Experimental Testing of Models to Determine Bending Modulus in Sperm Acrosomal Membrane Using Cell-Sized Vesicles of Controlled Mechanical Properties

B. Abstract

We propose to use a novel combination of atomic force microscopy (AFM) and micropipette techniques to study the mechanical properties of biological membranes, a materials problem that is of considerable interest to the biophysics community. The type of membrane vesicle to be studied is a good model system for the membranes of living cells, but unlike living cells, such as the sperm cell, the membrane mechanical properties can be adjusted in a predictable way by the lipid, cholesterol and protein content and the geometry is simpler. The sperm cell has been the subject of much research including an on-going Lawrence Livermore National Laboratory (LLNL) LDRD ERI Project as well as other LLNL research at BBRP, and the findings of the vesicle experiments will be relevant. The use of the micropipette to position the vesicle and to adjust its tension will allow more cogent AFM force-displacement measurements. The force-displacement measurements will not only serve as a proof-of-principle of a new technique for the characterization of biomembranes, but they will offer a means to validate the membrane mechanics model developed in the LDRD ERI Project, "Probing the Properties of Cells and Cell Surfaces with the Atomic Force Microscope".

Recent developments in atomic force microscopy now allow the “mapping” of molecular recognition events between single molecules using ligands attached to AFM tips for the recognition of protein receptors bound on rigid surfaces (1-4). Although “recognition mapping” on solid surfaces is of great interest, the real power is in the possibility of “recognition mapping” the surfaces of living cells and tissues. However, in living cells, the sensing elements are not attached to a rigid substrate. Instead, these sensing elements are physically and mechanically coupled with a flexible lipid bilayer membrane, which in turn may be associated with other flexible cellular structures. Thus, in order to successfully perform recognition mapping, “mechanical properties mapping” must be performed as well. Longo’s co-investigators at LLNL (Rudd, Balhorn, and McElfresh) are developing AFM experiments and supporting theory to perform “mechanical properties mapping” of the surfaces of living cells with a focus toward mapping the reorganization of bull sperm cell outer (acrosomal) membranes that occur during development (fig. 1)(5).

Longo’s main goal is to collaborate with the LLNL co-investigators for the purpose of testing experimentally their model for mechanical properties determination (in particular bending modulus) in cell membranes by running parallel and similar AFM experiments on synthetic lipid bilayer membranes of known bending modulus. Specifically, during one year, Longo’s graduate student will use the AFM to perform force-displacement measurements on single lipid component cell-sized (15-30 µm) vesicles of known bending modulus that are held in a micropipette (see fig. 8). The force-displacement data from the vesicles will be used to determine bending modulus by Rudd’s model that will then be compared to the known bending modulus. In addition, Longo will perform simple micropipette aspiration of vesicles formed of the main lipid components of the acrosomal membrane of bull sperm for determination of an average bending modulus that will be compared to the measurement on bull sperm by the LLNL co-investigators. There are many relevant extensions of this work (discussed in text) that could be undertaken in subsequent years.

All investigators on this proposal are in a good position to begin work. Rudd has developed the basic theoretical treatment of the AFM data to determine mechanical properties of cellular and synthetic membranes(5). McElfresh and Balhorn have performed the preliminary mechanical properties testing of the sperm acrosomal membrane (5). Longo’s laboratory is fully equipped and experienced in performing mechanical properties measurements by micropipette aspiration of lipid bilayer membranes and performing AFM of lipid bilayers in a liquid environment (6-9). To the investigators’ knowledge this is the first time that AFM and micropipette aspiration will be combined. Thus, we are in a good position to be the first group to substantiate and prove the validity of using the AFM to perform “mechanical properties mapping” on cellular membranes, which will pave the way for accurate “molecular recognition mapping” in living systems.
C. Scientific Text (4 pages in length)

Background and Goals

The matrix of cellular membranes is the lipid bilayer (fig. 2). This lipid bilayer structure self-assembles in aqueous solution and is a form of smectic liquid crystal. The lipid molecules are laterally mobile, the mobility can be long-ranged and mobility between both leaflets of the bilayer is possible. Lipid bilayers are mechanically elastic and can be stretched by approximately 4% before rupture (lysis). Because bilayers are extremely thin (3-5 nm), they are very easy to bend (very low elastic bending modulus). Micromechanical measurements on cell-sized lipid bilayered vesicles can be performed in a few laboratories (including the PI’s laboratory) to measure lipid bilayer mechanical properties (6, 7, 10-12).

![Fig. 2: Schematic of the lipid bilayer. Upper arrow point to atomistic representations of polar lipid head group and bottom arrow points to hydrophobic tail region](image)

Although the lipid bilayer is the fundamental structure of cellular membranes, real cellular membranes can be much more complex than the one-component lipid bilayer shown in figure 2. Mammalian cell membranes (fig. 3) contain lipids, lipopolysacharides, cholesterol, sphingolipids, membrane associated proteins, integral membrane proteins, and cytoskeletal attachments. In addition, recent studies indicate that the membrane is generally heterogeneous, containing domains in the size range of tens to hundreds of nanometers that seem to serve a variety of functions including bringing selected lipids and proteins into close association and controlling locally the rate and range of diffusion of membrane components. Local alterations in the lateral organization of a cellular membrane can result in a “cascade” effect such as in signal transduction in the cell that can radically alter cellular behavior.

![Fig. 3: Schematic of a typical mammalian cell membrane.](image)

Membrane-associated lipids and proteins often serve a sensory function and these sensory elements are known as ligands and receptors. The organization of sensing elements and their local membrane environment (for example they may be associated with membrane domains) serve vital roles in cell-cell interactions, cell signaling, and cell-surface interaction. In some cases, such as the sperm cell (which is the focus of this proposal), dramatic and rapid changes in the composition of membrane(s) and the location and distribution of receptor proteins occur throughout development (5). In others, slower and more subtle changes can lead to catastrophic cellular degeneration ultimately resulting in grave diseases such as Alzheimers, Parkinsons, cancer, or Multiple Sclerosis (13).

Recent developments in atomic force microscopy (AFM) now allow the detection of molecular recognition events between single molecules using ligands attached to AFM tips for the recognition of receptors bound on rigid surfaces (1-4). By monitoring the cantilever deflection during approach-retraction cycles while the position of the sample remains constant, unbinding forces (i.e the maximum force at the moment of receptor-ligand detachment) have been determined for various ligand-receptor pairs, including biotin-avidin (1), DNA bases (2), antibody-antigen (3), and cell recognition proteins (4). This has made it possible to use a single ligand molecule bound at the tip of an AFM cantilever to map the location of receptors bound on solid surfaces. Although “recognition mapping” on solid surfaces is of great academic interest, the real power is in the possibility of “recognition mapping” the membranes of live cells. Longo’s co-investigators at LLNL (Balhorn, Rudd, and McElfresh) are developing experiments and supporting theory to enable this “recognition mapping” method to be used in the study of the surfaces of living cells with a focus on mapping the reorganization of bull sperm cell outer (acrosomal) membranes that occur during development (5).

Moving recognition mapping onto living cells poses some particular challenges related to the fact that membrane associated sensing elements are not attached to a rigid substrate as was the case for the initial pioneering work mentioned above. Instead, these sensing elements are associated with a membrane, which in turn may be associated with a cytoskeleton, thus mechanically coupling the
energetic and mechanical properties of the sensing element, membrane, and cellular interior. In particular, mechanical coupling to the cytoskeleton can be problematic since it behaves in a complex visco-elastic manner. In the model system of this proposal, the acrosomal membrane of bull sperm, the coupling to the cytoskeleton appears to be negligible which will ultimately simplify the task of recognition mapping.

Thus, in order to successfully perform recognition mapping, “mechanical properties mapping” must be performed in parallel. Preliminary work on mechanical properties mapping of the bull sperm acrosomal membrane has been performed by the co-investigators at LLNL MRI (5). In this work, force-displacement measurements were performed locally using an AFM tip and cantilever to apply and measure force and deformation to the acrosomal membrane (fig. 4). Because the forces applied are small, the deformation can be considered as entirely due to membrane bending (no stretching). Models have been developed by Rudd at LLNL in order to translate the AFM data into values for local bending moduli.

Longo’s main goal is to collaborate with the LLNL co-investigators (Balhorn, Rudd, and McElfresh) for the purpose of testing their model for bending modulus determination of sperm acrosomal membrane. Longo will use AFM to perform force-displacement measurements on single lipid component vesicles of known bending modulus that are held in a micropipette (used to damp membrane thermal oscillations). The force-displacement AFM data from the vesicles will be used to determine bending modulus by Rudd’s model that will then be compared to the known bending modulus. In addition, Longo will perform simple micropipette aspiration of vesicles formed of the main lipid components of the acrosomal membrane of bull sperm for determination of an average bending modulus that will be compared to the measurement on bull sperm by the LLNL investigators. Natural extensions to the AFM work include the addition of cholesterol to stiffen the membrane and make it more “mammalian” and the use of two-component membranes containing single-component domains of controllable size in order to develop and test the capability to laterally resolve domains by their mechanical properties.

Techniques to Be Utilized Here

Micropipette Aspiration: Longo has approximately seven years of experience performing micromechanical measurements by micropipette aspiration of lipid bilayers. In this technique (fig. 5), the membrane of a vesicle or cell is aspirated into a very small pipette tip (inner diameter between 2 and 10 µm). A very wide range of membrane tension can be applied to the membrane (range from approximately .003 dyn/cm to 30 dyn/cm with an accuracy of 0.001 dyn/cm) (10). Area or volume changes of the vesicle can be monitored using optical microscopy with great accuracy. The technique can be used to determine mechanical properties in elastic membranes such as bending modulus, area compressibility modulus, lysis tension and properties such as yield stress, shear rate, and shear viscosity in viscous membranes. Longo has one micropipette set-up and is currently building another set-up. The equipment from those set-ups would be used and combined with the AFM housed in Longo’s laboratory and/or an AFM at LLNL MRI (already equipped with inverted optical microscope).
 Atomic Force Microscopy and Force Measurements: Longo has a total of approximately seven years of AFM experience. She has mainly focused on imaging of lipid bilayers that are deposited onto a solid support and maintained in an aqueous environment (8, 9, 14). More recently, she uses atomic force microscopy to characterize the formation and morphology of lateral domains in lipid bilayers (fig. 7), obtaining sub-nanometer height and several nanometer lateral resolution (8). The AFM characterization is being combined with diffusional measurements (using fluorescence photobleaching) to test theoretical relationships of two-dimensional diffusion in lipid bilayers containing obstacles to diffusion.

For the bending modulus measurements performed by the co-PIs at LLNL, the AFM tip is moved vertically into contact with the sample (in their case, the acrosomal membrane of bull sperm). After the AFM tip contacts the sample, the cantilever is deflected (fig. 4) and the deflection can be converted into the applied force (stress) and membrane displacement in the vertical direction (5). When applied to cells, certain assumptions (e.g. load on tip is a point-load) must be made and a model applied (e.g. as developed by Rudd) to determine the bending modulus from the force-displacement data. The performance of force-displacement measurements using AFM will be new to Longo’s lab. However, collaborations with the LLNL MRI group (in particular Balhorn and his close associate Mike Allen at Biometry), her background in micromechanical measurements, and experience working with the AFM in a fluid environment will allow her to move into this area.

Modeling: The membrane shape is governed by the response of bending, stretching and shearing to the applied force. In the proposed experiments, the external forces acting on the membrane arise from the AFM tip, the micropipette and the surrounding fluids. The AFM tip exerts essentially a point force on the membrane. The interior and exterior fluids exert normal forces due to the pressure difference across the membrane, which is especially important in the micropipette where it determines the projection length. The micropipette also exerts forces due to the constraint of the geometry of the tube. The new model developed at LLNL by Rudd uses techniques from solid mechanics to compute the deformation of the membrane under these external forces. Because the resulting equations are fourth order partial differential equations, a new type of finite element analysis has been employed in order to ensure proper convergence of the solution. A simpler model has also been developed for the case of an axially symmetric deformation. Both of these models allow the study of more complex deformations than typically studied in the literature, and in particular they go beyond pure micropipette aspiration. They are well suited for AFM indentation, with the cell or vesicle held either by a rigid substrate or with the micropipette.

Proposed work
Instead of using a bull sperm cell, Longo will perform AFM force-deflection measurements on bilayer vesicles, of known membrane mechanical properties, that will be held simultaneously in a micropipette at a low membrane tension (see fig. 8 for a schematic). Holding of vesicles in the pipette during AFM measurements is necessary and provides useful information: 1) when the AFM tip is pushing down on the vesicle, the accompanying area/volume ratio increase in the spherical section can
be easily accommodated by a decrease in the projection length in the pipette thus no stretching will occur (fig. 8B), 2) the holding pressure will be small enough that the membrane is not being stretched but large enough to damp out membrane fluctuations thereby eliminating area changes simultaneous to bending changes and fluctuation of the sample, 3) the change in the projection length in combination with the tip travel can be used to determine the geometry of the indentation into the vesicle (i.e. hemisphere of radius equal to the tip travel, cone of height equal to the tip travel, or geometry equal to the AFM tip). The force-displacement AFM data parallels the data obtained by the LLNL group on the acrosomal membrane of cells. Therefore, we will utilize the model of Rudd (and future developments of the model) to determine from our data, the bending modulus of the membrane and compare it to the actual bending modulus. In addition, as future mechanical testing modes that utilize AFM come on line, we can adopt and test these modes. For example, I have had some discussion with the LLNL MRI group of application of force on cells/vesicles through small magnets and subsequent AFM imaging to determine the shape of the indentation.

For those measurements discussed in the preceding paragraph, we propose to utilize vesicles of single component unsaturated phosphatidyl choline (PC) lipid such as 1-steryl, 2-oleoyl phosphatidyl choline (SOPC) which is a typical background lipid of cellular membranes, and then two component vesicles formed of unsaturated PC and cholesterol which will triple the bending modulus (at 50% cholesterol) (15). For both of these cases, the bending modulus has been previously measured using micropipette aspiration of vesicles and reported (10, 15) (see fig. 6 for measurements taken in Longo’s laboratory of bending modulus of SOPC vesicles, 0% ethanol).

Finally, we will determine through the literature the composition of the outer acrosomal membrane of bull sperm (16). Longo will form vesicles containing similar mixtures and then use micromechanical measurements to determine the average bending modulus. Nominally, this will give the LLNL group a basis for comparison to their bending modulus measurements on sperm acrosomal membranes.

Natural extensions for future years of collaboration include more complex membranes that will parallel more closely the bull sperm acrosomal membrane. For example, we intend to apply our techniques to vesicles composed of components with different mechanical properties (e.g. fluid lipid component and gel lipid component) that do not mix and instead form coexisting domains/grains (see figure 7). We can perform micromechanical measurements using micropipette aspiration alone to determine average mechanical properties, but the AFM force-deformation measurements may allow us to laterally probe (“mechanical properties mapping”) the mechanical properties of individual one-component domains that we can then compare to the pure components and the average of the mixed components. These measurements will fit in very well with diffusional and morphological measurements and manipulations that Longo has been performing on similar systems (fig. 7). It will be particularly interesting to compare microstructural properties (such as grain boundary density which Longo is learning to control) to bulk and discrete mechanical properties. Utilizing our current work on co-existing domains, we may be able to control the growth and regrowth of domains in these mixed membrane systems and map their mechanical properties (e.g. domain size and shape), thus paralleling similar future characterizations of membranes undergoing organizational changes such as the bull sperm acrosomal membrane during fertilization measured by the LLNL group (McElfresh, Balhorn, and Rudd).
D. References

1. List all relevant references, within one page.