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The Effect of Vesicle Shape, Line Tension, and Lateral Tension on Membrane-Binding Proteins

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THE EFFECT OF VESICLE SHAPE, LINE TENSION, AND LATERAL TENSION ON MEMBRANE-BINDING PROTEINS

A Dissertation Presented

by

Jaime B. Hutchison

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

DOCTOR OF PHILOSOPHY

May 2013

Department of Physics
THE EFFECT OF VESICLE SHAPE, LINE TENSION, AND LATERAL TENSION ON MEMBRANE-BINDING PROTEINS

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ACKNOWLEDGMENTS

I would like to thank my advisors, Professor Anthony D. Dinsmore and Professor Robert M. Weis, for the opportunity to work on this project and for their unwavering support and guidance throughout my time in graduate school. I could not have asked for better advisors. I would also like to thank my committee members, Professor Lori Goldner and Professor Chris Santangelo, for their comments, suggestions, and support. This work would not have been possible without the help of my colleagues in both the Dinsmore and Weis Labs. In particular I would like to thank Aruni Karunanayake M. for her guidance, advice, and encouragement.

I gratefully acknowledge support from the NSF through DMR-0907195 and the Integrative Graduate Education and Research Traineeship (IGERT) Program in Nanotechnology Innovation.

Finally, I would like to thank my friends and family. Zara Summers, thank you for being a constant source of friendship, understanding, and entertainment and for being an as-needed source of tough love. Joe Ryznic, thank you for your love and support and for reminding me not to take myself too seriously. This work is dedicated to my parents, David and Cindy Hutchison, and to my uncle, James Hutchison.
ABSTRACT

THE EFFECT OF VESICLE SHAPE, LINE TENSION, AND LATERAL TENSION ON MEMBRANE-BINDING PROTEINS

MAY 2013

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Directed by: Professor Anthony D. Dinsmore and Professor Robert M. Weis

Model membranes allow for the exploration of complex biological phenomena with simple, controllable components. In this thesis we employ model membranes to determine the effect of vesicle properties such as line tension, lateral tension, and shape on membrane-binding proteins. We find that line tension at the boundary between domains in a phase separated vesicle can accumulate model membrane-binding proteins (green fluorescent protein with a histidine tag), and that those proteins can, in turn, alter vesicle shape. These results suggest that domains in biological membranes may enhance the local concentration of membrane-bound proteins and thus alter protein function. We also explore how membrane mechanical and chemical properties alter the function of the N-BAR domain of amphiphysin, a membrane-binding protein implicated in endocytosis. We find that negatively charged lipids are necessary for N-BAR binding to membranes at detectable levels, and that, at least for some lipid species, binding may be cooperative. Measurements of N-BAR binding as a function of vesicle tension reveal that modest membrane tension of around 2 mN/m, corresponding to a strain of around 1%, strongly increases N-BAR binding. We attribute this increase in binding with tension to the insertion of N-BAR’s N-terminal amphipathic helix into the membrane which increases
the membrane area. We propose that N-BAR, which was previously described as being able to sense membrane curvature, may be sensing strain instead. Measurements of membrane deformation by N-BAR as a function of membrane tension reveal that tension can hinder membrane deformation. Thus, tension may favor N-BAR binding yet suppress membrane deformation/tubulation, which requires work against tension. These results suggest that membrane tension, a parameter that is often not controlled in model membranes but is tightly controlled in biological cells, may be important in regulating protein binding and assembly and, hence, protein function.
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CHAPTER 1

MEMBRANES: FROM NATURE TO THE LAB

The basic building block of any living creature is the cell. All of the myriad varieties of cells, from bacterial cells, to plant cells, to human cells, share a common ancestor thought to have originated about 3.5 billion years ago (Alberts et al. 2009). Billions of years of evolution have spawned a profusion of cellular shapes and functions, although they share a set of common purposes. All cells must be able to reproduce themselves, maintain homeostasis, take in matter and energy from the environment, and adapt to their surroundings. In order to accomplish these tasks some level of organization is required, from the relatively simple bacterial cells to the usually elaborate eukaryotic cells. The fundamental organizational tool that cells employ to carry out these tasks is the cell membrane. The cell membrane, with a thickness of 4-5 nm (Petrache et al. 2004) and a diameter on the order of microns, is essentially a two dimensional fluid. The cell membrane’s chief function is to act as a barrier between the cell and the outside world. Bacterial cells employ one (gram positive) or two (gram negative) cell membranes, which confine all of the materials the cell needs in order to carry out all the procedures necessary for life and reproduction, the DNA, ribosomes, etc. Eukaryotic cells are much more complex in their structure and contain not only an outer plasma membrane, but have evolved intracellular compartments, or organelles, each with their own membrane that separates them from each other and from the cytosol. The organelles have distinct functions, unique compositions, and notably varied configurations, from the flattened sack shape of the Golgi apparatus, to the rod-shaped mitochondrion, to the labyrinthine
tubules of the endoplasmic reticulum (ER)(Meer, Voelker, and Feigenson 2008) (See figure 1).

![Cell Diagram](image)

**Figure 1.** Artist depiction of an animal cell highlighting most of the organelles. From Biotechnika.org.

Although the various cellular membranes act as barriers, these barriers are neither static nor impermeable. The outer cell membrane must be able to take up raw materials from the environment and expel waste and synthesized materials to the environment, and the organelles must be able to transmit materials amongst themselves as cellular processes are carried out. Everything that comes into a cell or is created in the cell must be sorted and marked for delivery to a particular location. For example, proteins that are synthesized on the ribosomes studding the ER contain particular amino acid sequences called sorting signals that tell the cell which organelle it is destined for. Synthesized proteins can reach their destination organelles by three different processes. First, any protein marked for delivery to the nucleus enters through pores designed to accommodate
only specifically marked proteins. Second, proteins that are loose in the cytosol and marked for delivery to an organelle are aided by specific protein “chaperones” that allow them to unfold and pass through a given organelle’s membrane. Finally, proteins that have been synthesized in the ER and are destined for another organelle or the plasma membrane are carried by “transport vesicles” which bud and pinch off from the ER or Golgi apparatus and then fuse with the target organelle’s membrane (Alberts et al. 2009) (figure 2). A similar process of vesicle budding must occur at the outer plasma membrane whenever material needs to be expelled from or admitted to the cell (known as exocytosis or endocytosis). It is this process that we are concerned with in this thesis. How is the deformation of the various cellular membranes, in particular the budding of vesicles, accomplished?

![Figure 2](image_url)

**Figure 2.** Freeze-fracture views of Golgi cisternae and transport vesicles. Double arrows show vesicles in the process of budding off from the Golgi. Scale bar is 100 nm. Image reproduced from (Orci, Perrelet, and Rothman 1998)

Often, in order to identify the essential mechanisms of membrane transport, model cellular membranes (vesicles) are used instead of biological membranes. These
synthetic systems give the experimentalist control of the composition, size, tension, and other parameters, which are often unknown in a living cell. The simplest model membranes are composed of only a single lipid species, but biomimetic vesicles composed of multiple lipid species, cholesterol, and even membrane proteins are studied in order to understand their various roles in cellular function and how these components influence each other. Surprisingly, quite complex phenomena that mimic those seen in biological membranes have been observed in even the simplest, single-component membranes.

Early studies of single-component vesicles revealed some quite complex behavior even in a simple system. Vesicles made of 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) in water demonstrate dramatic shape changes, including budding and discocyte-stomatocyte transformation, in response to changes in temperature (Berndl et al. 1990) as seen in figure 1.3. DMPC vesicles in glucose can also exhibit spontaneous expulsion of daughter vesicles after irradiation by optical tweezers owing to changes in osmotic pressure (Bar-Ziv et al. 1996).

![Figure 3](image_url)

**Figure 3.** Discocyte-Stomatocyte transition in DMPC vesicles. Temperature increase from 43.8 to 44.1°C from left to right. Image reproduced from (Berndl et al. 1990)
As levels of complexity are added to the vesicle composition it is not surprising that the repertoire of interesting and complex phenomena that the vesicle displays expands. One area of great interest involves vesicles made of two lipid species and cholesterol, which can lead to spontaneous separation of the membrane into coexisting phases. This phase separation may mimic the heterogeneity of biological cells and the model systems that undergo phase separation allow us to probe both the mechanisms of phase separation and its effects on other membrane properties and functions.

The origin of phase separation lies in the structure of the lipids. Lipids consist of a hydrophilic head group and a hydrophobic hydrocarbon tail which allows them to self-assemble into vesicles in the presence of water. There are two distinct groups of lipids, saturated and unsaturated (figure 4). Saturated lipids have hydrocarbon tails in which each carbon atom is bound to two carbon atoms and two hydrogen atoms and are said to be “saturated” with hydrogen. Unsaturated lipids have hydrocarbon tails that are missing at least one hydrogen atom and thus contain at least one carbon-carbon double bond, causing the chains to kink.
Figure 4. Saturated and unsaturated lipids. (A) Structure of the saturated lipid PSM (left) and the monounsaturated lipid DOPC (right). (B) Space filling structure of PSM (left) and DOPC (right). From Avantilipids.com

Saturated lipids, with their straight hydrocarbon chains, are able to interact favorably with each other through van der Waals and other intermolecular interactions—essentially, they are able to pack well together (Dietrich et al. 2001). When vesicles are prepared with a mixture of saturated and unsaturated lipids and brought to a temperature, $T_{\text{mix}}$, they phase separate into regions that are rich in saturated lipids and regions that are rich in unsaturated lipids. The phases can be liquid, solid, or gel-like depending on the degree of order in the packing of their hydrocarbon tails, and can be readily observed by fluorescence microscopy if one lipid species is tagged with a fluorophore (figure 5).
Figure 5. Phase separated vesicle. Epifluorescence image of a vesicle composed of 2:2:1 DOPC:PSM:Chol with 0.8 mol% rhodamine DOPE showing phase separation at room temperature. The rhodamine dye partitions with DOPC into the liquid disordered phase while the dark areas are liquid ordered phases rich in PSM and cholesterol. Scale bar is 10 μm.

When phase separation was discovered in biomimetic vesicles it sparked a flurry of research into lipid “rafts”. The idea of lipid rafts was postulated in the 1970’s when Stier and Sackmann (Stier and Sackmann 1973) suggested that biological membranes were not homogeneous fluids as was previously thought, but rather had island or rafts of more highly ordered lipids dispersed in a less ordered phase. The field of lipid rafts has seen its fair share of controversy. There is still some question about whether or not lipid rafts have been observed, even indirectly, in biological membranes (Mukhopadhyay, Huang, and Wingreen 2008; Jacobson, Mouritsen, and Anderson 2007). Until a few years ago there was not even a consensus about what a lipid raft is; in 2006 at the Keystone Symposium of Lipid Rafts and Cell Function lipid rafts were finally defined as, “small (10-200nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes.” Even if we assume that rafts do exist in biological membranes, the link between the phenomenon of biological rafts and phase
separation in model membranes is tenuous at best as individual domains (rafts) in the latter are much larger and more stable than their biological counterparts. Regardless, the study of phase-separated vesicles has produced an abundance of interesting and useful data including phase diagrams for many lipid mixtures, and behavior of vesicles near critical points (Veatch and Keller 2005a; Honerkamp-Smith, Veatch, and Keller 2009). Evidence that biological cells use phase separation comes from “Giant Plasma Membrane Vesicles” or GPMV’s. In GPMV studies a portion of a biological membrane is chemically separated, or “blebbed”, from the cell so that its composition is similar to that of an intact membrane (Veatch et al. 2008; Tobias Baumgart, Hammond, et al. 2007). Researchers have found that GPMV blebs are at or near critical compositions and that fluctuations in composition are present at physiological temperatures (Veatch et al. 2008).

Incorporating membrane-binding proteins into vesicles allows for the study of the interactions that take place between proteins, the lipids that make up the membrane, and the mechanical properties of the membrane. Of particular interest are various families of membrane-binding proteins. Usually, the protein family that is chosen for experimentation is one which has been implicated in key cellular processes like endocytosis or exocytosis, signal transduction, cell respiration, or cell death. Some examples of membrane-binding proteins that have been utilized in conjunction with model membranes are: the pore-forming Equinatoxin (Schön et al. 2008), the proton pump Bacteriorhodopsin (Dumas et al. 1997), the Ras family of GTPase’s (Nicolini et al. 2006; Vogel et al. 2009), and the curvature sensing and inducing family of BAR proteins
(Peter et al. 2004; Takei et al. 1999). The BAR family of proteins will be the focus of much of this thesis, in part because of their established role in endocytosis.

Even the most complex model membranes or GPMV’s are missing several components that are present in intact biological membranes. The cytoskeleton, whose role in cellular structure and modulation is of paramount importance, is not included in even the GPMV experiments. There are, to date, no experiments that mimic the constant flux of materials, particularly proteins and lipids, into and out of biological cells. Finally, biological membranes often display compositional asymmetry between the two leaflets of the bilayer (Devaux 1991), and this is something that is usually not controlled in model vesicles.

There are many benefits to working with model membranes including the ability to control the composition of both the membrane and the surrounding environment. Giant unilamellar vesicles (GUVS) are particularly attractive as their size is comparable to that of the cell’s plasma membrane and allows for experimentation on single vesicles (Bagatolli 2006). As long as the differences between biological and model membranes are acknowledged, reductionist systems can be employed to answer many questions regarding interactions between specific proteins and lipids, the role that structural parameters, like curvature, play in these interactions, and the influence of mechanical parameters, like wall or line tension, on membrane morphology.
**Curvature: Introduction and energetics**

In order to attempt an understanding of the processes that govern the complex shape alterations in living cells or the shape transitions seen in synthetic membranes we must be aware of the physical principles that are responsible for controlling membrane structure. Viewed from the most simple perspective, a cellular membrane can be seen as a two dimensional elastic sheet in three dimensional space (Seifert 1997; Zimmerberg and Kozlov 2006). As such, the membrane's curvature at any point, P, can be described by two principal curvatures, $C_1$ and $C_2$. These principal curvatures are simply the inverse of the maximal and minimal radii of curvature at point P:

$$C_{1,2} = \frac{1}{R_{1,2}}$$

Where $R_{1,2}$ are defined as the radii of circles which, at point P, have the same tangent and curvature as the curves $C_{1,2}$.

![Figure 6. Maximal and minimal radii. Image reproduced from (Zimmerberg and Kozlov 2006)](image-url)
The principal curvatures can be used to define both mean and Gaussian curvatures:

Gaussian curvature \( G = C_1 \times C_2 \)

Mean curvature \( H = \frac{1}{2} (C_1 + C_2) \)

Although the choice of which sign to assign to the principle curvature is arbitrary as it depends on a definition of the surface normal, it has become convention in membrane biophysics to define a bulge outwards into the surrounding medium as having positive curvature (Zimmerberg and Kozlov 2006). Aside from planes, which have zero principle, mean, and Gaussian curvatures, there are three basic shapes commonly displayed in cellular membranes. First, cylinders, with positive mean curvature and zero Gaussian curvature, are seen in membrane tubules. Second, spheres, with positive mean and Gaussian curvature, are seen in transport vesicles. Finally, saddle shapes, with positive mean curvature and negative Gaussian curvature, are seen during cellular budding processes as a neck region develops between the original membrane and the bud.

In addition to the mean and Gaussian curvature, membrane shapes are also described by their spontaneous curvature, \( C_\omega \). Spontaneous curvature is the curvature that a membrane would assume under no stress. A monolayer composed of lipids whose shape is cone-like would have a negative spontaneous curvature, while a monolayer composed of inverted cone-like lipids would have a positive spontaneous curvature (Fig.7). A bilayer, however, has zero spontaneous curvature provided that the two leaflets are identical in composition. This symmetry between leaflets is often assumed for synthetic membranes, but is almost certainly not the case in biological membranes (Bretscher 1972; Alberts et al. 2009).
Figure 7. Cartoon of various lipid spontaneous curvatures. Lipids are arranged in order of decreasing spontaneous curvature from the inverted cone shaped lyso PC to the cone shaped DOPE. Image reproduced from (Strandberg et al. 2012)

In order for membranes to assume the varied and changeable configurations necessary for function, work must be done to accomplish the deformations. The accepted theory for describing the energetics of membrane deformation, developed by Helfrich (Helfrich 1973), encompasses the spontaneous, mean and gaussian curvatures, and the elastic properties of the membrane. By writing down relevant contributions to the membrane energy and then minimizing that energy we can determine a vesicle’s equilibrium shape.

\[ E_{\text{bind}} = \frac{1}{2}K \int dA \left( C_1 + C_2 - C_0 \right)^2 \]

Additional terms can be added to this description of vesicle membrane energy to account for compression of the inner leaflet and stretching of the outer leaflet (ADE model) (Miao et al. 1994), and for the incorporation of membrane active molecules, like membrane proteins. Chapter 7 will discuss this in greater detail.

**Mechanisms for changing curvature**

Vesicles are usually assumed to have zero spontaneous curvature due to the symmetry of the bilayer. However, if lipid species in one monolayer encounter a change
in composition, through peroxidation of one leaflet or the density of lipid molecules changes, a change in spontaneous curvature may result and lead to a global change in membrane shape (Parthasarathy and Groves 2006). Experiments performed on vesicles composed of an electrically neutral lipid, DMPC, in water showed that striking conformational changes, including budding and discocyte-stomatocyte transitions, upon changing the temperature of the vesicle solution (Berndl et al. 1990). It is proposed that these shape changes are due to local impurities in each monolayer which lead to differences in the thermal expansion between the monolayers. Essentially this means that as the temperature is increased the area of each monolayer changes asymmetrically and this leads to a conformational change via the bilayer couple model (Berndl et al. 1990; Döbereiner et al. 1997). Another way that membrane shapes can be altered by area differences in the monolayers is through osmotic stress. If a vesicle is deflated osmotically, water passes through the membrane and the rate of lipid flip-flop (lipids from one leaflet migrating to the other leaflet) can be uni-directionally enhanced due to water flow. This enhanced flip-flop can cause an increase in the area of the outer leaflet which, again, can change the vesicle’s conformation according to the bilayer couple model (Bruckner et al. 2009). A similar effect can be attained by exposing vesicles to pH gradients (Josephine B. Lee 1999). In this case, the pH gradient causes the translocation of particular lipid species from the outer to the inner membrane and vesicle deformation results in dumbbell or string of pearl conformations which are quite different from the vesicle’s initial quasi-spherical shape. In passing, we note that non-equilibrium effects such as these can be avoided by incubating vesicles for extended periods (24h or more) to allow equilibration of the lipids; this is discussed in detail in Ch.6.
Finally, for vesicles that contain more than one lipid species, phase separation can induce curvature changes. As described in Chapter 1, phase separation occurs as a vesicle is lowered through a particular temperature, $T_{\text{mix}}$. Although vesicles can display either solid-liquid coexistence or liquid-liquid coexistence depending upon the lipid species involved, their relative abundances, and the temperature of the sample, we will be concerned solely with liquid-liquid co-existence. There are several interesting consequences of liquid-liquid phase separation. The first is that phase separation can couple vesicle composition to vesicle curvature in the following way. There is a line tension (energy/length), $\sigma$, which acts along the boundary between the phases. The energy arising from this line tension is equal to the line tension times the perimeter of the boundary. The vesicle can minimize this energy in two ways. Diffusion and coalescence of the domains to form one large domain will lower the total boundary perimeter and thus the energy associated with the line tension. Second, the total perimeter can be further reduced if the domain bulges out from the vesicle as shown in figure 8.
**Figure 8.** (Left) Epifluorescence image of a vesicle composed of 2:2:1 DOPC:PSM:Chol with 0.8 mol% rhodamine DOPE displaying phase separation. (Right) Differential interference contrast image of the same vesicle showing budding of the liquid ordered (dark) phase in order to reduce the perimeter of the phase boundary. Scale bar is 10 μm.

A phase separated vesicle displaying this behavior thus contains domains that differ in both composition and curvature. Proteins or other molecules that bind membranes may prefer one domain or the other for a variety of reasons, or they may prefer the boundary between domains. In any case, phase separation has the ability to couple curvature and composition and this may affect the spatial organization of proteins in the membrane, and proteins, in turn, may affect membrane morphology (Roux et al. 2005; Manley et al. 2008; Mukhopadhyay, Huang, and Wingreen 2008)
CHAPTER 2
TECHNIQUES

GUV preparation

Lipids: All lipids used in LUV and GUV experiments are from Avanti Polar Lipids (Alabaster, AL) or Sigma Aldrich (St. Louis, MO). Lipids are either purchased in pre-dissolved in chloroform or purchased in powder powder and then dissolved in chloroform. Lipids are aliquoted and stored under nitrogen in a -20°C fridge.

Electroformation

Glass plates (25 x 75 x 1.1mm) coated on one side with indium tin oxide (ITO), $R_s = 15-25 \ \Omega$ were purchased from Delta Technologies (Loveland, CO, CG-60IN-S115). One copper wire was attached to each ITO plate with silver conductive epoxy (Chemtronics, Kennesaw, GA, CW2400).

If single component vesicles are desired, lipids can be spread directly onto ITO plates (2 plates per sample). If multiple component lipids are to be used, they should be mixed in a small glass vial. Homogeneity of composition from vesicle to vesicle can be increased if care is taken to properly mix the component lipids before spreading them on the ITO plates. We use a combination of gentle shaking of the vial, and mixing via Hamilton glass syringes. Lipids should be spread onto the ITO plates quickly and evenly as a thin, even layer of lipid seems to produce better quality (unilamellar, between 10 and 50 μm) vesicles. 10-25 μL of lipid is spread in a line at the top of an ITO plate with a glass syringe (Hamilton Company, Reno, NV), and the syringe tip is used to spread the
lipid down the length of the plate. The plate should quickly be inspected for buildup of lipid in any one spot. If lipid buildup is detected, a small amount of chloroform can be deposited on the plate, and the lipid can be re-spread. Once lipid has been spread on both plates they should be placed in vacuum for at least 2 hr to remove any residual solvent.

A Teflon spacer (homemade) is made to fit the ITO plates and has small holes directly opposite each other, meant to fit a 22 gauge needle (Becton Dickinson, Franklin Lakes, NJ). After solvent removal in vacuum, the Teflon spacer is lined with a thin layer of vacuum grease (Dow Corning, Midland, MI) and one plate is attached to the spacer with lipids facing in. The other side of the spacer is then lined with vacuum grease and the second plate is attached with lipids facing in. The chamber is secured with small clips and is filled with any non-ionic solution via one of the holes in the Teflon spacer. Care is taken so that the metal of the clips does not touch the sides of the sample chamber.

The sample chamber is placed in an oven for temperature control and a function generator is attached to the copper wires on the ITO plates. The voltage is set to 2.4 V peak to peak at 10 Hz, and vesicles are grown for 2 hr. Vesicles are slowly removed with a syringe through the same hole used for filling the sample chamber and stored in glass vials.

After vesicle preparation ITO plates are cleaned by being placed in a beaker filled with acetone and subjected to sonication for 10 minutes. The acetone is removed and placed with new acetone and the process is repeated. Finally, the ITO plates are washed and sonicated in DI H2O. The Teflon spacer is also cleaned with acetone and washed with DI H2O.
Gentle hydration

Thin Teflon discs (~1mm thick) are cut to fit in the bottom of 50 mL beakers and roughened with sand paper. Prior to use the discs are cleaned with soapy water, rinsed with copious amounts of DI H2O, and dried.

Teflon discs are cleaned with chloroform immediately prior to use and set on filter paper to dry. Meanwhile lipids are mixed in a glass vial (if multi-component vesicles are desired). 20-25 μL of lipids are deposited in a line at one edge of the Teflon disc via a glass syringe (Hamilton Company, Reno, NV) and the edge of the syringe is used to spread the lipids across the disc in one swift motion. Forceps are used to pick up the disc and place it, lipid side up, in a 50 mL beaker. The beaker is quickly put under vacuum for at least 2 hr to remove residual solvent.

In gentle hydration, lipids are pre-hydrated with water-saturated argon. An argon tank is attached to flexible tubing which is fed through a rubber stopper into a 1L beaker which is about 1/3 full of DI H2O. The beaker is set on a hot plate and kept at 37°C. Another piece of tubing has one end inserted through the rubber stopper about an inch into the beaker while the other end is connected to a large (1mL) pipettor tip (See Fig.9). About 30 minutes prior to removing the beaker from vacuum, argon should be bubbled gently through water at 37°C. Bubbling should be slow enough to see individual bubbles leaving the tubing.
**Figure 9.** Photo of gentle hydration equipment. Argon is bubbled through DI H20 at 37°C. Water-saturated argon flows into a beaker containing a lipid coated Teflon disc. The beaker is loosely covered parafilm.

After removing the beaker from vacuum, the pipette tip attached to the water-saturated argon should be placed inside the beaker (above the disc, but not making contact with it). A square of parafilm is placed loosely over the beaker such that oxygen can easily escape. Water-saturated argon is flown into the beaker for about 30 min. Meanwhile, 25 mL of desired buffer is prepared and brought to 37°C. The beaker is removed from the argon and about 20 mL buffer is slowly added to the beaker and the beaker is sealed with parafilm. The beaker is incubated in an oven overnight at 37°C.
The next day, the beaker is removed from the oven and allowed to come to room temperature. At room temperature a wispy, cloud-like formation should be visible in the fluid above the disc (this cloud is the vesicle suspension). The cloud is harvested with a glass pipette and stored in a glass vial.

**LUV preparation**

A 10 or 25 mL round-bottomed flask is cleaned with chloroform. Lipids are added to the flask and dried with nitrogen while the flask is being gently turned by hand. After the lipids have dried into a film, the flask is put under vacuum for at least 2 hr. After removing the flask from vacuum, 1 mL of buffer is added to the flask and multi-lamellar vesicles (MLVs) are formed by vortexing the flask. The MLV suspension is removed by a 1 mL syringe that fits the extruder (Avanti Polar Lipids, Alabaster, AL). The extruder is prepared with a polycarbonate membrane with pores of a defined size. The membrane is sandwiched between 4 filter supports (2 on each side) and 2 1 mL syringes (one containing the MLVs) are attached to the extruder. The MLV suspension is passed back and forth through the membrane 15 times. Vesicles are stored in glass vials.

As prepared, extruded LUVs will not be spherical unless they are prepared in DI H2O. In order to make LUVs spherical they should be prepared in glucose or another solute that is slowly membrane permeable and allowed to incubate at 37°C for at least 12 hours. See Chapter 6 for more details.
**Sedimentation assay**

Known concentrations of protein were incubated with known concentrations of lipid in the form of LUVs. Sedimentation of 75 μL samples was accomplished by centrifugation at 160,000xg for 30 minutes (Beckman Coulter, Brea, CA, model Optima TLX ultracentrifuge with a TLA 100 rotor). After centrifugation a 10 μL sample of the supernatant was taken and the rest was immediately removed with a pipette. The sediment (pellet) was then resuspended in a volume of buffer equal to that removed and a 10 μL sample was taken.

Separate aliquots (8 μL-12 μL) of both the supernatant and pellet (mixed 1:1 with protein loading buffer) were separately run on SDS-PAGE (10%) and quantified by the gel analysis tool of ImageJ. Any samples that bled into neighboring lanes of the SDS-PAGE gel were discarded. The band appearing in each lane corresponds to the MW of N-BAR and the integrated intensity of that band is determined. The fraction of bound protein was defined as the ratio of the integrated intensity of the pellet to the sum of the integrated intensities of the pellet and of the supernatant.

**Protein purification**

**C-His N-BAR purification**

Residues 1-256 of amphiphysin from *Drosophila melanogaster* in PGEX vector were [obtained from the MacMahon laboratory, Cambridge, UK]. These residues correspond to the N-terminal α-helix and the BAR domain of amphiphysin, which are together referred to as N-BAR. These residues were expressed as His-tagged proteins from a pETite Kanamycin vector in *E.coli* BL21 DE3 cells. Bacteria were grown at 37°C
until OD$_{600}$ was between 0.7-1.2, induced with IPTG (100 μM) and grown at 28°C for 4 hours. Cells were collected by centrifugation (Beckman Coulter, Danvers, MA, model: Allegra 6R centrifuge with GH-3.8 rotor) at 4°C for 30 min at 3500 rpm and resuspended in buffer A (50mM NaH$_2$PO$_4$, 300 mM NaCl, pH 7.5). Cells were lysed with lysozyme (1mg/5mL), gently shaken for 15 minutes, and sonicated for a total of 15 minutes in 5 minute bursts interspersed with icing. Lysate was centrifuged at 4° for one hour at 11,000 rpm, supernatant was collected and centrifuged (Beckman, Danvers,MA, model: LE-80k Ultracentrifuge with SW28 rotor) at 4°C for one hour at 35,000 rpm and the supernatant was collected. His-tagged N-BAR was purified via a Ni-Chelating HiTrap affinity column (loaded with 0.1M NiSO$_4$) (GE Healthcare Life Sciences, Piscataway, NJ, cat. No 17-0408-01). The column was equilibrated with buffer A, washed with buffer B (50mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM Imidazole, pH 7.5), and fractions were eluted with buffer C (50mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM Imidazole, pH 7.5). Purity of the protein was inspected by SDS-PAGE, and pure fractions were combined and dialyzed in buffer A. Protein concentration was determined with a Modified Lowry Protein Assay Kit and a bovine serum albumin (BSA) standard (Thermo Scientific, Rockford, IL, cat no. 23240.).

**GST N-BAR purification**

Residues 1-357 of amphiphasin from *Drosophila melanogaster* in PGEX vector were [obtained from the MacMahon laboratory, Cambridge, UK]. These residues correspond to the N-terminal α-helix and the BAR domain of amphiphasin, which are together referred to as N-BAR. These residues were expressed as GST-tagged proteins
with thrombin cleavage site from a PGEX Ampicillin vector in E.coli BL21 DE3 cells. Bacteria were grown at 37°C until OD_{600} was between 0.7-1.2, induced with IPTG (100 μM) and grown at 28°C for 4 hours. Cells were collected by centrifugation (Beckman Coulter, Danvers, MA, model: Allegra 6R centrifuge with GH-3.8 rotor) at 4°C for 30 min at 3500 rpm and resuspended in buffer A (50mM NaH₂PO₄, 300 mM NaCl, pH 7.5). Cells were lysed with lysozyme (1mg/5mL), gently shaken for 15 minutes, and sonicated for a total of 15 minutes in 5 minute bursts interspersed with iced. Lysate was centrifuged at 4°C for one hour at 11,000 rpm, supernatant was collected and centrifuged (Beckman, Danvers, MA, model: LE-80k Ultracentrifuge with SW28 rotor) at 4°C for one hour at 35,000 rpm and the supernatant was collected. Cell lysate was added to a GSTrap FF column (GE Healthcare Life Sciences, Piscataway, NJ, cat. No 17-5131-02) and GST tag was cleaved with thrombin (20 U/mL in 1X PBS). GSTrap was connected to a HiTrap Benzamidine FF column (GE Healthcare Life Sciences, Piscataway, NJ, cat. No 17-5143-01) and purified protein was eluted. Purity of the protein was inspected by SDS-PAGE and pure fractions were combined and dialyzed in PBS buffer.

**Labeling N-BAR with HiLyte Fluor 488 maleimide**

The HiLyte Fluor (AnaSpec, Fremont, CA, 81164) binds to the N-BAR through a cysteine-maleimide interaction. The thiol group on cysteine forms a stable carbon-sulfur bond with the maleimide. N-BAR from amphiphsin contains 2 native cysteine residues, one at position 66 and one at position 82. Using PCR mutagenesis we created an N-BAR construct with the cysteine at position 66 mutated to alanine and left the cysteine at
position 82 for dye attachment. N-BAR concentration is 100 μM for dye attachment. Dilute in HEPES-NaCl buffer (Per L: 4.766g HEPES, 3.843g NaCl, pH 7.6). Dye is dissolved in DMSO (10mg/mL). Dye is added to N-BAR dropwise while mixing so that ratio of protein:dye is 1:10. N-BAR dye mixture sits at room temperature for 2 hr covered in tin foil. Fresh glutathione (50 mM) is prepared in HEPES-NaCl buffer and added to N-BAR dye mixture for final molar ratio of dye: glutathione of 1:1. Dialyze overnight in 2L HEPES-NaCl buffer. Dialysis solution was switched several times the next day for a total buffer exchange of 4-5L.

**Superfolder GFP purification**

Superfolder GFP with a hexahistidine tag in a pET 28 plasmid with Kanamycin resistance was expressed in *E. coli* BL21 DE3 cells. Bacteria were grown at 37°C until OD₆₀₀ was between 0.7-1.2, induced with IPTG (100 μM) and grown at 28°C for 4 hours. Cells were collected by centrifugation (Beckman Coulter, Danvers, MA, model: Allegra 6R centrifuge with GH-3.8 rotor) at 4°C for 30 min at 3500 rpm and resuspended in buffer A (50mM NaH₂PO₄, 300 mM NaCl, pH 7.5). Cells were lysed with lysozyme (1mg/5mL), gently shaken for 15 minutes, and sonicated for a total of 15 minutes in 5 minute bursts interspersed with icing. Lysate was centrifuged at 4°C for one hour at 11,000 rpm, supernatant was collected and centrifuged (Beckman, Danvers, MA, model: LE-80k Ultracentrifuge with SW28 rotor) at 4°C for one hour at 35,000 rpm and the supernatant was collected. His-tagged N-BAR was purified via a Ni-Chelating HiTrap affinity column (GE Healthcare Life Sciences, Piscataway, NJ, cat. No 17-0408-01). The column was prepared by washing with 15mL of 20% ethanol, washing with 15 mL DI H20, and
loading with freshly prepared 0.1M NiSO₄. The column was then equilibrated with buffer A, the protein was loaded, washed with buffer B (50mM NaH₂PO₄, 300 mM NaCl, 10 mM Imidazole, pH 7.5), and fractions were eluted with buffer C (50mM NaH₂PO₄, 300 mM NaCl, 250 mM Imidazole, pH 7.5). Purity of the protein was inspected by SDS-PAGE and pure fractions were combined and dialyzed (10,000 MWCO) in buffer A.

**Micropipette aspiration**

Pipette tips made of borosilicate glass (World Precision Instruments, Inc, Sarasota, FL) are pulled with a pipette puller (KOPF, Tujunga, CA, model 720). Evenly broken tips of the desired inner diameter (5-10 µm) are made with a microforge (Narishige International USA Inc., East Meadow, NY, model MF-900) equipped with a 10X objective. Pipettes are backfilled with buffer via microfil syringes (World Precision Instruments, Inc. Sarasota, FL, MF34G-5) and attached to the aspiration tubing.

![Diagram](image)

**Figure 10.** Schematic of the micropipette aspiration system. The red circles correspond to open/closed valves. The green circles correspond to three way valves.
Suction pressure is applied by a computer controlled syringe pump (Harvard PHD 2000, Holliston, MA). The pressure is monitored by a pressure transducer (Validyne, Northridge, CA, diaphragm 3-34), read by a demodulator (Validyne, Northridge, CA, CD23), and sent to the computer.

A homemade sample chamber is loaded with 0.1 weight\% BSA and the pipette is slowly positioned inside the chamber. The pipette is zeroed (pipette pressure and reference pressure equal) by opening the front (sample) chamber to air, opening the front chamber to the back (reference) chamber, and opening the front chamber to the pipette. The front and back chambers are manually raised or lowered until small particles positioned near the pipette opening are neither sucked into or blown out of the pipette. If there are no small particles visible, a dilute solution of small beads may be added to the BSA solution. After the pipette is zeroed the syringe pump is calibrated via a Labview aspiration program (Heinrich and Rawicz 2005). The input to the pump is given in volume/time, but the input the user gives to the program is a desired pressure or pressure ramp. The calibration allows for the conversion of pressure input to volume/time output to the pump. Finally, the BSA solution is removed, and vesicle solution is added. While examining the sample for vesicles, a small positive pressure is applied so that vesicles are not accidentally aspirated. Once a suitable (unilamellar, approximately 20 mm diameter) vesicle has been located a small suction pressure is applied to capture the vesicle, and the experiment is performed. Once the experiment is complete the vesicle (if still intact) can
either be sucked completely into the pipette or pushed completely out of the pipette by application of positive pressure.
CHAPTER 3

THE EFFECT OF A MODEL MEMBRANE-BINDING PROTEIN ON PHASE SEPARATED VESICLES

In order to explore the ways that membrane properties, like line tension, may impact protein-membrane interactions, and, in turn, how proteins may alter the properties of vesicles, we introduce a model protein, GFP with a hexahistidine tag, to phase separated vesicles. A vesicle that exhibits liquid-liquid phase separation can lower its energy by deviating from its initial, spherical, shape. The line tension, $\sigma$, acting along the boundary between phases acts like a purse string, cinching the vesicle, while the membrane surface tension, $T$, opposes the cinching. The equilibrium shape is, in part, determined by a competition between these two tensions.

Because many proteins exhibit a preference for either the $L_o$ or $L_d$ phase, phase separation – or even concentration fluctuations that arise under single phase conditions (Veatch et al. 2008) – can provide a mechanism to sort particular lipids and proteins into domains in the membrane (K Simons and Ikonen 1997; Kai Simons and Vaz 2004; Sprong, van der Sluijs, and van Meer 2001; Edidin 2003). The line tension can also play a role in sorting or directing proteins. Dumas et al. (Dumas et al. 1997) showed that bacteriorhodopsin proteins accumulate at the boundary between $L_d$ and gel phases in model membranes. They proposed that the energy of the boundary, which arises from the mismatch of the lengths of the acyl chains of the two lipid species, is reduced by the presence of the protein. Since then, other examples of proteins that localize at domain boundaries have been reported, including ion channels (Alessandrini and Facci 2011; Seeger et al. 2009; Cannon et al. 2003), antimicrobial peptides (Guo, Smith-Dupont, and
Gai 2011), N-Ras (Nicolini et al. 2006; Weise et al. 2010), pore-forming proteins Equinatoxin II (Schön et al. 2008) and Bax (García-Sáez et al. 2007), and according to simulations, lipidated transmembrane WALP proteins (Schafer et al. 2011). The tendency of these and other proteins to accumulate at domain boundaries may enhance their function, as proposed for the ion channels and pore-forming Equinatoxin II.

Viewing this phenomenon at a continuum level rather than a molecular-scale level, the spontaneous adsorption of proteins at domain boundaries should accompany a reduction of the line tension of the boundary, $\sigma$. The magnitude of $\sigma$ (with units J/m) at the $L_d/L_d$ interface can influence the dynamics of phase separation and the size, number, and stability of domains (García-Sáez, Chiantia, and Schwille 2007). A recent study of living human HaCaT cells showed micrometer-sized $L_d$-type domains formed as buds; the authors proposed that the line tension between the buds and the surrounding membrane favors the formation of budded rather than coplanar domains (Vind-Kezunovic et al. 2008). Hence, it may be that the line tension plays a key role in living cells and that domain-boundary partitioning of membrane proteins may be important in biological function. The question then becomes: how much does $\sigma$ change as a result of protein binding to the domain interface? In the case of Bax pore-forming protein, the line tension at a membrane pore (distinct from a domain boundary) was reduced by 2.2 pN (or 40%) according to AFM measurements (García-Sáez et al. 2007). For liquid-liquid boundaries, the line tension is smaller and the effect of proteins may be correspondingly smaller and more difficult to measure. In the N-Ras studies (Nicolini et al. 2006; Weise et al. 2010), the authors indirectly inferred a reduction of $\sigma$ based on the change in height of the Lo phase, but a direct measurement of the magnitude of the change in $\sigma$ and an
understanding of how it depends on the nature of the proteins and their concentration remains an open problem. In order to explore the ways that membrane properties, like line tension, may impact protein-membrane interactions, and, in turn, how proteins may alter the properties of vesicles we introduced a model protein, green fluorescent protein (GFP), to canonical samples of phase-separated vesicles and observed dramatic changes in vesicle shape

**Methods**

**Lipids, GFP, preparation of GUVs**

 Dioleoyl-sn-glycero-3-phosphocholine (DOPC), N-palmitoyl-D-erythro-sphingosylphosphoryl-choline (PSM), cholesterol (Chol) and the nickel salt of 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel lipid) were purchased from Avanti Polar Lipids Inc. (Alabaster AL). Superfolder histagged GFP was isolated with nickel-nitrilotriacetic acid affinity chromatography as described in the literature (Pédelacq et al. 2006) and in Chapter 2 of this thesis. The superfolder variant of GFP essentially eliminates dimerization, even when in concentrated solutions (Pédelacq et al. 2006).

Poly-L-histidine (polyhistidine), used in some experiments (Sigma-Aldrich, St. Louis MO, cat. # P9386), had a 5,000 - 25,000 MW range and an average degree of polymerization of 86. Multiple batches of lipids were used over the course of the experiments.
GUVs were prepared by electroformation in 175 mM sucrose solution, using the technique of Angelova (Angelova et al. 1992) and described in Chapter 2 of this thesis. In all experiments, the DOPC:PSM:Chol ratio was 2:2:1; this proportion was maintained as the amount of nickel lipid was varied (1, 2.5, 5.0 or 10 mole percent). Vesicles composed of 2:2:1 DOPC:PSM:Chol at room temperature (25 °C) are known to exhibit coexistence of two distinct liquid phases, in which the L_d phase is rich in DOPC and the L_o phase is rich in PSM and Chol. We found that the area ratio of the L_o phase to the L_d phase ranged from 0.3 to 1 (0.6 ± 0.2). Our average value for the L_o/L_d area ratio is comparable to that of a published study of vesicles with this composition (Veatch and Keller 2005b)

The hydrocarbon chains of Ni-DOGS-NTA are identical to the hydrocarbon chains of DOPC and should partition into the liquid disordered phase.

![Figure 11](image.png)

**Figure 11.** Structure of DOPC and Ni-DOGS-NTA. DOPC (left) and Ni-DOGS-NTA (right) with identical hydrocarbon chains. [www.avantilipids.com](http://www.avantilipids.com)

**Microscopy of GUVs**

GUVs were observed with differential interference contrast (DIC) and epifluorescence microscopy using a Zeiss Axiovert 200 inverted microscope equipped
with a Plan-NEOFLUAR 100x oil objective and a Hamamatsu CCD camera. To observe
the fluorescence of GFP, a filter set with a 480-nm short-pass filter, a 505-nm dichroic
mirror, and a 535-nm long-pass filter (Chroma Technology Corp., Bellows Falls, VT)
was used with a 100 W mercury lamp. Video was recorded using S-VHS format (30
frames per second) and subsequently digitized. In separate fluorescence imaging
experiments, we also investigated the non-uniformity of the GFP fluorescence intensity
near the $L_v/L_d$ domain boundary.

To visualize GUVs and observe shape changes, an aliquot of sample was placed
into a 120$\mu$L sample chamber and imaged under DIC optics to confirm phase separation.
Once a suitable vesicle was located, a mock experiment was usually first performed: an
aliquot of (an isoosmolar) buffer (2.5-3.0 $\mu$L of 12 mM Na$_2$HPO$_4$, 70 mM NaCl, pH 6 to
7) was introduced to the sample chamber to verify that the change in shape did not occur
as a result of osmotic shock, flow-induced stress, or other nonspecific effects. Then an
aliquot of his-tagged GFP was added (2.5-3.0 $\mu$L 40 $\mu$M GFP in buffer). His-tagged GFP
was in excess under experimental conditions (the molar ratio of accessible nickel lipid to
GFP varied between 0.3 and 0.03). Following injection, the vesicle was imaged with
DIC (for shape analysis) and epifluorescence optics (to verify his-tagged GFP binding).
The sequence of events before and after the addition of GFP was captured on videotape.

Images selected for digitization were those video frames in which the vesicle was
in focus and the radii were at their largest, conditions under which the focal plane
corresponded most closely to the equatorial planes of the two phases. Using this
procedure, vesicle morphology was determined both before GFP addition and following
the addition of GFP, after the vesicles reached steady state. A time series from a typical experiment is shown in figure 12.

![Figure 12](image)

**Figure 12.** Microscope images of a phase-separated vesicle with 2.5 mol % nickel lipid during a GFP-binding experiment. (a,b) DIC images of the vesicle before and after adding GFP, respectively. (c) Vesicle outlines from (a,b) rotated and superimposed to show shape change. (d) Epifluorescence image of the same vesicle after adding GFP, demonstrating that the GFP partitions to the \( L_d \) phase. Brownian motion and convection produced the different orientations of the GUV in the three images. Scale bar: 10 \( \mu \)m.

**Shape Analysis of GUVs**

All of the vesicles chosen for analysis had shapes that were well-described by two spherical caps. This selection ensured that lateral tensions were sufficiently large for the
bending stiffness terms to be ignored (T. Baumgart et al. 2005; Allain and Amar 2005) and a simple, geometric analysis of the vesicles could be performed. Using a method developed by Tian (Tian et al. 2007) we start with a force/length balance in the plane of the domain boundary:

$$T_o \cos(\alpha) + T_d \cos(\beta) = \frac{\sigma}{R_b}$$

Where $T_o, T_d$ are the lateral membrane tension in the ordered and disordered domains respectively, $\alpha$ and $\beta$ are the tangent angles made by the ordered and disordered domains with respect to the domain boundary, $\sigma$ is the line tension at the boundary, and $R_b$ is the radius of the boundary (figure 13).

The definition of Laplace pressure, $P$ (excess pressure inside the vesicle) and a requirement of that it be the same throughout the interior of the vesicle gives:

$$P = \frac{2T_o}{R_o} = \frac{2T_d}{R_d}$$

Where $R_o, R_d$ are the radii of the spherical caps of the ordered and disordered domains respectively.

Combining these two equations in order to eliminate the lateral tension, $T$, results in:

$$\frac{\sigma}{P} = \frac{R_o}{2} \times \left\{ R_o \cos(\alpha) + R_d \cos(\beta) \right\}$$

This equation allowed us to measure the ratio of line tension to the Laplace pressure directly from the shape of the vesicle. Uncertainties were estimated from standard deviations computed for $R_o, R_d, \alpha$, and $\beta$ from at least two measurements. Error propagation was then used to calculate the uncertainties of the areas, volumes, and $\sigma/P$. 

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The analysis was performed before and after the addition of his-tagged GFP to obtain two independent measurements of $\sigma/P$, and thereby the change of $\sigma/P$ upon GFP binding.

![Image](image.png)

**Figure 13.** (a) Optical microscopy (DIC) image of a vesicle, with brightness and contrast enhanced. (b) The same image overlaid with circles and squares for obtaining radii ($R_d$, $R_o$, $R_b$) and angles ($\alpha$, $\beta$). Also depicted are the tensions ($T_o$, $T_d$, $\sigma$) that participate in the force balance.

**Results**

**Change in $\sigma/P$ upon addition of his-tagged GFP**

The nickel lipid, like DOPC, has oleic acid acyl chains, so it is also expected to partition to the L_d phase. By varying the amount of nickel lipid during vesicle preparation (between 1 and 10 mol %), we controlled the amount of his-tagged GFP bound to the L_d-phase. Three images from a typical experiment are shown in Figure 12. Figure 12a is a DIC image of a GUV before the addition of his-tagged GFP. The overall shape of a vesicle containing a L_o and an L_d domain resembles a sphere with a bud. (The external shape of these two-phase vesicles resembles two merged soap bubbles but differs in an important respect: the merged soap bubbles have an internal membrane that separates the small and large bubble; the vesicle does not.) The ‘pinched waistband’
corresponds to the Lće-Lće circular line interface. After his-tagged GFP was introduced, over a time interval ranging from a few tens of seconds to two minutes, we observed that GFP bound to the vesicle (by epifluorescence) and the vesicle morphology changed and reached a new steady state (DIC image, Figure 12b). Figure 12c illustrates the shape change more clearly by superimposing the outlines of the vesicle shown in (a) and (b). Figure 12d shows GFP fluorescence from the Lće domain. Figure 13 shows a DIC image of a phase-separated GUV focused at the equatorial plane, and (in Figure 13b) overlaid with the geometric constructions used to measure the three radii: \( R_d \) (from the red-dashed circle) \( R_o \) (from the green-dashed circle) and \( R_b \) (from a line drawn between the points of intersection of the red and green circles), as well as \( \alpha \) and \( \beta \) and the tensions \( (T_d, T_o, \sigma) \) involved in force balance. From a collection of images like these, the \( \sigma P \) was computed using Equation 3, which yielded a range of \( (2-10) \times 10^{-11} \text{ m}^2 \). To compare this result to the literature, we assume a line tension of 1 pN, (Kuzmin et al. 2005; García-Sáez, Chiantia, and Schwille 2007; Tobias Baumgart, Hess, and Webb 2003) and thereby obtain \( P = 0.01-0.05 \) Pa, and estimates for \( T_o \) and \( T_d \) of \( \sim 10^{-4} \) mN/m (via Equation). These values are similar to tensions and pressures reported previously for GUVs (Tobias Baumgart, Hess, and Webb 2003). Figure 14 shows a plot of the percentage change of \( \sigma P \) as a function of nickel lipid concentration. The plot shows data for all vesicles that could be described as sections of spheres (as described above). Each column in the plot corresponds to an experiment conducted on a single vesicle, and the error bars correspond to two measurements of the same vesicle. While there is scatter in the data, there is a clear trend toward increasing reduction in \( \sigma P \) with increasing nickel lipid concentration. In some cases the effect is dramatic, with a relative decrease in \( \sigma P \) of approximately
100%. These cases corresponded to final morphologies that were nearly spherical, with little or no bulge in the ordered domain. In most cases, the interior volumes of the vesicles were found to be the same before and after addition of the his-tagged GFP solution, within measurement uncertainty. These data are represented with the gray-filled columns in figure 14. However, in 8 out of 40 cases, there was a distinguishable change in interior volume; these latter data are represented with open columns.

**Figure 14.** Plot of the measured percent change in line tension over Laplace pressure (σ/P) due to GFP binding in vesicles with different nickel lipid concentrations. The gray-filled columns correspond to changes in σ/P of vesicles in which the interior volume remained unchanged, within experimental uncertainty. The unfilled columns correspond to vesicles with a discernible change in volume.
The average responses as a function of the incorporated nickel lipid concentration (mol%) are shown in figure 15. The percent change in $\sigma P$ increased monotonically as the concentration of protein binding sites increased. In this analysis, only those vesicles whose interior volume remained unchanged were considered. (As discussed below, these vesicles have constant pressure $P$, so that the change in $\sigma P$ refers only to $\sigma$.) For nickel lipid concentrations of 1, 2.5, 5 and 10 mole percent, the average change in $\sigma P$ was $1\pm 4\%$, $-16\pm 5\%$, $-32 \pm 8\%$ and $-46 \pm 13\%$, where the uncertainties are standard errors of the mean. The systematic dependence on the nickel lipid concentration indicates that the concentration of bound GFP determines the magnitude of the shape change.

Figure 15. Average percent decrease in $\sigma P$ as a function of the nickel lipid concentration. The unfilled data points correspond to his-tagged GFP and were derived from the constant-volume vesicles of Fig.3. The curve is the best fit of the data to the model described in the text, and the dashed curves show the 95% confidence range for the fitted parameters. The filled circles show the decrease in $\sigma P$ produced by poly-l-histidine.
Geometric constraints may play a role in restricting the possible morphologies, or at least in determining what shapes will minimize the total free energy. In general, a change of shape from one set of \([R_o, R_d, R_h]\) to another can change total interior volume \(V\), total surface area \(A\), and the relative areas of the two domains, \(A_o/A_d\). Given the three parameters \((R_{od, b})\), we cannot in general expect that all three quantities \(A, V\) and \(A_o/A_d\) can be held constant (as expected from basic mathematical principles and verified by direct geometric calculations). From the data, we find that among the 53 measured vesicle changes that preserved \(V\), the majority (80%) also conserved \(A\) within our estimated uncertainty and a further 13% were nearly conserved (i.e., within twice our estimated uncertainty). Among those that conserved \(V\), 56% showed increasing \(A_o/A_d\) and the others showed decreasing \(A_o/A_d\).

To explain the scatter in the data shown in Figure 14, we looked for correlations between either \(\sigma P\) or the change in \(\sigma P\), and either the initial vesicle surface area (both total and by domain), initial vesicle volume or initial vesicle “bulginess” \((\alpha+\beta)\), but found no statistically significant correlations.

**Change in \(\sigma/P\) upon addition of polyhistidine**

To assess whether the change in \(\sigma/P\) arises generally from a binding interaction between the nickel lipid and a histidine tag, we performed the same procedure using polyhistidine instead of his-tagged GFP. (2.5 \(\mu\)L of ~40 \(\mu\)M polyhistidine in pH 5.5 buffer was added.) In these experiments, the vesicles contained either 2.5 mol % or 5 mol % nickel lipid. As with the his-tagged GFP, we identified the vesicles that maintained \(V\)
(and hence \(P\)), and found their average change in \(\sigma/P\). For 2.5 and 5 mol \%, we found an average decrease of 27 \(\pm\) 10\% and 40 \(\pm\) 9\% respectively (Figure 16). These data are consistent with the idea that it is the chelation of nickel lipid with the hexahistidine tag (independent of GFP) that generates the decrease in \(\sigma/P\), and that the larger valency of polyhistidine produces a greater change in \(\sigma/P\) (figure 16).

![Figure 16](image.png)

**Figure 16.** Plot of the measured percent change in line tension over Laplace pressure \((\sigma/P)\) due to polyhistidine binding in vesicles with different nickel lipid concentrations. The gray-filled columns correspond to changes in \(\sigma P\) of vesicles in which the interior volume remained unchanged, within experimental uncertainty, while the unfilled columns correspond to vesicles with a discernible change in interior volume.

**Mechanism for change in \(\sigma/P\)**

We now discuss the mechanism of the change in vesicle morphology and in \(\sigma P\).

First we note that vesicles that had constant \(V\) should also have constant pressure \(P\).
Because membranes are permeable to water, but not to larger molecules such as sucrose, there exists an osmotic pressure, $\pi$, across the membrane $\approx k_B T(N/V - c_o)$, where $k_B T$ is Boltzmann’s constant, $T$ is the absolute temperature, $N$ is the number of solute molecules inside the vesicle volume $V$, and $c_o$ is the concentration of solute outside the vesicle. In equilibrium, $P$ (the difference in pressure across the membrane) balances $\pi$. Assuming that the vesicles did not lyse during GFP binding, $N$ remained constant, so a constant $V$ corresponds to constant $\pi$ and $P$. Therefore, in these cases we equate a change of $\sigma P$ with a change of $\sigma$ only. Two possible mechanisms were initially considered to explain the change of line tension. The first posits that GFP binding changes the compositions of the two phases and thus the chemical potentials of the species, and as a result, the line tension. While we cannot absolutely rule out this mechanism, we expect that a change in the chemical potentials that is sufficient to reduce $\sigma$ by up to 46% would also change the relative areas of the $L_o$ and $L_d$ phases systematically. While the relative areas either increased or decreased in 34% of the cases, these changes were not systematic and did not depend upon the nickel lipid concentration. Another possibility is that GFP binding causes an increase in membrane lateral tension which, in turn, alters the phase boundary. To examine this possibility we monitored the area of single-phase vesicles before and after GFP binding and found no change in area (figure 17).
Figure 17. Plot of the measured percent change in area of single phase vesicles with 10% nickel lipid due to GFP binding.

Instead, we propose that $\sigma$ is reduced by the accumulation of GFP-bound nickel lipid at the one-dimensional boundary between the $L_o$ and $L_d$ domains. Direct evidence of this accumulation is provided by digital-camera fluorescence images of GUVs having 1 mol% nickel lipid. The results varied from vesicle to vesicle, yet we found evidence of enhanced GFP fluorescence at the domain boundary in six images of vesicles. In images of 4 vesicles containing 5 mol% Ni DOGS, however, the same deconvolution process showed no fluorescence enhancement at the boundary. GUVs were imaged using a 63x oil-immersion objective in epifluorescence mode with a CoolSNAP HQ$^2$ CCD camera (Photometrics Inc., Tucson, AZ). Images were deconvolved with Huygens Essential.
software (Scientific Volume Imaging BV, Hilversum, NL) and surface plots of intensity were created with ImageJ.

Figures 18-23 show the original and deconvolved fluorescence images of six vesicles. GFP is bound to the nickel lipid partitioned into the \( L_d \) phase, so the \( L_o \) phase appears dark. The vesicles thus have the appearance of a bite taken out at the location(s) of the \( L_o \) domain(s).

**Figure 18.** Deconvolution analysis of GFP fluorescence. (A) Original image. (B) Deconvolved image. (C) Surface plot of intensity in deconvolved image, scale is arbitrary.
**Figure 19.** Deconvolution analysis of GFP fluorescence. (A) Original image. (B) Deconvolved image. (C) Surface plot of intensity in deconvolved image, scale is arbitrary.

**Figure 20.** Deconvolution analysis of GFP fluorescence. (A) Original image. (B) Deconvolved image. (C) Surface plot of intensity in deconvolved image, scale is arbitrary.
Figure 21. Deconvolution analysis of GFP fluorescence. (A) Original image. (B) Deconvolved image. (C) Surface plot of intensity in deconvolved image, scale is arbitrary.

Figure 22. Deconvolution analysis of GFP fluorescence. (A) Original image. (B) Deconvolved image. (C) Surface plot of intensity in deconvolved image, scale is arbitrary.
Figure 23. Deconvolution analysis of GFP fluorescence. (A) Original image. (B) Deconvolved image. (C) Surface plot of intensity in deconvolved image, scale is arbitrary.

In the Figures 18 to 23 panel A is the original image, panel B is the deconvolved image, and panel C is a surface plot of the intensity in the deconvolved image. Each image shows some evidence of enhanced fluorescence at the Lc/Ld boundary. The shapes of the vesicles are varied and some appear to have vesicles captured inside them, which we think may have occurred during the process of sedimentation and resuspension. Despite the fact that the enhancement is not the same in all vesicles, and that there is more noise in some images than others, all the images show enhanced GFP emission from the Lc/Ld boundary.

We used test images to test against deconvolution artifacts and to estimate the buildup in intensity required for detection via deconvolution. These images were then
deconvolved by the same procedure used for the experimental images. To create the test images, we first made a three-dimensional image of a one-pixel-thick vesicle with a “bite” taken out of it. This image was created in IDL (Interactive Data Language; ITT Visual Information Solutions, Boulder, CO). At the boundary of the “bite,” the intensity was enhanced by a factor that we varied between 1 and 4.2. (An enhancement of 1 represents no enhanced fluorescence and should correspond to a control image with no accumulation of GFP at the boundary). The resultant image was convolved using an estimate of the point-spread function of our microscope. Noise was then added to each pixel in the amount \( yI^{1/2} \) where \( I \) is the intensity of a given pixel and \( y \) is a random number drawn from a Gaussian distribution with mean of zero and a standard deviation of 1. Finally, a slice through the middle of the image was taken and deconvolved using the Huygens Essential deconvolution software. Figure 24 illustrates the deconvolution of a test image with no enhancement at the boundary. Figure 25 illustrates the deconvolution of a test image with an intensity enhancement of 4.2 at the boundary. The test images show both that the deconvolution procedure does not artificially introduce a buildup at the boundary where none exists, as evident in Figure 24, and that an enhancement of 4.2x should be detectable by our deconvolution procedure (figure 25). Enhancements of 1.5 and 2x did not show a detectable buildup of intensity at the boundary.
Figure 24. Deconvolution of a test image with no enhancement at the boundary. (A) Original image. (B) Deconvolved image. (C) Surface plot of intensity in deconvolved image; scale is arbitrary.

Figure 25. Deconvolution of a test image where intensity is enhanced at the boundary by a factor of 4.2. (a) Original test image. (b) Deconvolved test image. (c) Surface plot of intensity in deconvolved image; scale is arbitrary.

The localization of proteins at one-dimensional domain boundaries is analogous to the adsorption of surfactants or colloidal particles at two-dimensional liquid
boundaries, like those found in oil-water emulsions (Alessandrini and Facci 2011; Nicolini et al. 2006; Du et al. 2010; Pieranski 1980). In three-dimensional suspensions of colloidal particles, it is well known that adsorption at a liquid boundary can lower the total interfacial energy and reduce the measured interfacial tension (Pickering 1907; Pieranski 1980). In these cases, the particles need not have an amphiphilic structure. Instead, adsorption to the boundary is driven by the reduction in the contact area between the two liquid phases, provided that the particle does not have too strong a preference for one phase over the other. A similar phenomenon may contribute to the reduction in \( \sigma \) at the domain interfaces in fluid membranes. In the case of bacteriorhodopsin, the affinity for the domain boundary was based on the thickness of the hydrophobic region of the protein (Dumas et al. 1997). In our case, the affinity for the boundary may arise from the fact that both poly-L-histidine and 6×his-tagged GFP may bind multiple nickel lipids, thereby creating small lipid-protein clusters. In a previous study of his-tagged GFP, Nye and Groves provided evidence that 10×his-tagged GFP bound with mono- and polyvalent interactions to nickel lipid in supported lipid bilayers (i.e., one GFP to multiple lipids), with polyvalent binding remaining stable for hours (Nye and Groves 2008). The changes in structure and/or orientation of the nickel lipid acyl chains generated upon binding histidine are, to our knowledge, not known in detail. However, it is reasonable to speculate that GFP binding could change the splay in the acyl chains, which would in turn modify the hydrophobic length. According to the model of Kuzmin et al. (Kuzmin et al. 2005), this modification would reduce \( \sigma \) which depends on the square of the hydrophobic height mismatch. Alternatively, the separation between the nickel lipid headgroups may be altered when more than one lipid binds to a single histidine tag. In
this case, the GFP-nickel lipid cluster may act as an entity that is distinct from the unbound nickel lipid and has reduced solubility in the $L_d$ phase. Finally, we note that even if the packing of nickel lipid were unaffected by multivalent binding to a histidine tag or polyhistidine, there could be an entropic drive to bind clusters to the interface between domains. A cluster of $N$ particles would lose approximately the same amount of translational entropy as a single lipid, but it would cover a larger length at the boundary and more strongly reduce the line energy. We expect the affinity of a protein-lipid cluster for the boundary to increase with its size, because a larger cluster covers a greater length of boundary. This model is qualitatively consistent with the greater reduction of $\sigma P$ produced by poly-$l$-histidine compared to his-tagged GFP: each poly-$l$-histidine molecule contains $\sim 80$ histidine residues (on average), while each GFP histidine tag has only six residues.

As a further test of this domain-boundary affinity model, the data were fit to a simple adsorption equation, (Langmuir 1918; Adamson and Gast 1997) corresponding to a Langmuir adsorption equilibrium between a two-dimensional ideal gas of particles – clusters of GFP-bound nickel lipids in the $L_d$ phase – and particles bound to the one-dimensional $L_d$-$L_o$ boundary. Bound clusters of diameter $l_0$ were modeled as objects whose centers of mass are confined to within a distance $h$ of the domain boundary. Bound clusters have an internal energy lower than the clusters in the surrounding two-dimensional gas by the amount $\varepsilon$. This binding energy $\varepsilon$ should be proportional to the reduction of the line energy, $l_0\sigma$. We then approximate $\Delta\sigma$, which is the change in energy per length, as the product of $\varepsilon$ and the number of bound clusters per unit length. Assuming for simplicity that $h=l_0$, the model leads to
\[
\Delta \sigma / \sigma = \varepsilon (l_0 \sigma)(x \cdot \exp[-\varepsilon k_B T])/(1+x \cdot \exp[-\varepsilon k_B T]),
\]

where \(l_0\) is the cluster diameter and \(x\) is the cluster mole fraction, taken to be equal to the nickel-lipid mole fraction. In principle, each hexahistidine tag can bind between one and six nickel lipids (although the steric hindrance among the GFP might prevent this); we therefore set \(l_0\) equal to two lipid diameters. If the line tension is assumed to be \(\sim 1\) pN, we obtain \(l_0 \sigma \approx 1.6\) pN-nm, which is \(\sim 0.3\) \(k_B T\) at 298 K. For simplicity, we set \(l_0 \sigma = 0.3\) \(k_B T\), which then allows us to use the equation to fit the data in figure 15 to a single parameter, \(\varepsilon k_B T\). The fit was done using the nonlinear least-squares method (Origin Software, OriginLab Corp., Northampton MA). The quality of the fit is decent (solid line in Figure 13) indicating that the functional form of the model is reasonable. The best-fit value for \(\varepsilon k_B T\) is \(-0.80 \pm 0.04\). Varying the value of \(l_0 \sigma\) by a factor of 3 did not significantly change the quality of the fit and yielded \(\varepsilon\) values in the range of \(-0.4\) to \(-1.5\) \(k_B T\). Hence, we find \(\varepsilon\) values that are on the order of \(\sim l_0 \sigma\), providing evidence that the model is self-consistent.

**Conclusions**

In summary, we have investigated the binding of a model membrane-associating protein, histidine-tagged GFP, to a nickel lipid in \(L_v/L_d\) phase-separated giant unilamellar vesicles. The binding reduced the line tension at the boundary between \(L_v\) and \(L_d\) phases by up to 46\%, depending on the mole fraction of nickel lipid. To our knowledge, this is the first time that such measurements have been made. An adsorption model with a single adjustable parameter provides a reasonable fit to the data with a best-fit binding energy of approximately \(-1k_B T\) for a lipid-protein cluster at the boundary between phases. Our
results suggest that proteins that bind to multiple lipids or cause local reorganization of
the lipid composition should exhibit an affinity for the boundaries of domains in
membranes. This affinity reduces the line tension and thereby lowers the energy cost of
forming a domain. Finally, the localization of proteins at the domain interface may
further enhance their clustering, which may be important for their function.

In general, one might expect that proteins with a more complex internal structure than
his-tagged GFP might modify membrane shape by additional mechanisms. Membrane
proteins with an amphiphilic structure (such as lipidated N-Ras) provide one example
which has been addressed in the literature (Nicolini et al. 2006; Weise et al. 2010).
Membrane proteins that deform membranes upon binding may also have an affinity for
domain boundaries when a bud is formed, owing to the large negative Gaussian curvature
at the boundary. The effect of protein shape in determining protein interaction with
domain boundaries is an interesting topic for future work and will be discussed in
Chapter 8.
CHAPTER 4
BAR PROTEINS: CONSENSUS AND CONTROVERSY

The work discussed in the following chapters addresses the question of how protein shape affects the binding of membranes and subsequent remodeling of the membrane’s shape. In particular, we focus on the effects of membrane composition, initial membrane shape, and applied tension. We focus on the class of biologically relevant membrane binding proteins known as BAR. Because BAR has a striking banana like shape, exhibits a strong tendency to deform membranes, and plays a role in endocytosis, it is a widely studied model system- a kind of hydrogen atom of membrane-curvature effects.

In this chapter we provide an overview of the BAR family of proteins. In subsequent chapters we describe our measurements of the role of lipid composition and membrane tension in binding of BAR to membranes.

Introduction to the BAR superfamily of proteins

The BAR superfamily of proteins, named for a homologous region found in three related amphipathins; BIN1, Amphiphysin, and RVS167 (Sakamuro et al. 1996) are found in many organisms across species and kinds, and are implicated a variety of biological processes relating to dynamic membrane remodeling (Peter et al. 2004). These processes include cellular trafficking: the movement of material between organelles or between the inside and outside of a cell, organelle biogenesis, T-tubule formation, cell migration, cell signaling, and cell division (Ren et al. 2006). In vivo studies involving proteins that have been genetically mutated to remove their native BAR domains speak to
the key role that they play, where mutation or inactivation often leads to abnormal membrane dynamics (Munn and Aspenström 2010; Milosevic et al. 2011). Deletion of the BAR domain in APPL1 knocks out its ability to bind the GTPase Rab5 which, in turn, is necessary for the proper localization of APPL1 to transport vesicles (Habermann 2004; Miaczynska et al. 2004). ASAP1 (a protein that hydrolyzes GTP and is involved in the trafficking of endosomes) mutants missing BAR domains lose their ability to associate with tubular structures and also affect the rate of cellular spreading (Nie et al. 2006). Reduction of BAR domains in the cytosol of rat brains results in a depletion of synaptic-like microvesicle formation (Ringstad et al. 1999; Schmidt et al. 1999). Amphiphysin BAR, which this thesis focuses on, is enriched in the mammalian brain and is involved in clathrin-mediated endocytosis (Lichte et al. 1992; Wigge et al. 1997; Yamada et al. 2007).

In vitro experiments focusing on interactions between BAR domains and other proteins known to play key roles in membrane trafficking demonstrate the morphological changes that BAR family members induce both alone and in concert with partner proteins. A morphological study of liposome tubules coated with either dynamin, BAR, or dynamin+BAR clearly show that the structures formed by dynamin+BAR hybrids resemble structures found in vivo (figure 26).
Figure 26. Positive staining of thin sections of liposomes tubulated by either amphiphysin (b) dynamin (d) or amphiphysin + dynamin (f). Image (g) shows positive staining of thin sections of a synaptic membrane in brain cytosol demonstrating its morphological similarity to structures formed by amphiphysin + dynamin. Scale bar is 100 nm. Image reproduced from (Takei et al. 1999).

Furthermore, similar studies regarding the interaction between BAR and clathrin coats show that without BAR domains very few membrane tubules are coated with clathrin (Takei et al. 1999). Taken together, these studies point strongly to the role of BAR proteins as a crucial link between clathrin-mediated budding and dynamin-mediated fission of membrane vesicles.

BAR may also act as a link between budding of the cellular membrane and the reorganization of the underlying cytoskeleton. Recent experiments have shown that BAR domains frequently are positioned in the amino acid sequence of proteins in the BAR family next to a domain that is responsible for binding neural Wiskott-Aldrich syndrome protein (N-WASP), a protein that regulates the actin cytoskeleton during endocytosis (Tsujita et al. 2006). These studies paint a picture of the BAR superfamily’s importance in many cellular processes that require deformation of the membrane, whether it be in endosome formation in the Golgi complex, generation of clathrin-coated pits, outer membrane endocytosis, or the production of neuronal microvesicles.
Mutations of the genes that encode for BAR family domains can have serious consequences, as improper BAR function has been indicated as a factor in several diseases including acute myelogenous leukemia, renal cancer, Huntington’s disease (Fricke, Gohl, and Bogdan 2010), PAPA syndrome (Wise et al. 2002), and stiff-person syndrome (De Camilli 1993).

**Structure of BAR superfamily members**

The BAR superfamily can be divided into three main subgroups or families. The N-BAR domain of *D. melanogaster*, which was the first superfamily member to be characterized (Peter et al. 2004), is about 245 residues long. Each monomer consists of 3 coiled-coil alpha helices. While the protein probably resides in monomer: dimer equilibrium *in vivo* (Bhatia et al. 2009), the consensus is that the membrane-bound form is a homodimer. The homodimer has a banana-like shape with several regions of positive charge on the concave face. The concave face is moderately curved with an arc depth of about 30 Å (Henne et al. 2007). Members of this family often possess N-terminal amphipathic helices that are disordered in solution but become helical when inserted into the membrane (Gallop et al. 2006). Domains with this feature are called N-BAR domains, those without are simply called BAR.

The BAR domain of human FCHo2, termed F-BAR or extended FCH, is about 274 residues long. Each monomer of F-BAR consists of 5 α-helices in a coiled coil formation. Like N-BAR the F-BAR domain is expected to bind to membranes in dimer form. The F-BAR dimer, like N-BAR, has a banana-like shape when viewed from the side but has a tilde shape when looked at from the top or bottom that distinguishes it from N-BAR. The F-BAR module has regions of positive charge
decorating (Henne et al. 2007). Unlike N-BAR the F-BAR module also has a region of negatively charged residues running inside a central groove in the concave face. All helices of the F-BAR domain appear to be intrinsically ordered and no change in helicity is seen upon membrane-binding (Henne et al. 2007; Gallop et al. 2006; Shimada et al. 2007).

The third and final subgroup contains I-BAR modules. The I-BAR domain of human IRSp53 is about 250 residues long. Each I-BAR monomer is formed by 4 coiled-coil α-helices, 3 extended and 1 shorter helix at the C-terminus. The general shape of the I-BAR module differs from the banana-like shape of both N-BAR and F-BAR, it is zeppelin shaped and has no concave faces. The central region of the dimer consists of a bundle of 6 α-helices. Helices 2 and 3 extend past the central region to form the ends of the dimer (Millard et al. 2005). I-BAR domains have a large percentage (~50%) of hydrophobic residues that form the dimer interface so I-BAR is thought to exist in dimer form both in solution and when membrane-bound. Like N-BAR and F-BAR, the I-BAR module has regions of positive charge, in this case they are located at the extreme ends of helices 2 and 3 (Millard et al. 2005). Various members of the three main subgroups of the BAR family are shown below in figure 27.
Figure 27: Architecture of BAR domains and their role in membrane deformation. a–e Crystal structures of BAR/N-BAR domains: the arfaptin BAR domain (1I49) (a), the Drosophila melanogaster amphiphysin BAR domain (D-Amph) (1URU) (b), the endophilin III BAR domain (2Z0V) (c), the PX-BAR domain from SNX 9 (3DYT) (d), and the BAR-PH domain from APPL1 (2Q12) (e). f–h Structural insights into F-BAR domain architecture: the F-BAR domains of FBP17 (2EFL) (f), syndapin1 (2X3X) (g), and yeast Syp1 (3G9G) (h). i Crystal structure of the IRSp53 I-BAR domain (1WDZ). j Membrane deformation by BAR/N-BAR, F-BAR, and I-BAR domains. BAR and F-BAR domains induce positive membrane curvature, whereas I-BAR domains generate negative membrane curvature. Amphipathic α-helices are indicated by red arrows in (b), (c) and (h). The additional amphipathic α-helix within the endophilin BAR domain is indicated by an orange arrow in (c). Crystal structures were generated with Pymol. Image reproduced from (Rao and Haucke 2011)
Figure 28: Structure of the *Drosophila* amphiphysin BAR domain with the surface colored by electrostatic potential (red, $-10 \text{kT}^{-1}$, blue, $+10 \text{kT}^{-1}$). Image reproduced from (Peter et al. 2004)

Within each subgroup there are, of course, many members—there are more than 60 members in the human proteome alone (Rao and Haucke 2011). Evolutionary conservation studies, in which the genetic sequence of a particular protein is compared against the sequences of possible family members, show that although there is only weak conservation of the sequence there is a strong similarity of the 3-dimensional structure of most BAR family members (Frost, Unger, and De Camilli 2008). In addition to evolutionary conservation studies, proteins that include a BAR domain can be examined for any other nearby motifs that they may have in common. Indeed several regions of homology have been identified in close proximity to the BAR domain of various proteins (Peter et al. 2004). Of particular note are motifs responsible for binding specific lipids or protein partners that also participate in membrane reorganization. These motifs include but are not limited to the PH (Pleckstrin homology) and PX (Phox homology) domains which bind phosphoinositides (particularly PIP$_2$ and PIP$_3$ in the case of PH and PIP$_3$ in the case of PX) (D. S. Wang and Shaw 1995) (Karathanassis et al. 2002), SH3 domains which bind dynamin (Rasmussen et al. 1998), N-WASP domains which regulate actin (Martinez-Quiles et al. 2001), and the GTPase activating domains RhoGAP and ArfGAP.
(Tcherkezian and Lamarche-Vane 2007). Even though there is wide variety in the genetic sequences of BAR family members the similarity of their structures leads to an understandable similarity in their function- almost all family members are involved in cellular trafficking and membrane deformation in some respect. The presence of other domains that have also been implicated in membrane reorganization, often in close proximity to BAR motifs, coupled with the fact that BAR domains are conserved not only among different species but among members of different kingdoms suggests that BAR and BAR-like domains may have evolved as a general principle for the deformation of biological membranes.

**BAR’s role in curvature sensing and induction**

BAR domains are thought to have two main functions- curvature sensing (binding preferentially to membranes of a particular curvature Fig 29) and curvature alteration (Fig 30) (Habermann 2004). It is generally accepted that both electrostatic interactions between negatively charged lipids and positive residues on BAR and hydrophobic interactions between the membrane and BAR’s amphipatic helix will have some role to play in BAR’s functions. However, the mechanisms by which BAR domains sense curvature and alter curvature, particularly the former, have been the subject of some controversy (Madsen et al. 2010).
Figure 29. Curvature sensing by BAR domains. Brain liposomes of different intrinsic membrane curvatures were prepared and sequentially extruded through polycarbonate membranes with pores of 0.8, 0.4, 0.1, and 0.05 μm. (B) Liposome sedimentation assays were normalized by setting the amount of binding to the largest liposomes to 100%. The PtdIns(4,5)P$_2$-binding protein Dab2 does not affect curvature and serves as a control for the available surface area of the liposomes. Inset: an example gel showing the liposome sedimentation results for oligophrenin BAR + PH. P, pellet; S, supernatant. (C) Liposome sedimentation assays as in (B) showing the effect of deleting the N-terminal amphipathic helix in amphiphasin1. Figure reproduced from (Peter et al. 2004)
Figure 30: Tubulation of brain lipid extract vesicles induced by the addition of endophilin BAR. Scale bar is 100 nm. The inset shows tubules induced by the addition of endophilin BAR (left), amphiphysin BAR (center), and dynamin (right). Scale bar is 70 nm. Image reproduced from (Masuda et al. 2006)

Historically there have been three methods commonly employed to study BAR curvature sensing. The first method is a bulk technique in which BAR protein is incubated with vesicles that have been extruded through pores of a defined size (typically producing vesicles of between 50 and 800 nm), and then centrifuged or suspended in a gradient. Centrifugation allows for the separation of bound versus unbound protein so that the fraction of bound protein can be compared to vesicle size (curvature). There are two main drawbacks to this method. First, the extrusion method produces vesicles that are not completely monodisperse, particularly for vesicles of larger diameter (> 100 nm). Second, the technique averages over vesicles and assumes that the initial state of the vesicles in the sample is uniform. As discussed later on in this chapter this assumption may not be correct. However, in chapter 6, I will suggest a method to make this assumption more tenable. The second method involves creating regions of large curvature by using optical tweezers to pull cylindrical tubules out of a giant vesicle. By controlling
the pulling force, tubules of varying diameters can be created, and binding of fluorescently labeled BAR can be quantified. Finally, in the past few years, a method called the SliC assay has been developed to image individual vesicles and determine both vesicle size and intensity of bound, fluorescently labeled protein. One advantage of this method is that any size polydispersity that would be averaged out in bulk methods is accounted for. However, this method assumes that vesicles are spherical as made. As will be discussed in chapter 6, this assumption is not always correct. These methods, used in conjunction with strategic mutations to either the positive residues on BAR’s concave face or the amphipathic helix of N-BAR, have been employed by various groups in order to determine the mechanisms responsible for curvature sensing.

Bulk centrifugation techniques examining BAR binding to vesicles of varying curvature performed on BAR domains with either a truncated or completely deleted N-terminal amphipathic helix have produced the following results: A complete loss of binding (Farsad et al. 2001), or a reduction in overall binding but an increase in curvature sensing (Peter et al. 2004; Henne et al. 2007). In fact, one study found that curvature sensing was only present in BAR domains without an N-terminal amphipathic helix (Peter et al. 2004). This phenomenon has generally been explained by reasoning that if the energetic benefits of protein-membrane interactions are not enough to pay the cost of bending the membrane, the protein may still be able to sense a particular curvature and bind to it preferentially. Thus, BAR domains with an insertable amphipathic helix are able, through protein-membrane interactions, to generate the curvature they prefer and so don’t bind preferentially to a particularly curved vesicle, but when the amphipathic helix
is removed they must prefer pre-curved vesicles as they can no longer deform the vesicle to suit their tastes.

Bulk centrifugation techniques examining BAR binding to vesicles of varying curvature performed on BAR domains as a function of salt concentration have produced the following results: A loss of endophilin binding with an increase in salt concentration (from 120 to 1000 mM KCl) (Farsad et al. 2001), and a displacement of BAR but not N-BAR with an increase in salt concentration (from 150 to 250 mM NaCl) (Henne et al. 2007).

Individual measurements made with the SLiC assay found that disrupting BAR’s amphipathic helix knocked out curvature sensing, and that an N-terminal amphipathic helix without the BAR domain senses curvature just as well as the full protein (Bhatia et al. 2009). In addition, in disagreement with bulk measurements, SLiC experiments on BAR with disruptions to the N-terminal amphipathic helix show no curvature sensing (figure 31).
Figure 31. Membrane curvature sensing of BAR domains is dominated by the N-terminal amphipathic helix. (C) Membrane curvature sensing graph for eNBAR-F10E measured at 740 nM. The point mutation disrupting the formation of the N-terminal AH impaired severe membrane curvature sensing. The concentration was increased to get sufficient binding. (F) Membrane curvature sensing of aNBAR was also severely impaired by mutations in the N-terminal peptide (aNBAR 3xE), binding measured at 4 μM. Thus, membrane curvature sensing by BAR domains seems to originate from the insertion of the N-terminal AHs in the bilayer. Image reproduced from (Bhatia et al. 2009)

This phenomenon is explained by positing that highly curved membranes have more amphipathic helix binding sites in the form of defects in the membrane (regions where the hydrophobic core of the bilayer is exposed to solution). Further evidence for this hypothesis is provided by experiments where packing defects were filled with lysolipid and N-BAR curvature sensing ability was subsequently lost. These individual experiments also found that removing positive residues from BAR’s concave face had no effect on sensing. The authors argue that bulk measurements intent on quantifying curvature sensing should be approached with caution for the following reasons. They
found that there is a large heterogeneity both in binding (less than 10% of all vesicles interrogated had any detectable bound protein) and in binding kinetics (times until detectable binding was seen ranged from minutes to hours). Bulk experiments, however, cannot distinguish between the density of bound protein and the fraction of vesicles with any bound protein. Importantly, they also find that the fraction of vesicles with detectable binding decreased with increasing curvature, thus bulk measurements may underestimate curvature sensing efficiency. While this may explain why curvature sensing ability appears to be lost for BAR domains containing an N-terminal amphipathic helix, it does not explain why BAR (without the helix) does sense curvature according to bulk measurements. The authors address this point by suggesting that mutations to either the N-terminal amphipathic helix, or positive residues on the concave face, may greatly impact fractional binding and thus confuse bulk results. We will return to these results in chapter 6.

The picture for membrane deformation or tubulation by BAR family members is less contentious and seems to depend on both electrostatics and helix insertion. Several groups have made BAR constructs with a deleted amphipathic helix and found a reduction in tubulation (Gallop et al. 2006; Blood, Swenson, and Voth 2008). Simulations show that without the amphipathic helices the BAR domain simply doesn’t attach well or closely enough to drive tubulation, but if extra negatively charged lipids are added to the membrane then both binding and tubulation are restored (Blood, Swenson, and Voth 2008). Mutations either removing positive residues (Bhatia et al. 2009; Gallop et al. 2006; Peter et al. 2004) or adding negative residues (Masuda et al. 2006) found that tubulation was either reduced or knocked out.
In Chapter 6 I will suggest a different approach to curvature sensing that may shed light on some of the unresolved issues discussed above. However, I will first discuss the effect of lipid composition on N-BAR’s ability to bind membranes.
CHAPTER 5

THE EFFECT OF LIPID COMPOSITION ON N-BAR BINDING TO LUVs

Lipid composition is highly regulated in biological membranes (Sprong, Van der Sluijs, and Van Meer 2001). There are several strategies that cells may employ to adjust their composition in response to environmental cues: changes can be made to lipid headgroups, changes can be made to acyl chains, or acyl chains can be exchanged thus creating new lipid species while maintaining the average chain composition (Rilfors and Lindblom 2002). Unsurprisingly, lipid composition can have a marked impact on membrane proteins. Both protein kinase C and phospholipase C exhibit enhanced activity when the membrane is enriched in non-lamellar phase forming lipids like DOPE (Epand 1996; Ruiz-Argüello, Goñi, and Alonso 1998), while bacteriorhodopsin reacts oppositely-activity is depressed when the membrane is enriched in DOPE (Curran, Templer, and Booth 1999). Phospholipase C activity decreases with increasing amounts of Sphingomyelin and cholesterol. Finally, Equinatoxin II requires a phase separating composition for proper function (Schön et al. 2008). We expected that understanding the interactions between membrane binding/remodeling proteins and membrane components will be key to understanding cellular processes like endocytosis.

Here we examine the role that membrane composition plays in amphiphysin N-BAR’s membrane-binding ability. Although there is evidence that WRP F-BAR binds preferentially to PIP, PIP₂, and PIP₃ (Carlson et al. 2011), to date the role of lipid composition on BAR binding has not been systematically explored. In this work we keep the lipid hydrophobic hydrocarbon chains fixed and vary the headgroup composition (figure 32)
**Figure 32.** Lipids used in binding vs. composition experiments. DOPS: 1,2-dioleoyl-sn-glycero-3-phospho-L-serine
DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine
DOGS: 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-l-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)
PIP2: 1,2-dioleoyl-sn-glycero-3-phospho-(1′-myo-inositol-4′,5′-bisphosphate) (ammonium salt)
SM:N-palmitoyl-D-erythro-sphingosylphosphorylcholine
Chol: Cholesterol

**Methods and materials**

**Protein purification**

Two N-BAR constructs were used in these experiments. Residues 1-256 of amphiophysin from *Drosophila melanogaster* in PGEX vector were [obtained from the MacMahon laboratory]. These residues correspond to the N-terminal α-helix and the BAR domain of amphiophysin, which are together referred to as N-BAR. These residues were expressed as His-tagged proteins from a pETite Kanamycin vector in *E. coli* BL21 DE3 cells (see Chapter 2 for details). The cysteine residue at position 66 was previously mutated to alanine to facilitate attachment of a maleimide dye at position 82, however, all
protein used in these experiments was unlabeled. Before use in binding experiments, N-BAR in buffer A was diluted with deionized water to achieve a final protein concentration of 4 µM and buffer concentration of 200 mOsm, and gently centrifuged for 10 min at 10,000 rpm (Eppendorf, Hamburg, Germany, model 5415 D) to get rid of any large protein aggregates.

GST-tagged N-BAR was purified with a GS Trap HP affinity column (GE Healthcare Life Sciences, Piscataway, NJ, cat. No 17-5281-01) (see Chapter 2 for details).

**Lipids, GFP and the preparation of LUVs**

Dioleoyl-sn-glycero-3-phosphocholine (DOPC), N-palmitoyl-D-erythro-sphingosylphosphoryl-choline (SM), cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-myo-inositol-4′,5′-bisphosphate) (ammonium salt) (PI(2)), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (LysoPC), and the nickel salt of 1,2-dioleoyl-sn-glycero-3-[(N-[5-amino-1-carboxypentyl]iminodiacetic acid)succinyl] (nickel lipid) (DOGS) were purchased from Avanti Polar Lipids Inc. (Alabaster AL). Multiple batches of lipids were used over the course of the experiments.

Lipids were mixed in solution, dried under argon, and placed under vacuum for at least 2 h to remove residual solvent. Multi-layer vesicles (MLVs) were formed by rehydrating with 1 mL 300 mM sucrose or 299 mOsm NaCl + 1 mOsm DTT and vortexing. Large unilamellar vesicles (LUVs) of diameter approximately 100 nm were prepared by extruding (Avanti Polar Lipids Inc., Alabaster AL; cat. no. 610000) 15-20
times through a polycarbonate membrane (Whatman, Piscataway, NJ, cat. No 7060-4701) with a pore size of 100 nm (See Chapter 2 for details).

**Sedimentation assay**

Sedimentation assays were used to quantify the amount of N-BAR bound to the vesicles. The LUVs are buoyant in suspension but become denser than the solution when N-BAR binds to them. The amount of NBAR in the sediment and the supernatant are then measured by gel electrophoresis.

LUVs, after extrusion, are diluted 1:1 in either isoosmolar sucrose or NaCl+DTT. A fixed amount of N-BAR was then incubated for 10 minutes with a known quantity of LUVs for a final protein concentration of 14 μM. The final protein:lipid ratio was ~1:100. Sedimentation of 75 μL samples was accomplished by centrifugation at 160,000xg for 30 minutes (Beckman Coulter, Brea, CA, model Optima TLX ultracentrifuge with a TLA 100 rotor). After centrifugation a 10 μL sample of the supernatant was taken and the rest was immediately removed with a pipette. The sediment (pellet) was then resuspended in a volume of buffer equal to that removed and a 10 μL sample was taken.

Separate aliquots (8 μL) of both the supernatant and pellet (mixed 1:1 with protein loading buffer) were separately run on SDS-PAGE (10%) and quantified by the gel analysis tool of ImageJ. Any samples that bled into neighboring lanes of the SDS-PAGE gel were discarded. The band appearing in each lane corresponds to the MW of N-BAR and the integrated intensity of that band is determined. The fraction of bound protein was defined as the ratio of the integrated intensity of the pellet to the sum of the integrated intensities of the pellet and of the supernatant.
Results

Control experiments

We began with several experiments to determine an appropriate ratio of protein:lipid, to determine an appropriate protein-LUV incubation time, and to check the linearity of our method of analysis.

The linearity of our analysis was checked by loading controlled volumes of pure N-BAR protein into each gel lane (figure 33). The gel was then examined with the gel analysis tool of ImageJ which provides the integrated intensity (area in arbitrary units) for each lane. The result is consistent with a linear response. A total sample volume of 8 mL was loaded into each gel lane in all subsequent experiments.

![Graph showing the relationship between volume of protein loaded (μL) and area (arbitrary units).](image)

**Figure 33.** Check of the linearity of the gel analysis tool of ImageJ. The plot shows the integrated intensity (area) of each band in an SDS-PAGE gel. Each band was loaded with a different volume of pure N-BAR protein.
To ensure that there is enough lipid available for complete N-BAR binding, we tested both protein constructs as a function of the total number of moles of lipid present during LUV preparation (figure 34). The total amount of lipid (µmoles) in each preparation was resuspended in 1mL of 300 mOsm sucrose. It is assumed that all dried lipid is reconstituted during the resuspension process. LUVs were diluted 1:1 in isoosmolar salt or sucrose and 36 µL of this solution was used in each experiment. For this set of experiments we chose an LUV composition that demonstrated strong (~100%) binding; 9:1 DOPC:DOGS and incubated each sample with a fixed amount of protein. We found that 100% binding was possible with 6 or more µmoles of total lipid (108 nmoles of lipid per experiment) for a final protein:lipid ratio of 1:50 (this is the ratio of protein to exposed lipid - i.e. lipid on the outer leaflet). To ensure that complete binding was possible all subsequent experiments were performed with 12 µmoles of lipid (216 nmoles lipid per experiment) for a final protein:lipid ratio of approximately 1:100.
Figure 34: Plot of the percent of N-BAR (residues 1-256 + His-tag) found in the pellet as a function of the total moles lipid used in the LUV preparation. LUVs were composed of 9:1 DOPC:DOGS. The filled data points correspond to LUVs diluted in 300 mM sucrose. The unfilled data points correspond to LUVs diluted in 299 mOsm NaCl + 1 mOsm DTT.

Recent work (Bhatia et al. 2009) has suggested that the time required for appreciable binding may vary from minutes to hours from LUV to LUV. We checked for this phenomenon by varying the N-BAR-LUV incubation time prior to performing the sedimentation assay (figures 35 and 36). These time course experiments were performed with two different LUV compositions, one that demonstrated strong binding (DOPC:Ni-DOGS) and one that demonstrated weaker binding (DOPC:DOPS). We find, at the protein concentrations employed here, no such heterogeneity in binding. All subsequent experiments were performed after an incubation time of 10 minutes.
Figure 35: Plot of the percent of N-BAR (residues 1-256 +His tag) found in the pellet as a function of the incubation time of the protein with LUVs. LUVs were composed of 9:1 DOPC:Ni-DOGS. The filled points correspond to LUVs diluted 1:1 in 300 mOsM sucrose. The unfilled points correspond to LUVs diluted 1:1 in 299 mOsM NaCl + 1 mOsM DTT.
Figure 36: Plot of the percent of N-BAR (residues 1-256 +His tag) found in the pellet as a function of the incubation time of the protein with LUVs. LUVs were composed of 9:1 DOPC:DOPS. The filled points correspond to LUVs diluted 1:1 in 300 mOsm sucrose. The unfilled points correspond to LUVs diluted 1:1 in 299 mOsm NaCl + 1 mOsm DTT.

Binding as a function of LUV composition

**His-tagged N-BAR, residues 1-256**

We examined the impact of lipid composition on the membrane binding ability of two different N-BAR constructs; residues 1-357 from drosophila and residues 1-256 with a C-terminal hexahistidine tag from drosophila. For his-tagged N-BAR, as shown in Fig 37, LUV composition significantly alters the membrane binding affinity.
**Figure 37.** Plot of the percent of N-BAR (residues 1-256 + His-tag) found in the pellet as a function of membrane composition. The filled data points correspond to experiments performed in sucrose buffer while the unfilled data points correspond to experiments performed in salt buffer (same as in the caption of the previous figure). Compositions are as follows, from left to right: 90:10 DOPC:DOGS, 79:10:11 DOPC:DOGS:SM, 90:10 DOPC:PIP2, 90:10 DOPC:DOPS, 88:12 DOPC:SM, 90:10 DOPC:LysoPC, 90:10 DOPC:DOPE, 50:50 DOPC:DOPE, pure DOPC.

The amount of N-BAR pelleted out with the LUVs ranges from nearly 100% to 0% depending on the composition. The his-tag present at the C-terminus of N-BAR used in these experiments should bind specifically to the nickel chelating lipid DOGS regardless of N-BAR’s ability to bind the membrane, so compositions comprising DOGS should act as positive controls (Nye and Groves 2008). Indeed, both lipid compositions comprising DOGS, 90:10 DOPC:DOGS and 79:10:11 DOPC:DOGS:SM, show binding of between 91% and 100%. LUVs comprising 90:10 DOPC:PIP2 also show strong
binding of between 80% and 99%. These results agree with prior research demonstrating the preference of N-BAR for the negatively charged lipid PIP2 (Munn and Aspenström 2010). The results for LUVs comprising 90:10 DOPC:DOPS are less straightforward. Four samples prepared in 300 mOsm sucrose buffer demonstrated strong binding of between 85% and 98% while two samples prepared in the same buffer showed only moderate binding of between 16% and 18%. While the reason for this discrepancy is not known, it is possible that slight differences in composition from various LUV preparations may be responsible. N-BAR binding to DOPC:DOPS LUVs is strongly dependent on the molar ratio of DOPC:DOPS as will be discussed later. The four samples that demonstrated strong binding were from a single preparation, and the two samples that demonstrated moderate binding were from a separate preparation. Two DOPC:DOPS samples that were diluted in 299 mOsm NaCl +1mOsm DTT buffer showed low binding of between 2% and 4%.

Two samples comprising 90:10 DOPC:LysoPC were examined and demonstrated strong binding (58%) in one case and no detectable binding in the other case. The reason for the wide range of binding in these two cases is unknown and more data for this composition is needed. Of the five samples comprising 90:10 DOPC:DOPE, four of them showed weak binding of between 1% and 3% while one sample show high binding of 84%. Samples that showed weak binding of between 0% and 6% include LUVs comprising 88:12 DOPC:SM, 50:50 DOPC:DOPE, and pure DOPC. These low binding compositions did not demonstrate any dependence on the buffer used.
**Figure 38.** Plot of the percent of N-BAR (residues 1-357) found in the pellet as a function of membrane composition. The dark blue data points correspond to experiments performed in sucrose buffer while the light blue data points correspond to experiments performed in salt buffer. Compositions are as follows, from left to right: 90:10 DOPC:PIP2, 90:10 DOPC:DOG, 79:10:11 DOPC:DOG:SM, 90:10 DOPC:DOPS, 88:12 DOPC:SM, pure DOPC.

Figure 38 shows the amount of N-BAR pelleted out with the LUVs for the construct without a C-terminal his-tag. Like the his-tagged N-BAR, the amount of N-BAR bound to the LUVs is strongly dependent on the LUV composition with binding ranging from 0% to 100%. LUVs comprising 90:10 DOPC:PIP2 show strong binding ranging from 62% to 100%. LUVs comprising DOG, 90:10 DOPC:DOG and 79:10:11 DOPC:DOG:SM, also demonstrate strong binding ranging from 71% to 100%. In this case the strong binding cannot be due to interactions between the nickel chelating lipid, DOG, and histidine as this N-BAR construct does not contain a histidine-tag. LUVs
comprising 90:10 DOPC:DOPS show weak to moderate binding. DOPC:DOPS LUVs
diluted in 300 mOsm sucrose showed essentially no binding, while those diluted in 299
mOsm NaCl + 1mOsm DTT showed 25% binding. LUVs comprising both 88:12
DOPC:SM and pure DOPC showed weak binding of between 0% and 15%.

**The effect of lipid charge on N-BAR binding**

While there is some scatter in the data, there is a clear trend towards strong
binding to negatively charged lipids and weak to no binding to zwitterionic lipids. The
net charge on PIP2 depends on local pH and interactions with proteins, but should be
between -3 and -5 (McLaughlin and Murray 2005). The net charge on both DOGS and
DOPS is -1. All other lipids used are zwitterionic and most of these show very weak
binding. For both N-BAR constructs and both buffers (sucrose and salt), the LUVs
comprising PIP2 and DOGS demonstrate binding of between 62% and 100%. The strong
binding seen with these negatively charged membranes is to be expected based on the
regions of positively charged amino acids on N-BAR’s membrane-active face and agrees
with past experiments and simulations. What is unexpected is the response of N-BAR to
LUVs comprising DOPS. DOPS has the same formal charge as DOGS, yet the binding of
N-BAR to LUVs containing DOPS is not always strong. In particular, N-BAR binding to
DOPS LUVs diluted in salt buffer is weak. A 300 mOsm NaCl solution corresponds to a
Debye length of around 0.4nm and may be expected to interfere with N-BAR binding
provided that binding is due to electrostatic interactions. However, if this were the case,
one would expect the same interference to occur for the other negatively charged lipids,
DOGS and PIP2, yet, in most cases, this is not true.
In order to further explore the binding of N-BAR to vesicles containing DOPS, we incubated his-tagged N-BAR with LUVs comprising DOPC and DOPS and varied the amount of DOPS from 2.5 mole% to 10 mole% (figure 39).

Figure 39. Plot of the percent of his-tagged N-BAR (residues 1-256) in the pellet as a function of LUV composition. All LUVs are composed of DOPC and DOPS, with the mole percent of DOPS increasing from 2.5% to 10% from left to right. All experiments were performed in 300mOSm sucrose buffer

As the mole percent of DOPS increases from 2.5% to 7.5% the percent of N-BAR in the pellet increases roughly linearly. At 2.5 mole% DOPS the average percent of protein in the pellet is 8% ± 2%. The uncertainty is the standard error of the mean of the four separate measurements. At 5 mole% DOPS the average percent of protein in the pellet is 17% ± 3%. At 7.5 mole% DOPS the average percent of protein in the pellet is 27% ± 5%. The linear increase in binding with mole% DOPS suggests that, at low DOPS
concentrations, N-BAR binding depends simply upon the concentration of binding sites (negatively charged DOPS lipids) such that if you double the amount of negatively charged lipid, from 2.5% to 5% for example, the amount of protein bound should double (as it does- from 8% to 17%). However, as the amount of DOPS is increased to 10 mole%, rather than seeing double the amount of bound protein for the 5 mole% case (34%), we see a large jump to 93% ± 5% protein bound. The sigmoidal shape of the binding plot is, instead, suggestive of cooperative behavior and resembles a model described by Hinderliter (Hinderliter and May 2006), however, in this case the area fraction for bound N-BAR exceeds the area fraction of DOPS lipids. This type of membrane-mediated cooperative binding has been seen or at least suspected before, as with the C2A domain of synaptotagmin (Hinderliter and May 2006), and is particularly relevant for membranes that contain mixed lipid species in which one of the component lipids has a higher affinity for the protein than the other - as is the case here with N-BAR binding preferentially to negatively charged lipid species. Due to the different binding affinities for particular lipids, proteins may be able to locally demix the lipids and thus recruit more protein to the region rich in negatively charged lipids (J. Wang et al. 2002). Thus, even if protein-protein interactions are not present, protein-protein interactions mediated by the membrane may arise. Although experiments with simple proteins with regions of positive charge caused no demixing for monovalently charged lipids, these experiments have not been performed with BAR family members (Golebiowska et al. 2006). Similar experiments could be performed with other negatively charged lipids both mono and polyvalent, particularly PIP2 which has previously been shown to be sequestered by MARCKS, in order to see if cooperative binding by N-BAR occurs
generally with mixed membranes containing both zwitterionic and negatively charged lipids or depends on the specific lipid species present in the membrane.

Non-electrostatic effects

While electrostatics have a clear effect on N-BAR’s ability to bind membranes, there are other factors that may also impact binding. In particular, the N-terminal amphipathic helix of N-BAR domains is able to insert into the hydrophobic core of the membrane, and, as discussed in Chapter 4, this feature has been proposed to impact binding. One possible lipid characteristic that may influence N-BAR binding through the insertion of an amphipathic helix is spontaneous curvature. Based on the relative areas of lipid headgroup to lipid acyl chain lipids have a spontaneous curvature—either slightly negative, like DOPC, or slightly positive, like DOPS. When lipids with a negative spontaneous curvature are incorporated into the outer leaflet of a vesicle it imposes packing stress in the leaflet as the lipid, in order to prevent exposure of its hydrocarbon chains to the aqueous environment, must conform to the positively curved leaflet. The amount of packing stress imposed on the membrane will depend on how much the vesicle curvature departs from the spontaneous curvature of the lipid (Davies et al. 2001). If the amphipathic helix of N-BAR inserts into the outer leaflet it can relieve some of the packing stress and thus, compositions including lipids with more highly negative spontaneous curvature, like DOPE, may promote N-BAR binding. As seen in figure 37 we examined membranes comprising both 9:1 DOPC:DOPE and 1:1 DOPC:DOPE. While one sample showed strong binding, 84% bound to 9:1 DOPC:DOPE membranes, the remaining 6 samples show very weak binding of less than 5%. We also examined
membranes comprising a lipid, LysoPC, with positive spontaneous curvature expecting that it would act to reduce packing stress and disfavor binding. We found that one sample showed weak binding of around 0%, but that the other sample showed moderate to high binding of 60%. The reason for the discrepancy in these results is not known but might arise from a possible tendency of lipids with very different spontaneous curvatures to segregate and create vesicles with a dispersity of mole fractions. More experiments are needed to tease out the effect of lipid spontaneous curvature on N-BAR binding.

We also examined the effect of cholesterol on N-BAR binding. Because cholesterol can condense lipids and fill defects in the membrane (Kahya and Schwille 2006) it is possible that its presence in vesicles could disfavor the insertion of N-BAR’s amphipathic helix and reduce binding. We incorporated varying mole fractions of cholesterol into lipid compositions that demonstrated both strong and weak binding and the results are shown in figure 40.
Figure 40. Plot of the percent of his-tagged N-BAR in the pellet as a function of cholesterol content. Lipid compositions used were (from left to right): (90:10 DOPC:DOGS), (80:10:10 DOPC:DOGS:Chol), (70:10:20 PC:DOGS:Chol), (90:10 DOPC:DOPS), (80:10:10 DOPC:DOPS:Chol), (70:10:20 DOPC:DOPS:Chol), (88:12 DOPC:SM), (80:10:10 DOPC:SM:Chol), (Pure DOPC), (90:10 DOPC:Chol)

For the compositions tested there is no discernible effect from cholesterol. The compositions that demonstrated strong binding with no cholesterol (DOGS- or DOPS-containing membranes) continued to demonstrate strong binding with up to 20 mole% cholesterol. The compositions that demonstrated weak binding with no cholesterol (DOPC or DOPC:SM membranes) showed no increase in binding with 10 mole% cholesterol incorporated into the membrane. In order to rule out the possibility that cholesterol influences N-BAR binding, a composition that shows moderate binding of
around 50% should be doped with cholesterol and the effect on binding should be monitored. It may be that the compositions tested here bind so strongly that we are simply not sensitive to any effect of cholesterol.

**Conclusions**

There are two main characteristics of N-BAR that are thought to be crucial to biological function; the positive amino acid residues on the concave face of the protein and the N-terminal amphipathic helix that inserts into the membrane’s outer leaflet. As was discussed in Chapter 4, there is some controversy regarding the relative importance of these two features. The experiments discussed here demonstrate that the presence of negatively charged lipids is crucial for N-BAR binding and that each N-BAR appears to interact cooperatively with multiple anionic lipids. We incorporated both negative and positive spontaneous curvature lipids and cholesterol into the LUVs in order to either enhance or disfavor the insertion of the N-terminal amphipathic helix and found no clear impact on binding. For the protein concentration (14 μM), protein:l lipid ratio (1:100), and lipid compositions used here the electrostatic contribution to N-BAR binding is clear. The next chapter will turn the focus from chemical properties of the membrane to mechanical properties of the membrane.
CHAPTER 6

THE EFFECT OF MEMBRANE TENSION ON N-BAR BINDING

Membrane tension, like membrane composition, is controlled in biological cells. Membrane tension regulates functions that involve membrane deformations, typically through interactions between the membrane and the cytoskeleton via motor proteins, like myosin (Gauthier, Masters, and Sheetz 2012; Boulant et al. 2011; Nambiar, McConnell, and Tyska 2009). Because tension is highly controlled and plays a key role in membrane deformation, we expect that it will have a large impact upon BAR function. Here we investigate the effect of membrane tension on the binding of N-BAR to LUVs of a single diameter. This enables us to separate the effect of tension and strain from the effect of curvature. The results point to the importance of membrane strain in N-BAR binding, and may explain prior literature results.

Methods and materials

To determine the effect of osmotic stress on N-BAR binding to LUVs we prepared 100nm DOPC:PIP (9:1) LUVs by extrusion. All LUVs were prepared in 200 mOsm glucose solutions and incubated at 37°C for at least 24 hours to allow them to “round up” after the extrusion process. LUVs were then diluted 1:1 in either hyperosmotic or hypoosmotic solutions between 363 and 10 mOsM for a final concentration of 45 mg/mL and allowed to equilibrate for 30 min. This corresponds to initial osmotic pressure differences of between -70 and 75 mOsM. We used two different osmolytes, NaCl and glucose. LUVs were then incubated with 1 μM N-BAR in solution
and a sedimentation assay was performed. Any N-BAR that binds to LUVs will pellet out
during centrifugation while unbound protein remains in the supernatant. The percent of
N-BAR bound to the LUVs, which corresponds to the percent of protein found in the
pellet, can then be analyzed by SDS-PAGE.

Lipids, preparation and characterization of vesicles

Vesicles were made from a mixture of unsaturated zwitterionic phosphocholine
lipid and a minority of anionic phosphatidylinositol. We used 1,2-dioleoyl-sn-glycero-3-
phosphocholine (DOPC), purchased in powder form and dissolved in chloroform(Avanti
Polar Lipids Inc., Alabaster AL; cat. no. 850375), and L-α-phosphatidylinositol-4-
phosphate (Brain, Porcine) (PIP), purchased in powder form and dissolved in
chloroform(Avanti; cat. no. 840045). All LUVs discussed here were composed of 9:1
molar ratio of DOPC:PIP. Lipids were mixed in solution, dried under argon, and placed
under vacuum for at least 2 h to remove residual solvent. Multi-layer vesicles (MLVs)
were formed by dehydrating with 1 mL 200 mM glucose and vortexing, this mixture
yields a lipid concentration of 90 μg/mL. Large unilamellar vesicles (LUVs) of diameter
approximately 100 nm were prepared by extruding 15-20 times through a polycarbonate
membrane (Whatman, Piscataway, NJ, cat. No 7060-4701) with a pore size of 100 nm.

The filtered suspension of LUVs was used for further experiments either directly
after preparation or, alternatively, after incubation at 37°C for at least 24 h. As we will
show later (and as pointed out previously (Mui et al. 1993)) allowing a 24 hour
incubation is a crucial step in obtaining repeatable and interpretable results.
Dynamic light scattering at 632.8 nm was used to measure the size and monodispersity of the incubated LUVs (Malvern Instruments, Worcestershire, UK, Model Zen3600 Zetasizer). The mean size, by number was 104 nm diameter (figure 41).

![Size Distribution by Number](image)

**Figure 41.** Dynamic light scattering measurement of incubated LUVs at 632.8 nm.

**Controlling Osmolarity**

The osmotic pressure of the solution on the exterior of the LUVs was tuned by adding either glucose or NaCl solution of known concentration. Separate solutions of glucose and of NaCl were prepared at the highest concentration to be used, 350 mOsm, then syringe-filtered through a 0.2 μm filter, and then diluted with de-ionized water to the desired concentration. Osmalalities of all solutions were measured by a Vapro vapor pressure osmometer (Wescor Inc, Logan, UT, model #5600). All solutions were used within 48 hours of preparation; over this period concentration changes owing to evaporation were not detectable by the osmometer thus ensuring that any concentration changes due to evaporation were undetectable within the error of the osmometer.
Protein purification

Residues 1-256 of amphiphysin from *Drosophila melanogaster* were [obtained from the MacMahon laboratory]. These residues were expressed as His-tagged proteins from a pETite Kanamycin vector in *E. coli* BL21 DE3 cells. The cysteine residue at position 66 was previously mutated to alanine to facilitate attachment of a maleimide dye at position 82, however, all protein used in these experiments was unlabeled. Details about the purification procedure can be found in Chapter 2. Before use in binding experiments, N-BAR in buffer A was diluted with deionized water to achieve a final protein concentration of 4 μM and buffer concentration of 200 mOsm, and gently centrifuged 10 min at 10,000 rpm (Eppendorf, Hamburg, Germany, model 5415 D) to remove large protein aggregates.

Sedimentation assay

Sedimentation assays were used to quantify the amount of N-BAR bound to the vesicles. The LUVs are buoyant in suspension but become denser than the solution when N-BAR binds to them. The amount of NBAR in the sediment and the supernatant are then measured by gel electrophoresis.

For sedimentation assays a fixed amount (25.7 μL) of protein was incubated between 5 and 10 minutes with a known quantity of LUVs in salt or glucose buffers of known osmolarity for a final protein concentration of 1 μM. Sedimentation of 75 μL samples was accomplished by centrifugation at 160,000xg for 30 minutes (Beckman Coulter, Brea, CA, model Optima TLX ultracentrifuge with a TLA 100 rotor). After centrifugation a 10 μL sample of the supernatant was taken and the rest was immediately
removed with a pipette. The sediment (pellet) was then resuspended in a volume of buffer equal to that removed and a 10 μL sample was taken.

12 μL aliquots of both the supernatant and pellet (mixed 1:1 with protein loading buffer) were separately run on SDS-PAGE (10%) and quantified by the gel analysis tool of ImageJ. Any samples that bled into neighboring lanes of the SDS-PAGE gel were discarded. The fraction of bound protein was defined as the ratio of the integrated intensity of the pellet to the sum of the integrated intensities of the pellet and of the supernatant. Variations in defining a baseline in the ImageJ gel analysis tool resulted in <1% variation in the percent of bound protein.

In the data reported here, the protein:lipid ratio is 1:40. Maintaining this ratio is important in the sedimentation assays. When the ratio is decreased to 1:400, the bound fraction as determined from sedimentation is saturated near 100% because of the very small quantity of N-BAR remaining in the supernatant. In this regime, osmotic-pressure-induced variations in binding cannot be detected.

**Results**

Figure 42 shows the percent of protein found in the pellet as a function of the initial difference in osmotic pressures inside and outside the vesicles. The solute concentration in the vesicles’ interior was 200 mOsm and, in these experiments, the exterior was tuned over the range of 10 to 33 mOsm. Uncertainty in $f_0$ were measured from 4 experiments with one applied osmotic gradient. The standard deviation of these measurements was 10% and we assume that this fractional uncertainty is the same for all data.
From the ideal gas/Van’t Hoff/Morse equation, this corresponds to initial osmotic pressure differences of between $-1.7 \times 10^5$ Pa and $1.9 \times 10^5$ Pa. Over the course of the incubation and sedimentation assays (~30 min), solute molecules in the vesicle are retained but water permeates, so that the vesicle volume can change in response to the exterior osmotic pressure (Mui et al. 1993; Polozov et al. 2001).

Noting that the $f_b$ is constant (within error) near $\Delta c = 0$, we define a reference bound fraction $f_b^0$ as the mean of the 4 data points with $\Delta c$ between $-12\text{mOsm}$ and $+5\text{mOsm}$: $f_b^0 = 0.110 \pm 0.005$. At this binding fraction, we can estimate the fraction of vesicle surface that is covered by bound N-BAR as follows: The surface area of N-BAR is approximately $3600 \ \text{Å}^2$ (Bhatia et al. 2009) and the area per lipid of DOPC is $72.5 \ \text{Å}^2$ (Petrache et al. 2004) from the known initial protein:l lipid ratio we find that $f_b^0$ corresponds to approximately 12.5% area coverage.

The most striking result in figure 42 is that the N-BAR binding increases dramatically, by a factor of up to six, when membranes are made tense ($\Delta c > 0$). For the data with glucose as osmolyte, we find a maximum $f_b = 0.72 \pm 0.07$. This bound fraction corresponds to an approximate close packing of BAR on the membrane (approximately 70% area coverage). While the other data points with $\Delta c > 10 \ \text{mOsm/kg}$ show scatter, the trend of sharply increasing $f_b$ is quite clear.
Figure 42. Plot of the fraction of bound N-BAR as a function of the applied osmotic difference. All LUVs are 100 nm extruded vesicles that were incubated in glucose at 37°C for at least 24 hours prior to experiments. The square data points correspond to LUVs diluted into hypertonic(-) or hypotonic(+) glucose solutions. The triangle data points correspond to LUVs diluted into hypertonic or hypotonic NaCl solutions. The star data point corresponds to a reversibility experiment in which the LUVS were exposed to an initial applied osmotic difference of 37 mOsm/kg (data point not shown as the protein:lipid ratio is dissimilar from the other data points on this plot), and was then exposed to an applied osmotic difference of -25 mOsm/kg.

Under more hypertonic conditions ($\Delta c > 60$ mOsm/kg), we find that $f_b$ decreases to approximately the value measured in unstressed LUVs, $f_b^0$. We attribute this reduction of $f_b$ to rupture (lysis) of the vesicles, following which the solute concentration inside the vesicles could be rapidly reduced toward the value of the exterior concentration. In this regime, the osmotic imbalance $\Delta c$ is not known except that it must be closer to zero. It has been shown that lysis is not an all-or-nothing event, rather there is a series of lytic events in which the vesicle releases a portion of its contents, and reseals at a lower
tension (Mui et al. 1993). Thus, it may be that LUVs exposed to applied gradients of 70 mOsm/kg are experiencing lytic events and the actual membrane tension is not known. We will return to this point below, in the context of a discussion of the tensions arising from Δc.

Under hyperosmotic conditions (Δc < 0), figure 1 shows quite different behavior: the bound fraction fb increases smoothly. Under the most hyperosmotic conditions probed with glucose, fb increased to 0.23 ± 0.04, approximately a two-fold increase relative to the zero-tension reference value, fb0. The hyperosmotic data show less scatter than the hypoosmotic data and indicate a smooth increase in fb as Δc becomes more negative. It has been shown that osmotic shrinkage can lead to protrusions (regions of high curvature) (Disalvo et al. 1996). N-BAR has been shown to bind preferentially to highly curved membranes, so it may be that at large negative osmotic gradients LUVs display regions of high curvature thus slightly increased binding. We return to a discussion of this point below.

Role of solute species

We repeated the above experiments using NaCl to adjust osmolarity rather than glucose. A comparison of the data obtained by addition of NaCl (▲) rather than glucose (■) reveals no significant difference. While a specific interaction between glucose and the lipids or N-BAR was not expected, the comparison of different solutes is nonetheless helpful in demonstrating that the effect of Δc is chiefly from osmotic pressure. Specifically, the lack of a difference between NaCl and glucose suggests that the electrostatic interaction between the N-BAR and lipids is not essential for the tension
response. For the NaCl data with $\Delta c = 25$ mOsm/kg, we find a high binding fraction of $f_b = 0.60 \pm 0.06$ with $[\text{NaCl}] = 250$ mOsm (or 125 mM) which corresponds to a Debye length of about 1nm. May be expected to impact N-BAR’s ability to bind membranes if the electrostatic contribution of positive residues on N-BAR interacting with PIP’s negatively charged headgroups was the dominant factor involved in binding. This result agrees with previous research showing that increasing salt concentration decreases BAR binding but not N-BAR binding (Henne et al. 2007) and suggests that the dominant factor influencing binding in these experiments is N-BAR’s N-terminal amphipathic helix.

**Reversibility**

To test the reversibility of N-BAR binding, we first incubated N-BAR with vesicles in hypoosmotic solution ($\Delta c > 0$; making the vesicles tense) then in hyperosmotic solution ($\Delta c < 0$; making the vesicles floppy). In one experiment, LUVs were exposed to $\Delta c = 37$ mOsm/kg (glucose), $f_b$ was measured, and then vesicles and N-BAR were incubated in $\Delta c = -25$ mOsm/kg (glucose) for 30 min and $f_b$ was measured again. After the first step, we found $f_b = 0.11 \pm/-.01$, but since the protein:l lipid ratio was 1:20 the results cannot be directly compared to the data in figure 41. (In our control experiments, a higher protein:l lipid ratio reduced the measured bound fraction.) After the second incubation step, we found $f_b = 0.040 \pm .004$; in this case the protein:l lipid ratio was 1:40, the same as that of the other measurements of figure 42. This measurement is represented in Fig. 42 by the star. This bound fraction is close to (albeit slightly less than) the zero-tension reference value $f_b^0$. More importantly, the bound fraction is substantially less
than that of the initial $\Delta c$ of 37 mOsm/kg, which indicates that N-BAR re-equilibrated by desorbing from the vesicles during the second 30-min incubation period.

Additional reversibility experiments were performed with an initial protein:lipid ratio of 1:40 and a final ratio of 1:80. In these cases, if binding were *irreversible*, then the amount of protein in the pellet would not change upon dilution of the buffer solution. Thus, the fraction of protein found in the pellet would not change, but the amount of protein found in the 65 µL supernatant would decrease two-fold and the apparent $f_b$ would *increase*. We found, however, that binding decreases. Two samples incubated initially at $\Delta c = 40$ mOsm/kg showed bound fractions of 0.72 and 0.54 (1:40 protein:lipid ratio). After incubation in $\Delta c = -38$ mOsm/kg for 5 min, we measured bound fractions of 0.48 and 0.25 for the same samples, respectively (1:80 protein:lipid ratio). Again, these data are not shown in figure 42 because of the different protein:lipid ratio. While we cannot perform a reversibility experiment while maintaining protein:lipid ratio throughout, taken together the three sets of data suggest that N-BAR binding is at least partially reversible and likely fully reversible if incubation time is sufficient (~ 30 min or more).

**Results with as-made LUVs (not pre-swelled)**

Importantly, the response of N-BAR binding to LUVs that have not been allowed to incubate for at least 24 hours in a glucose solution after extrusion is markedly different from those that have incubated for at least 24 hours. Experiments performed with LUVs tested directly after extrusion show no increase in binding associated with applied hypoosmotic solutions (figure 43). Instead, there is possibly a slight trend of decreased
binding as the applied osmotic gradient becomes hypotonic. For applied osmotic
gratings of between -25 and 20 mOsm/kg most vesicles show binding of between 10% 
and 30%. We attribute this difference to changes in vesicle shape rather than changes in
tension from osmotic stress. Mui et al. showed that extruded LUVs in sugar or salt
solutions, often assumed to be spherical, are instead mostly discoid. This departure from
sphericity is caused by the extrusion process. If LUVs are then exposed to a hypoosmotic
solution they will first “round up” to spheres without experiencing an osmotic pressure or
tension (Mui et al. 1993). Because membranes are slowly permeable to glucose, LUVs
prepared in glucose solutions, and not exposed to any osmotic gradients, will naturally
round up over time in order to minimize bending forces without paying for osmotic
forces associated with volume changes. This ensures that the initial shape of extruded
LUVs is spherical, and that any hypoosmotic gradients applied are tensing the vesicles
rather than simply changing their shape. If the vesicle’s initial shape is different from a
sphere and we apply increasingly hypoosmotic gradients, the LUVs become more and
more spherical, but do not experience changes in tension. Indeed, Mui et al. showed that
100nm EPC:Chol LUVs are able to experience applied osmotic gradients of up to 1780
mOsm/kg without experiencing any tension. Thus, the small decrease in binding with
hypoosmotic gradients reflects N-BAR’s preference for more highly curved, or less
spherical, vesicles. These results imply that the initial tension and shape of extruded
vesicles is an important experimental parameter.
Figure 43. Plot of the percent of N-BAR in the pellet as a function of the applied osmotic difference for 100 nm LUVs used without incubation in glucose.

Discussion

Pressure-tension conversion

This model applies to vesicles exposed to hypoosmotic gradients. The calculated tensions in these experiments range from 0 to 4.9 mN/m, and the dramatic increase in N-BAR binding corresponds to tensions between 1 and 2 mN/m. Tensions above ~4.5 mN/m correspond to decreased N-BAR binding and may be due to vesicle lysis. It has been shown that lysis is not an all-or-nothing event, rather there are a series of lytic events in which the vesicle releases a portion of its contents, and reseals at a lower tension. While a lysis tension of 4.5 mN/m is slightly lower than the reported lysis tension of 9.9 mN/m for pure DOPC LUVs (Olbrich et al. 2000), this difference may be due to composition (9:1 DOPC:PIP), or the interaction of N-BAR with the membrane.
Recent MD simulations of Dil labeled DPPC bilayers under tension show that tension causes a change in acyl chain orientation and a decrease of lipid density at the headgroup area (Muddana et al. 2011). A decrease in lipid density in the headgroup region will expose some of the hydrophobic core of the membrane to solvent and is analogous to creating defects in the membrane. Membrane defects have previously been suggested as being responsible for increased N-BAR binding to more highly curved vesicles because N-BAR’s N-terminal amphipathic helix is able to fold more readily in regions with membrane defects (Bhatia et al. 2009). Simulations of PS:PC bilayers with varying curvature have shown that defect area and defect area fraction both increase as membrane curvature goes from flat to 0.02 nm⁻¹ (Cui, Lyman, and Voth 2011). While previous research into N-BAR binding has focused on the response to curvature (modulated by vesicle size), our results, which describe vesicles of one size only, suggest that the important membrane property in determining N-BAR binding is areal strain \( \frac{\Delta A}{A_0} \) of the outer leaflet. Strain may arise from either increased curvature, where the inner leaflet is compressed and the outer leaflet is stretched, or from increased tension, as with osmotic swelling. From the simulation data published by Voth et al (Cui, Lyman, and Voth 2011), the calculated strain of a 100nm vesicle, given by the area per lipid, defect area, and defect area fraction, is 0.02. We also calculated strain in our osmotically stressed vesicles:

\[
\alpha = \frac{\Delta P r}{2K}
\]

where \( \alpha \) is the strain, \( \Delta P \) is the applied pressure difference, \( r \) is the vesicle radius, and \( K \) is the area expansion modulus. The applied pressure difference of the case with highest N-BAR binding is \( 9.7 \times 10^4 \) Pa, the vesicle radius is 50 nm, and the area
expansion modulus is 200 mN/m, giving strain of 0.012. This value of strain is commensurate with the value of the strain calculated from simulation data.

**Area expansion upon binding**

In addition to interpreting the increase in binding of N-BAR to osmotically tensed vesicles as a function of defects, one could also take a continuum approach. If binding increases with applied tension, then something must be increasing the membrane area upon binding. Because N-BAR binding seems to be reversible we can use a Boltzmann argument to estimate the change in area on binding:

$$\text{Probability of binding} \propto e^{-\frac{\Delta E}{kT}}$$

If binding increases by a factor of 5 upon tensing then $\Delta E = 2kT$. The binding energy should be proportional to $\sigma \Delta A$, where $\sigma$ is the tension and $\Delta A$ is the change in area. For a tension of 2 mN/m this corresponds to an area change of 4 nm$^2$ per bound protein. This area change is quite similar to the area of N-BAR’s amphipathic helix, 6 nm$^2$ (Cui, Lyman, and Voth 2011), and suggests that the insertion of the amphipathic helix is responsible for the increased binding seen in tensed vesicles. These results are similar to the response to tension seen with “molecular harpoons”- molecules that are able to sense bilayer stress (Naka, Sadownik, and Regen 1992). Increased binding or activity in stressed membranes has been seen with the antifungal drug Amphotericin B (Wolf and Hartsel 1995), and with phospholipase A2 (Lehtonen and Kinnunen 1995).

These results imply disagreement with other bulk sedimentation experiments (Peter et al. 2004; Gallop and McMahon 2005; Henne et al. 2007) that find BAR domains, rather than N-BAR domains, sense curvature and attribute curvature sensing to
favorable electrostatic interactions with membranes that more closely match the proteins intrinsic shape. Although we did not test the effect of tension on the binding of BAR (without the N-terminal amphipathic helix) we predict that BAR would not be sensitive to membrane tension (or curvature). Indeed, measurements on individual vesicles by the SliC assay (Bhatia et al. 2009)(Hatzakis et al. 2009) found no curvature sensing ability for BAR domains lacking an N-terminal amphipathic helix. The authors attributed the lack of BAR curvature sensing and the ability of N-BAR to sense curvature on the increase in packing defects found in curved membranes which favors amphipathic helix insertion and folding. However, our results show that curvature changes are not needed for increased binding, and that changes in tension alone can accomplish binding increases. These results also highlight the need for understanding the initial membrane tension which is usually not considered or controlled.
CHAPTER 7

THE EFFECT OF MEMBRANE TENSION ON CURVATURE INDUCTION BY N-BAR

N-BAR has another function besides binding to membranes, it is also implicated in deforming membranes by causing tubulation. While lateral tension may favor binding as shown in the previous chapter, it may disfavor tubulation. In order to examine this possibility we control membrane tension osmotically and monitor the resultant tubulation by N-BAR.

The accepted theory for describing the energetics of membrane deformation, developed by Helfrich (Helfrich 1973), encompasses the spontaneous, mean and gaussian curvatures, and the elastic properties of the membrane. Starting from the Helfrich description, we can estimate the energy that is needed to pull a tube of length, L, and diameter, d, from a vesicle with initial radius, R₀, and initial tension, T₀.

![Diagram of vesicle deformation](image)

**Figure 44.** Initial vesicle of radius R₀, and vesicle of radius R with a cylinder of length L and diameter d pulled out of it.

We start with the assumption that the volume of the vesicle remains fixed throughout the pulling of the tube. We assume that the osmotic pressure “clamps” the volume by making the work of volume changes much larger than other contributions to
the energy. We also assume that the tube has length L, is cylindrical (of diameter d), and terminates in a hemispherical cap:

\[
\frac{4}{3}\pi R_0^3 = \frac{4}{3}\pi R^3 + \pi L \left(\frac{d}{2}\right)^2 + \frac{2}{3}\pi \left(\frac{d}{2}\right)^3
\]

1)

Part of the energy comes from the bending of the membrane as the tubule is formed. The ability of the membrane to resist bending is described by the bending rigidity modulus, K. The energy of the membrane due to bending is described by the Canham-Helfrich form:

\[
E_{\text{bend}} = \frac{1}{2}K \int dA \left(C_1 + C_2 - C_0\right)^2
\]

2)

We assume that the spontaneous curvature \(C_0 = 0\) because of the symmetry of the two leaves of the bilayer. The change in energy is therefore described by:

\[
\Delta E_{\text{bend}} = \pi d \left(L + \frac{d}{2}\right) \frac{1}{2}K \left(\frac{2}{d}\right)^2 - \frac{1}{2}K \left(4\pi R - \pi \frac{d^2}{4} - 4\pi R_0^2\right)
\]

3)

The first term is due to the cylindrical tube and hemispherical cap, and the second term is due to the spherical vesicle. This term can be written:

\[
\Delta E_{\text{bend}} = \frac{\pi K}{2} \left(L \frac{dL}{R_0^2} + 2 - \frac{d^2}{2R_0^2} - \frac{d^2}{4R^2}\right)
\]

4)

The next contributor to the energy is the tension in the membrane. Pulling a cylinder of length, L, against tension, T(L), requires:

\[
\Delta E_{\text{tension}} = \int_{0}^{L} \pi dL \ T(L)
\]

5)
Here we assume that we start with an initial state where the spherical vesicle is under some tension, $T_o$. As the length of the cylinder increases the tension increases according to:

$$\Delta T = \frac{k \Delta A}{A_0}$$

6)

Where $k$ is the membrane stretching modulus. Thus,

$$T(L) = T_o + \frac{k \Delta A}{A_0}$$

7)

The initial area is simply the area of the initial spherical vesicle, and the final area is the area of the (now smaller) spherical vesicle minus a hole where the tubule protrudes, plus the area of the cylindrical tubule with a hemispherical cap, giving:

$$\frac{\Delta A}{A_0} = \frac{4 \pi R^2 - \frac{\pi d^2}{4} + \frac{\pi d}{4} (L + \frac{d}{2}) - 4 \pi R_0^2}{4 \pi R_0^2}$$

8)

The assumption that the volume remains fixed allows us to use equation (1) to replace $R$ with $R_o$.

$$R^2 = \left( R_0^3 - \frac{3}{16} \frac{d^2 L}{16} - d^3 \right)^{\frac{2}{3}}$$

9)

Because $d << R_0$ we can approximate this relationship as:

$$R^2 \approx R_o^2 \left( 1 - \frac{d^2 L}{8 R_o^3} - \frac{d^3}{24 R_o^3} \right)$$

10)
\[ \frac{\Delta A}{A_0} = -\frac{d^2 L}{8 R_0^3} - \frac{d^2}{24 R_0} - \frac{d^3}{16 R_0^2} + \frac{d}{4 R_0^2} \left( L + \frac{d}{2} \right) \]

Returning to equation (5) we can now write the energy term from tension as:

\[ \Delta E_{\text{tension}} = \pi d LT_0 + \pi d k \int_0^L dL \left( -\frac{d^2 L}{8 R_0^3} - \frac{d^3}{24 R_0^2} - \frac{d^2}{16 R_0} + \frac{d}{4 R_0^2} \left( L + \frac{d}{2} \right) \right) \]

\[ = \pi d LT_0 + \pi d L k \left( \frac{dL}{8 R_0^2} + \frac{d^2}{16 R_0} - \frac{d^2 L}{16 R_0^3} - \frac{d^3}{24 R_0^3} \right) \]

We can also use equation (10) to rewrite the bending term (4) in terms of \( R_0, d, \) and \( L \):

\[ \Delta E_{\text{bend}} = \frac{\pi K}{2} \left( \frac{L}{d} - \frac{dL}{R_0^2} + 2 - \frac{3d^2}{4 R_0^2} \right) \]

Combining equations (12) and (13) gives the total:

\[ \Delta E_{\text{total}} = \frac{\pi K}{2} \left( \frac{L}{d} - \frac{dL}{R_0^2} - \frac{3d^2}{4 R_0^2} + 2 \right) + \pi d LT_0 + \pi d L k \left( \frac{dL}{8 R_0^2} + \frac{d^2}{16 R_0} - \frac{d^2 L}{16 R_0^3} - \frac{d^3}{24 R_0^3} \right) \]

In order to determine which, if any, terms are negligible we must deal with the two parameters \( K \) and \( k \) which have different units and magnitudes. A rough relationship between the two moduli is:

15) \( K \approx kh^2 \)

Where \( h \) is the thickness of the membrane. For the example of DOPC, \( h \) is approximately 4nm, so that \( h \ll R \). Replacing \( K \) with \( kh^2 \) in order to compare the orders of magnitude of these terms gives:

\[ \Delta E_{\text{total}} = \pi kd L \left( \frac{h^2}{2 d^2} - \frac{h^2}{2 R_0^2} + \frac{3dh^2}{8 R_0^2} - \frac{h^2}{8R_0^2} + \frac{dL}{8 R_0^2} - \frac{d^2 L}{16 R_0^3} - \frac{d^3}{24 R_0^3} \right) + \pi d LT_0 \]

Collecting terms for easier comparison gives:
\[ \Delta E_{\text{total}} = \pi d L k \left[ \frac{h^2}{d^2} \left( 2 + \frac{d}{L} \right) + \left( \frac{d^2}{R_0^2} \right) \left( 1 - \frac{3d}{8L} \right) + \left( \frac{L}{d} \right) \left( \frac{1}{16} - \frac{24d}{24R_0} \right) \right] + \pi d L T_0 \]

While it is possible that \( \frac{d}{L} \approx 1 \), both \( \frac{h^2}{d^2} \) and \( \frac{d^2}{R_0^2} \) are \( \ll 1 \). The only non-negligible terms are:

\[ \Delta E_{\text{total}} = \pi d L k \left[ \frac{h^2}{d^2} \left( \frac{1}{2} + \frac{d}{L} \right) + \left( \frac{d^2}{R_0^2} \right) \left( \frac{L}{8d} + \frac{1}{16} \right) \right] + \pi d L T_0 \]

After reinserting the stretching modulus \( K \), we get the final result keeping only the leading order terms:

\[ \Delta E_{\text{total}} = \pi d L K \left( \frac{1}{2d^2} \right) + \pi d L k \left( \frac{dL}{8R_0^2} + \frac{d^2}{16R_0^2} \right) + \pi d L T_0 \]

The first term is from bending, the second from tension associated with pulling area out of the vesicle at constant volume, and the final term is from pre-existing tension. Now that we have a general expression for the energy needed to pull out a tubule we can use it to explore some of the tubule’s properties. One property that is immediately discernible from the energy equation is that there is a competition between the tension and bending terms. Smaller tube diameter is beneficial to the tension components of the energy, but is detrimental to the bending term. Within this model, we can calculate the equilibrium radius by minimizing the energy with respect to tubule diameter for a given tubule length:

\[ \frac{\partial E}{\partial d} = -2\pi L K + \frac{2k\pi dL^2}{8R_0^2} + \frac{3k\pi d^2L}{16R_0^2} + \pi LT_0 = 0 \]

Provided that the initial vesicle size is much larger than the tubule, we can treat the

\[ \frac{1}{R_0^2} \]

terms:

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\[ d = \sqrt{\frac{2K}{T_0}} \quad \rightarrow \quad r_{\text{tube}} = \sqrt{\frac{K}{2T_0}} \]

21) We can also determine the force needed to pull out a tubule of a given radius, by micropipette aspiration for example, by adding a force term \((-fL)\) to the energy and minimizing with respect to the tubule length:

\[ f = 2\pi \sqrt{2KT_0} \quad \rightarrow \quad f = \frac{2\pi K}{R_0} \]

22) This relationship between force and tubule radius has been used to determine the bending moduli of various lipid species (Waugh and Hochmuth 1987; Bo and Waugh 1989)

Because we are ultimately interested in interactions between the membrane and membrane-binding proteins, it is necessary to add a term to the energy to account for bound protein:

\[ \Delta E_{\text{total}} = \pi dKL \left( \frac{1}{2d^2} \right) + \pi dLk \left( \frac{dL}{8R_0^2} + \frac{d^2}{16R_0^2} \right) + \pi dLT_0 - \pi dL\rho \Delta \mu \]

23) Where \( \rho \) is the number density of proteins bound to the tubule, and \( \Delta \mu \) is the difference in chemical potential of a protein bound to the spherical vesicle and that of a protein bound to a tubule of diameter, \( d \). Performing an energy minimization with respect to tubule length, \( L \), while holding tubule diameter, \( d \), fixed gives a prediction for the equilibrium length of a tubule:

\[ \frac{\partial E}{\partial L} = \frac{\pi K}{2d} + \frac{\pi kd^3}{4R_0^2} + \frac{\pi kd^3}{16R_0^3} + \pi dT_0 - \pi d\rho \Delta \mu = 0 \]

24) This gives the equilibrium value of \( L \):

\[ L = \frac{4R_0 \rho \Delta \mu}{kd} - \frac{4R_0^2 T_0}{kd} - \frac{d}{4} - \frac{2KR_0^2}{kd^3} \]

25)
We expect that membrane tension is a key contributor to the deformation, or lack thereof, of vesicles by membrane-binding proteins, as tubule formation requires work against tension. By placing a restriction on the length of a tubule, namely that it be greater than zero, we can glean some information about the difference in chemical potential needed for membrane-binding proteins to be able to form a tubule:

\[
\rho \Delta \mu \geq T_0 + \frac{kd^2}{16 R_o^2} + \frac{K}{2d^2}
\]

26)

Again, if we regard the vesicle as a reservoir for the tubule this reduces to:

\[
\rho \Delta \mu \geq T_0 + \frac{K}{2d^2}
\]

27)

It is important to note that the difference in chemical potential between proteins bound to a spherical vesicle versus proteins bound to a tubule should depend on the tubule diameter. Often, proteins that tubulate vesicles have an intrinsic curvature that they appear to impose on the tubules, thus the diameter, \(d\), of a tubule is set by the protein. For members of the BAR family of proteins this diameter ranges from 50-70 nm (Takei et al. 1999). If we start with a floppy vesicle where \(T_0 = 0\), we can get a rough estimate of the difference in chemical potential needed for tubule formation. Here we use a typical value for the bending modulus of \(4-15 \times 10^{-20}\) J (Marsh 2006). The number density of BAR proteins (F-BAR) on tubules has been determined through electron microscopy to be roughly \(1/70\) nm\(^2\) (Frost et al. 2008). These values give a value for \(\Delta \mu\) of \(4-14 \times 10^{-22}\) J or roughly 0.1 to 0.4 k\(b\)T. Recent experiments measuring binding isotherms of BAR on vesicles of varying diameter showed a gain in free energy of just over 1 kT for binding to 100 nm diameter vesicles vs. 700 nm vesicles. According to our rough estimate of the energy required to form a tubule, even this relatively small gain in energy should be
sufficient to form tubules. However, if the initial membrane tension is greater than zero, the difference in chemical potential may not be enough to initiate tubulation. In order to examine the effect of membrane tension on tubulation by N-BAR we made giant unilamellar vesicles tense or floppy osmotically, incubated them with N-BAR, and monitored any tubulation by fluorescence microscopy.

**Methods and materials**

**Lipids and GUV preparation**

Dioleoyl-\textit{sn}-glycero-3-phosphocholine (DOPC), Brain extract, the nickel salt of 1,2-dioleoyl-\textit{sn}-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DOGS), and 1,2-dipalmitoyl-\textit{sn}-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhod PE) were purchased from Avanti Polar Lipids Inc. (Alabaster AL) and used without modification.

GUVs were prepared by electroformation in 175 mM sucrose solution, using the technique of Angelova \textit{et al.} and described in detail in Chapter 2. In all experiments, the DOPC: Brain extract: DOGS ratio was 8:1:1 with 0.8mol\% Rhod PE. After electroformation, GUVs were diluted 1:1 in either 160, 165, 170, 180, 185, or 190 mM glucose and allowed to equilibrate for 1 hour. GUVs were then incubated with N-BAR (residues 1-252 from drosophila, final concentration of 15 \(\mu\)M) for between 10 minutes and 1 hour.

**Microscopy and analysis**

GUVs were observed with epifluorescence microscopy using a Zeiss Axiovert 200 inverted microscope equipped with a Plan-NEOFLUAR 100x oil objective and a Hamamatsu CCD camera. A filter set with a 480-nm short-pass filter, a 505-nm dichroic
mirror, and a 535-nm long-pass filter (Chroma Technology Corp., Bellows Falls, VT) was used with a 100 W mercury lamp. After GUVs were incubated with N-BAR and allowed to equilibrate for a set period of time, a series of still images was taken for each sample, resulting in images of between 50 and 100 vesicles for each osmotic pressure gradient. Vesicles were then analyzed by eye to determine if and how many tubules were present in each vesicle.

**Results**

Giant unilamellar vesicles (GUVs) with a composition of 8:1:1 DOPC:Brain extract:DOGS with 0.8 mol% Rhod PE were electroformed in 175 mM sucrose. The Rhod PE partitions to the Lα phase (Tobias Baumgart, Hunt, et al. 2007). The GUVs were then diluted in hypertonic glucose solutions (180, 185, or 190 mM) or hypotonic glucose solutions (170, 165, or 160 mM). GUVs were allowed to equilibrate for one hour and then incubated with N-BAR for a final protein concentration of 15 μM. GUVs were then imaged in epifluorescence and analyzed by eye to determine if tubules were present. Sample images are shown in figure 45.
Figure 45. Sample epifluorescence images of GUVs. (A) GUVs exposed to a hypotonic gradient of -0.06 atm -no N-BAR was added. (B) GUVs exposed to a hypertonic gradient of 0.06 atm. (C) GUVs exposed to a hypertonic gradient of 0.12 atm. (B) and (C) were incubated with 15 μM N-BAR. Arrows highlight examples of tubulation.

As shown in figure 46, vesicles that were exposed to hypertonic glucose solutions (floppy) had a greater percent of vesicles with tubules than vesicles that were exposed to hypotonic solutions (tense). Applied osmotic gradients can be converted to osmotic pressure differences through the Morse equation:

$$\Delta \pi = (C_{in} - C_{out}) \cdot RT$$

where $\Delta \pi$ is the initial osmotic pressure difference, $C_{in}$ and $C_{out}$ are the molar concentrations inside and outside the GUVs, $R$ is the ideal gas constant, and $T$ is the temperature.
**Figure 46.** Plot of the percent of vesicles with at least one tubule as a function of the initial applied osmotic pressure difference. The line at 6% of vesicles tubulated corresponds to control experiments with no N-BAR. All other data points correspond to vesicles which were incubated with 15 μM N-BAR for one hour.

The initial osmotic pressure differences for vesicles exposed to hypertonic solutions were 0.06 atm, 0.12 atm, and 0.18 atm and led to 40%, 46%, and 54% of vesicles being tubulated respectively. The initial osmotic pressure differences for vesicles exposed to hypotonic solutions were -0.06 atm, -0.12 atm, and -0.18 atm and led to 12%, 16%, and 12% of vesicles being tubulated respectively. The electroformation process, while often producing spherical vesicles, does occasionally produce vesicles that are markedly non-spherical and may have shapes that resemble tubules, and this departure from sphericity may be exacerbated by applying a hypertonic gradient. In order to account for this, a control experiment was performed in which vesicles were incubated with the largest
hypertonic gradient, allowed to equilibrate for one hour, and images of these vesicles were taken without any N-BAR present. We found that 6% of vesicles had tubule-like protrusions in the absence of N-BAR. While GUVs made tense osmotically showed marginally more tubulation than the control, GUVs made floppy osmotically showed markedly more tubulation than the control.

In order to examine the dynamics of tubule formation we incubated GUVs with N-BAR and took images every 10 minutes for half an hour. As shown in figure 47, in most cases tubulation continued to increase over the course of 30 minutes, particularly for vesicles made floppy osmotically.

![Graph showing the percentage of vesicles tubulated as a function of time with N-BAR and initial pressure difference.](image)

**Figure 47.** Plot of the percent of vesicles with at least one tubule as a function of the incubation time with N-BAR and the initial applied osmotic pressure difference.

Two GUV samples exposed to hypotonic environments, those exposed to pressure differences of -0.06 atm and -0.18 atm, showed essentially no increase in tubulation as a
function of time while the remaining sample exposed to a hypotonic environment (-0.12 atm) showed a modest increase in tubulation from around 8% to 21%. All three samples exposed to hypertonic environments showed increased tubulation over the course of 30 minutes. GUVs exposed to an initial osmotic pressure gradient of 0.06 atm went from having tubules in 6% of vesicles to having tubules in 35% of vesicles. GUVs exposed to an initial osmotic pressure gradient of 0.12 atm went from having tubules in 4% of vesicles to having tubules in 50% of vesicles. GUVs exposed to an initial osmotic pressure gradient of 0.18 atm went from having tubules in 7% of vesicles to having tubules in 24% of vesicles.

**Discussion**

As discussed in Chapter 3 the pressure difference across a spherical vesicle can be related to membrane tension via Laplace’s Law:

\[ \tau = \frac{\Delta P r}{2} \]

where \( \tau \) is the membrane tension, \( \Delta P \) is the pressure difference between the inside and the outside of the vesicle, and \( r \) is the vesicle radius. Here \( \Delta P \) is given by:

\[ \Delta P = (C_{in} - C_{out}) \cdot RT \]

where \( C_{in} \) and \( C_{out} \) are the concentrations of the solutions, in mol/L, inside and outside the vesicle, \( R \) is the ideal gas constant (0.08206 L atm mol\(^{-1}\) K\(^{-1}\)), and \( T \) is the temperature in Kelvin. For the vesicles exposed to hypotonic solutions with osmotic pressure differences of 0.06, 0.12, and 0.18 atm the corresponding changes in tension are 30, 60, and 90 mN/m respectively. The experiments described in Chapter 6 coupled with micropipette aspiration experiments (Evans et al. 2003) suggest that these tensions are likely to be lysing the vesicles. This has significant implications for vesicles made tense osmotically.
When vesicles lyse it is not an all or nothing event, rather, a small pore opens up, the vesicle loses some of its contents, and then reseals. This series of events will continue until the vesicle tension is below lysis tension. Thus, the data corresponding to tubulation of vesicles made tense osmotically should be approached with some caution as the actual membrane tension is unknown. Aside from questions about possible lysis there is another issue regarding the osmotic properties of vesicles that impacts these experiments. Electroformation, like other vesicle formation techniques, does not allow for control over initial membrane tension. If electroformed vesicles are imaged as prepared there are often a range of shapes seen, both spherical and non-spherical. As discussed in Chapter 6, if extruded LUVs are prepared in glucose solution and allowed to sit for 10s of hours they will gradually round up to spheres to minimize bending energy without paying for osmotic changes. While the extrusion process causes most if not all vesicles to be aspherical, the same forces should apply to non-extruded GUVs, thus, initially non-spherical GUVs may round up to spheres if prepared in glucose and allowed to sit for some period of time. We tested this hypothesis by electroforming 9:1 PC:PIP with 0.8mol% Rhod PE GUVs in 200mM glucose and imaging them with Epifluorescence over the course of one day. Images were then analyzed to determine whether or not their shape was well-described by a sphere. As seen in figure 48, the percent of non-spherical GUVs does decrease over time.
**Figure 48.** Plot of the percent of GUVs with non-spherical shapes as a function of time. GUVs were electroformed in 200mM glucose and incubated at 37°C.

The implication of figure 48 is that electroformed vesicles that are not allowed to round up over time do not have well-defined initial tensions or shapes. While vesicles exposed to hypertonic environments are not subject to the issues raised regarding osmotic lysis, it is necessary to acknowledge that their initial shapes and tensions are unknown. What we can say with some certainty is that vesicles exposed to hypotonic environments, regardless of the possible lysing, are at higher tensions than vesicles exposed to hypertonic environments, and that the vesicles exposed to hypertonic environments show increased tubulation.

In order to try to separate the effect of tension or osmotic pressure on tubulation from the effect of initial vesicle shape and tension we electroformed 9:1 DOPC:PIP GUVs in 202 mM glucose and allowed them to round up for 2 days. GUVs allowed to
incubate in glucose for this period of time were essentially all spherical and should be at zero tension. We then diluted the GUVs 10:1 into glucose solutions of either 203 mM or 197 mM glucose and allowed them to equilibrate for 30 minutes. These concentration differences correspond to osmotic pressure differences of -0.008 atm and 0.04 atm respectively, and a tension of ~2mN/m for the hypotonic case. Equilibrated vesicles were then incubated with fluorescently labeled N-BAR (labeled with HiLyte Fluor 488 maleimide) for 30 minutes. A series of epi-fluorescence images was taken for each sample and vesicles were examined for tubulation. Aside from allowing for better control over GUV initial tension and shape, these experiments employed fluorescently labeled N-BAR rather than fluorescently labeled lipid which enables the disentanglement of binding and tubulation. In the experiments that used unlabeled N-BAR it was impossible to tell if a vesicle displaying no tubules had no binding, or binding but no tubulation. One feature that was present in the previous experiments, but was even more pronounced here is that binding of N-BAR to vesicles is often not homogeneous. As shown in figure 49, there are often regions of increased fluorescence intensity throughout the vesicle, and that when tubules are present they often emanate from these regions of increased intensity. These regions of nonhomogeneous intensity suggest that bound N-BAR recruits other bound N-BAR proteins. While tubules are often associated with regions of increased fluorescence intensity, there are also many cases where there is an increase in fluorescence but no tubule (or at least no tubules that are long enough to be discerned). The results of tubulation experiments performed on GUVs starting from an initially spherical state are as follows: GUVs exposed to a hypotonic environment (-0.008 atm, or 2 mN/m) had 20% of vesicles with at least 1 tubule, 70% of vesicles had no tubules but did have regions of
increased fluorescence intensity, and 10% of vesicles had no tubulation and homogenous fluorescence, GUVs exposed to a hypertonic environment (0.04 atm) had 29% of vesicles with at least 1 tubule, 57% of vesicles had no tubules but did have regions of increased fluorescence intensity, and 14% of vesicles had no tubulation and homogenous fluorescence.

![Fig. 49](image)

**Figure 49**: Epi-fluorescence images of labeled N-BAR binding to 9:1 DOPC:PIP GUVs. (A) GUV exposed to a hypertonic environment displaying inhomogeneous fluorescence intensity, (B) GUV exposed to a hypotonic environment displaying both tubules and inhomogeneous fluorescence intensity

While the sample size is small (about 10 vesicles each) these results suggest that the difference between tubulation in tense vs. floppy vesicles may not be as pronounced as the data without pre-swelling vesicles implies.
CHAPTER 8
SUMMARY AND FUTURE WORK

The experiments described in this thesis provided new insights into how membrane composition, morphology, and stress affect the behavior of membrane-associating proteins. With synthetic large unilamellar vesicles (LUVs), we have the advantage of being able to tune composition and tension and we have explored both of these parameters. With the larger GUVs, we can visualize the morphology more accurately than with LUVs, which allowed us to investigate the effect of phase separation (model lipid rafts) and deformation (e.g. tubulation). GUVs offer the additional advantage of reversible control of applied tension (via pipette aspiration), which will potentially allow experiments to probe new questions beyond the scope of this thesis. This chapter provides an overview of the results of this thesis along with new questions that arose and suggestions of new experiments that take full advantage of the capabilities of synthetic vesicles.

Phase separated vesicles

We added histidine-tagged GFP to phase separated vesicles and observed a subsequent reduction in line tension at the phase boundary of up to 46% (Chapter 3). By varying the concentration of nickel lipid in the vesicles and monitoring the ensuing changes in line tension we were able to fit the data to a Langmuir adsorption equation and estimate the binding energy of a histidine-GFP-lipid cluster to the phase boundary to be approximately -1k_B T. These results demonstrate how phase separation can lead to an accumulation of proteins at the boundary between phases and, in turn, how proteins can
alter line tension and vesicle morphology. We suggested in Chapter 3 that, in the case of histidine-tagged GFP, the accumulation at the boundary may be due to differences in acyl chain configuration upon histidine-lipid binding. Changes in chain configuration may lead to height mismatch between the histidine-lipid clusters and the unclustered lipids in the L₀ and L₄ phases. Height mismatch and changes in lipid order present at the boundary between phases have also been proposed to induce defects in the membrane (Bhagat and Sofou 2010; Kruijff, Gier, and Hoogeveest 1984). This increase in packing defects at the boundary between phases has interesting implications for proteins, like N-BAR, that contain amphipathic regions that insert into the membrane via defects.

Balance between membrane lateral tension and phase boundary line tension determines the overall shape of a phase-separated vesicle. Such vesicles need not be simply spherical (Fig.49). As BAR domains have been implicated in curvature sensing one might imagine that they would prefer the more highly curved neck region between phases as a binding site, either because of curvature itself or because of an increase in packing defects arising from increased curvature. Taken together, these effects of phase separation (curvature changes and increased defects) imply that binding of N-BAR to phase separated vesicles may be increased at the boundary between phases.
Figure 50. Superimposed two-photon image of a phase separated vesicle. The L₀ phase is labeled with perylene (blue) and the L₄ phase is labeled with rhodamine-DPPE (red). The arrow marks the neck region where the two phases meet. Scale bar is 5 μm. Image reproduced from (Tobias Baumgart, Hess, and Webb 2003)

Our exploratory experiments in introducing N-BAR domains to phase separated vesicles do indeed seem to indicate that something interesting may be occurring at the boundary between phases. The images seen in figure 51 show a phase-separated vesicle composed of 2:2:1 DOPC:PSM:Chol with 2.5 mol% Ni-DOGS and 0.8 mol% dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine DOPE). The rhodamine DOPE partitions with DOPC to the liquid disordered phase.
Figure 51. Epifluorescence images of tubulation of a phase-separated vesicle induced by N-BAR. 175 \mu M N-BAR was flowed into the sample container at time \( t_0 \) and three representative images showing fine tubulation were selected at \( t = t_0 + 3 \text{s} \), \( t = t_0 + 6.9 \text{s} \), and \( t = t_0 + 11 \text{s} \). The double arrows in panel (a) show the boundary between phases. The single arrow in panel (b) shows the location of the initial tubulation. Scale bar is 10 \mu m.

175\mu M purified N-BAR (residues 1-357) was flowed into the sample chamber and then diffused down the length of the chamber. Because of the gradient of N-BAR concentration at the location of this vesicle is not known, but can be estimated as 15 \mu M. Images of the vesicle were obtained using a microscope with a 100x/1.3 objective and a CCD camera; the images were recorded on VHS videotape (file name “9-14-09 add NBAR”)

There are several interesting features of the fine tubulation seen initially in panel (b). First, the shape of the tubule(s) seems consistent with those seen previously in membranes that have been deformed by members of the BAR superfamily (Saarikangas et al. 2009). Second, it appears that the material being recruited for tubule formation is coming from the liquid disordered domain, as the tubule is fluorescent and there is a
decrease in the area of the liquid disordered domain as the tubule(s) grows. This makes sense intuitively as the bending modulus of the disordered domain should be smaller than that of the ordered domain (T. Baumgart et al. 2005) so it should be easier to deform.

Third, the tubule(s) seems to be originating from the boundary between phases. This result, and two later experiments of the same type showing similar features, suggests that phase boundaries may be important factors in determining the localization of tubule formation. Fourth, not only does the tubule formation appear to initiate at the phase boundary, but it also originates from one small region of the boundary. This may point to tubulation beginning as a nucleation-like process which relies on multiple BAR proteins forming a sufficiently large aggregate before tubulation is stable.

Additional evidence for buildup of N-BAR at the boundary between phases is shown in Fig. 52 where fluorescently labeled N-BAR was incubated with Brain polar extract (Avanti Polar Lipids, Alabaster, AL, 141101P) GUVs. Brain polar extract GUVs (unexpectedly) phase separate at room temperature. In some cases, buildup of labeled N-BAR can be seen at the boundary between phases.
Figure 52. Epifluorescence images of Brain polar extract GUVs with 1.4 mM NBAR HiLyte. (A) Brain polar extract GUV with bound N-BAR HiLyte (same construct as in Chapter 6) clearly showing phase separation at room temperature. (B) Brain polar extract GUV with bound N-BAR HiLyte showing increased fluorescence at the phase boundary (arrows).

There are several strategies that could be employed in order to further explore the relationship between phase separation and tubulation. While preliminary experiments suggest that tubulation may be originating at the boundary between phases it is difficult to make a conclusive statement about this due to the nature of the epifluorescence imaging technique used. In epifluorescence background fluorescence from outside of the focal plane is detected, lowering the resolution particularly in the z direction. If we use confocal microscopy, which employs pinholes to block out of focus light, we can visualize slices through the vesicle without background fluorescence from the vesicle’s northern and southern hemispheres. If slices of the entire vesicle are taken they can be reconstructed into a 3D image which should show in greater detail where vesicle tubulation is originating. Spinning disk confocal systems employ an array of pinholes
rather than a single pinhole and thus allow for faster imaging which may be necessary
given the speed with which tubules grew in our preliminary experiments (see figure 50).
The Andor Revolution® spinning disk laser microscopy system is able to image 60
vertical slices per second. If we want to reconstruct a 50 μm diameter vesicle by a slice
every 0.5 μm we can do so in just over 1.5 seconds (figure 53).

![Figure 53. Reconstructed 3D confocal image of a phase separated vesicle. Image taken with an Andor Revolution® (Andor, South Windsor, CT) spinning disk laser microscopy system.](image)

In order to determine the impact of defects on N-BAR protein binding and
tubulation, the acyl chain length of the component lipids should be varied. If greater
differences in chain length lead to more or larger defects at the phase boundary we may
be able to see an impact on N-BAR binding and tubulation. Unsaturated PC lipids are
available with acyl chains of between 14 and 24 carbons in length, and saturated PC
lipids are available with acyl chains of between 3 and 24 carbons in length (Avanti Polar
Lipids, Alabaster, AL) so there is room for a testing of a wide variety of chain mismatch.
**N-BAR binding and tubulation**

The mechanisms by which N-BAR and other BAR family members bind to and tubulate membranes have been under debate for several years. Some data suggests that the curved shape of the BAR domains, in addition to the regions of positively charged amino acid residues on the concave face of the membrane, confer curvature sensing ability to the protein. Other data suggests that the N-terminal amphipathic helix, which can insert into the membrane, is responsible for curvature sensing (Gallop et al. 2006; Blood, Swenson, and Voth 2008). Finally, recent experiments with a mutant of epsin1-hexahistidine that is missing an insertable helix or GFP-hexahistidine demonstrate that vesicles containing Ni-DOGS-NTA (20 mol%) can be tubulated by proteins that have neither a curved shape nor an amphipathic helix (Stachowiak et al. 2012). In this case, the tubulation is thought to occur because of an increase in lateral pressure arising from collisions between bound proteins. The area available for the proteins to diffuse in can be increased by deformation of the membrane.

The results presented here, in Chapters 5-7, paint a complex picture of interactions between N-BAR proteins and lipid membranes. We found that the presence of negatively charged lipids was necessary for the binding of N-BAR to vesicles (Chapter 5), lending support to the theory that electrostatics are key to binding and possibly curvature sensing. However, our experiments exploring the role of tension in N-BAR binding (Chapter 6) show that N-BAR binding to membranes increases when the membrane is tensed. We posit that membrane binding to tense membranes increases either due to membrane area increases upon N-terminal amphipathic helix insertion or due to an increase in defects in tense membranes, and hypothesize that BAR domains
without an N-terminal amphipathic helix will not bind more avidly with increasing membrane tension. Indeed, if the BAR induces local curvature of the membrane, then binding should involve work against the tension and hence the affinity would decrease with tension. In addition, the data suggest that N-BAR may not be sensing curvature per se, but instead sensing strain. We incubated osmotically inflated or deflated GUVs with N-BAR and, while the results need to be treated with caution (Chapter 7), they suggest that tubulation is hampered by membrane tension. In sum, our data suggests that negatively charged lipids are necessary for N-BAR binding, but that the N-terminal amphipathic helix confers strain sensing upon the protein. It may be that the positively charged residues on N-BAR’s concave face act only to position the protein into close association with the membrane, and once close enough, the N-terminal amphipathic helix senses membrane mechanical properties. Tubulation may occur only when enough protein has accumulated to sufficiently lower the membrane tension. There are several experiments that could be performed to check these hypotheses. First, BAR constructs (lacking an N-terminal amphipathic helix) could be used in tension as a function of binding experiments like those described in Chapter 6 in order to see if sensitivity to tension is lost, as predicted. Second, more binding experiments with different lipid composition should be performed, particularly with lipids of varying spontaneous curvature. Lipids such as DOPE that have negative spontaneous curvature may increase membrane defects and, if this is the mechanism responsible for N-BAR’s tension sensing ability, binding may be enhanced by increased DOPE concentration in the membrane. Third, endophilin N-BAR, another member of the BAR superfamily, has an appendage in addition to the N-terminal amphipathic helix which also insert into the membrane.
(Masuda et al. 2006). If endophilin N-BAR were in experiments described in Chapters 6 and 7, the results may be even more dramatic than those seen with amphiphysin N-BAR, as endophilin may be able to further increase membrane area and thus be more sensitive to tension. Finally, for precise control over membrane tension, and the ability to interrogate individual vesicles, micropipette aspiration could be employed to examine the role of tension in both binding and tubulation.

**Micropipette aspiration**

Micropipette aspiration is a powerful tool that allows for tension control of individual giant unilamellar vesicles. This tension control, coupled with information about the vesicle geometry, allows for measurements of many of the membrane's mechanical properties. The tension control could also be used to perform experiments with N-BAR in order to determine the precise relationships between membrane tension and N-BAR binding, and membrane tension and tubulation for individual vesicles. In micropipette aspiration a glass pipette with an inner diameter of a few microns is positioned by micromanipulators into close contact with the object to be interrogated, in this case giant unilamellar vesicles with diameters between 10 and 50 microns. The pipette is filled with water and is attached to a reservoir, a pressure transducer, and a syringe pump. There is a reference reservoir attached to the other side of the pressure transducer and open to the atmosphere. A pressure differential between the pipette and the reference reservoir (and sample chamber) is set by the syringe pump and measured by the pressure transducer (see Chapter 2 for more details). Once a pressure differential is set, a vesicle nearby the pipette will be partially aspirated into the pipette (figure 54), and
by analyzing the geometry of the aspirated vesicle we can determine the tension in the membrane:

\[
\sigma = \frac{PR_p}{2(1 - \frac{R_p}{R_v})}
\]

Where \( \sigma \) is the membrane tension, \( P \) is the suction pressure, \( R_p \) is the radius of the pipette, and \( R_v \) is the radius of the vesicle.

![Figure 54. Hoffman optics image of an aspirated GUV.](image)

Computer control over the syringe pump driving the pressure differential allows the user to input a desired pressure differential or a pressure (tension) ramp. A vesicle can be aspirated at a desired suction pressure, labelled N-BAR can then be introduced to the sample chamber, and the amount of binding (as measured by fluorescence intensity) can be quantified as a function of the vesicle tension. Once N-BAR binding vs. tension has been quantified, the tension can be stepped down until tubulation occurs. Alternatively,
tubulation could be initiated at low or zero tension, and the tension could gradually be stepped up until tubulation is halted or reversed.

Preliminary experiments coupling N-BAR with micropipette aspiration uncovered a technical issue that has not yet been resolved. In order for vesicles to be aspirated properly, the pipette needs to be coated so that the lipids do not adhere to the glass walls of the pipette. Typically, before any vesicles are added to the sample chamber, the chamber is filled with a bovine serum albumin (BSA) solution and the pipette is allowed to sit in the BSA solution for several minutes. The negatively charged BSA solution allows the vesicle to slide in and out of the pipette without adhering to the pipette walls. However, when N-BAR is added to the sample chamber it adheres to the negatively charged BSA, clogging the pipette, and also making it impossible to see the aspirated section of the vesicle (figure 55).

Figure 55. Epifluorescence image of a pipette tip coated with N-BAR-HiLyte.
We have tried silanization with dimethylchlorosilane, and a combination of silanization and BSA, but to date, either the vesicle does not adhere but the N-BAR does, or the N-BAR does not adhere but the vesicle does. If these issues are resolved, micropipette aspiration should allow for precise measurement of the effect that membrane tension has on both N-BAR binding, and tubulation by N-BAR.
APPENDIX

N-BAR MUTATION STRATEGIES

We will create fluorescent N-BAR proteins using PCR mutagenesis. There are several strategies that we are employing in order to create these constructs, the details of which are given below.

Plasmid containing pGEX/Dm_NBAR (from drosophila melanogaster) as a GST fusion protein was a gift from Harvey McMahon. The nucleotide sequence with GST in blue is as follows:

**ATG**TCCCCTATACTAGGTTTATGGAAAAATTAAGGGGCTCTTGTGCAAACCACCTCGACTTCTTTTGGAAATATCT
TGAAGAAAATATGAAGAGCATTTTGTATGAGCCGAGTGAAGGTGATAAATGCGAAGAAACAAATGGAATTCTCTAGCCAA
AATGGAAGTGTTTCTCCAAATCTTCTTTATATGGTATGTTGATGTTAAATTAACAGATCTCATGCGC
ATCATGCATTACAGCTGAACAGCAACATGATTGGGCTCTTCATGCGAATCCAGAGACTGCTTACGACAT
TGCAAGTAGGGCCTCTTCTGCAAGGCTTCATGGCCTTTACAGCACATGACGATCGTACCCGACTTTG
ACGCTGTAGACGCTTATGACGCTTCTGCAAGGCTTCATGGCCTTTACAGCACATGACGATCGTACCCGACTTTG
AGCTGTAGACGCTTATGACGCTTCTGCAAGGCTTCATGGCCTTTACAGCACATGACGATCGTACCCGACTTTG
ACGCTGTAGACGCTTATGACGCTTCTGCAAGGCTTCATGGCCTTTACAGCACATGACGATCGTACCCGACTTTG
ACGCTGTAGACGCTTATGACGCTTCTGCAAGGCTTCATGGCCTTTACAGCACATGACGATCGTACCCGACTTTG
ACGCTGTAGACGCTTATGACGCTTCTGCAAGGCTTCATGGCCTTTACAGCACATGACGATCGTACCCGACTTTG
ACGCTGTAGACGCTTATGACGCTTCTGCAAGGCTTCATGGCCTTTACAGCACATGACGATCGTACCCGACTTTG
ACGCTGTAGACGCTTATGACGCTTCTGCAAGGCTTCATGGCCTTTACAGCACATGACGATCGTACCCGACTTTG
ACGCTGTAGACGCTTATGACGCTTCTGCAAGGCTTCATGGAC
A mutation was introduced at nucleotide 1716,1717 (AC → TA) to create a stop codon (TAA).

The translated sequence of the GST-NBAR fusion with stop codon is as follows:
The GST tag is in black, the N-BAR (including an underlined linker) is in red, and two native cysteine residues are in blue. The last few residues in bold is an extra linker region that is available for mutation.

Alignment of the nucleotide and protein sequence:

cttggttccgcagtggatcctccaggaattcgatagaccgaaataaagcgataatgttggcc
LVRGSPGIRMTENKGLMALAAATCTTCTTCAAAACCACGCTGGAGCCTGCCAAAGGAAGAGATTCTCACAAGAAGTTGAGGGGGAC
KSVCQKKEILGQNGLKTGCTGATCGCACTGCAGATGAATCTTCTGCAGCTACCTGGAACACTTCATCGCCAGCGA
VDRTEFIDDDHLNNFRQQGCGAGTGCACACAGATCATTGATGGATTCCGTGTTCGAGATCTACAGCACCACAATGGGCAGC
QAASKTLMDSECEIYEPOWSGTTACGATGCCCAGACACAAACCGGTTGCCTCGGAAAGTCTTGGGCAGCTTGTTCG
GYDALQAQTGASESLWADFAACAAATTTGGGGATCAATCTTCATTCGGCTTACACACATACGCTGACATTTCCCGAA
HKLGDQVLPLNTYGTQFPEATGAAGAAAGTAGAAGAAGCAGCCAAACCGGATCTTGATTACGATGCGCAACACACMKKVEKRNKLDYGQHR
TCGTTCAGAATCTCAGCAGGCAAACCCCAAAGAGTGATGTCAAACTACCCAA
SFPQLANANKRKKDDVKLTKGGACCGAAGCAGCTGGAGGAAGCTAGACGCCACCTCAGAAATCTCAGAACACGGAACCTCAG
GREQLEEARTYELNETLHACGAGTGGCGGCAATTTGATAGACTTACTGTTTCGTGTCACCAACTTGCAACACA
DELPAELYDSPILFLVTNLQTCTTCTGGCCACAGAGCAGATTGTTCCACAACGGAACCGCTAAGATTTACTCGAAGCTGGAG
LFATEQFVENETAKIFYELESGCAATCGTGCAAAAATTGGCCACAGAATCGACCGCGGCTTCAATACGCTACGCAAGC
AIVDKLAQESQRGNTLRKQACAGCAGATCCATCAATCGAGCCACATGGTACGTCGCCAGTAATAAGTAAAACAT
TSNPIK

The blue residues are native cysteines which will be utilized in creating fluorescent BAR. Maleimide dyes will bind to any exposed cysteine residues in the protein. There are two
strategies we will employ in order to attach one maleimide dye molecule to each BAR monomer.

**C-His NBAR construct**

For ease of purification and to allow control experiments with Nickel lipids, we also constructed Histidine tagged N-BAR. The same maleimide attachment procedures can be used with the His-tagged construct.

**Primers**

<table>
<thead>
<tr>
<th>Vector</th>
<th>N-Bar</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insert</strong></td>
<td>5'</td>
</tr>
<tr>
<td><strong>5'</strong> - GAA GGA CAT ATA CAT ATG ACC GAA AAT AAA GGC ATA ATG CTG GCC AAA TCT GTT-3'</td>
<td>Met Thr Glu Asn Lys Gly Ile Met Leu Ala Lys Ser Val</td>
</tr>
<tr>
<td><strong>N-Bar</strong></td>
<td>5'</td>
</tr>
<tr>
<td><strong>Vector</strong></td>
<td>ACG CTA CGC AAG CAA ACA AGC AAT CCC ATC AAG CAT CAT CAC CAC CAT CAC-3'</td>
</tr>
<tr>
<td>5' - AAT</td>
<td>3'</td>
</tr>
<tr>
<td>5' - ATT</td>
<td>3'</td>
</tr>
<tr>
<td>5' - GTG ATG GTG GTG ATG ATG CTG GAT GGG ATT GCT TGT TGT TTG CTT GCG TAG GTG GTG GTA GTG-5’</td>
<td></td>
</tr>
</tbody>
</table>

Introduce into pETite C-His Kan Vector (2235 bp, From Lucigen Website). The insert is residues 1-256 of N-BAR amphiphysin (*italics* blue; the primer overlap with vector is *boldface* red). The resulting vector sequence (petite_NBar_C-His) is saved at the Lablife website:
We introduced the stop codon after the His tail. During the primer design the stop codon was incorporated.

**Mutation of C66A and C82A.**

Two cys sites are in red (bold).

**Nucleotide sequence of GST-NBAR ORF in pGEX/D_Am_Bar (Dm_NBAR) plasmid**

**Mutation introduced at nucleotide 1716, 1717 (AC → TA) to create stop codon (TAA)**

```
ATGTCCTCCTATATAGGTTATGGAAATTAGGGGCTTGTGCAACCCACTCGACTTTTTTGGAGATATTCT
TGAAGAGAAAAATAGAAGAGCATTTATGTAGGACCGAGTGAACTTGAGTAAGATGGCCAAGCCATTTTCGAA
ATCATACTGTTATATAGCAGACAAAGCTATGTTGGGTGTCTTCAAAAGAGCGTGCAAGATTTCAA
TGCTGAAAAGCGGTGTGGTTTGGGATTGATAGATACGCTGGTTGCGAGATTGCTATAGAAGACCTGTGAAAC
TCTCAATGGTTTTTCTTAGCAAGCTACCTGAAATGCTGAAATGCTGGAAGATCCTTATATGCTAATAAAA
CATATTTAATATTGCTCATGTAACCACATCTGACTCTCATGTTGGTTGTAGCGCTTGTGTTGGTATTGTATAGCA
TGGACCCAATGCTCTGCTAGGGTTTCCACCAATTGAGTTTGTGGAGATTAAAAATAGGTTGAGCTACATCCAA
ATTGATAAGCTATCTGAAATCCGAAAGTAGATCAGATGACGGCCTGAGGGCTGGAAGACGCTTTTGAG
GGTGCACCTCCTCCAAAATTCCGATCTGTCCTGGCAGATCCTCAGGACCAATTCGAGGGAGATTATCCAT
GGCGAAAAGTGACGACTCGCCGGAGTAAATCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG
GAGCTATCTAATCAGGACAGCTGGAAATCCTTCCGAAAATGAAAGAAAATAGAGAAGAACGGCCAAGGGCT
GATTACATGCTGAAATCCGAAATGCTGGAAGATCCTTATAGTCTACAAACAAAGCGGAGCAATGCTGAGGAG

136
GCAGTCCCGTGCAGAATGGGTTGACCACCCAAATCATTTGGAGCGTCCCGAGCTCAGTGGTCTGAATGCGA
GTGCCAAGGGCAACCACACTCGAGCGGCGCGCATCGTGACTGA

**Strategy 1:** We will remove the cysteine at residue 66, leaving the cysteine residue at position 82 available for maleimide binding. We chose this mutation instead of the converse because structural data suggests that the residue at position 82 is more exposed than the residue at position 66. All mutations will be done using PCR mutagenesis, wherein mutations are introduced in the primer.

Wild type sequence in region of cys 66 (blue): 5’-tcataagatgttgcgccccgcac-3’
Forward primer sequence to change cys 66 to alanine:

5’ – tacataagagcagttcgccgccgcac – 3’

Reverse primer:

5’ – gtgcggcggaactgctttatgta – 3’

After PCR mutagenesis was perfomed, the plasmid was sent to Genewiz for sequencing to confirm the proper mutation:

**5’3’ Frame 1**

```
XXXXXXXXGCSXLVPRGSPIRMetTENKGI MetLAKSVQKHAG
RAKEKILQNLGKDRTADEIFDDHLNNFNQASANRL
QKEFNNYIRAVRAQAAASKTLMetDSVEYEIPQWSGYD
ALQAQTGASESLWADFAHKLGDQVLIPLNTYTGFPE
MetKVKVEKRNKRLLIDYGQRHFSQNLQANANKRKKDDV
KLTKGREQLEARRTYEILNTELHDEXXALYDSRILFLV
TNLQTLFATEQVFHNETAKIYSELEAIVDKLATESQRGS
NTLRKQTSPNIK Stop
```

Pink highlight shows that cys 66 is now alanine. The red highlight shows cys 82 remains for maleimide dye attachment.

Strategy 2 involves changing both cys 66 and cys 82 to alanines, and then inserting a new cysteine at residue 252 (in the linker region at the end of the BAR domain).

Strategy 3 involves removing both native cysteines and adding 4 cysteines back (in the linker region at the end of the BAR domain). This will allow for the binding of Flash dye
to the domain as it requires a tetracys binding site. Strategies 2 and 3 have not been completed yet.

In order to check maleimide binding we introduced maleimide fluorescein to purified Cfrag protein which has a cysteine residue in approximately the same degree of exposure as residue 82 in N-BAR. We then ran an SDS-PAGE gel of the Cfrag-maleimide-fluorescein complex and checked for fluorescence with a UV source:

**Figure A1.** SDS-PAGE gel under UV light. Gel shows 2 lanes with maleimide fluorescein dye attached to the protein Cfrag (red arrows). The other lanes contain Cfrag without maleimide fluorescein attached as a control.
Figure A2. SDS-PAGE gel under UV light showing C-His N-BAR with HiLyte 488 maleimide attachment. From left to right the dye:protein ratio is 20:1, 15:1, 10:1, 5:1, 2:1, 1:1.
REFERENCES


