**1. Introduction**

**1.1**. A. *Basic biology of membranes and membrane fusion*. Biological membranes consist of lipids and proteins, with the lipids self-organized into sheets and the proteins embedded into or bound to the sheets. The lipid sheets are arranged as a “bilayer,” two lipid monolayers with lipid acyl chains (i.e., hydrophobic tails) directly abutted against each other and sequestered from the aqueous solutions bathing the two sides of the membrane. The lipid bilayer membrane appeared early in evolution [Cavalier-Smith, 1987] and provides the barrier between interiors and exteriors of all eukaryotic (nucleus-containing) cells and their organelles. The architectural organization of the lipid bilayer is critical to life, and the functioning of proteins embedded in membranes is intrinsically connected to the bilayer structure in which they reside. The electrical insulation provided by the lipid bilayer is critical for the generation and propagation of action potentials in neurons; the battery created by voltage across mitochondrial membranes drives the production of ATP, the currency of cellular energy; the transduction of extracellular signals such as hormones and growth factors into intracellular messages some of which are direcly derived by enzymatic cleavage of bilayer lipids; these and virtually every other membrane process rely critically on the integrity of the lipid bilayer structure [Textbook]. Despite its superficial simplicity, the lipid bilayer is a complex structure. Lipids are asymmetric in all directions, are in rapid motion, rotating, for example, 109 times/sec [ref], and both the mass and electron density profiles are heterogeneous along the thickness (1.5 ­– 2 nm) of each lipid monolayer [ref].

Membrane fusion is the central process of many vital cellular functions such as neurotransmitter release in the brain, insulin release from the pancreas, and trafficking of materials between organelles. Fusion is also the means by which many viruses (e.g., HIV, hepatitis, flu, Ebola) infect cells. On the molecular level, fusion results in the joining of two aqueous compartments and the continuity of two formerly separate membranes. Fusion of membranes is not a spontaneous process. Membranes are stable and when two lipid bilayers are experimentally forced against each other, they generally do not fuse. Fusion proteins embedded in the membranes supply the required energies for lipid rearrangements. Proteins also provide the specificity of fusion: the ability of one membrane to selectively fuse to another. But it is the lipids that confer the fluidity necessary for membranes to deform into configurations that lead to fusion. For fusion to happen, lipids must temporarily leave the bilayer arrangement for a non-bilayer configuration. To obtain a physically deep understanding of membrane fusion, the movements and reorientations of the lipids must be determined.

B. *Steps of fusion: hemifusion, pore formation, pore expansion*. There is extremely strong experimental evidence that in an early step of fusion, two initially separate membranes merge to create a configuration known as “hemifusion” (Kemble et al., 1995; Mel et al., 1995; Chernomordik et al., 1998; Rothman; Shing; McMahon;). At hemifusion, contacting proximal lipid monolayers of each bilayer have merged, but the distal monolayers remain distinct (Fig. 1). An hour-glass-shaped “stalk” is the initial hemifusion connection. Stalk geometry has been experimentally deduced by X-ray crystallography [Huang]. In the stalk structure, the distal monolayers have just begun to contact each other. When the stalk expands laterally, the more extensive contact of distal monolayers creates a “hemifusion diaphragm.” A hemifusion diaphragm is a pure lipid bilayer devoid of proteins [ref]. At its circumference, the diaphragm connects to the two original membranes, creating a ‘Y’ configuration. Formation of a pore in the hemifusion diaphragm establishes aqueous continuity, and the initial transfer of aqueous contents now occurs (Fig. 1). It has been experimentally shown, for several cellular systems, that some states of hemifusion can lead to fusion pore formation whereas other states of hemifusion cannot [refs]. But the reasons are unknown and this remains a fundamental question in the field of membrane fusion.Forces that could cause lipids to rearrange into a fusion pore are known [kozlov], but the lipid rearrangements and the consequences of water movement that must accompany pore formation are unknown. The initial fusion pore must expand to permit the transfer of large molecules, such as proteins (e.g., insulin), from intracellular vesicles to the extracellular space; for virus, pore expansion permits its genetic material to pass into cytosol, initiating infection. The physics of this expansion has been theoretically modeled [Chiz, Jackson], but pore geometry was fixed (as a toroid) and consequences of a non-zero aqueous viscosity were ignored.

C. *Past theoretical investigations*. Mathematical modeling of membrane fusion has included microscopic all-atom simulations of molecular dynamics, mesoscopic Monte Carlo calculations, and macroscopic approaches of continuum membrane mechanics. Each has its range of applicability. All-atom simulations have the merit that, in principle, they could eventually (subject to the accuracy of the force fields) explicitly yield the motion of every atom during steps of fusion. But the time scales of these simulations are quite limited, presently on the order of a s [ref; Ash et al., 2004; Bahar et al., 2010; Spruce et al, 1991]; this is much too short to compare to experimental results. Mesoscopic calculations can cover a wide time range, but the coarse grain description of lipids and surrounding water may not capture essential properties of the system [Stevens et al., 2003]. Continuum models have the merit that they cover all relevant time ranges and lead to predictions that can be experimentally tested. But continuum theories cannot reveal phenomena caused by molecular interactions that are atypical of the average continuous material. Lipid interactions are dominated by volume exclusion and van der Waals dispersion forces, forces that are roughly the same for all lipid species. Therefore, lipid complexes that are functionally isolated from the overall monolayer are unlikely to drive fusion. Overlap between the approaches can be quite fruitful—since their predictions can be compared—especially when predictions are experimentally testable. Generally, there is overlap between coarse grain and continuum models both spatially and temporally [K. Kremer].

The continuum mechanical description of membranes predicted that the ability of two membranes to hemifuse should depend on the spontaneous curvature of the contacting leaflets (Markin et al., 1984. recent): hindered by positive spontaneous curvature and promoted by negative curvature. This was confirmed experimentally [refs], showing that the predictions of continuum mechanics could be “translated” into experimentally controlled variables. Biologists could therefore powerfully use theory to investigate phenomena without the need for them to comprehend the complexity of mathematical equations [refs].

But classical continuum formalisms used to date have limitations. They assume that membranes are at equilibrium, and thus time courses of changes during fusion are not calculated. They also assume that elastic energies are conserved. Movements of membrane and aqueous solutions, however, must cause energy dissipation and this can, in fact, be large in membrane processes. For example, we have shown that in osmotic swelling and lytic bursting of vesicles (e.g., as occurs in hemolysis), more than 70% of the elastic energy stored in the vesicle membrane at the time of pore formation converts to heat after the Laplace pressure within the vesicle has collapsed (Ryham et al., 2012). Because cellular environments are viscous, energy is dissipated during virtually every biological process.

Fortunately,several continuum approaches have been developed over the last twenty years or so—phase field (Du et al., 2009), level set (Osher et al., 2002, et al., Osher 2003, Sethian, 1986), boundary integral (Sohn et al., 2010, Vlahovska et al., 2011), immersed boundary (Fogelson and Guy 2007, Lowengrub et al., 2007), and direct monolithic/splitting (Guidoboni et al., 2009; Pasquali and Scriven, 2002)—that do not assume equilibrium and that account for energy dissipations. These methods have been successful in describing many and complex phenomena of condensed physical matter, but they have not been applied to biological processes. Field theories—systems of differential equations derived from a characterization of an energetic structure— are potentially a powerful way to self-consistently describe steps in membrane fusion.

D. *The phase field approach*. The forces within condensed matter are consequences of their material properties and are described by Navier-Stokes equations, a form of Newton’s second law of motion. These forces cause the shape of a diffuse interface (in our case, the membrane) to change, which in turn generates a reactive force that tends to restore the prior shape. In terms of energy (rather than forces), this is the principle of virtual work which is central to the phase field method.

 In classical continuum mechanics, the geometry of a structure such as a fusion pore is assumed, and its elastic energy is calculated. In contrast, phase field theory iteratively adjusts geometry until minimum energy is found. In practice, this can be quite important: we have found that the energies of the commonly assumed geometry of a fusion pore (a toroid) is much larger than the energy of the minimal surface (see Specific Aims, Aim 4). That is, phase field methods find shapes of minimal energy; classical methods do not.

 The phase field method describes energy dissipation through the principle of “maximum dissipation.” In essence the principle states that the sum of kinetic and elastic energies is converted to heat as fast as possible, maintaining constant temperature. For incompressible media (such as water and membranes), the phase field method allows the system to evolve so that elastic (and kinetic) energy decreases through the pathway of steepest descent that is consistent with the geometry at each moment.

 Also, in the phase field method, an interface is not assumed to be a mathematical surface of zero thickness, but a bulk hydrodynamic material of small thickness, as is the actual case for biological and model bilayer membranes. The changes in energy of a diffuse interface as its shape varies are accounted for by both the Helfrich elastic energy and a continuously varying phase field parameter, ***x*,*t*) as given by the Ginzburg-Landau form of energy (Du et al. 2004; de Giorgi, 1991; R\”oger and Sch\”atzle, 2006). The principle of virtual work gives rise to a set of partial differential equations (PDEs). The solutions directly yield the forces and velocities of matter at each point in space over time.

E. *Similarities between bilayers, liquid crystals, and interfaces; the use of phase field.* The Ginzburg-Landau formalism has had considerable success improving prior theories for liquid crystals and phase field methods have made it possible to model complicated interfaces between immiscible liquids. These methods can be ported to and adjusted for problems in membrane biology. Lipids within bilayers display positional and directional orders that resemble those of liquid crystals. The classical continuum theories for liquid crystals [Leslie, ref; Ericksen, ref; de Gennes 1974] have been generalized (Lin and Liu 1995; Shen et al. 2002) by phase field and other modern energy minimization methods, and this has led to reliable predictions of liquid crystal dynamics (Liu and Shen, 2001; Liu and Walkington, 2000). By adapting the phase field approach to bilayer membranes, the positional and directional orders of lipids can be calculated as a function of time, yielding the lipid motions that lead to the steps between intermediate states of fusion.

 Biological membranes differ from liquid crystals, however, in that membranes are fluid and create an interface with water. The phase field method has been successfully used to calculate the dynamics of shape changes of interfaces in response to applied forces, between immiscible fluids (Yang et al., 2008). The phase field method does not pre-assign fixed geometries to interfaces, but rather employs time-dependent PDEs that account for physical properties of the fluids, such as their viscosities, to describe the fluids and the interfaces between them. This yields, as a function of time, the geometry of interfaces as an output of the calculations, strategies that can be applied to many biological processes in addition to membrane fusion.

F. *String method*. Phase field finds the minimum energy once a system is within a basin; a different method is needed to calculate the pathway to move from one basin to a higher basin. Biological systems can move energetically uphill since there are many ways to supply energy. For example, prior calculations have shown that, in general, the energy of a stalk is larger than the energy of separate membranes [ref]. The “string method” finds the path that requires the least energy for a system under an external force (e.g., as supplied by fusion proteins) to go from the minimum of one basin to a higher minimum of another basin—the “mountain pass problem.” The path of least energy is, by definition, the one whose tangents are everywhere normal to the gradients of the energy functional. Past efforts by biophysicists have assumed that barriers are surmounted through a favorable confluence of thermal fluctuations, but this is seldom the biological situation. The string method treats the pathway as a greased string that adjusts until the least energy is found for steady state flow. At steady state, the external force that must be applied to surmount the barrier in a given time is exactly the force that is needed to constrain the system to the given path. Regardless of whether every step of fusion uses the path of least energy, determining this path will yield the features that are energetically optimal and will make explicit predictions as to possible molecular functions of fusion proteins in inducing reconfigurations of lipids.

**1.2 Intellectual Merit**

1. A major challenge in the large field of mathematical biology has been to generate paradigms in cellular biology that can be experimentally tested. Classical continuum mechanics has been stalled in advancing biological understandings because it assumes states of equilibrium and particular geometries, and it ignores energy dissipation. Our approach calculates geometries, and does not assume equilibrium, procedures that will overcome the present bottleneck in the accurate depiction of changes in membrane shape and topology during fusion.

2. The solutions of the PDEs that determine forces and velocities everywhere will lead to the development of novel numerical schemes that can be applied to a large class of minimization problems.

3. The geometries of the stalk and fusion pore that yield minimum energies will be outputs, rather than inputs, of the model, permitting even investigators who continue to use traditional continuum approaches to benefit, as they can start with physically realistic configurations.

4. Topological changes are defining features of the process of membrane fusion. Traditional theoretical approaches cannot describe these changes. Combining the Helfrich Hamiltonian, as generalized by phase field, (for the energetic profiles of membranes) and the string method, developed in the last few years, will yield pathways for surmounting energy barriers between topological states. These calculations are a means, not previously available, to obtain descriptions of major aspects of biological membrane fusion. Once other investigators derive the energetic profiles generated by proteins, our adaptation of the string method can be applied to a large class of cell biological processes.

5. The experimental techniques that can monitor lipid orientation—such as nuclear magnetic and electron spin resonance—cannot isolate the small fraction of membrane area that participates in fusion from the bulk of the membranes. The same limitation applies to calorimetric techniques that yield energy changes. The application of phase field theory to yield the dynamics of lipid reorientations in space and time and associated energy changes would reveal the events at the localized site of fusion. This would be a major contribution to the fusion field.

6. Biology poses problems that will require the development of new areas of mathematics. The geometric ‘Y’ created at the junction of a hemifusion diaphragm and the two initial membranes is not a true mathematical surface, but rather is a “rectifiable set,” and so traditional methods applied to interfaces are not directly applicable. The finite element tools we develop (see Aim 3) can lead to new mathematical avenues for rectifiable sets. Also, in using new representations of bilayer structures in Aim 4, we foresee new areas in the calculus of variations, including exploring the existence and regularity of a surface and director field that minimize the sum of splay and tilt energies. The study of these nonlinear and domain-dependent problems will require the development of new mathematical tools that go beyond combining theories of liquid crystals and harmonic maps (Hardt et al. 1986) with those of bending and Willmore energy minimizers (R\”oger and Sch\”atzle, 2006; Willmore, 1993; Simon, 1993).

7. Local and non-local interactions together determine the energy of a lipid bilayer. By combining phase field and liquid crystal theory we will be able to account for both types of interactions, and thus the lipid reorienations that lead to changes in membrane topology can be calculated (see Procedures, Aim 2). This new formalism will also benefit other mathematicians interested in describing biological materials which are not true surfaces, but thin elastic phases.

8. Phase field methods can provide a mathematical description of the heterogeneity of the lipid bilayer portion of a membrane across its thickness. Once we accomplish this for the lipids of the membrane, the method can be extended to account for the proteins embedded in the bilayer by introducing additional phase parameters. This would considerably increase computational complexity, but would provide an initial way to include membrane proteins and biological actions in new physical models.

**2. Specific Aims**

1. *The string and phase field methods will be combined to calculate the energetically most favored pathway toward membrane hemifusion*.

2. *The phase field method and liquid crystal theory will be used to create a new membrane model that will determine lipid rearrangements during the formation of a pore in a single lipid bilayer.*

3. *Finite element methods will be used to determine the location where a fusion pore is most likely to form within a hemifusion diaphragm*.

4. *Steepest descent and force balance equations will be used to calculate the rate of growth of a fusion pore*.

**Aim 1.** *Rationale.* Some pathways are energetically much more expensive than others. We will determine lipid dynamics and energetics for the path of least energy between independent and hemifused membranes. Traditionally, biophysicists have calculated which events are likely to happen when only thermal fluctuations are present. The string method provides a means to discover how external forces effect the transition to hemifusion. Combining the string method, to move between energy basins, with a phase field approach, to reach energy minima within a basin, the minimum energy needed to reach hemifusion will be obtained. These calculations will allow us to generate an animation of the lipid motions that give rise to the stalk and hemifusion diaphragm structures in a way intelligible to non-mathematicians.

*Procedures.* There exist a few methods for calculating a path of least energy, and each has its advantages. The string method (E et al. 2002) is one such method. It has been used primarily to analyze problems in which a dynamical system jumps from one metastable state to another due to thermal fluctuations (E et al. 2002; refs). As an important consequence of the intrinsic definition of the string, the method avoids the stiffness of differential equations that often arises in other methods when successive points on a path are separated by equal distances. It also has good convergence properties, and it can be performed by a splitting procedure [ref].

The string method has been used primarily to analyze problems in which thermal fluctuations drive a dynamic system from one metastable state to another [ref]<<Fred: we are repeating ourselves here>>. The string method, however, is well suited for calculating how a deterministic system is able to surmount an energy barrier even though the agent driving the system over the energy barrier is unknown. We will calculate the energy barrier separating independent parallel bilayers from the hemifused state.

 A hypothetical transition path (although not a minimal one) will be defined by geometrically evolving the initial parallel membranes to the Y-shape of the hemifusion diaphragm , yielding a family of one parameter functions Since membrane configurations are identified by field variables, the configurations may be linearly averaged to provide an initial path. To find the least-energy path, these points are subject to a coordinated, modified gradient descent dynamic whereby points move in the direction of steepest descent, but movement is altered tangentially to adjust for changes in the distance between the points. The benefit of this procedure is that the steady-state path is everywhere normal to the energy contours and hence it is the least-energy path. <<Rolf: it is **the** least energy path and not **a** least energy path?? Fred: it is a least energy path>>

The phase space of the least energy path provides the set of membrane geometries. This space includes the initial planar and final hemifused configurations, as well as all possible deformations and topological changes to these configurations. Using a formalism borrowed from the phase field and liquid crystal tradition, we will identify membrane geometries by two field variables: one phase field function identifies the location of the interface and the second field, a director field, identifies the orientation and length of the lipid molecules. The energy landscape as a function of membrane geometry is defined by a Ginzburg-Landau functional encoding energies associated with the lipid deformations and with steric effects occuring inside the bilayer. Further details of the functionals and the field variables are given in Aim 3.

By graphically plotting the field variables, we will be able to see how the initially parallel bilayer interfaces deform and then apposing monolayers undergo the topological change of merging into the stalk complex. Similarly, we will be able to see how the lipid directors reorient while minimizing the deformation energy. The increase in energy is provided by the evolution of the membrane configuration along the least energy path, allowing us to precisely calculate candidate energy barriers and predict what forces might be involved in these deformations.

In practice, the field variables are discretized by finite differences over a two dimensional grid. On the order of a hundred thousand grid points will provide sufficient spatial resolution. We expect that we will observe meaningful changes to the field variables with hundreds of time steps and will consequently use on the order of a hundred points along the string. This will provide sufficient resolution to obtain the sought saddle-point energy landscape.

To speed up computation time, we will employ a splitting scheme where a gradient descent is performed on each point on the string for a few steps and then the equidistant points constraint is enforced. Since these steps may be done independently, we will be able to employ a parallelization whereby each processor handles one of the points on the string. Because any given string is not far from its equilibrium position on the path, the convergence will not be overly lengthy.

**Aim 2.** *Rationale.* Modeling the pathway for pore formation in a continuous single bilayer involves multi-scale forces and changes in topology. Both are difficult to capture by a comprehensive mathematical theory, but they arise in many areas of membrane biophysics. The phase field method traditionally uses a label to identify bulk material regions, thereby avoiding the explicit identification of regions with varying topology. However, this method applies only to membrane surfaces that separate two (inside and outside) fluid compartments. When a pore is present in a vesicle, there is one continuous aqueous compartment. In the hemifusion configuration, there are three separated aqueous compartments (two intracellular and one extracellular solution for hemifused cells). Also, the phase field model implicitly assumes that the lipids are parallel to the surface normal and that opposing monolayers deform identically, but this does not apply for the morphologies of a pore or hemifusion. A different approach is needed to accommodate the actual situation. We will depart from traditional phase field methods, and instead combine the elements of liquid crystal and phase field theory in an original way to describe a membrane as a thin, ordered material. The directional order of the lipids occurs over a diffuse region, which we refer to as an “ordered diffusive interface” (ODI). The ODI model is flexible enough to describe virtually all known membrane configurations. Our proposed ODI model couples field variables through the energy functional. It has precedents in prior membrane modeling studies of bending energy minimizing vesicles [Du et al., 2004, 2005], vesicles in fluids (Du et al. 2005, 2007; Kim and Lowengrub 2005), multicomponent membranes (Wang and Du, 2006; Lowengrub et al, 2007) and calculating topological indicators (Du et al., 2005, 2007). But ODI describes a much broader range of lipid deformations, including those of stalk creation and pore nucleation in membranes. The idea behind the ODI model is to define a mean field bilayer energy with local and nonlocal interactions.

*Procedures*. The primary local interactions, similar to those of liquid crystal theory, consist of lipid deformations at the lipid-water interface. To account for these interactions we define

Here is the lipid orientation for a lipid director , and is the phase field parameter labeling the lipid phase as and the water phase as In prior theories, the bilayer is a level *surface*; we label the lipid core of the bilayer and the lipid directors by *bulk* field variables. The first term is the splay energy density where is the bending modulus and the spontaneous curvature. The second term is the tilt energy with modulus . Tilt measures the degree to which the lipids are aligned with the normal of the water-lipid interface. Splay and tilt are multiplied by a surface energy density because lipid molecules impart a directional order only at the surface of the water-lipid interface, close to the neutral surface. Unlike liquid crystal molecules, lipid molecules can stretch and compress. This will be accounted for by including a term to restrict the range of stretch/compression; a surface tension constant will account for the lipid-water interface surface energy. The above energy functional is not an exhaustive description of a biological membrane, but it does encode the essential, local interaction energy of a piece of lipid monolayer. Additional effects such as twist, spatially varying spontaneous curvature, volumetric and surface incompressibility, temperature, and electrostatic dependencies, can be incorporated by modification of the integrands. We will numerically stabilize the energy functional to ensure its coercivity and avoid non-physical singularities.

The nonlocal interactions are what really distinguish membrane bilayers from liquid crystals. These interactions are steric effects stemming from the formation of voids (interstices) and interdigitation of lipid molecules. It is the combination of these two functional relationships which gives the bilayer a thickness:

The calculation of the void penalty is based on the formula for finding the minimum of a function: where The formula assigns an exponential weight to a point in the bilayer core if it does not lie on a lipid molecule. Such a point represents a void. The interdigitation term assigns a repulsive potential between the hydrophilic head group of one lipid and the hydrophobic tail group of another. Our definition of the nonlocal interaction yields a bilayer thickness by joining monolayers along their tail groups. Our approach is, to our knowledge, a completely new strategy to model membranes. All the necessary physics is contained in the functional relationship of the total energy—the sum of the local and nonlocal energies—with the field variables, allowing us to determine how the lipids rearrange in pore formation, and to calculate the energies involved. The energy functional is encoded numerically using finite differences; we take advantage of symmetry by assuming an axial symmetric configuration.

Experimentally, increasing surface tension of a membrane (e.g., by osmotic swelling of vesicles) induces pore formation, relieving stresses on the membrane. By treating surface tension as an increasing parameter while simultaneously allowing the system to evolve along the path of steepest descent with respect to the ODI energy, we can faithfully imitate the experimental procedure of creating pores and examine the behavior of the in-silico bilayer. Increasing surface tension will produce one of two effects: either the bilayer remains intact because the initial planar configuration lies in a shallow energy basin, or the lipids spontaneously deviate from parallel order, most likely along the axis of symmetry, leading to a small pore.

If, in experimental reality, a bilayer is locally stable, pore formation would occur as a consequence of thermal fluctuations. That is, the small holes that form in the bilayer would be a result of thermally induced in-plane lipid motions. But thermal fluctuations are not incorporated in continuum models. If the bilayer is locally stable in the simulation, we would employ the string method to find the minimum energy path connecting the planar and punctured state, yielding pore formation as a result of thermal fluctuations. We would also determine the depth of the basin by a stability analysis on the total energy’s second derivatives, and use this determination to ascertain what reasonable perturbations to the lipid order and lipid-water interface are needed to push the bilayer out of the basin.

**Aim 3.** *Rationale.* Identifying the reasons some states of hemifusion lead to pore formation while others do not could have important consequences in our understanding of the biological factors controlling fusion, and would shed light on processes as diverse at neurotransmitter release and viral infection. One parameter that may control the energy for pore formation is the angles of the ‘Y’. We will analyze how pores form in hemifusion diaphragms, and determine the energy barrier against pore formation as a function of the pore’s location, either within the interior or along the rim of the diaphragm. Because of their Y-shaped cross sections, hemifused membranes are not mathematical surfaces. Problems of minimization of energy of shapes that are not surfaces are not commonly addressed by mathematicians, and they pose significant analytical challenges. We have developed a novel finite element representation of a membrane. In essence, each monolayer of a bilayer will be represented by a piecewise linear map. The functional for the energy of the hemifusion diaphragm will be calculated and the shape of least energy and lipid orientations for this shape will be read from the minimizers. We will develop a means to define finite elements over a space with nontrivial topology. We refer to it as a “topological finite element” (TFE) method (Nilima Nigam) The TFE method will allow us to determine the shapes of the boundary between the unfused portions of the two membranes and their connections to the hemifusion diaphragm, the ‘Y,’ before and after pore formation.

*Procedures*. If a pore is not situated in the center of a hemifusion diaphragm, the system is not axially symmetric. A two-dimensional finite-element method to approximate membrane shape avoids the computational difficulties presented by a fully three-dimensional calculation. There remains, however, the problem that a hemifused membrane cannot be parametrized over a single planar domain. Rather, two annular domains are needed to represent the unfused portions of the effector and target membrane and a circular domain is needed to represent the diaphragm. To connect the domains, we will use a gluing procedure which not only reduces the problem to two dimensions, but also yields the position of the membrane monolayers and the orientation of the lipid molecules.

 Developing mutliple ways to parameterize a bilayer has considerable merit, both biologically and mathematically. Lipid monolayers are essentially incompressible in area and volume, and thus have constitutive relations that are quite different from other fluid interfaces. For this Aim, we will parameterize the mid-plane (the surface where the lipid tail groups meet) and neutral surfaces by piecewise linear (PL) functions. (The neutral surface is the surface for which the deformations of splay and tilt are independent of each other. Experimentally, it lies along the glycerol backbone of lipids, just below head groups [ref].) This parameterization implicitly eliminates the formation of voids, which can arise in some monolayer representations, causing energetic quandaries [refs].

The coupling between the neutral and mid-plane surface is provided by the incompressibility condition: for a constant area per head group, the infinitesimal ratio of volume to area is equal to the height of a planar monolayer. A penalty method enforces this constraint. The functional



provides a mean square measure of the monolayer incompressibility. The neutral surface is composed of triangular elements with area and where is the volume of the prism spanned between the neutral and mid-plane surface. For large values of the monolayer becomes effectively incompressible.

The total energy of the membrane consists of the splay and tilt energy densities summed over the TFE surface. We do not expect the monolayers to undergo sharp deformations in the shape calculations, and thus we anticipate that a uniform conforming mesh will be sufficient to resolve minimizers. In fact, the minimal energy shapes observed in our preliminary axially symmetric studies were quite smooth. But if sharp gradients do form and require resolution, an equipartition of energy algorithm will be utilized whereby the mesh generator will be passed through with a weight proportional to the energy density possessed by each triangle, and this will be used to further subdivide the mesh. Convergence tests will be performed to ensure that the shapes are stable with respect to the mesh parameter. Our version of bilayer minimization involves first order, elliptic terms, and here finite element convergence theory is well established (Textbook).

 The model is complete once boundary conditions are specified. The Y-shaped junction is formed by requiring the values of the neutral and mid-plane surfaces to agree on the boundaries of their respective domains. In a similar fashion, the pore is introduced by inserting a hole in the originally circular diaphragm domain. In practice, the boundary condition is affected by identifying nodes in the mesh adjacency matrix. Thus, we will be able to extend the planar PL functions to a nonplanar domain. Using this model, we will encode arbitrary membrane shapes with and without a pore, a major improvement over prior studies where the membrane shape was assumed [ref]. Furthermore, necessary physicalities such as incompressibility are built into the model. Because the hemifusion diaphragm is only a few nanometers in diameter, hydrodynamic forces are small and should be less consequential than the thermal fluctuations that occur within the diaphragm. If, however, we were to unexpectedly find otherwise, the TFE representation can be incorporated into a fluid mechanical immersed boundary method.

Physical outcomes (e.g., What type of hemifusion diaphragm leads to pore formation?) are highly sensitive to energy gradients. Thus, it is of the utmost importance to accurately calculate energy dependencies as functions of several physical parameters. The TFE method will allow us to determine these dependencies precisely, because it enables us to determine detailed information about membrane shape: the angles of the Y-junction, the geometry of the unfused membrane, the profile of the pores, the orientation of lipid molecules, and where energy is concentrated. The difference in energy (after pore) (prior to pore) will allow us to predict the most likely site of pore formation and how the energies depend on factors such as angles of the Y, spontaneous curvature, surface tension, and diaphragm diameter.

The one potential difficulty that could arise from a TFE analysis would occur if, mathemtically, there is no minimal or metastable pore position, or if the ’Y’ shape is not stable. This result could reflect experimental reality, or could be a consequence of incorrect physical assumptions placed in the model. If these instabilities occur, pore position will not be obtainable, but the solution of the gradient flow equations will still yield the time courses for a pore to reach its minimum energy.

**Aim 4.** *Rationale*. The growth of fusion pores is an energetically uphill process. Mathematically, fusion pores would shrink without an external force because the energy of an hourglass-shaped membrane is asymptotically proportional to its radius. Extensive experimental and theoretical investigations have not yet determined the nature of the external forces biology provides for pore growth to occur. In addition, pore growth must be damped by hydrodynamic forces: when fusion pores grow, the displacement of the viscous membranes and the surrounding aqueous medium water must produce dissipations which slow the growth of the fusion pore. However, past theoretical treatments of pore growth have neglected consequences of aqueous viscosity. We have recently shown experimentally that ignoring aqueous viscosity is certainly an incorrect assumption: increasing aqueous viscosity from the normal 1.1 cP (Fig. ??, light line) to ~30 cP (heavy line) significantly slows pore growth.

*Procedures.* In order to model the fusion pore, we will use an axially symmetric version of the bilayer model from Aim 3 to describe the pore and its energy, again parameterizing by piecewise linear functions. The splay and tilt energy density are integrated over the surface and the penalty formulation is included in the total energy to enforce the incompressibility condition. This method has the advantage that we can numerically track an extensive portion of the membrane using relatively few unknowns in the equations of motion.

A primary objective is to determine what experimental factors promote pore growth. Changing the value of a parameter may change the minimal energy shape of the membrane. If it does, the minimal energy of the new shape is greater than the minimal energy of the original shape. But there is no way to know, *a priori*, how a change in a parameter’s value will affect the energy landscape. In other words, explicit calculations are needed to determine how changes in forces alter the energy landscape. Because fusion proteins can alter local material properties of lipid mono- and bilayers, we will introduce a material label and use the label to define a spatially varying spontaneous curvature or varying surface tension. We will solve the equations of motion by setting the surface velocity fields (for the mid-plane, distal, and proximal surfaces) proportional to the first variation of the modified bilayer energy. Similarly, we will study the effect of applying inhomogeneous Dirichlet and Robin conditions to the director and lipid surface, respectively. This will model changes in contact angles caused by proteins inserting, either partially or fully, into the bilayer.

To study the effect of viscous dissipation on fusion pore dynamics, we will include the velocity field of the external fluid. As is common for fluid-interface problems, the challenge is to couple the flow field with the bilayer as defined on different spatial grids in a way that dissipates the total energy of the system. We will use an idea similar in spirit to the immersed boundary method [ref]. But rather than define a force on the velocity grid by convolution, as in the immersed boundary method, we will define the force implicitly on a test vector field. Specifically, if is the total energy of the membrane and is a piecewise linear + bubble or piecewise quadratic finite element velocity function, we define the force by the equation . The term is the standard inner product over the computational domain, and is the following operation: shift each vertex in the piecewise linear description of the bilayer by the value at the vertex; the derivative is numerically differentiated.

The in-plane viscosity of a lipid membrane is at least one hundred times that of water. Moreover, membrane monolayers are not stuck to each other, but may slide against each other (i.e., intermonolayer friction). To account for these forms of friction, we define a dissipation functional where is the domain over which the integrand is computed, is the fluid velocity, and is a spatially varying tensor field defined by the position of the bilayer and the orientation of the directors. (We still need to write down the equation for ). The momentum balance equation



is derived from the maximum dissipation principle. It states that the sum of conservative and dissipative forces is zero. Combining the momentum balance equation, the fluid incompressibility condition, and the condition that the bilayer moves with velociy **u** yields the equations of motion.

As part of our mathematical description of pore expansion to determine how biology could control of fusion pore growth, we will obtain realistic energetic values of fusion pores. Our preliminary calculations show that energies of pore geometries that have been assumed in the past are tens of higher than for the minimal energy pore shape. Because lower energies greatly enhance the likelihood that a pore will enlarge, our calculations show that fusion proteins can exert much smaller forces to promote pore expansion than has been realized. We will obtain characteristic asymptotic bilayer shapes for fusion pores; biophysicists may then incorporate these shapes into their own calculations of pore growth. This will be a significant advance over pore geometries assumed in the past and still used today [Chiz; Jackson].

We have proposed three different models of lipid bilayers (ODI for Aims 1 and 2, TFE for Aim 3, and a surface representation for Aim 4), choosing according to the problem posed. The question naturally arises: is our choice of mathematical representation purely of convenience, in which the outcomes of more complex calculations would still be the same? All three of our bilayer representations can, with varying degrees of computational difficulty, be used to predict pore growth. We will therefore also use ODI and TFE representations for this Aim to confirm that all lead to essentially the same predictions. If any differences arise, we will determine why they occur.

**3. Past Developments Leading to the Present Proposal.** In this section, we describe some prior theoretical studies of the PI using classical continuum mechanics to describe aspects of membrane fusion.

*A pathway for stalk formation*. To determine the energy barrier that must be surmounted for hemifusion to occur, we postulated a specific pathway and then calculated the consequences. Electron microscopy shows that membranes approach each other locally by protruding into “nipples” [refs]. Hydrophobic surfaces attract at small distances with a characteristic length of ~1 nm (refs), and so we considered the appearance of two hydrophobic patches—one within each of the apposing monolayers of two bilayers which we envisioned were biologically created by fusion proteins—directly opposite each other as a function of distance *l* between the tips of the nipples. The hydrophobic energy (*dWf*) favorable for attraction is aided by the work performed by the protein, –*Fpdl*, and opposed by the repulsive energy of hydration (*dWh*). We calculated the energy barrier, *W*, that must be surmounted for the hydrophobic patches to merge into a stalk, where *W = Wh + Wf + Fpl*. We obtained *Wh* from a standard equation [ref]. We estimated the energy provided by a fusion protein as *Fp ~ (Wp – Wn)/Lp*, where *Lp* is a length that characterizes protein movement during conformational changes and *Wp* is the energy released by these conformational changes. The process of membrane merger follows from the calculated energy surface which has a saddle-shaped topology; the equipotentials of *W(rf, l)* are shown in Fig. ??. When the membranes are separated by a large distance, hydrophobic patches do not form and the membranes do not attract. If the nipples approach each other through a fluctuation, hydrophobic patches form, promoting greater approach and in turn the radius of the hydrophobic patch (*rf*) become larger. In this process of positive feedback, as *l* decreases further, patches become larger and at a critical *l*, the hydrophobic attraction dominates and the tips of the nipples merge. The height of the energy barrier separating the membrane and the stalk for the most favored pathway of our model was between 35 and 40 kT.

 In this study, we used traditional methods, which necessitated the imposition of a physical assumption on the system, here apposed hydrophobic patches, to drive hemifusion. Using the string method, a priori assumptions will not be needed and the lipid reorientations, including possibly the creation of hydrophobic patches, will be output consequences of the calculations (Aim 1).

*Growth of fusion pores*. We have explored how the physics of membrane bending controls the growth of a fusion pore, including some consequences of energy dissipation caused by lipid movements. We assumed that a pore has a toroidal shape and that the distance between the two original flat membranes outside of the torus, $2H$, remained fixed. In order for the pore to expand, a net influx of lipid from the planar membranes into the wall of the pore must occur because the surface area of the toroid increases. This requires a redistribution of lipid between the planar and toroidal portions of the membranes. Because we assumed a specific geometry, we were able to calculate steady state lipid velocities and thereby obtain the associated dissipated energy, using experimental values of membrane viscosity.

 We used Lagrange’s equations with dissipation (Goldstein, 1950) to describe motion in the system. We separated into two terms, one for dissipation due to shear of lipid movement within a monolayer (i.e., intramonolayer friction) and the other due to intermonolayer friction. The symmetry properties of the lipid velocity distributions results in the dissipation rates caused by both shear and relative friction to separate into dissipation caused by lipid flow through the pore (sometimes referred to as trans-pore flux) and dissipation caused by pore growth.

We showed that trans-pore flux and pore growth are independent of each other. This conceptually useful result occurs mathematically because terms associated with pore expansion and lipid flow appear additively in expressions for the energy and the dissipation function, without cross-multiplication terms. Physically it occurs because the trans-pore flux of lipid between the two membranes does not lead to an increase in the surface area of the pore. We also showed that the pore velocity,$\frac{dR}{dt}$, is given by where *(R)* is the effective line tension of the fusion pore, $W\_{b}$ is the energy required to bend the appropriate portions of the two planar membranes into a curved pore, and ** is the sum of tensions applied to each of the membranes. For bending energy alone (i.e., ** = 0), the pore will close. The effective line tension is given by . The equation for growth of the fusion pore is formally the same as the expression for velocity of a pore within a single bilayer membrane with effective two-dimensional viscosity of  (Refs, Deryaguin and Gutop, 1962; Deryaguin and Prokhorov, 1981). Whereas line tension of a pore within a single bilayer is usually assumed to be independent of pore radius, our explicit calculations showed that the line tension ** of a fusion pore is dependent on pore radius, *R*. The explicit equations we derived for movement in radius space have the form of standard Langevin equations, showing that the growth of a toroidal pore can be thought of as a quasiparticle that both diffuses and migrates in radius space in response to applied forces.

Aim 4 of the present application proposes to calculate pore growth in a more systematic fashion, without assuming or fixing pore geometry and calculating the consequences of accounting for aqueous velocity. We will allow fusion proteins to alter spontaneous membrane curvature at the site of the pore, and by deriving the corresponding Langevin equations determine whether pores can grow without having to impose external forces such as tension.

**4. Broader Impacts.**

*Promotion of learning.*Our collaborations have engaged undergraduates from Fordham University. So far, seven students have been mentored and trained--during the course of two summers—through participation in our research program. When students enter the program, they think they do not have the background to contribute to solving the problems we propose. We discovered that they have compartmentalized their classroom experiences and think of the knowledge they have learned only in terms of the courses they learned it in. The biological problems these mathematics students are presented with in our program are certainly beyond the textbook exercises they have expertly learned to solve. After we have interactively and iteratively “translated” the biology into physical language and mathematical equations, they begin to realize that they have already learned, through their various course work, many of the tools they now need to think “outside of the box.”

 During the course of the program, the students learn that they do not have to completely master a subject before they can apply it. We found that their first inclination is to systematically go through an entire book chapter by chapter, as in course work, because they think this is necessary to correctly apply a mathematical technique to their research problem. We teach them a new way to learn: to pull out the understanding they already have in a range of areas, identify what additional information they need and gather it, and integrate all this knowledge into a construct that is their own. By the end of the research collaboration, these students have started thinking independently; they have acquired an understanding of what professional scientists and mathematicians actually do; and they have made a transition from passive participants in their own education to becoming contributing investigators in current, ongoing real-world research questions. Students have been pleased, even amazed, that they have learned and accomplished so much in so little time. The boundaries between courses have been broken, and connections have been made that previously they did not imagine. Our students have told us of the tremendous satisfaction they’ve derived from this experience, and some have said that it has been the best summer of their lives. Since our approaches have been highly successful, we propose to continue this type of mentoring program as part of the present application.

*Training.*Our students will be directly involved in answering the scientific questions of the present proposal. As have students in the past, they will learn enough classical differential geometry, differential equations, biology, and physics to make meaningful contributions to our reaseach program. They will become familiar with aspects of the cell biology of membrane fusion, thermodynamics, how to calculate an Euler-Lagrange derivative, and develop an enhanced appreciation of the utility of the physical principle of energy minimization. As direct contributions to the goals of the present proposal, they will write original mathematical software to solve differential equations and process data. For routine problems, such as solving systems of ODEs, they will program in Octave, a Matlab-like software. Within the context of this high-level programming language, they will become familiar with declaring variables, loops and conditionals, declaring functions, and calling built-in routines. They will program in C for more elaborate problems, such as finding the minimal energy shape of a fusion pore and finite difference schemes, and will solve large scale, linear systems of equations. They will obtain and install/compile the software and thereby learn to work in a UNIX-Terminal type environment. They will document their findings in LaTeX, will prepare presentations and posters by using the Beamer class, and use GNUPLot to graphically portray data.

Over the course of the subsequent academic year, they will present their work in undergraduate research symposia. Projects that yield publishable results will be followed by an abstract to the annual Biophysical Society Meeting. The students will be encouraged to be the presenters and will be co-authors of professional journal articles.

*Teaching.* The summer students meet with the Investigators for in-depth discussion on a daily basis. They make a biweekly presentation of the work in progress and receive feedback from the senior personnel. They attend Journal Clubs and Seminars that are ongoing in the Dept. of Molecular Biophysics and Physiology at Rush, gaining awareness of research areas in biology. So that students enhance their understanding of the scientific area in which they are engaged, they spend time viewing laboratory experiments and process the derived experimental data. We instituted this laboratory aspect this past summer, and it greatly aided the students’ ability to understand biophysical/biological research papers and to associate mathematical outputs with experimental results. The physics and biological proficiency at Rush combined with the mathematical expertise of Dr. Ryham provide the students with an everyday intellectual richness that they do not normally experience during their academic year.

*Opportunities.* This past year, 11 students applied for the program. We required each prospective student, as part of the application process, to review some pertinent biophysical and mathematical material beforehand. Based on the effort they put into understanding the material and on their successes in Mathematics courses at Fordham, three of the students were chosen by Dr. Ryham.

We especially welcome participation by underrepresented minorities. Through active recruiting, we have maintained and will continue to maintain an equal participation rate by women—of the seven students in the past two years, three have been women and one of them (an immigrant from Belarus) has begun a Ph.D. program in mathematics. One of the students this past summer, a Mexican-American, now plans to apply for a Ph.D. in mathematics. Our students have also included a Korean and an Albanian immigrant. All three students of this past summer, entering their senior year at Fordham, now plan to apply to programs for advanced degrees.

**Results of recent papers from the collaboration of the senior investigators with summer students.** *Aqueous viscosity is the primary source of friction in lipidic pore dynamics* (ref)*.* Membrane viscosity is at least 100 times greater than water viscosity. It has consequently been assumed that membrane viscosity generally dominates dissipative processes and aqueous viscosity is relatively unimportant. For example, past investigators describing the growth of a pore within a liposome have always made this assumption [refs]. We have shown that this assumption is not valid, and, in fact, causes essential physics to be missed.

 Experimentally, the dynamics of pores within a giant liposome (> 20 m in diameter) under pressure follows a three-phase pattern (Fig. ??, crosses). In the first observed phase after a pore forms, pressure within the liposome induces rapid pore enlargement. The pressure also causes an outflow of the internal aqueous solution. As the force of the ever decreasing pressure becomes balanced by pore edge energy, the pore radius reaches its maximum value. In the second phase, the pore slowly shrinks as the pressure promoting pore enlargement becomes less than the edge energy which promotes contracture. When the pressure has effectively collapsed, the third phase, rapid pore closure, is observed. The long-standing theory in the field (referred to as BGS, based on original authors’ initials) accounted for experimental data through fitting parameters, but ignored what turns out to be a dominant limiter of pore expansion—aqueous viscosity.

We (including one of the summer students) formulated a new theory that quantitatively matches data and does so by using experimentally measured aqueous and membrane viscosities, without any free parameters. We accounted for energy dissipation in the aqueous and membrane solutions through the equation $η\_{s}rr^{'}+ 2η\_{m}r^{'}= σr- γ$ , where $r(t)$ is pore radius, $r^{'}= \frac{dr}{dt}$, $σ $is membrane surface tension, $η\_{s}$is the viscosity of the aqueous solution, and $η\_{m}$is membrane viscosity. The first term $Cη\_{s}rr^{'}$is critical: it accounts for the lateral stresses generated on the bilayer as water movement shears along the dilating or shrinking pore. $C$ is a coefficient independently obtained by directly calculating the friction for a changing radius of a circular hole in a two-dimensional sheet surrounded by water. We invoked conservation of mass—the rate of volume of the internal solution leaving a liposome of radius $R$,$-\frac{d}{dt}\left[\left(\frac{4}{3}\right)πR^{3}\right]$, is equal to the flux through the pore—and conservation of lipid before and after pore formation. This led to the equations necessary to determine the three unknowns, $r, R,$ and $σ$.

The fundamental difference in predictions between BGS and our theory, which we named DAV to emphasize the **d**ominance of **a**queous **v**iscosity, is shown in Fig. ??. It shows the experimental record of pore dynamics for a liposome 20 m in radius surrounded by an aqueous solution with viscosity, $η\_{s}$= 32 cP, along with the BGS and DAV fits to the experimental data. BGS has to use $η\_{m}$= 1,000 Poise in order to obtain sufficiently slow kinetics in stages I (fast enlargement) and III (rapid closure). This is an inordinately large $η\_{m}$, about three orders of magnitude greater than experimental values. An artificially large membrane friction is necessary because BGS ignores the shearing of water that occurs as the membrane slides against the aqueous solution during changes in pore radius. DAV theory (solid line) accounts for the time course of pore radius using $η\_{m}$= 1 P, a realistic value for lipid bilayer membranes [ref]. Although both DAV theory and BGS account for the experimental data quite well, they utilize very different values for the physical parameter $η\_{m}$.

The importance of accounting for aqueous friction becomes strikingly apparent when it is varied, as we show in Fig. ?? for $η\_{s}$= 1.13 cP. The curves for both DAV and BGS use their respective values of $η\_{m}$of Fig. ??. Clearly, DAV accurately describes the experimental pore dynamics (crosses), whereas BGS predicts a significantly slower change in pore radius in both the opening and rapid closure stages. For DAV theory, both $η\_{m}$ and $η\_{s}$are true physical parameters, set by their experimental values, and are independent of each other; DAV theory can be directly compared to experimental data.

*A dynamic model of open vesicles in fluids* [ref]. We solved the same problem as above, using the same parameters, but did so through a phase field treatment in order to benchmark the phase field approach for problems in membrane biophysics. A full mathematical treatment has been presented [Rolf]. At its physical essence, we defined a Hamiltonian as the sum of Helfrich, phase field, and lipid alignment terms, given as

We coupled discrete force equations with the Navier-Stokes equations of fluid motion by first expressing kinematic relationships for the field variables in both the aqueous and membrane media. We then calculated forces from variational derivatives. This phase field approach quantitatively yielded the same relationships of pore radius as a function of time as did DAV theory. This supports phase field theory as a reliable formalism to describe phenomena in membrane processes. Field theory has the merit that it yields forces (Fig. ??) and velocities over the entire space, allowing physical mechanisms to be inferred.