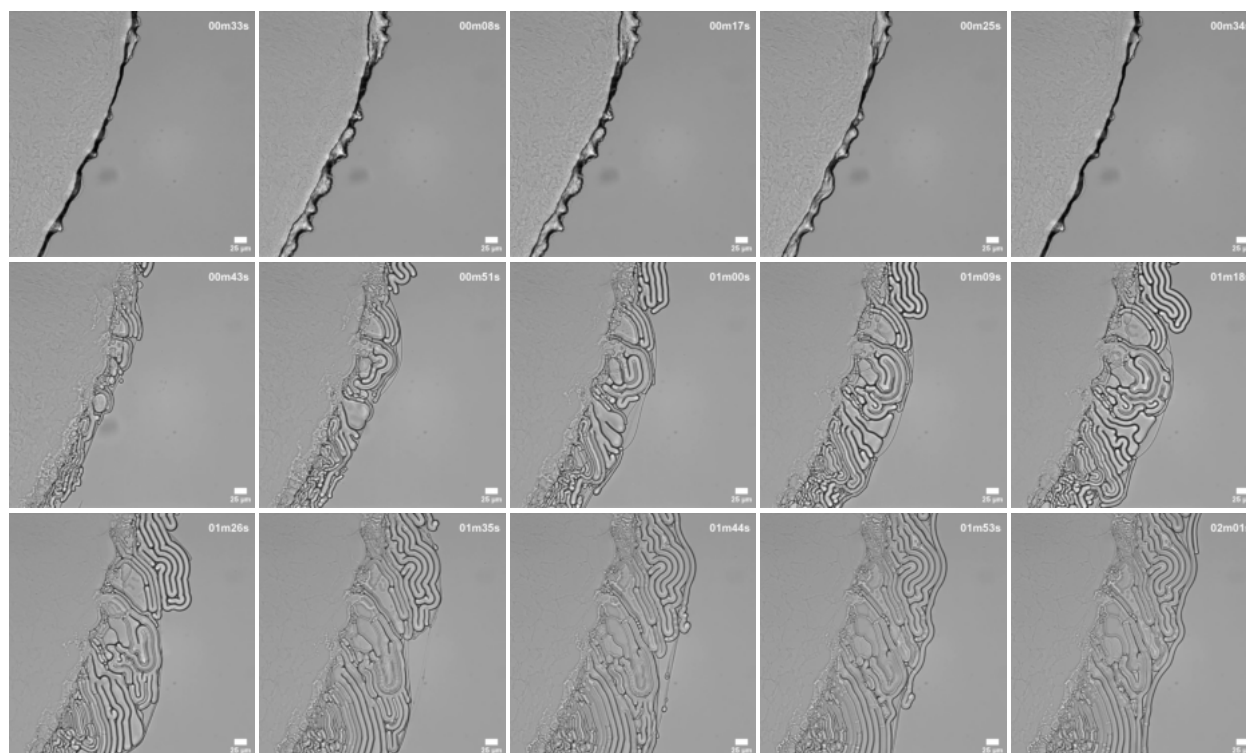


Figure A.1: Time-lapse movie comprising brightfield microscopy images of the hydration of a dry plaque of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) doped with 1 mol% fluorescently labelled lipid, namely, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss-Rho-PE) using 30 μ L of deionized water. The movie documents the dynamics of formation, growth, and quasi-steady-state morphologies revealing bundles of myelin figures formed from the edge of the initial plaque

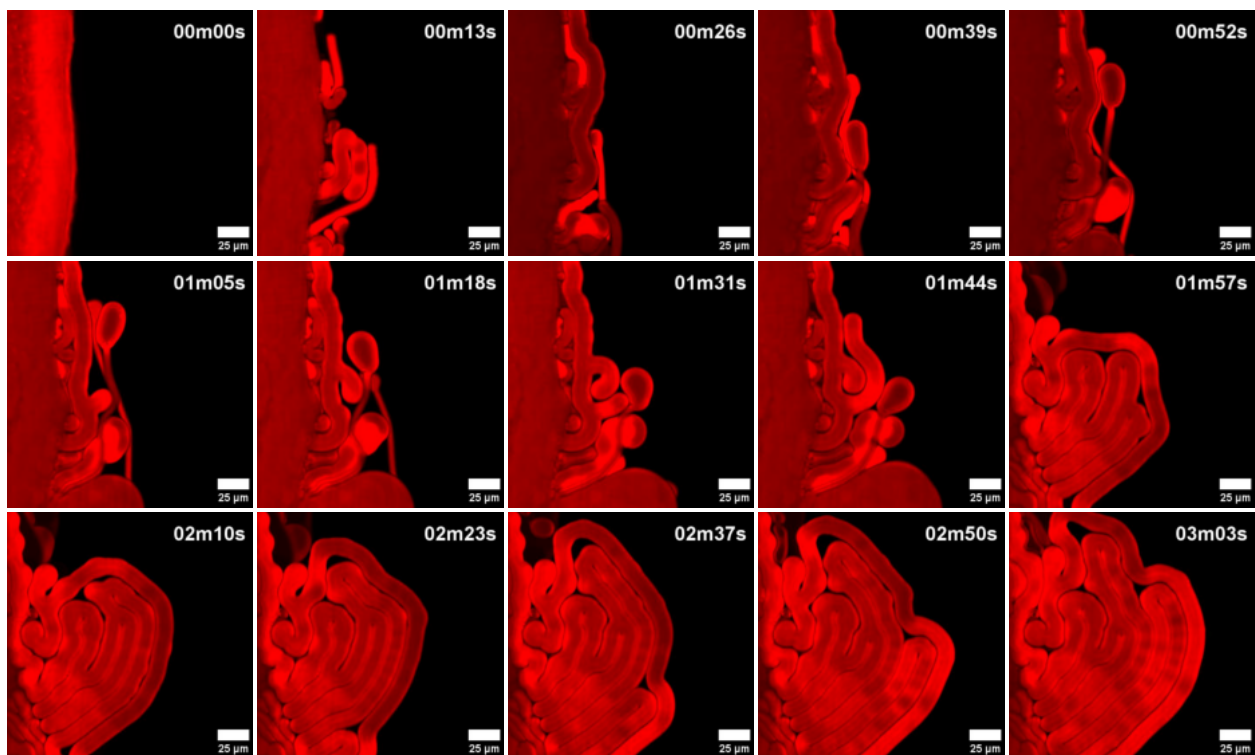


Figure A.2: A time-lapse movie of confocal fluorescence microscopy images. Sample details are identical to those described in Figure A.1

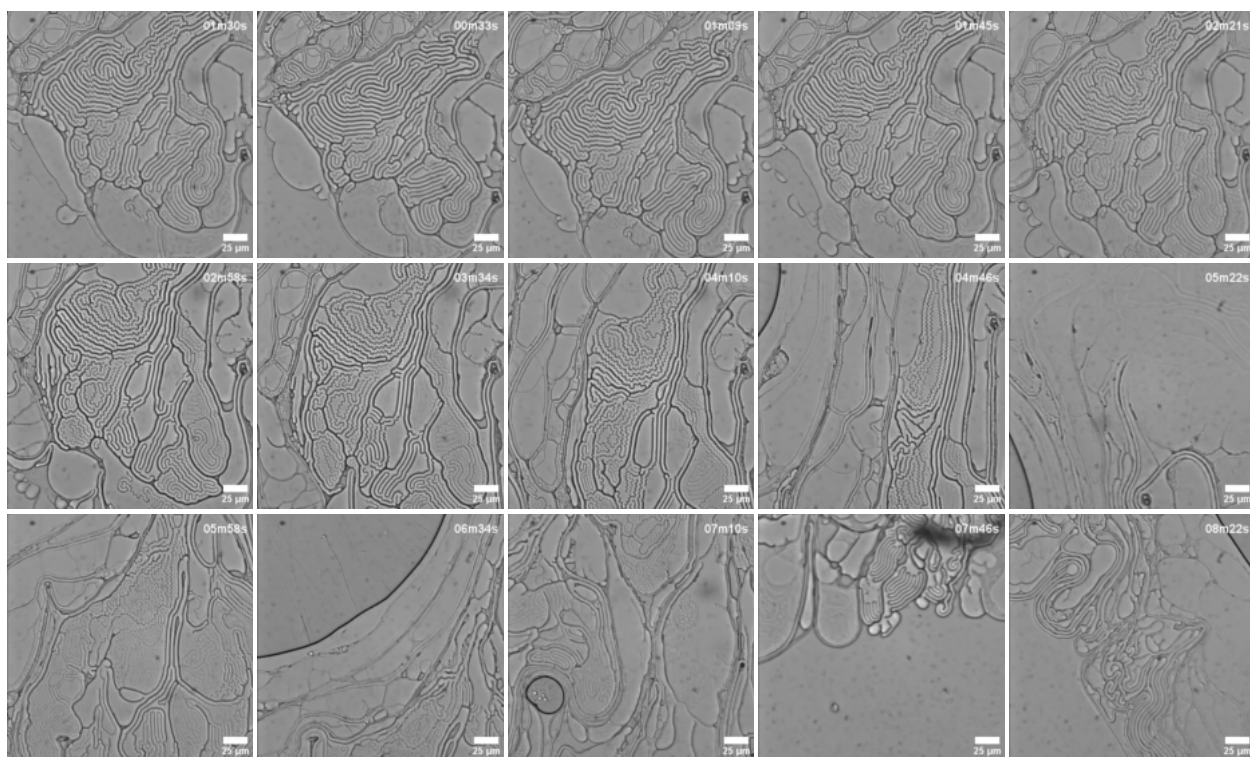


Figure A.3: Time-lapse brightfield microscopy movie showing dehydration of preformed myelin figures generated from a plaque of POPC after initial hydration with 30 μ L of deionized water. The movie captures the temporal evolution of myelin dehydration.

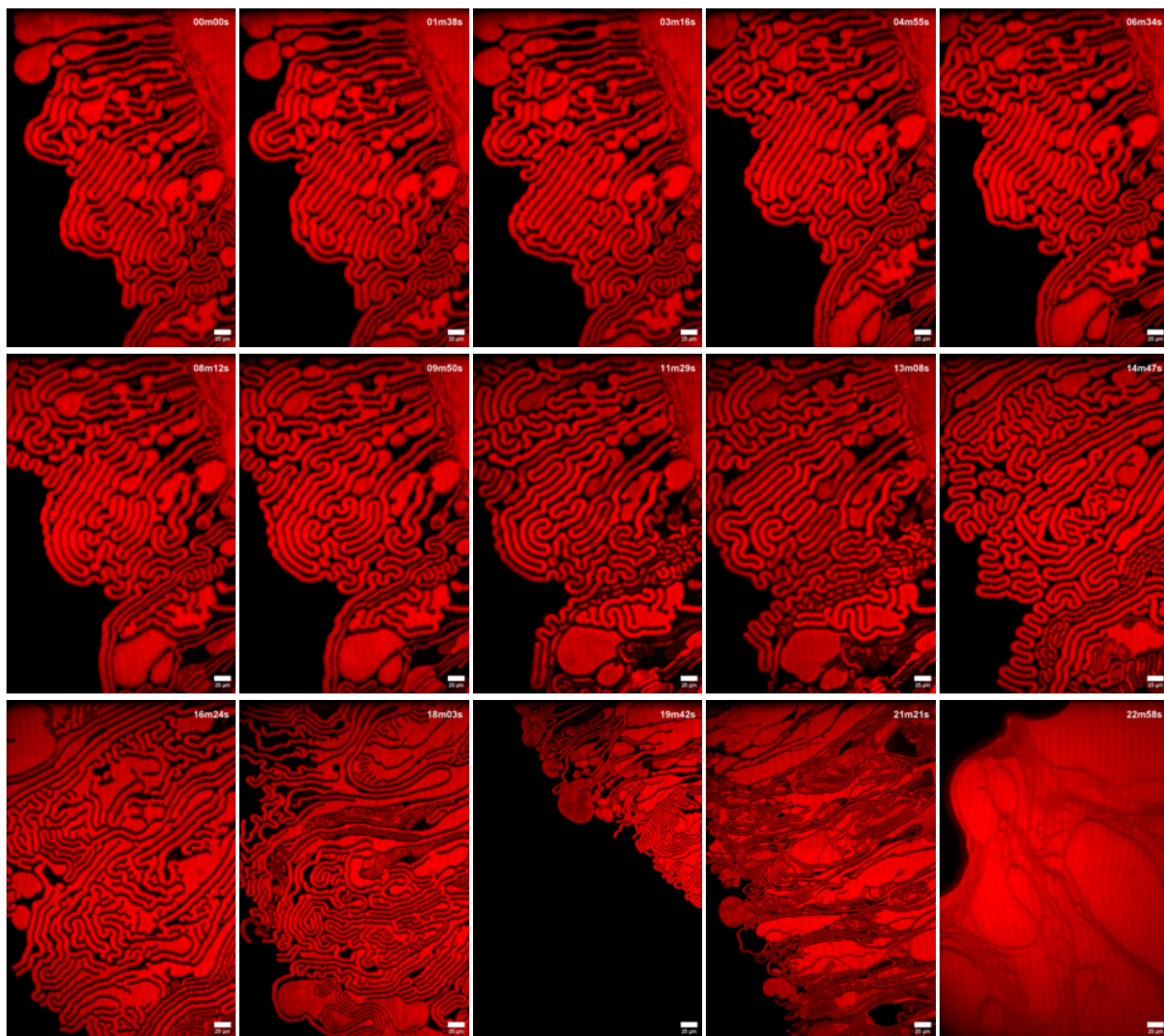


Figure A.4: Time-lapse confocal fluorescence microscopy movie showing dehydration of preformed myelin figures generated from a plaque of POPC doped with 1 mol% Liss-Rho-PE after initial hydration with 30 μ L of deionized water. The movie captures the temporal evolution of myelin dehydration.

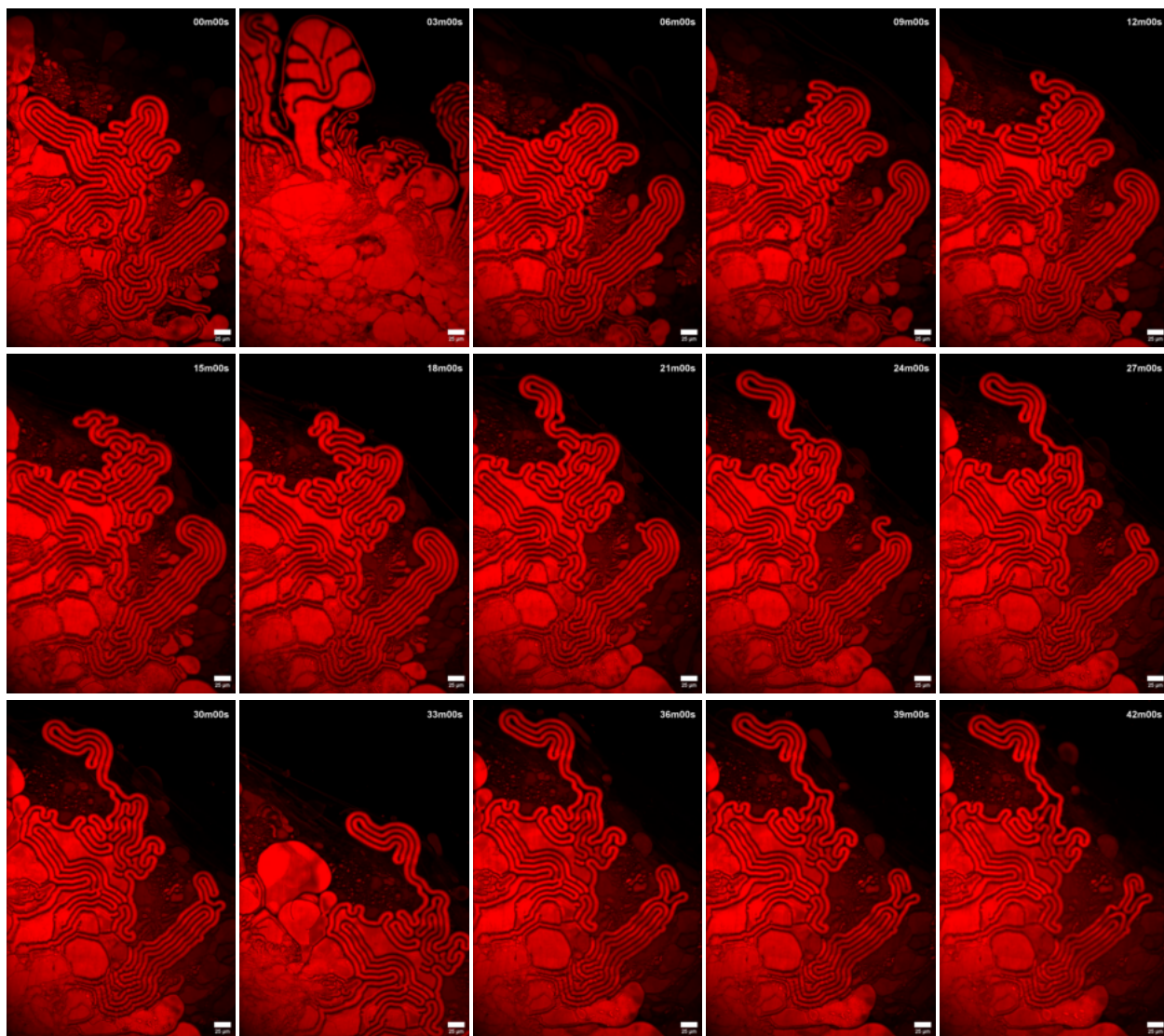


Figure A.5: Time-lapse confocal fluorescence microscopy movie of secondary hydration of pre-formed myelin figures formed from a POPC plaque doped with 1 mol% Liss-Rho-PE using 30 μ L of 1000 mM sucrose, showing the absence of corrugations and long-term structural stability.

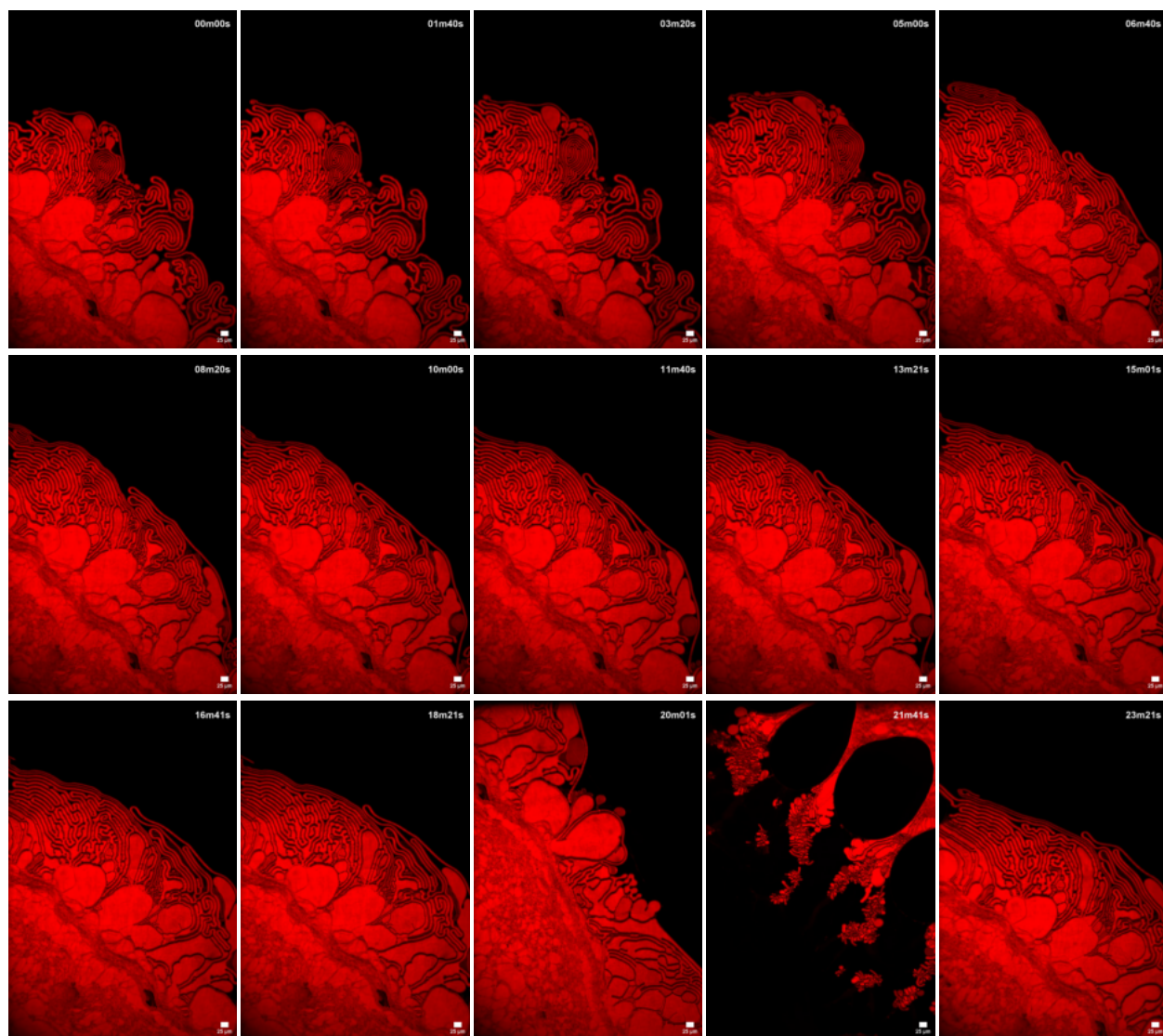


Figure A.6: Time-lapse movie of a compilation of confocal fluorescence microscopy images of secondary hydration of preformed myelin figure plaque of POPC doped with 1 mol% Liss-Rho-PE with 30 μ L of 30 w/w% glycerol. This movie reveals the absence of detectable surface corrugations on preformed myelin figures.

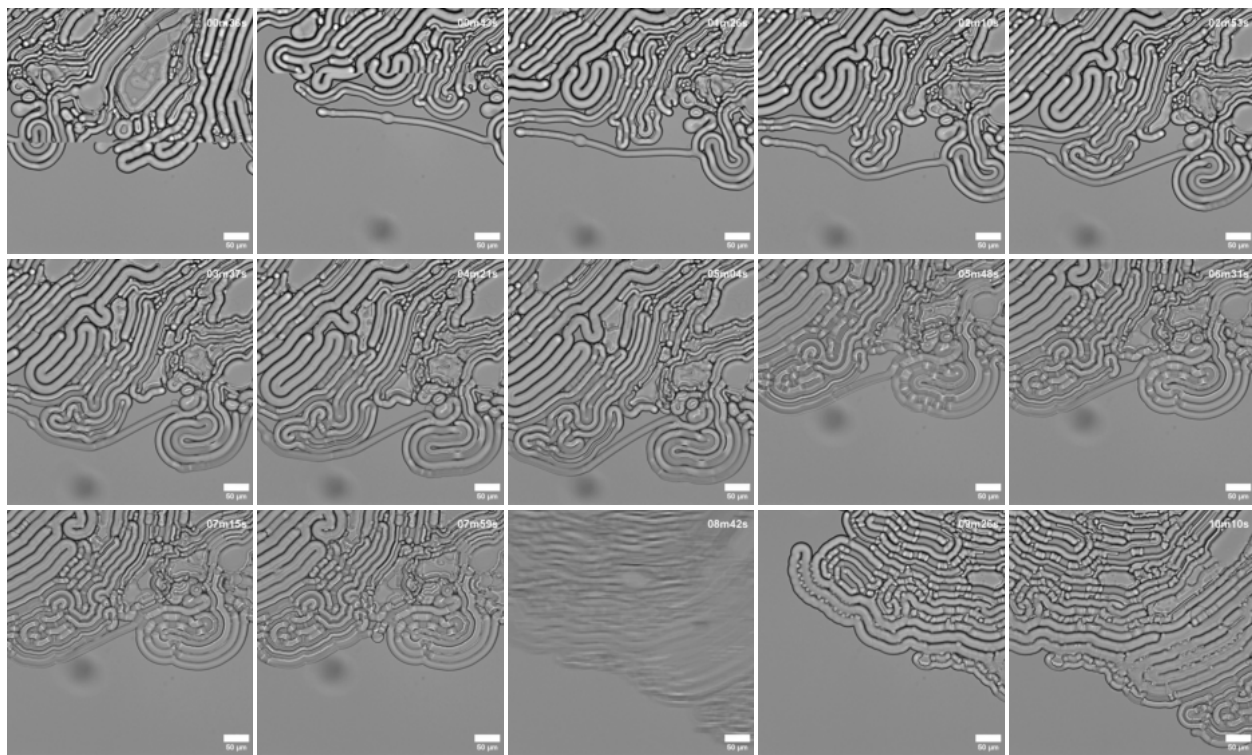


Figure A.7: Time-lapse confocal fluorescence microscopy movie of secondary hydration of pre-formed myelin figures formed from a POPC plaque doped with 1 mol% Liss-Rho-PE using 30 μ L of PEG8K (12.7 mM, $\Pi = 0.266$ MPa), showing corrugation (wrinkling) of the myelin structures.

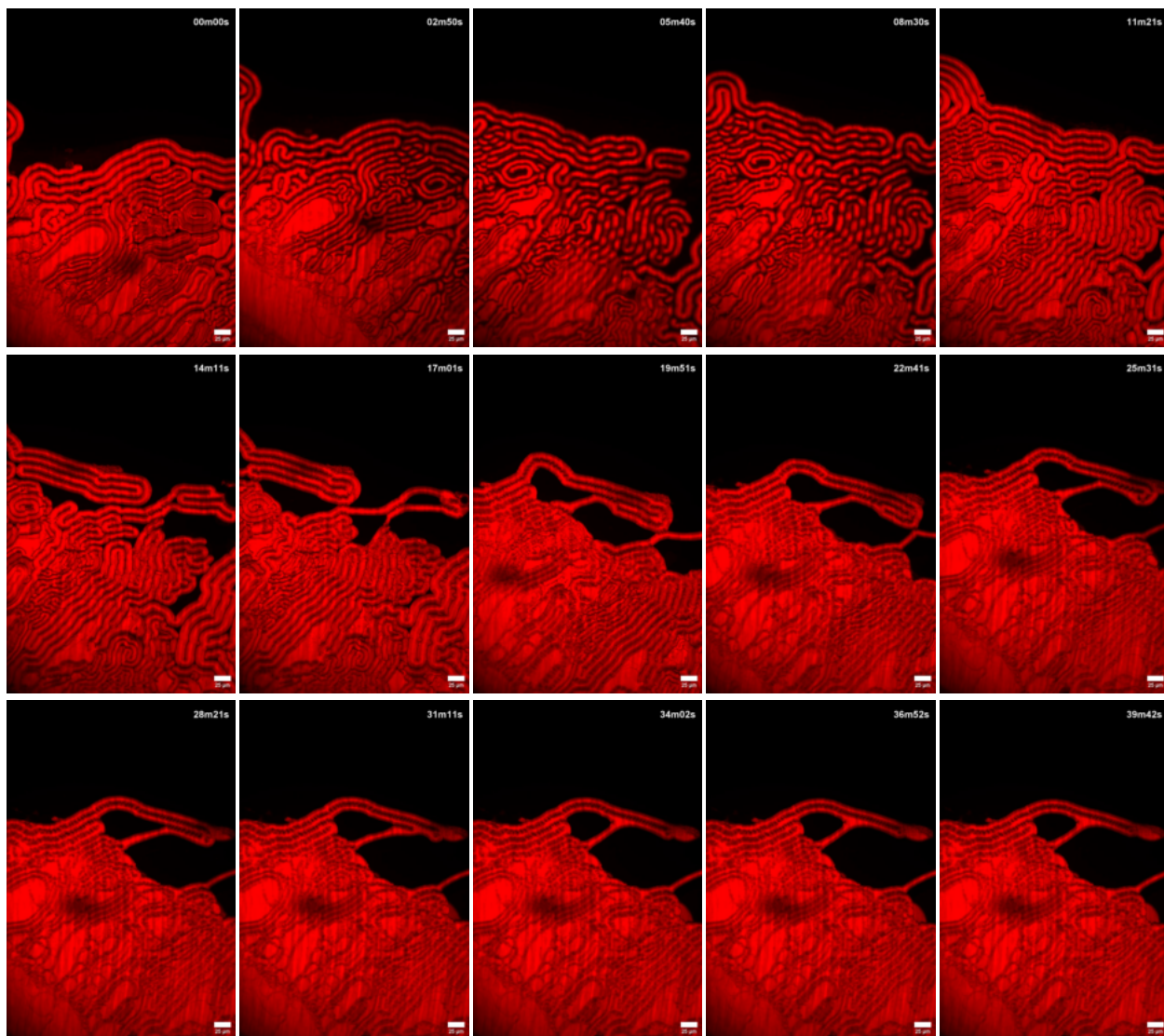


Figure A.8: Time-lapse confocal fluorescence microscopy movie of secondary hydration of pre-formed myelin figures formed from a POPC plaque doped with 1 mol% Liss-Rho-PE using 30 μ L of PEG8K (37.5 mM, $\Pi = 2.15$ MPa), showing corrugation (wrinkling) of the myelin structures.

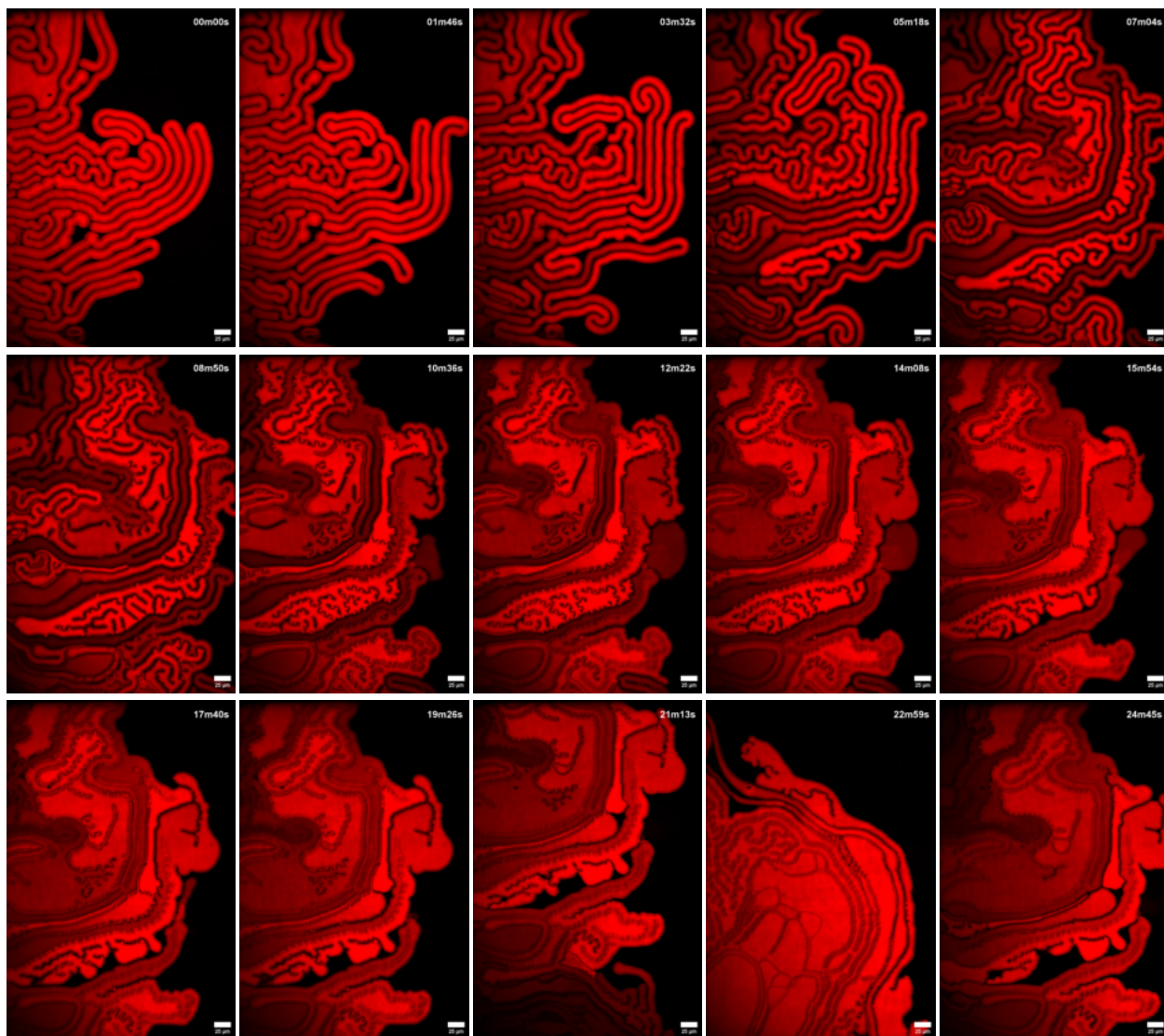


Figure A.9: Time-lapse confocal fluorescence microscopy movie of secondary hydration of pre-formed myelin figures formed from a POPC plaque doped with 1 mol% Liss-Rho-PE using 30 μ L of DEX10K (55.6 mM, $\Pi = 2.15$ MPa), showing corrugation (wrinkling) of the myelin structures.

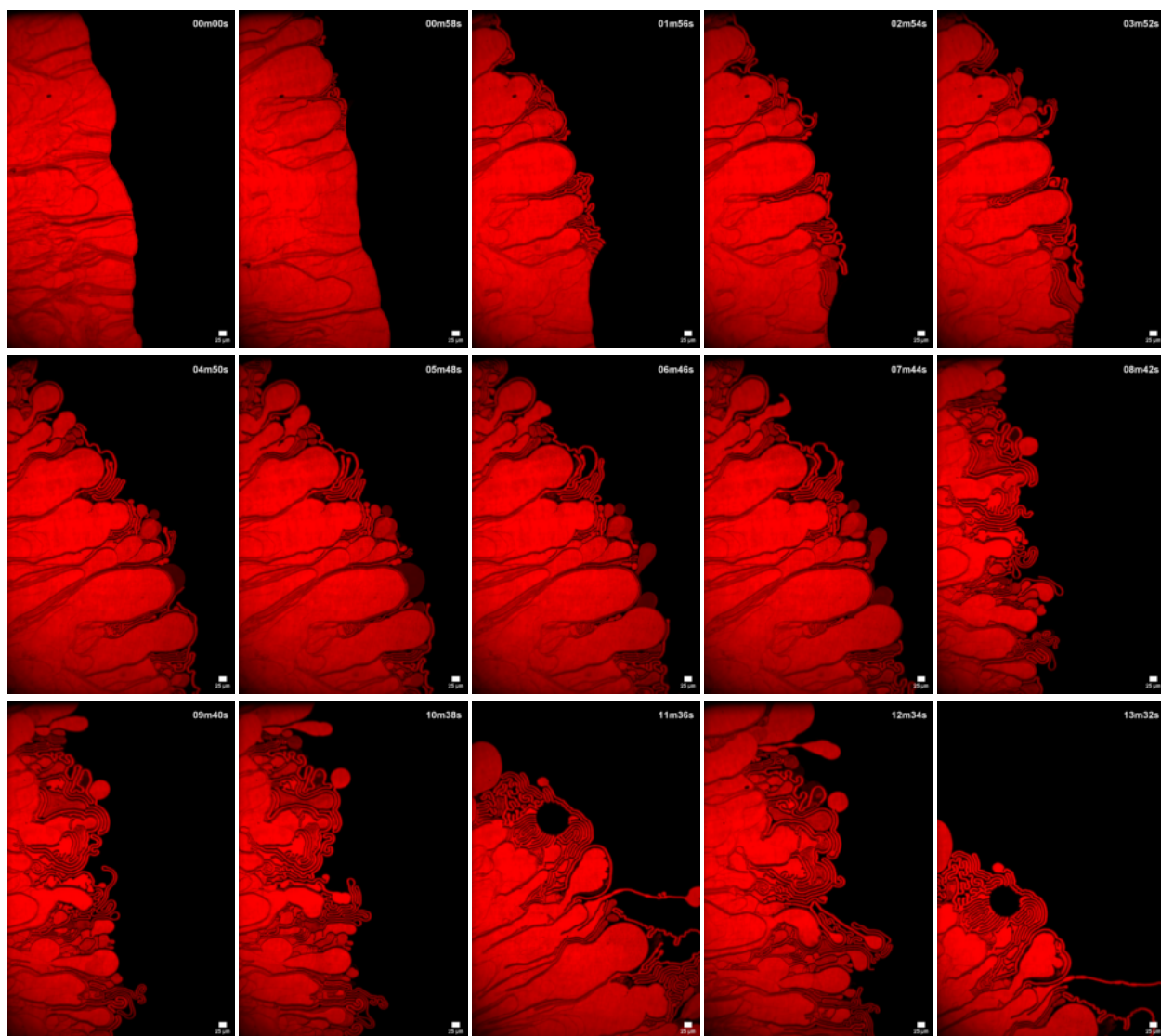


Figure A.10: Time-lapse movie comprising brightfield microscopy images of hydration of a dry plaque containing POPC, 10% cholesterol, and ~ 1 mol% Liss-Rho-PE using $30\ \mu\text{L}$ of deionized water. The movie documents the formation and propagation of myelin bundles from the edge of the plaque.

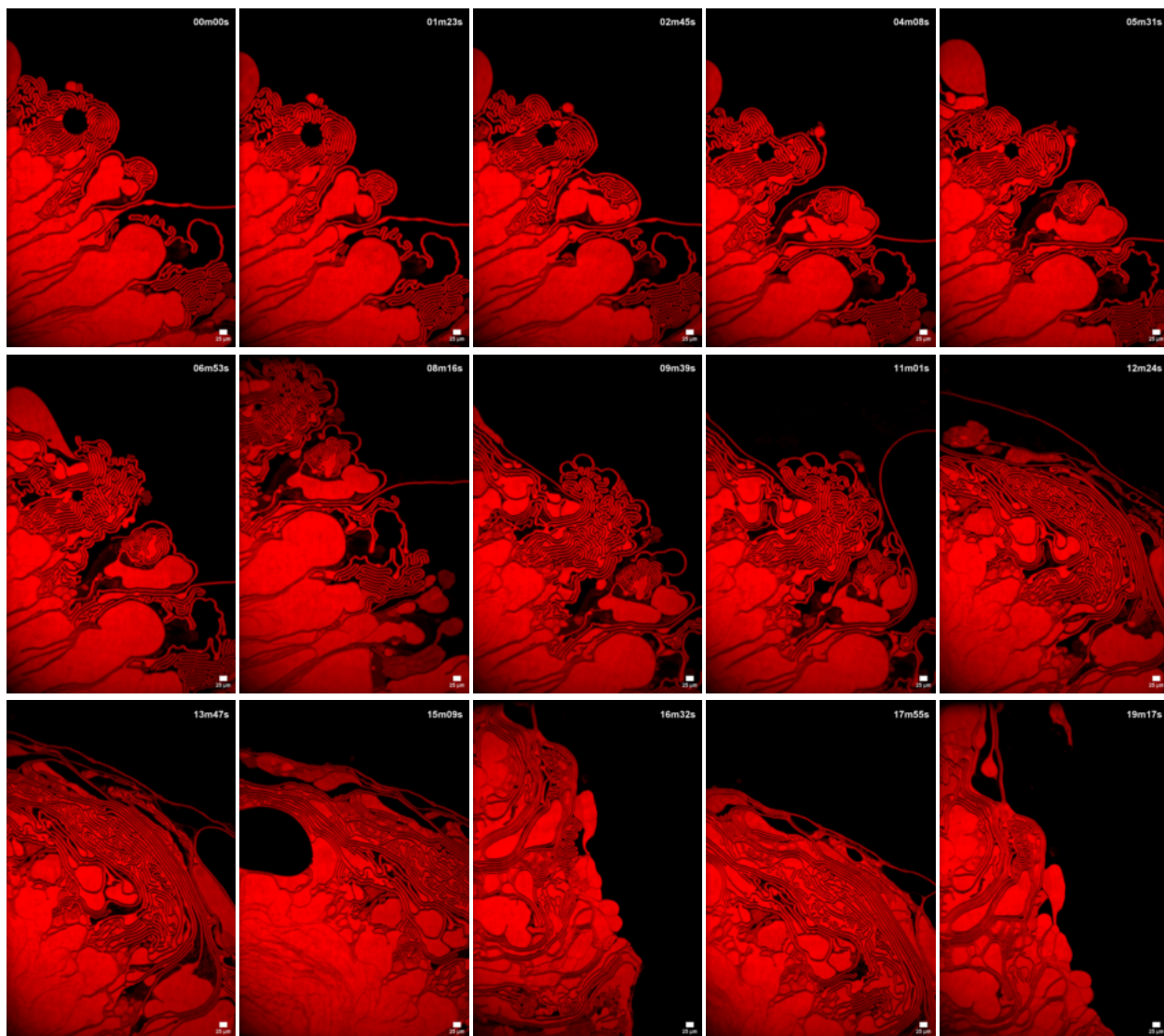


Figure A.11: A time-lapse movie of confocal fluorescence microscopy images. Sample details are identical to Figure A.10. Corrugation (wrinkling) seen in myelin figures subjected to secondary hydration using 30 μ L PEG8K 37.5 mM.

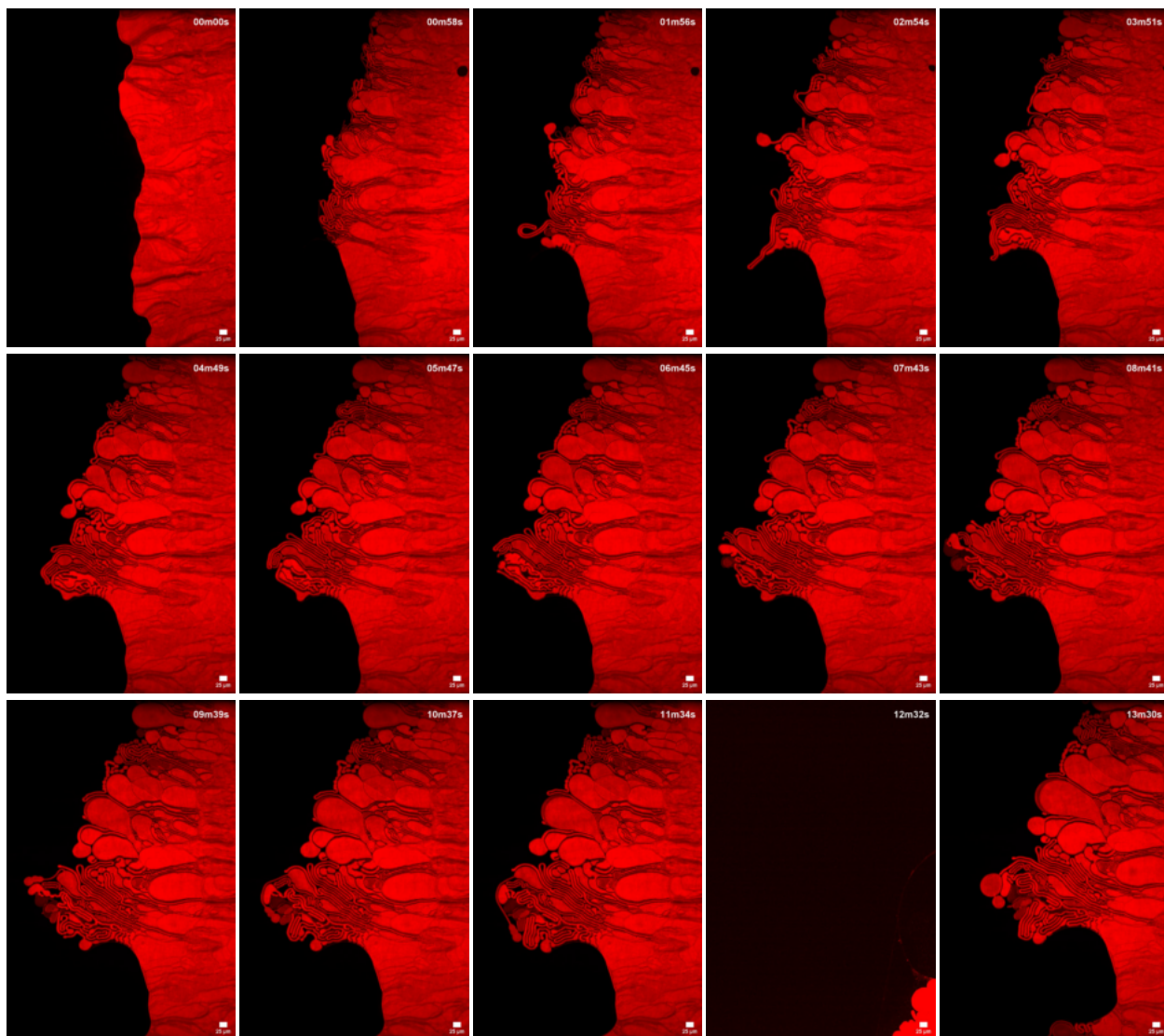


Figure A.12: Time-lapse movie of a compilation of brightfield microscopy images of hydration of a dry plaque $\sim 75\%$ POPC, 25% cholesterol, and ~ 1 mol% Liss-Rho-PE using $30\ \mu\text{L}$ of deionized water. The movie reveals the formation and propagation of bundles of myelin figures from the edge of the plaque.

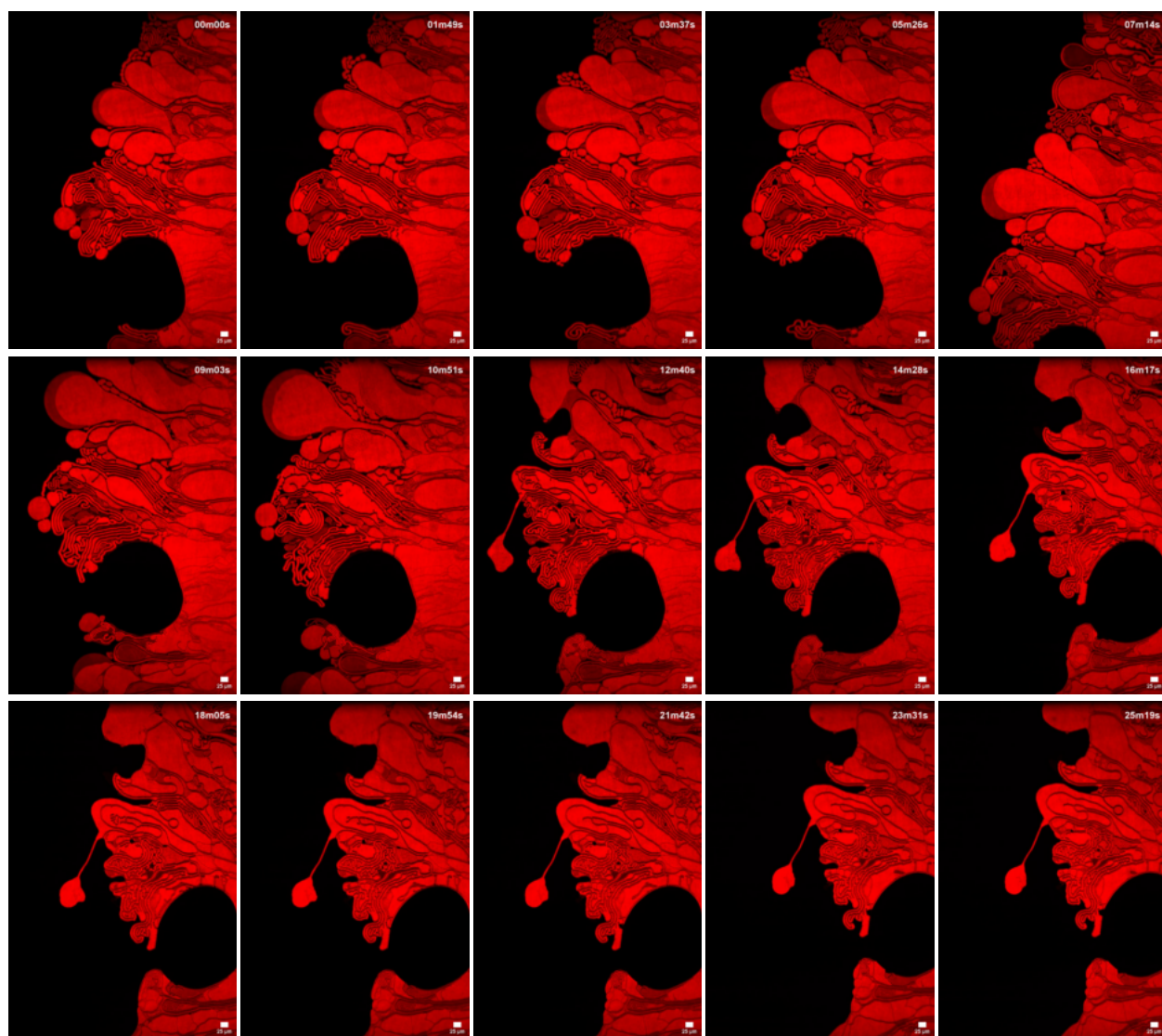


Figure A.13: Time-lapse confocal fluorescence microscopy movie under conditions identical to Figure A.12, showing corrugation (wrinkling) of myelin figures during secondary hydration with 30 μ L of PEG8K 37.5 mM.

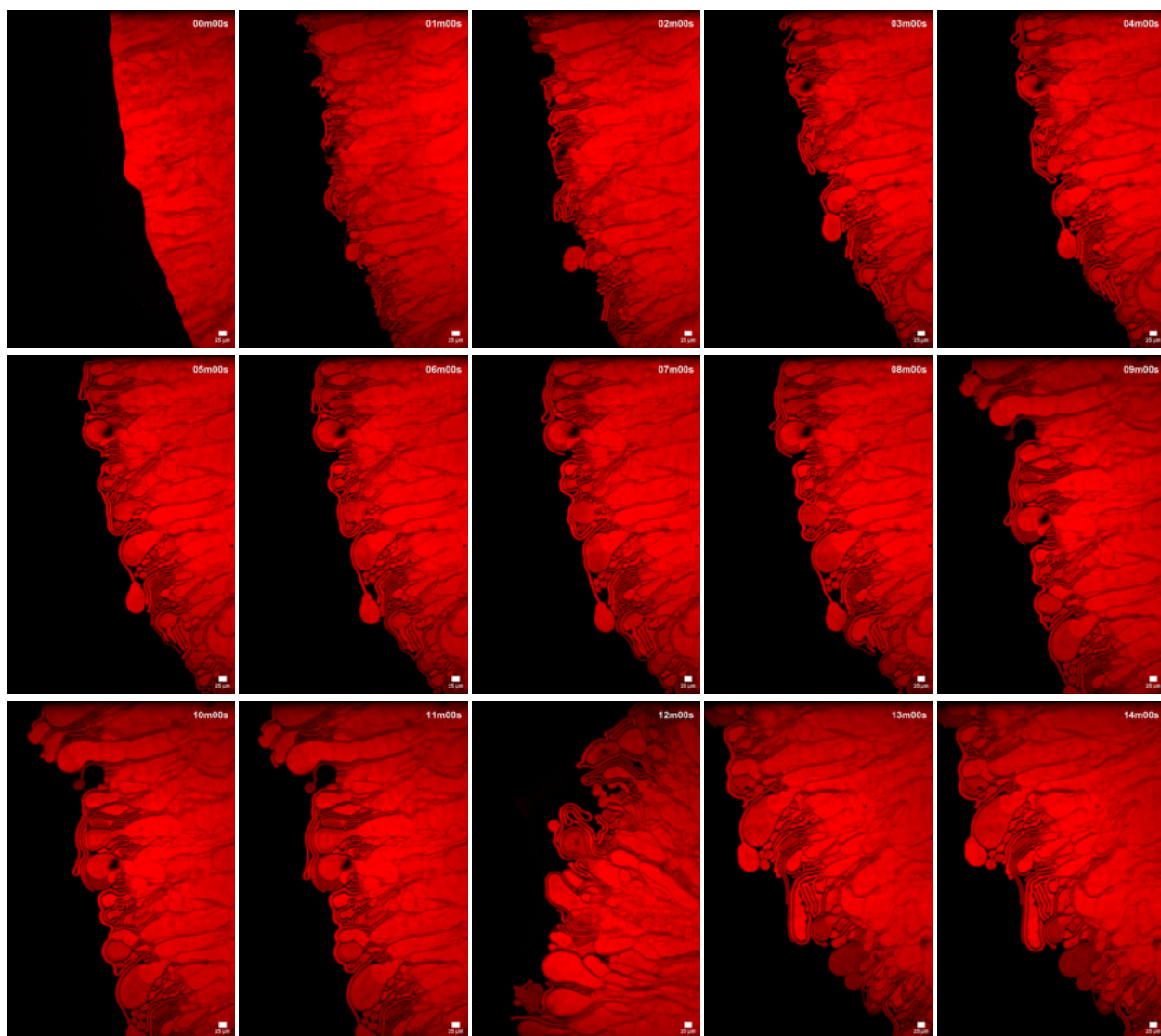


Figure A.14: Time-lapse brightfield microscopy movie showing hydration of a dry plaque composed of 60% POPC and 40% cholesterol, doped with <1 mol% Liss-Rho-PE, upon addition of $30\ \mu\text{L}$ of deionized water. The movie captures the emergence and propagation of bundled myelin figures from the plaque edge.

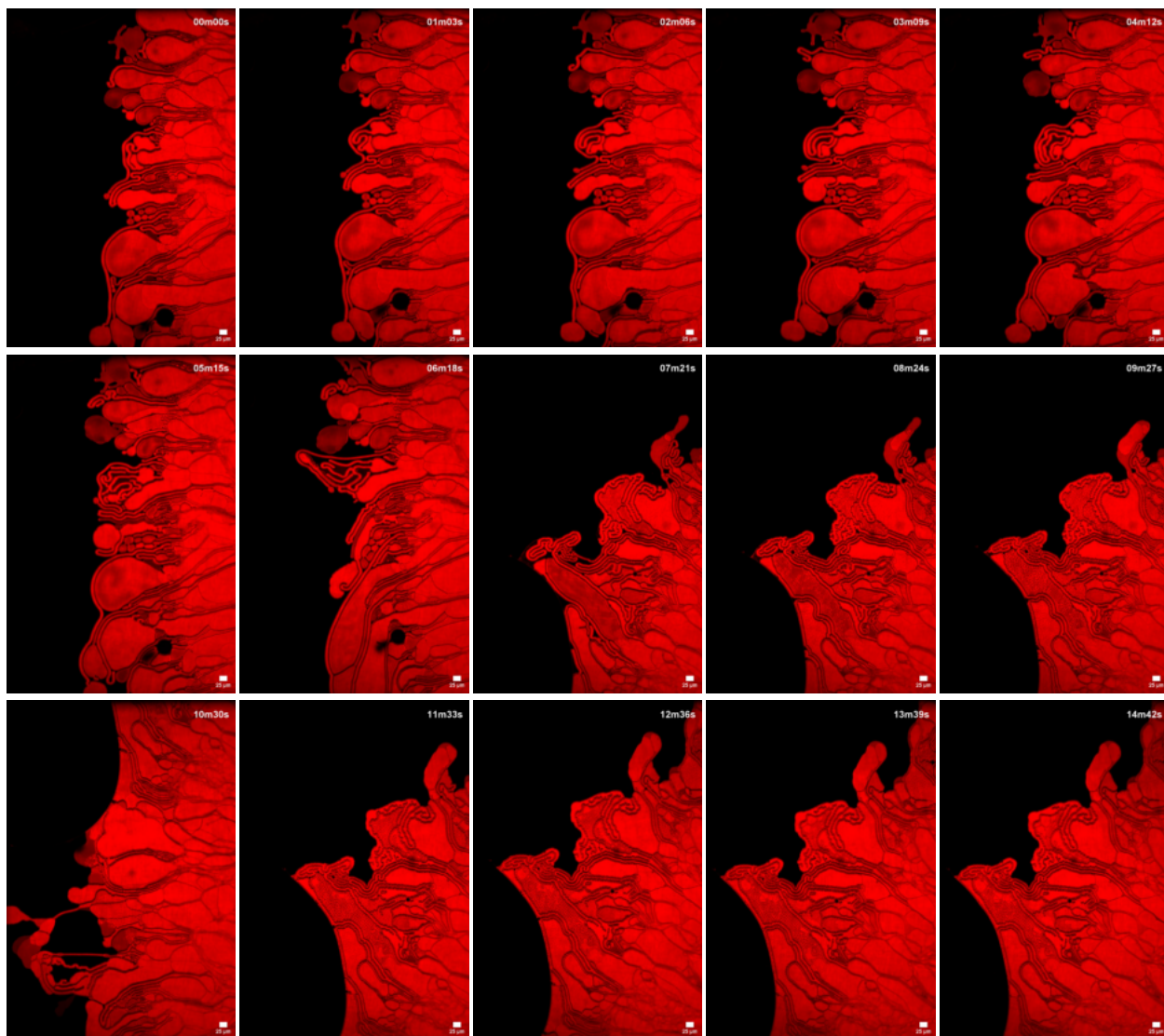


Figure A.15: A time-lapse movie of confocal fluorescence microscopy images. Sample details are identical to Figure A.14. Corrugation (wrinkling) seen in myelin figures subjected to secondary hydration via 30 mL PEG8K 37.5 mM.

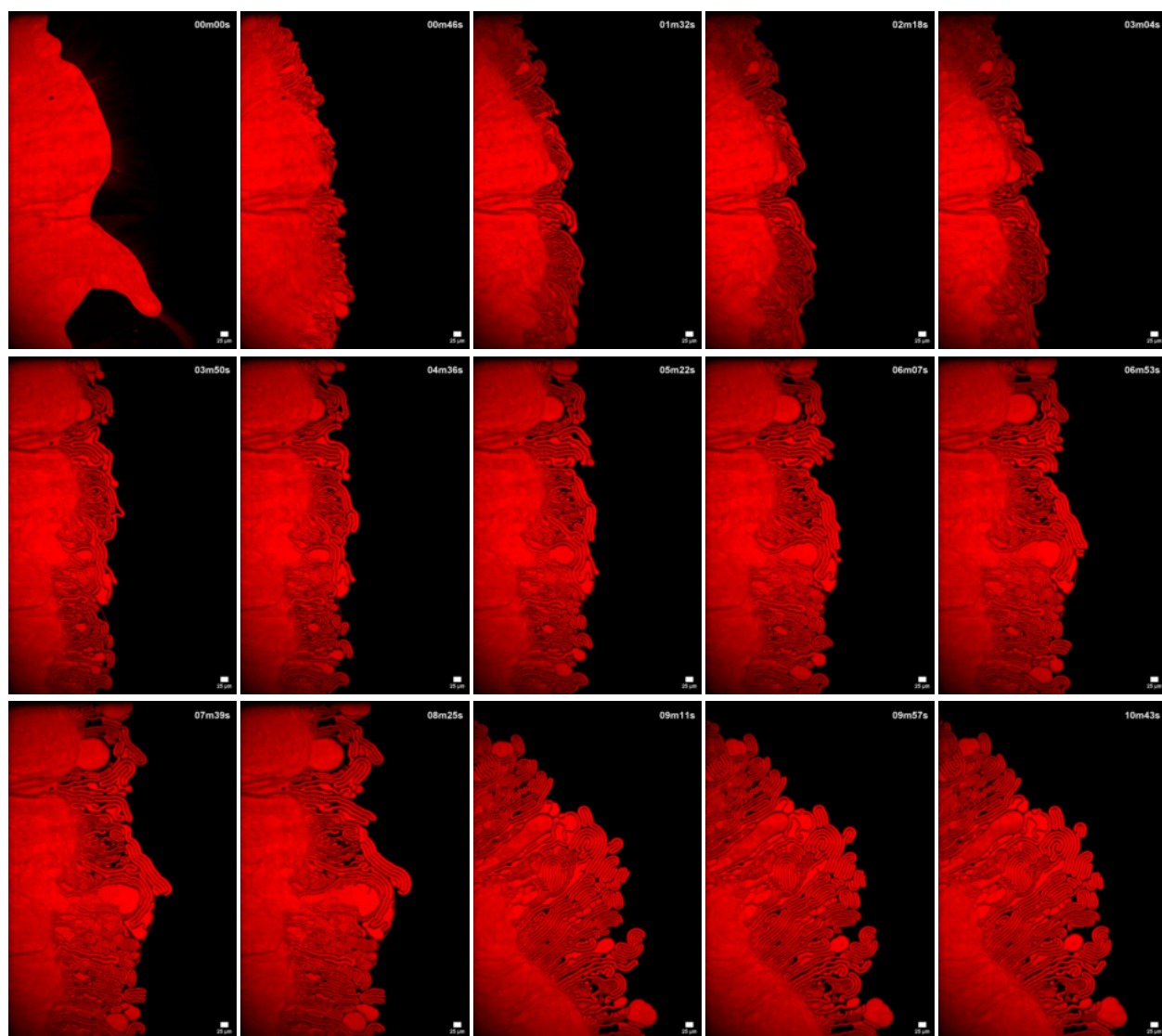


Figure A.16: Time-lapse movie comprising brightfield microscopy images of hydration of POPC doped with Liss-Rho-PE1 mol% using 30 μ L of deionized water. The movie documents the formation and propagation of myelin bundles from the edge of the plaque.

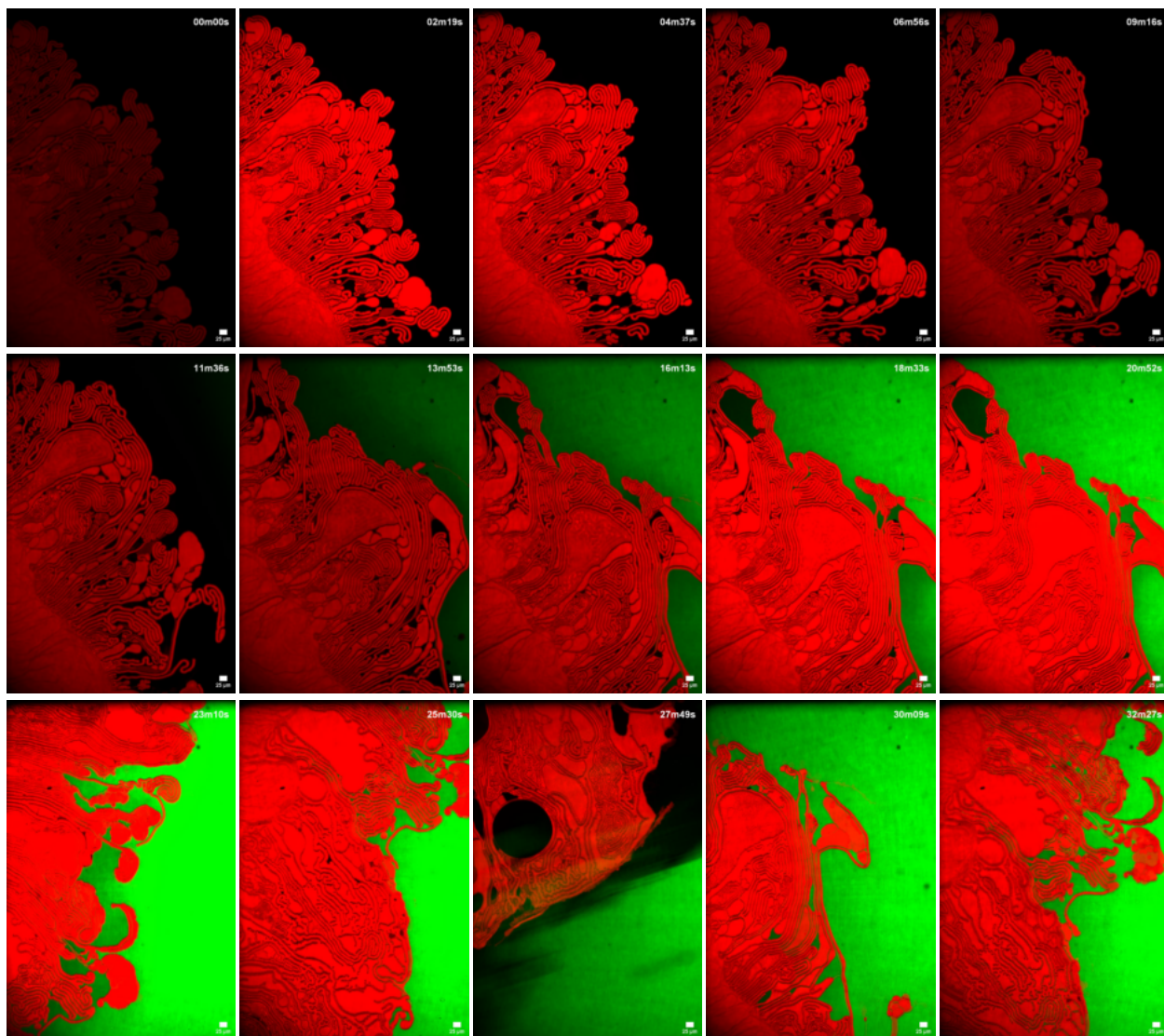


Figure A.17: Time-lapse confocal fluorescence microscopy movie of secondary hydration of pre-formed myelin figures formed from a POPC plaque doped with 1 mol% Liss-Rho-PE using 30 μ L of DEX10K (55.6 mM, $\Pi = 2.15$ MPa; 1000 : 1 *unlabeled* to *labeled* FITC – Dex10K), showing corrugation (wrinkling) and exclusion of osmolytes from the corrugated regions.

Appendix B

Actin Interplay with ATPS

Supplementary Material

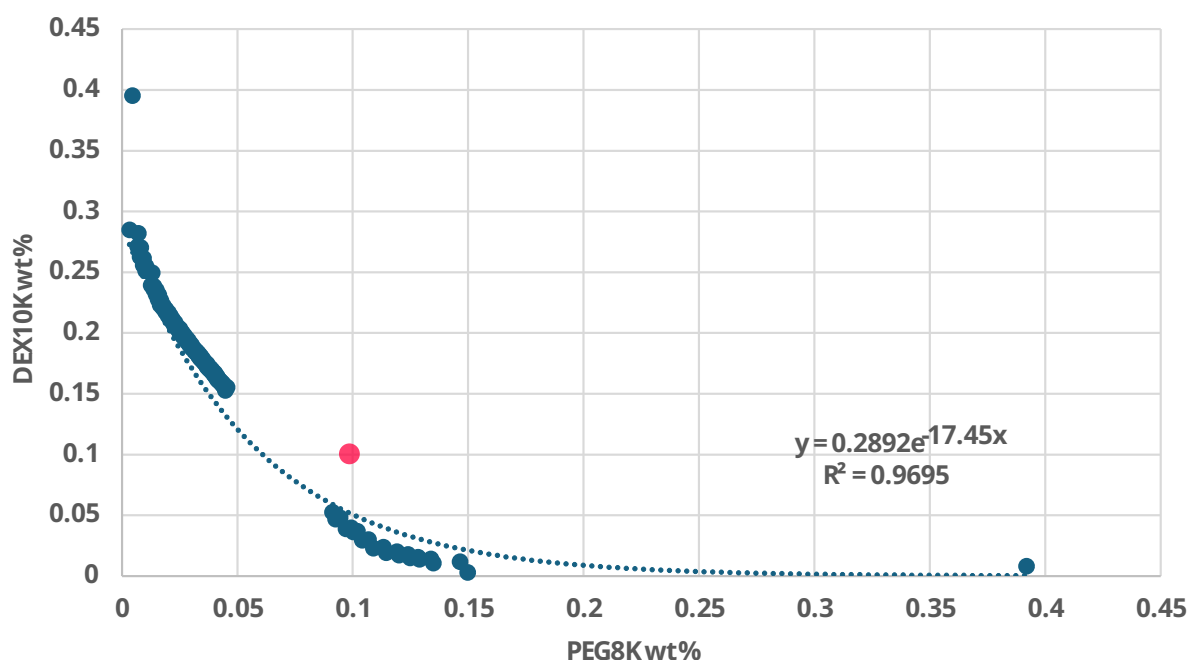


Figure B.1: Binodal curve constructed by the cloud point method¹²⁴ for dextran (DEX 10K) paired with polyethylene glycol (PEG8K), plotted as polymer mass fraction against one another. Curve represents the phase boundary separating the single-phase mixed region (below the curve) from the two-phase separated region (above the curve). Phase boundaries were determined by visual turbidity assessment at increasing polymer concentrations. The working concentrations used in this study ,PEG 8K and DEX 10K each at 10 wt%, is indicated by red dot.

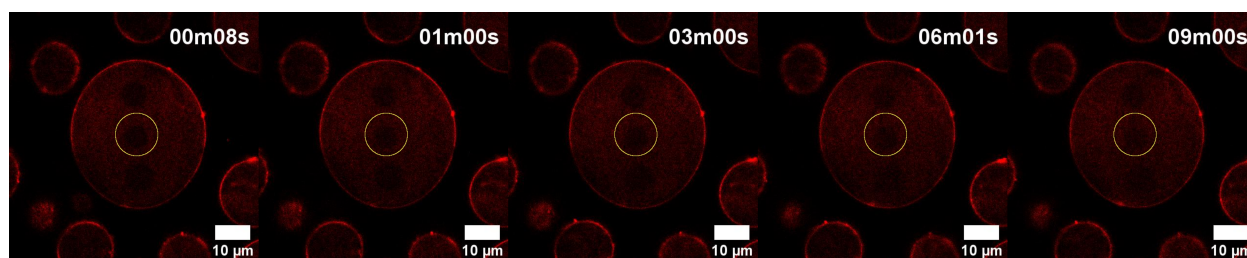


Figure B.2: Representative fluorescence recovery after photobleaching timelapse of a DEX-rich droplet containing 0.2 mg actin (7:1 unlabeled to rhodamine-labeled ratio) under 1xG buffer conditions. Recovery plateaus between 0.6–0.7 of the pre-bleach intensity, consistent with a network-constrained, largely immobile actin state within the crowded droplet interior. Scale bar = 10 μm

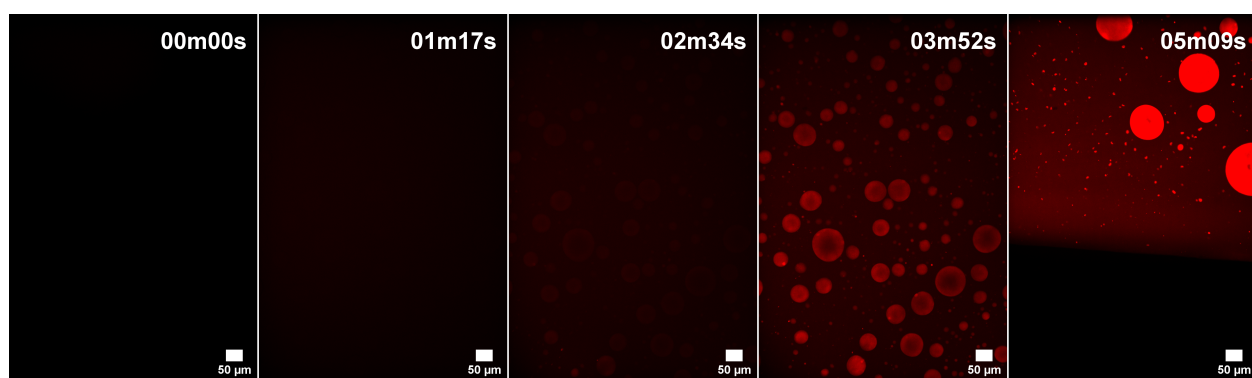


Figure B.3: Representative time-dependent accumulation of rhodamine phalloidin signal inside DEX-rich droplets containing 0.2 mg unlabeled actin under 1xG buffer conditions, following addition of 100 mol% rhodamine phalloidin. Frames are shown over a representative acquisition window prior to stage drift, demonstrating the initial penetration of phalloidin into the droplet interior and its binding to filamentous actin. Despite the shortened acquisition window relative to the 1xF condition, the visible accumulation of phalloidin signal inside the droplets confirms that crowding-driven assembly under 1xG buffer conditions produces phalloidin-accessible filaments in the absence of conventional polymerization triggers. Scale bar = 50 μm

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