MEMBRANE MICRODOMAINS REGULATE FOCAL ADHESION SIZE IN BOVINE AORTIC ENDOTHELIAL CELLS

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ABSTRACT

Cell membranes were once thought to be functionless, lipid bilayers with little lateral movement within the membrane. We now know that plasma membranes contain different kinds of lipids with varying degrees of hydrophobicity and disorder. It is believed that these lipids arrange themselves into lipid-ordered and lipid-disordered domains, and the height mismatch between these domains causes the ordered domain to sit rest slightly higher than the other lipids, like a “raft”. These “rafts” have been implicated as platforms for cellular signaling. However, their role in mechanotransduction has not been fully elucidated. The goal of this experiment was to perturb the cellular membrane by addition of chemicals which may selectively assemble into certain lipid domains and to quantitatively analyze the focal adhesion dynamics under those conditions. The additives used were benzyl alcohol, Triton-X, and vitamin E. Bovine aortic endothelial cells were transfected with green fluorescent protein tagged to focal adhesion kinase and visualized using total internal reflection microscopy (TIRF-M). The quantitative analysis was completed by the Focal Adhesion Analysis Server. The results showed that Vitamin E and Triton-X both decreased focal adhesion size in the entire cell. Benzyl alcohol increased focal adhesion size in the entire cell. These findings elucidate the role of membrane microdomains in mechanotransduction. The results also showed that benzyl alcohol doubled focal adhesion size at the leading edge of the cells. These findings indicate that benzyl alcohol may play a role in integrin clustering by modifying the cell membrane.
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Chapter 1

Introduction

It was previously thought the plasma membrane was a homogeneous bi-lipid layer with little lateral movement within the membrane. This passive view of the plasma membrane has since been replaced with a more realistic view of a two-dimensional protein lipid composite (Owen, Magenau, Williamson, & Gaus, 2012). In this most current model, the membrane is separated into ordered and disordered domains based on their lipid composition. Saturated sphingolipids tend to self-associate in the membrane due to their hydrophobicity; they also co-localize with cholesterol to form “rafts” which rest on top of the rest of the lipid bilayer (Simons & Ikonen, 1997). These rafts were first discovered due to their insolubility to the detergent Triton X-100 and have since been found to have many other distinguishing properties. These sphingolipid-rich rafts were also found to be platforms for cell signaling such as proliferation, differentiation and migration, because of their ability to bind proteins. Therefore, it is thought that rafts play a significant role in the signal transduction cascade.

Cell Interaction with the Substrate

The mechanotransduction pathway, that is the pathway in which cells convert mechanical stimuli to intracellular signals, is not fully mapped or understood. However, it has been shown that cells respond and interact with their substratum and are influenced by its chemistry, topography, and mechanical properties (Cretel, Pierres, Benoliel, & Bongrand, 2008). The
cascade that occurs when a cell contacts a substance and begin to form adhesions is complex and involves many kinases, adapter proteins and proteins. This occurs over multiple length and time scales and the interdependence and these molecules is also not completely understood. Lo and other researchers found in 2000 that when fibroblasts were plated on materials of different stiffness, the fibroblasts migrated towards the material with stiffness that most matched their intrinsic environment, indicating that cells can sense their environment (Lo, Wang, Dembo, & Wang, 2000). They also found that on stiffer surfaces the cells generated stronger traction forces and spread more. Therefore the ability of cells to sense their environment and react to it has been shown. In this experiment, we propose that sphingolipid-rich rafts play a role in the mechanotransduction cascade by providing a platform for focal adhesion formation. **Figure 1** shows two membrane rafts and the their connection to the transmembrane receptors called integrins. Lipid rafts can be identified by the ganglioside marker, GM1. This figure was drawn by Daniela E. Fuentes (Fuentes & Butler, 2012).

**Figure 1: Organization of Lipid Rafts and Integrins.** Ordered microdomains, characterized by the presence of cholesterol and sphingolipid self associate in the lipid membrane to form lipid rafts. Some of the lipid rafts are attached to integrins via the the actin cytoskeleton, while others diffuse freely in the plasma membrane.
Integrins and their Ligands

Integrins are transmembrane adhesion receptors with an intracellular domain that binds to adapter proteins and an extracellular domain that binds to extracellular matrix (ECM) components such as fibronectin, vitronectin and laminin. When integrin binds to an ECM component, such as fibronectin, its intracellular domain is activated which begins the signal transduction cascade. Previous studies have found that the affinity of integrins for their ECM ligands influences integrin clustering. More recently researchers have found that the recruitment of adapter proteins and kinases to the intracellular domain of integrins is necessary for integrin clustering. And furthermore by binding to a membrane lipid, some adaptors can even increase their affinity for an integrin and initiate a conformational change of the integrin (Wang, Bi, Ampah, Zhang, et al., 2013). Other researchers have shown that cholesterol and sphingolipids (two key components of lipid rafts) play a role in the regulation of integrins (Pande, 2000). Furthermore, Caswell et al showed the lipid rafts can regulate integrin clustering. All of these results point to the importance of the lipid membrane in regulating integrin clustering (Caswell, Vadrevu, & Norman, 2009).

Focal Adhesions and Lipid Rafts

In 2014, Wang, Bi, Ampah, Ba, et al. studied the interdependence of focal adhesions and lipid rafts. They found that compromising the integrity of the lipid rafts caused the inhibition of focal adhesion disassembly, implicating lipid rafts as key players in disassembly of focal adhesions. They developed a model for the disassembly of focal adhesions and the role that lipid rafts play. They found that lipid rafts can control the disassembly of FA by “controlling the
dynamics of the actin cytoskeleton-mediated integrin internalization and focal adhesion protein dephosphorylation” (Wang, Bi, Ampah, Ba, et al., 2013). These findings agree with the previous hypothesis that lipid rafts play a key role in focal adhesion formation and thus the mechanotransduction cascade.

Another study performed in 2002 by Leitinger and Hogg found a similar dependence between lipid rafts and integrins. This study used Jurkat cells to investigate adhesion and dependence on intact rafts. Jurkat cells with no cholesterol depletion were stimulated to adhere to fibronectin. Then researchers disrupted the lipid rafts by adding methyl-β-cyclodextrin (MβCD), which depletes the raft of the essential component, cholesterol. This method of disrupting rafts through MβCD has been shown to be successful in other studies (Harder & Kuhn, 2000). After the addition MβCD, adhesion levels were significantly lowered to background levels. They also showed for the specific integrin α4β1, that lipid rafts were required for integrin clustering in the Jurkat cells. Thus the major findings of this report show that there is a significant dependence of integrin clustering and binding to fibronectin on lipid rafts (Leitinger & Hogg, 2002).

Focal Adhesion Formation

Focal adhesion formation has both a spatial and a temporal dependence. Strictly from a temporal perspective, the first molecules to contact the surface are glycosaminoglycans (GAGs), which initiate the contact. GAGs are long unbranched polysaccharides, which are attached to the cells surface and are the first part of the cell to contact any surface. The contacts formed by these GAGs are very brief and are replaced by integrin-mediated contacts within tens of seconds
to minutes. The contact first begins as a focal complex, then with time matures to a nascent focal adhesion and finally a mature focal adhesion or a mature fibrillar adhesion in some cases (Zaidel-Bar, Cohen, Addadi, & Geiger, 2004). Each of these is defined by its timing, composition and morphology.

Nascent FAs have a special role in transmitting force to the cell. They have been found to be the main generators of propulsive forces in the cells. Nascent FAs are typically small adhesions near the leading edge of the cell, that mature over time to become focal adhesions, as more integrins are recruited (Beningo, Dembo, Kaverina, Small, & Wang, 2001). Beningo et al, have shown that these nascent focal adhesions are responsible for generating traction forces and that as the nascent focal adhesion matures to a focal adhesion, the traction forces decrease.

New Unpublished Study Investigating Force between a Cell and ECM

In Dr. Butler’s mechanobiology lab at Penn State, a talented P.h.D. student, Seoyoung Son, is investigating the forces between an ECM-coated bead and an integrin in the cell membrane. In this setup, the cells are attached to glass, and the ECM functionalized bead is brought into contact with the cell for a brief amount of time (1-2 seconds), and then pulled away. Because the functionalized bead is caught in the optical trap during this time, the optical trap can measure the force required to remove the cell from the bead. This force is indicative of the number of integrin-ECM interactions formed during the brief contact with the functionalized bead. Preliminary results have shown that benzyl alcohol may affect this process. When benzyl alcohol was added to the cells, the force required to remove the cell from the bead doubled, indicating that BA may recruit a second integrin to the focal complex. These preliminary results
from the Butler lab at Penn State indicate that BA may be active in new focal complexes that are beginning to form. In this experiment, we would like to further this research by investigating if BA may be active in nascent focal adhesions which can be detected by total internal reflection fluorescence microscopy (TIRF-M) at the leading edge of cells.

**Molecules Involved in Focal Adhesion Formation**

Using fluorescent tags attached to proteins, many researchers have studied the timing and spatial orientation of focal adhesions. Zaidel-Bar et al found that some of the earliest molecules to enter the focal complex are $\alpha_\nu\beta$-3 integrin and phosphotyrosine, which are shortly followed by talin and paxillin. After that, vinculin, $\alpha$-actinin enter the focal complex and focal adhesion kinase (FAK) and VASP follow after that (Zaidel-Bar et al., 2004). This matures to a nascent focal adhesion and finally a mature focal adhesion as more integrins are recruited to this focal complex. **Figure 2** shows the important mechanotransduction proteins and adaptors involved in an integrin binding to the ECM.
Figure 2: Major mechanotransduction adaptors and proteins. The first molecules to enter the focal complex are αβ-3 integrin and phosphotyrosine. Talin and paxillin are next, followed by vinculin and α-actinin. Lastly and focal adhesion kinase (FAK) and VASP come into the focal complex.

**Role of Focal Adhesion Kinase (FAK)**

Focal adhesions may appear like static structures, but in fact they are dynamic associations of proteins, each with a specific timing and effect. This complex that is forming on the inside of the cell begins to attach to the actin matrix of the cell, which gives it the ability to transduce the extracellular mechanics into an intracellular signaling cascade. The role of focal adhesion kinase (FAK) in this process is fairly well documented to date. FAK is a cytoplasmic tyrosine kinase, which associates with integrins at focal adhesions. FAK is in the later stages of focal adhesion formation and phosphorylates upon adhesion to the integrin, which allows it to associate with SRC and further the signaling cascade (Li et al., 2002). In a paper published in 2002, Li et al
proved that FAK was correlated with focal adhesion dynamics. This finding makes FAK an ideal marker to study the dynamics of focal adhesions.

One way to track the movement of FAK and thus study the dynamics of focal adhesions globally, is to fluorescently label FAK and then study its movements under fluorescence microscopy. It can be assumed that the movements of FAK correspond to the movements of the focal adhesions; however, it must be taken into account the FAK arrives in the later stages of focal adhesion formation. Focal adhesion kinase can be fluorescently tagged by green fluorescent protein (GFP). The plasmid for this new fluorescently tagged protein, must be inserted into the cells so that they will express this protein naturally. Then once this protein has been expressed, the cells can be imaged under fluorescent microscopy and the focal adhesion dynamics quantified.

**Total Internal Reflection Fluorescence Microcopy (TIRF-M)**

Under immunofluorescence, green fluorescently-tagged focal adhesion kinase (GFP-FAK) will be visible freely floating in the cytoplasm and also at the focal adhesions, attached to integrins. To isolate the GFP-FAK co-localized to the integrins, total internal reflection fluorescence microscopy (TIRF-M) was used. This specialized kind of fluorescence microscopy requires an objective with a very high numerical aperture so that it bends the incident angle of light at a high angle. The refractive index between the glass and the cell culture media dictate how light is refracted or reflected at that interface. When the incident angle of light is high enough, it reaches the critical angle, and all the light is reflected. This reflection causes an evanescent wave to travel through the cell culture medium. The evanescent wave is
exponentially decaying and therefore only excites the fluorescent molecules up to about 200 nm into the medium (Axelrod, Davidson). This is ideal for visualizing the GFP-FAK that is localized to the focal adhesions, and eliminating the background fluorescence caused by GFP-FAK in the cytoplasm.

**Partitioning of Lipids into Liquid Ordered and Liquid Disordered Domains**

Because the lipid rafts in the plasma membrane are hypothesized to play such a key role in cell signaling, partitioning of the membrane into ordered (rafts) and disordered domains has been studied using “domain selective lipid analogues DiI-C12 and DiI-C18” (Tabouillot, Muddana, & Butler, 2011). DiI-C12 and DiI-C18 are fluorescent dyes which associate into the cell membrane and laterally stain it, allowing them to visualize the cell membrane. The researchers in our lab found that DiI-C12 selectively associated with the liquid disordered domain and DiI-C18 selectively associated with the ordered domain. They also found that these membrane microdomains were differentially sensitive to shear stress. Using these results, our lab began to study how to perturb the membrane using non-lipid amphiphiles to modulate the liquid-ordered and liquid-disorder domains. They were able to either stabilize or destabilize the microdomains by adding these non-lipid amphiphiles to giant unilamellar vessels (GUVs) and quantify the results with the Dil-12 and Dil18 (Muddana, Chiang, & Butler, 2012). The three additives used in the above stated study were \( \alpha \)-tocopheral (vitamin E), Triton-X-100, and benzyl alcohol. Each additive had a different result on membrane partitioning and stabilization. **Figure 3** shows the way that DiI-C12 and DiI-C18 partition selectively into the membrane. It was adapted from
Figure 3: Partitioning of DiI-C12 and DiI-C18 into the cell membrane. DiI-C12 selectively partitioned into the liquid-disordered phase and DiI-C18 selectively partitioned into the liquid-ordered phase. With the addition of a non-lipid amphiphile, DiI-C12 and DiI-C18 were used to quantify the changes in membrane microdomains.

Non-Lipid Amphiphiles Affect Domain Separation

The three additives vitamin E (VE), Triton-X (TX), and benzyl alcohol (BA) will now be referred to by their abbreviations. Each lipid amphiphile added to the giant unilamellar vessels (GUVs) selectively partitioned to a specific domain. Because there exists a height mismatch between the liquid-ordered and liquid-disordered domain, interfacial tension is created between these two domains. The underlying belief to this study being that, if a lipid amphiphile increased the interfacial tension, then it would also increase the tendency of the GUVs to form domains.
Conversely, a decrease in interfacial tension would result in a decreased tendency to form domains. The research on the usage of each additive will now be reviewed individually and compared to the results found by Muddana, Chiang and Butler.

**Vitamin E Effect on Phase Separation**

![Vitamin E Structure](image)

*Figure 4: Vitamin E Structure. Vitamin E is a larger molecule and assembles into the cell membrane due to its hydrophobic and hydrophilic portion. This assembly allows it to affect the phase separation in the plasma membrane.*

The literature does not all agree regarding Vitamin E and its structural partitioning into the plasma membrane. The above stated study by Muddana, Chiang and Butler found that Vitamin E mostly partitioned to the domain boundaries, which decreased interfacial tension between liquid-disordered and liquid-ordered phases and thus reduced the tendency to form domains (Muddana et al., 2012). This study was completed in GUVs, (model membranes). Other studies have found that VE has a “membrane stabilizing effect” in endothelial cells by decreasing the membrane fluidity (Van Aalst, Burmeister, Fox, & Graham, 2004). This specific study used fluorescence recovery after photobleaching (FRAP) to determine membrane
microviscosity of cells incubated with vitamin E and then conjectured that membrane microviscosity is inversely related to membrane fluidity. Other researchers studying model membranes found that vitamin E actually partitioned into the disordered domain. They found that \( \alpha \)-tocopheral selectively partitioned to areas that were rich in the non-raft polyunsaturated fatty acids (PUFA) phospholipid as opposed to the sphingomyelin rich areas (Atkinson, Harroun, Wassall, Stillwell, & Katsaras, 2010).

These findings along with the lipid raft hypothesis provide the background to study the addition of vitamin E into bovine aortic endothelial cells. Lipid rafts have been shown to be cell-signaling platforms and especially important for integrin regulation and clustering. The antioxidant properties of VE have been studied extensively; however, the effect it has on endothelial cell focal adhesions has not been investigated. Studies by Muddana, Chiang and Butler in GUVs have found that VE decreased phase separation of liquid-ordered and liquid-disordered domains. Therefore, in this study, it is hypothesized that the addition of vitamin E to cultured endothelial cells will decrease the average size of focal adhesions, by decreasing raft coalescence.

**Triton-X Effect on Phase Separation**

Triton-X was first used as a detergent to extract lipid rafts from cell membranes, so the initial thought about Triton-X might be that it partitions to the liquid-ordered domains. But, according to the study by Muddana, Chiang, and Butler in GUVs, Triton-X partitioned uniformly to both ordered and disordered domains. They also found that it increased the order of the ordered phase more significantly than the disordered phase which led to slight increase in phase separation (Muddana et al., 2012). This slight increase in phase separation could be attributed to
a slightly greater height mismatch between the liquid-ordered and liquid-disordered domains, increasing the interfacial tension, and thus slightly increasing the tendency to form domains.

Figure 5: Triton X structure. Triton X is a very long molecule with repeating units. We used Triton X-100, which means the molecule has 100 parts of the repeating section. Triton X also has a hydrophilic and hydrophobic part of the molecule which allows it to assemble into the plasma membrane and affect phase separation.

There are many studies, investigating the detergent abilities of TX, but more recent findings have studied its ability to affect lipid rafts. One such study, completed in large unilamellar vesicles, quantified domain formation through differential scanning calorimetry and pressure perturbation calorimetry (Heerklotz, 2002). They found that addition of Triton-X increases microdomain formation of the liquid ordered phase, or lipid rafts (Heerklotz, 2002). Although Triton-X was originally used as a detergent to discover these lipid rafts these recent studies show that it affects lipid raft formation.

They also echo the difficulty that other researchers have been observing in studying these lipid rafts. That is that these lipid rafts are fluid, heterogenous and dynamic. They may appear, grow, disassemble and reassemble based on the requirements of the membrane or other unknown factors. This is what makes them so difficult to qualify and quantify. These findings along with the lipid raft hypothesis warrant the testing of Triton-X in bovine aortic endothelial cells. TX has been used for years as a detergent, and more recently it has been shown to affect lipid rafts, but the affect on TX on focal adhesions size and formation has not been studied. According to
the literature results, from both Heerklotz, and Muddana, Chiang and Butler, Triton-X appears to mildly increase phase separation. Based on these results and the belief that lipid rafts act as cell-signaling platforms, it is hypothesized that the addition of Triton-X into endothelial cells will slightly increase the size of focal adhesions.

**Benzyl Alcohol Effect on Phase Separation**

![Benzyl Alcohol Structure](image)

**Figure 6: Benzyl Alcohol Structure.** Benzyl alcohol is a relatively small molecule with a benzene ring attached to a hydroxyl group. The hydroxyl group is the hydrophilic end of the molecule which the benzene ring is the hydrophobic part. This amphipathy of the molecule and its small size allow it to easily assemble into the cell membrane and affect phase separation.

Lastly, Muddana et al found that benzyl alcohol had the most significant effect by strongly partitioning into the disordered phase where it decreased membrane thickness, increased hydrophobic mismatch and increased interfacial tension thus leading to increased tendency to form domains (Muddana et al., 2012). Benzyl alcohol’s preference for ordered or disordered membrane microdomains has not been studied as extensively as Triton X-100 and vitamin E, however there are studies documenting it as a membrane fluidizing agent. In a study conducted at University of California, San Diego, researchers showed that addition of benzyl alcohol increased membrane fluidity in endothelial cells, as measured by fluorescence of a molecular probe that associated into the cell membrane (Haidekker, Heureux, & Frangos, 2000). Another study conducted by Yamamoto and Ando found that the addition of benzyl alcohol to endothelial
cells caused an increase in the diffusion coefficient and decreased the order of both ordered and disordered phases (Yamamoto & Ando, 2013). Thus the role of benzyl alcohol as a membrane fluidizer has been elucidated.

Based on these results, it is clear that benzyl alcohol partitions into the membrane and effects lipid raft coalescence. However, it has not been studied how this change in membrane fluidity will affect focal adhesion formation. These results along with lipid rafts being cell-signaling centers, provide the background for benzyl alcohol to be tested in bovine aortic endothelial cells. Based on the results provided by Muddana et al that showed increased phase separation after addition of benzyl alcohol and other research showing benzyl alcohol as a membrane fluidizing agent, it is hypothesized that the addition of benzyl alcohol in endothelial cells will increase focal adhesion size.

**Objective and Goals of Experiment**

The objective of this experiment is to study the effects of these three additives: Vitamin E (α-tocopheral), Triton X-100, and benzyl alcohol on focal adhesions in bovine aortic endothelial cells. These three additives have all been shown to perturb the membrane and affect the formation of lipid rafts in the plasma membrane of cells. There has also been found an interdependence between lipid rafts and integrin clustering and regulation. Therefore, by perturbing the membrane and lipid raft formation, we propose to modulate focal adhesion formation. The effects of these additives on endothelial cell membranes will be studied by transfecting the endothelial cells with green fluorescent protein-tagged focal adhesion kinase (GFP-FAK), adding the 3 non-lipid amphiphiles, monitoring the focal adhesions by live imaging
under total internal reflection microscopy (TIRF-M), and analyzing the FA dynamics by the focal adhesion analysis server (FAAS).
Chapter 2

Materials and Methods

The goal of this experiment is to understand the way that the cell membrane can modulate focal adhesion formation through membrane perturbations. The first step in this is to culture live bovine aortic endothelial cells. These cells were then transfected with GFP-FAK, allowed to recover, and then imaged under TIRF-M. Individually, the non-lipid amphiphiles were added and time-lapse images were recorded every one minute. The control fluid was CO\textsubscript{2} independent media (optimal for imaging) and the experimental fluid was CO\textsubscript{2} independent fluid containing an additive in the micromolar to millimolar range. The imaging chamber was temperature-controlled with minimal fluctuations during experimentation, because temperature changes can affect the focus of the microscope and the health of the cells. This imaging chamber was the Delta T Culture Dish Temperature Controller made by Bioptechs, which allowed for precise temperature control of cells without interfering with the imaging. The focal adhesions were tracked by an online server, called FAAS, supported by a laboratory at University of North Carolina.

The cells used in this experiment were bovine aortic endothelial cells (BAEC) from Sigma Aldrich, passage 3 through 7. They were maintained in culture and passaged every 3-4 days. At 70-90% confluence, the cells were transfected with (GFP-FAK). This fluorescently tagged kinase localizes to focal adhesions, which allows detection and quantification size and distribution of focal adhesions. The cells were given 2 days to recover from the transfection, and were then transferred to the imaging chamber that was coated with fibronectin. Fibronectin increases adhesion and spreading by mimicking the endogenous environment that endothelial cells typically adhere to.
Then the fluorescent microscope was configured for total internal reflection microscopy (TIRF-M). This requires a high aperture objective to create the angle necessary for total internal reflection of light. TIRF-M illuminates the first 200 nm into the cell which comprise the cell membrane. The wave does not penetrate further than ~200 nm into the cell and therefore the GFP-FAK in vessels (not localized to focal adhesions) will not fluoresce, eliminating the background fluorescence.

Once the cells were prepared for experimentation, the experimental mediums were prepared. There were 3 test conditions and 2 controls. The three experimental mediums were CO$_2$ independent media with one of the additives at the following concentrations: Benzyl alcohol 10 mM; Triton-X 50µM; and Vitamin E 50µM. The two control mediums were one with just CO$_2$ independent media, and one with CO$_2$ independent media and ethanol, as a vehicle control.

Then the microscope was set up for TIRF microscopy, with a PCO camera for taking the images. Once the focus was set, BAECs were imaged at 1 minute intervals for a set amount of time. Then the media was exchanged with a pipette to the experimental medium and imaging continued at 1 minute intervals. At the end of this time period, the time-lapse images were combined into a stacked TIFF file in ImageJ.

This file was uploaded to the focal adhesion analysis server (FAAS), operated by the Gomez lab at UNC. This server analyzes the birth, death, size, migration, alignment and other static and dynamic parameters of focal adhesions. The parameters of interest in this experiment were focal adhesion size and distribution.
Part 1: Cell Culture and Maintenance

Cells used in this experiment were bovine aortic endothelial cells (BAECs) from VEC Technologies (Rensselaer, New York). Passages 3-7 were used in this experiment and were thawed according to given directions. They were maintained in culture in MCDB-131 complete medium (VEC Technologies, Rensselaer, New York) supplemented by 10% FBS. Cells were maintained in an incubator at 37°C, with 5% CO2- 95% air and passaged every 3-4 days at 70-80% confluence.

Cells were seeded at 5 x 10³ cells/cm² in T25 flasks. For passaging of cells, BAEC were rinsed with PBS and detached from the plate with 0.5% EDTA-Trypsin (GIBCO, Grand Island, NY). Cells were then incubated for about 2 minutes until they became round and detached. EDTA-Trypsin was then aspirated and complete growth medium was added; then cells were centrifuged at 1000 rpm for 5 minutes. A pellet and liquid suspension remained after centrifugation. The liquid was aspirated and cells were reconstituted in complete growth medium. Cells were counted using a hemacytometer and plated into 24 well plates for transfection. Cells were plated at a density of 10 x 10⁴ cells per well of the 24-well plate for transfection.

Part 2: Transfection with GFP-FAK

The transfection reagent used was Lipofectamine 2000 (Invitrogen), and Opti-MEM (GIBCO, Grand Island, NY) reduced media serum was also used. Transfection occurred 24 hours after cells were plated in 24 well plates. All reagents were warmed to 37°C and cells were transfected at 70-90% confluence. The Lipofectamine 2000 protocol was used and the optimum
transfection amounts found by an optimization were 500 ng of the plasmid (a gift from Song Li, Ph.D., University of California, Berkeley) and 1.5 μL Lipofectamine per well in a 24 well plate. These were incubated in 500 μL Opti-MEM for four hours. At the end of the four hours, the media was switched to complete growth medium. Cells were placed in the incubator to recover for 48 hours.

Part 3: Preparation of Additives

The three additives used were Vitamin E, Triton-X, and benzyl alcohol. VE (Pfaltz and Bauer, Waterbury, CT) and TX (MP Biomedicals) were dissolved in PBS, and then added to CO2 independent media (Thermo Fischer Scientific, Grand Island, NY), at 50 µM concentrations. BA (Sigma Aldrich) was dissolved in ethanol, and added to CO2 independent media at a concentration of 10 mM. 1 mL of each experimental solution was prepared for experimentation. Additionally, a 1 mL solution of CO2 independent media was prepared containing the same amount of ethanol used to dissolve BA. This was used as a vehicle control. Another control was simply 1 mL of CO2 independent media.

Part 4: Coat delta T dish with fibronectin

A stock solution of 5.0 μg/mL of fibronectin was made in PBS and 500 μL of this solution was placed in a Delta T dish, resulting in a 5 mM solution. This was kept under the hood for 45 minutes. At the end of 45 minutes, excess fibronectin was aspirated and the cells were immediately added to the fibronectin-coated Delta T dish.
Part 5: Live Imaging of Cells under TIRF-M

Part 5a: Set up of Delta T System

The Bioptechs Delta T system was used to maintain the cells at 37°C during live imaging. This Delta T dish was placed into the standard stage adaptor which connects to the temperature controller. The Delta T system functions by applying heat directly to the underside of the Delta T dish which is coated with an electrically conductive material (Bioptechs, 2015). Figure 7 shows the Delta T dish used to image cells (adapted from the Bioptechs website) (Bioptechs, 2015). After the cells had been added to the fibronectin-coated Delta T dish, they were allowed to adhere for 2-4 hours before imaging.

Figure 7: Delta T Dish Used for Imaging. The Delta T dish provided an optimal surface for cell adhesion and imaging. It has a coating on the bottom of it that allows for heat to be evenly distributed to the entire surface. Connected with the temperature controller, the system keeps the cells at 37°C during imaging.
Part 5b: Set up of TIRF-M

The Olympus IX71 Inverted Microscope for live cell imaging was used with an attached PCO camera. Melles Griot Argon Ion Laser was used for total internal reflection fluorescence microscopy (TIRF-M) to visualize the focal adhesions. The wavelength of light used for TIRF microscopy was 488 nm (blue light)—this is the excitation wavelength for GFP. A high aperture oil-immersion objective (PLAPO 60 × /1.45 NA, TIRFM-2, WD 0.15 mm) was used with n = 1.515 oil on the objective. The focus plane of the cells was first found in epifluorescence. Then the angle of light hitting the aperture, was adjusted, which changed the incident angle of light until it approached the critical angle. It was evident from the angle of the visible refracted laser beam when the light underwent total internal reflection. This was also seen by observing the fluorescence of the cells as the incident angle of light was changed. When the light began to totally internally reflect, the background fluorescence of the cells disappeared.

Part 5c: Imaging Conditions and Addition of Additives

After the cells had been focused under TIRF-M, the first image was taken at time t=1. Images were manually taken every minute under normal conditions for 13-24 minutes. When an image was taken, the aperture was opened, the cells were focused again and the image was acquired. After acquiring an image, the aperture was closed again to reduce photobleaching of the GFP-FAK. After the allotted amount of time, the CO2 independent media was pipetted out of the Delta T dish and exchanged for a test condition or a control condition. The images were subsequently taken after that, every minute.
This process was repeated with a new set of cells for the other control and test conditions. These results were compiled into 5 different stacked TIFF files and uploaded to the FAAS for further analysis.

**Part 6: Analyzing results using Focal Adhesion Analysis Server (FAAS)**

As stated before, results were composed of a stacked TIFF file of time-lapse images, which was uploaded to the FAAS. The server then sent back the analyzed data to perform statistics on. In this experiment, size of focal adhesions was identified as a main predictor of changes in membrane microdomain formation. It has been found by our lab in preliminary studies that such additives can assemble themselves into the phospholipid bi-layer of the cells and affect the size and grouping of focal adhesions. The FAAS returns a folder with many different analyses of the data. One important spreadsheet contains the unique number identifier of each focal adhesion along with the size of the focal adhesion over time. If a focal adhesion has not been identified yet or has disappeared from the visualization field, the spreadsheet will report NaN for that time. Another important folder contains an .svg for each time period which has each focal adhesion outlined with the unique number identifier next to it. This allows individual tracking of each focal adhesion by its unique number identifier over time. **Figure 8** shows an example of one of these images.
Figure 8: Example of FAAS results. This image shows an example of how the focal adhesion analysis server analyzes focal adhesion size. An outline is traced around each fluorescing area, that the software deems to be a focal adhesion and the enclosed area is calculated. A unique number identifier is given to each focal adhesion so that the size may be tracked, over time.

Part 7: Statistical Techniques

This study used the statistical software package GraphPad Prism 6 to analyze the size and distribution of focal adhesions before and after the additive. A cumulative distribution function (CDF) was plotted for the focal adhesion size of the time point right before the non-lipid amphiphile was added, and the last time point taken. These two time points are referred to as the before and after time points. The x-axis on these plots represent the size of the focal adhesions in pixels and the y-axis is the CDF for the before and after times. The distribution shifted very minimally, to the left or to the right in all cases. If the distribution shifted minimally or not at all, it indicated that the before and after distributions were very similar or the same, as thus the additive or control condition had no effect. If the distribution shifted to the right, it indicated that
the focal adhesion size was larger and the two distributions were different due to the affect of the additive. If the distribution shifted to the left, it indicated that the focal adhesion size was smaller and the two distributions were different due to the affect of the additive.

These shifts were analyzed by a specialized type of t-test called the Kolmogorov-Smirnov test. This test analyzes two cumulative distribution functions to determine if they are statistically different. The null hypothesis here being that the two samples are from the same distribution and the alternative hypothesis that they were taken from different distributions. P-values were calculated from this test to determine statistical significance.

**Part 8: Analysis of BA action at the leading of the cell**

The preliminary results found by Seoyoung Son, a P.h.D. student in Dr. Butler’s mechanobiology lab, about the action of benzyl alcohol in focal complexes that are formed immediately after initial contact with the cell warrant a special analysis of this condition. The lab results show that BA is important in recruiting integrins to this focal complex, but it is not clear, if they act in nascent focal adhesions which are found at the leading edge of the cell. As discussed in the introduction, there is an .svg file which contains the unique number identifier for each focal adhesion. This .svg file will be referred to as the lineage file. There is a different lineage file for each time point that has all of the focal adhesions for that time point outlined and identified by their unique number identifier. This allows for a researcher to locate the focal adhesions which are at the leading edge of the cell. For analysis of this BAEC under benzyl alcohol, the leading edge of the cell was first located (Figure 9). The red line is drawn to emphasize the location of the leading edge. The area of focal adhesions at the leading edge was
analyzed every five minutes to see the changes over time in average FA size and distribution. This was accomplished by finding the lineage file for a given time point, locating the FAs at the leading edge, and recording their areas as found in the area file returned by FAAS. This process was repeated for each time point. An average FA size for each time point was calculated, and the distribution was determined as well.

Figure 9: BAEC leading edge. The entire cell is shown here with all the focal adhesions outlines. The leading edge was chosen as the area with small, pinpoint focal adhesions. It can also be identified by viewing the time lapse images to determine which direction the cell is crawling. The leading edge of the cell is shown here by the red outline.
Chapter 3

Results and Discussion

Preliminary Results taken with permission from lab of Dr. Peter Butler:

These results were from the paper published in 2012 by Muddana, Butler and Chiang. These tests were run in our lab in bovine aortic endothelial cells (Figure 10, B) and GUVs (Figure 10, A). As referenced before, the initial tests were conducted in GUVs; however, a preliminary test on bovine endothelial cells was also conducted and not published with the paper. The first row of photos depicts the phase separation studies using quaterny and ternary mixtures of DOPC, DPPC, and cholesterol. Lo stands for liquid ordered phase, and Ld stands for liquid disordered phase, as discussed in the introduction. The first image in Figure 10, A, is the control image showing phase separation between Lo and Ld phases. When Vitamin E was added to these mixtures, in the second picture of row A, it led to mixing of the gel and liquid phase, which resulted in a uniformly mixed phase. When Triton-X was added to the GUVs, the result closely mirrors the control image, but does appear to slightly promote domain formation (Muddana et al., 2012).

Both Triton-X and benzyl alcohol allowed for phase coexistence of gel and liquid phase. But as seen below, they resulted in different morphologies. Benzyl alcohol appeared to have a stronger effect on phase separation. As seen in the far left picture of row A, BA increased phase separation significantly (Muddana et al., 2012).
Figure 10: Preliminary Results from study by Muddana, Chiang and Butler, 2012. A) Published Results of microdomain studies conducted in giant unilamellar vessels (GUVs). B) Unpublished results of microdomain studies in endothelial cells. Studies show that Vitamin E decreases phase separation and promotes mixing of the Lo and Ld phases. Studies also show that Triton X and Benzyl Alcohol promote phase separation.

The second row of images shown in Figure 10 depict the addition of the three additives to bovine aortic endothelial cells. The mean size and number of focal adhesions were calculated using ImageJ. With the addition of Vitamin-E, the number of focal adhesions increased and the mean size decreased, indicating that there was decreased phase separation. In Triton X, the number of focal adhesions also increased, but the size of focal adhesions doubled, leading to inconclusive results about the effect of Triton X. Lastly, with the addition of benzyl alcohol, a dramatic change in morphology was observed. The mean size of focal adhesions increased significantly and the number of focal adhesions decreased, indicating increased phase separation.
These results shed some light on the issue but present an incomplete picture of focal adhesion dynamics in endothelial cells (Muddana et al., 2012).

The major limitation with this method of studying the effect of additives is that each of the four images come from a different endothelial cell. Thus, it is difficult to compare number of focal adhesions and average size between different cells and obtain an accurate result. Additionally, these images represent a static view of focal adhesions, whereas researchers understand that focal adhesions are very dynamic structures that model and remodel on the order of microseconds. One image of a cell after an additive was added was not significant data to report that the non-lipid amphiphile affected focal adhesion dynamics. More studies were needed to capture the dynamic nature of focal adhesions and their remodeling due to the addition of vitamin E, benzyl alcohol or triton X. A time-lapse study, with observation of the cells before and after amphiphile addition was needed to understand the time dependence and effect of these non-lipid amphiphiles on focal adhesion dynamics.

The following results are from a study I conducted in bovine aortic endothelial cells. These cells were cultured and transfected as described in the methods section. Then they were imaged under TIRF microscopy to illuminate the focal adhesions. Images were captured every one minute and the images were captured for a total time ranging from 30-60 minutes. Due to the difficulty of photo bleaching, some imaging times were shorter than others. The additive was added about halfway through the imaging time and results collected before and after addition. Because BA was dissolved in ethanol, the addition of pure ethanol at the same concentration as the other experiment was used. The control used was a change from CO₂ independent media to more CO₂ independent media to simulate the temperature change and exposure to air when the media was
switched. As explained in the methods, results were analyzed by FAAS and cumulative distribution functions plotted below in Figure 11.

In Figure 11, the x-axis represents focal adhesion size in pixels and the y-axis represents the percentage of focal adhesions, out of 100. The blue line represents the time before the additive was added to the cells and the red line represents the time after. A cumulative
distribution function calculates the percent of focal adhesions that are smaller than or equal to a certain focal adhesion size. For example, at \( y = 50 \), 50\% of the focal adhesions are equal to or smaller than the corresponding x-value for each condition. By looking at the shifts in the distributions, one can see whether the distribution contains smaller or larger focal adhesions after the addition of an additive. For the control condition, imaging media was simply exchanged for the identical media, using the same procedure as the other experimental conditions. Ethanol was used as a vehicle control because benzyl alcohol was dissolved in ethanol. The ethanol concentration used was the same amount required for the benzyl alcohol test condition. Each individual result will now be discussed in detail.

**Effects of Vitamin E on Focal Adhesion Size Distribution**

As seen in Figure 12, the distribution shifted to the left (from blue to red), with the addition of vitamin E. This indicates the distribution of focal adhesions in the cell shifted towards smaller areas of focal adhesions. After the addition of vitamin E, there were a higher proportion of smaller focal adhesions, and a lower proportion of larger focal adhesions. The two distributions, before and after, are shown to be statistically different with a p-value of 0.006. This quantitatively shows that Vitamin E has a significant effect on focal adhesion size. This is consistent with the hypothesis that VE decreases interfacial tension, disperses microdomains, and thus decreases focal adhesion size.
Figure 12: Distribution of focal adhesion size before and after the addition of Vitamin E. Vitamin E was hypothesized to reduce the tendency of the cell membrane to form microdomains and thus decrease the focal adhesion size. This can be seen by the shift left of the CDF after the addition of vitamin E. This shift indicates that a higher percentage of the focal adhesions are smaller. The t-test showed that the two CDFs come from different populations (p-value = 0.0006).

Effects of Triton-X on Focal Adhesion Size Distribution

Triton X appears to have had a similar effect as Vitamin-E, which is inconsistent with the initial hypothesis. The distribution of focal adhesion size shifted to the left after the addition of Triton-X. This indicates the focal adhesion size has shifted towards the smaller size focal adhesions and away from the larger focal adhesions and overall that the mean focal adhesion size decreased. This is inconsistent with what was previously suggested as a hypothesis for Triton-X. As discussed in the introduction, Triton-X was hypothesized to increase phase separation and lipid raft formation. The initial results from Muddanna et al showed that Triton-X partitioned equally to both phases but increased order in the ordered phase more significantly. However, as seen in Figure 12, the focal adhesions have become smaller. The K-S t-test showed that the two
distributions are statistically different (p=0.0076). These results suggest that at this concentration, TX is decreasing the tendency to form microdomains or rafts in the endothelial cells.

Figure 13: Distribution of focal adhesion size before and after the addition of Triton-X. Triton-X was hypothesized to increase the tendency of the cell membrane to form microdomains and thus increase the focal adhesion size. This hypothesis is inconsistent with the results. The results show a shift left of the CDF after the addition of Triton_X. This shift indicates that a higher percentage of the focal adhesion are smaller in the after distribution. The t-test showed that the two CDFs come from different populations (p-value = 0.0076).

Effects of Benzyl Alcohol on Focal Adhesion Size Distribution

Benzyl alcohol had the opposite effect of VE and TX on focal adhesion size distribution. After the addition of benzyl alcohol, the percentage of smaller focal adhesions decreased, while the percentage of larger focal adhesions increased (Figure 14). This is consistent with the initial hypothesis that addition of benzyl alcohol would cause increased phase separation, leading to coalescence of lipid rafts and thus increased focal adhesion size. The distribution of focal
adhesion size shifts to the right, indicating an increase in the proportion of larger focal adhesions and a decrease in the proportion of smaller focal adhesions, and an overall increase in the average focal adhesion size. The K-S t-test showed that the two distributions are statistically different (p=0.0076), meaning that benzyl alcohol has a significant effect on focal adhesion size. These results suggest that BA increases phase separation and lipid raft coalescence, resulting in larger focal adhesions.

Figure 14: Distribution of focal adhesion size before and after the addition of benzyl alcohol. Benzyl alcohol was hypothesized to increase the tendency of the cell membrane to form microdomains and thus increase the focal adhesion size. This hypothesis is consistent with the results shown here. The results show a shift right of the CDF after the addition of benzyl alcohol. This shift indicates that a higher percentage of the focal adhesion are larger in the after distribution. The t-test showed that the two CDFs come from different populations (p-value = 0.0033).

**Effects of Ethanol and Control Solution of Focal Adhesion Size Distribution**

The distribution of focal adhesion size under the control condition remained relatively unchanged. This confirms that the test conditions, such as being imaged for about an hour, and
the accompanying photobleaching, do not affect the measurement of the focal adhesion size. The t-test confirmed that the two CDF’s come from the same distribution (P-value = 0.1291). Additionally, the vehicle control condition, ethanol, also remains relatively unchanged after addition of ethanol. The distribution, shown in Figure 15B, for ethanol does appear to shift to the right. However, the t-test validates that the two CDFs come from the same distribution (P-value = 0.1107). This confirms that the vehicle ethanol did not effect focal adhesion size formation. It also confirms that the slight change in temperature and exposure to air during the switch to a different solution did not affect focal adhesion formation. If focal adhesion area were to change in either of these cases, then that effect would be confounding the data.

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 15:** Distribution of focal adhesion size for control and vehicle control conditions. A) Control Condition. The red line represents the time before the media was changed. Distributions before and after changing the media are not statistically different (P-value = 0.1291). B) Ethanol Control. The red line represents the time before the media was changed to ethanol containing media. Distributions before and after changing the media are not statistically different (P-value = 0.1107).

**Benzyl Alcohol Effect on the Leading edge**

This study was also interested in determining the effect of benzyl alcohol at the leading edge of the cell where new adhesions are being made. Our lab has some preliminary data that shows benzyl alcohol is active in focal complexes; therefore, we are interested in knowing
whether it is active in nascent focal complexes as well (detectable under TIRF-M). Nascent focal adhesions are found at the leading edge of the cell, which explains why we are studying benzyl alcohol at the leading edge. The effect of benzyl alcohol at the leading edge of the cell was determined using the focal adhesions residing at the leading edge of the cell. This is only a small proportion of the total focal adhesions and is in contrast to the above analysis of focal adhesion size which analyzed the focal adhesions in the entire cell. Because the range in focal adhesion size is inherently large, the graph depicts a large range of values and the standard deviation of the mean is quite large (not reported here). Instead, the mean and the standard error of the mean are reported here to determine if the means are different. The trend in average focal adhesion size (shown by the blue dots) is evident (Figure 16). After the BA is added at 14 minutes, the mean focal adhesion size gradually doubles, from about 50 pixels to about 100 pixels. The standard error of the mean also increases. This is because as the focal adhesion become larger, there are fewer focal adhesion to count, which means a smaller sample size and thus a larger standard error of the mean. These results support the hypothesis that benzyl alcohol is active in focal complexes (previous data) as well as in nascent focal adhesions (Figure 16).
Figure 16: Effect of benzyl alcohol at the leading edge of the cell. The addition of benzyl alcohol to bovine aortic endothelial cells doubles the mean focal adhesion size. The mean and standard error of the mean are plotted here to show how focal adhesion area changes over time. The focal adhesion area was calculated for the focal adhesions at the leading edge of the cell every 5 minutes. Benzyl alcohol was added at 14 minutes, as indicated by the arrow.

Visualizations

The next set of images was included in the data set returned from the FAAS for each set of conditions. These images show how the software tracks an individual focal adhesion through each frame, during the experimentation. An outline surrounds each focal adhesion in a given frame. Then in the next frame, any size changes of the focal adhesion are marked by a different size or shape of the outline.

Figure 17 shows how an individual focal adhesion is tracked from one frame to the next, with each rectangle representing a different frame. The frames are taken one minute apart. The focal adhesion of interest here is outlined in green, and tracked through each frame. The additive is added in the fourteenth frame, as shown by the pink arrow. Before this time point, the size of
this focal adhesion remains steady. But after the addition of vitamin E, the focal adhesion size begins to decrease, due to the effect of VE assembling into the cell membrane. This image represents the size change of one focal adhesion of time, but is indicative of the larger global effects seen by the distribution shift to the left.

Figure 17: Vitamin E individual FA representative size change. Each rectangle represents a different time point, and they are each one minute apart. Vitamin E is added in the 14th frame, indicating that it was added to the solution at 14 minutes. The image shows that the focal adhesion, outlined in green remains a steady size until the addition of vitamin E at which point, it decreases in size. This is only one focal adhesion, but is representative of the global changes seen by the distribution shift.
Figure 18: Triton X individual FA representative size change. Each rectangle represents a different time point, and they are each one minute apart. Triton X is added in the 26th frame, indicating that it was added to the solution at 26 minutes. The image shows that the focal adhesion, outlined in green remains a steady size until the addition of TritonX at which point, it decreases in size. This is only one focal adhesion, but is representative of the global changes seen by the distribution shift left.

Figure 18 displays the changes in size of an individual focal adhesion over the course of the experiment. In this experiment, Triton-X was added in the 26th frame, as indicated by the pink arrow. The size of the focal adhesion, outlined in green, is relatively constant until frame 26 when the Triton X is added. After this, it begins to decrease due to the effect of Triton-X assembling into the cell membrane and affecting microdomain size. This is inconsistent with the hypothesis outlined in the introduction that stated that the addition of TX to BAECs would increase focal adhesion size. This image represents the size change of one focal adhesion of
time, but is representative of the larger global effects seen by the shift towards smaller focal adhesions.

Figure 19: Benzyl Alcohol Individual FA representative size change. Each rectangle represents a different time point, and they are each one minute apart. Benzyl alcohol is added in the 15th frame, indicating that it was added to the solution at 15 minutes. The image shows that the focal adhesion, outlined in green, remains a steady size until the addition of benzyl alcohol at which point, it increases in size. This is only one focal adhesion, but is representative of the global changes seen by the distribution shift to the right.

**Figure 19** shows the size change of a representative focal adhesion before and after addition of BA to BAECs. Each frame represents a different time point during experimentation, and the frames are one minute apart. The additive was added in the 15th frame, or 15th minute, where the pink arrow indicates. The focal adhesion of interest is the one outlined in green, which begins to increase after the addition of benzyl alcohol to the cell. This image represents
the size change of one focal adhesion of time, but is representative of the larger global effects seen by the shift towards larger focal adhesions.

Representative size changes for the control and ethanol conditions are not shown here because the size remains the same over the course of experimentation.
Chapter 4

Discussion and Future Work

Conclusion

The objective of this experiment was to perturb the cellular membrane through the use of three non-lipid amphiphiles known to affect domain separation, and observe the changes in focal adhesion size. The hypotheses were that the addition of vitamin E would decrease focal adhesion size, and that the addition of Triton X-100 and benzyl alcohol (individually) would increase focal adhesion size. Bovine aortic endothelial cells were cultured, and transfected with GFP-FAK. Then they were imaged under TIRF microscopy and changes in focal adhesion size were analyzed by the focal adhesion analysis server. The results were the vitamin E did significantly decrease focal adhesion size, as shown by a specialized t-test on the before and after distribution of focal adhesion size (P-value = 0.0006). Triton X significantly decreased focal adhesion size, as quantified by the aforementioned t-test (P-value = 0.0076). This was inconsistent with the hypothesized result. Benzyl alcohol significantly increased the focal adhesion size, again as quantified by the specialized t-test (P-value = 0.0033). Analysis of the control and vehicle control condition showed no significant difference between the before and after distributions (P-values > 0.1). Further time-dependent analysis of benzyl alcohol at the leading edge of the cell showed that the addition of benzyl alcohol doubled the mean focal adhesion size at the leading edge of the cell.

The above results show the feasibility of the link between non-lipid amphiphiles and focal adhesion dynamics. Research has shown that these 3 non-lipid amphiphiles affect microdomain formation by assembling into the cell membrane and increasing or decreasing
interfacial tension between the liquid-ordered and liquid-disordered domains. This change in interfacial tension leads to an increased or decreased tendency to form microdomains in the cell membrane. As this paper has demonstrated, addition of these non-lipid amphiphiles to bovine aortic endothelial cells increases or decreases focal adhesion size as well. Therefore, the conclusion may be drawn that membrane microdomains can regulate focal adhesion formation.

**Strengths of this Study**

The strengths of this study are many. For one, we were able to evaluate a single cell over time, and thus characterize the focal adhesion size in a single cell before and after addition of the non-lipid amphiphiles. This technique eliminates the added variability of comparing the focal adhesion size from 2 different cells (one control and one experimental). Secondly, we were able to gather and analyze data (focal adhesion area), from all of the focal adhesions in a cell, which resulted in a large number of focal adhesions. This created a large sample size of focal adhesions; however, there is still a very large size distribution of focal adhesion area that is inherent to the cell. Third, using the lineage file from the FAAS, we were able to identify the location of specific focal adhesions and track their size over time based on the unique number identifier assigned to each focal adhesion. This opens the door to analyzing the rates of assembly or disassembly of focal adhesions in the future. Transfection of bovine aortic endothelial cells was also a strength of this study, because transfection efficiency was high with this technique and the cells appeared to be healthy after transfection. Lastly total internal reflection fluorescence microscopy (TIRF-M) was a powerful microscopy technique used to
image the focal adhesions and eliminate background fluorescence. This produced high quality, accurate images of the cells.

**Limitations of this Study**

Despite all of the many strengths of this study, there were significant limitations imposed on this study. For example, only one cell was analyzed for each condition with no replicates. This is a limitation because the effect seen in that one case could be an exception or an unhealthy cell. Secondly, focal adhesions and microdomains are both very dynamic structures that organize, assemble, and disassemble on the scale of microseconds to seconds. Capturing images every 1 minute may not have allowed us to fully quantify the time dependent nature of these focal adhesions. Additionally, we are not sure about the time scale that the amphiphiles assemble into the membrane so this could be faster than we are imaging as well. Third, bovine cells are a good analogue and were useful in this study, but there are still limitations that go along with using animal cells and trying to extrapolate the results from that to a human model.

Fourth, some photobleaching did occur during experimentation, which may have led to some smaller focal adhesions not being recognized in the later time periods by the tracking software that FAAS uses. Along with that, in analyzing the images from the FAAS, I found that some larger focal adhesions that looked like they should be two separate focal adhesions were actually categorized as one focal adhesion by the tracking software. This may have caused a focal adhesion to have a large area, when it should have been categorized as two focal adhesions with relatively smaller areas. Hopefully, this problem with the software was uniformly distributed across all time points and conditions. But if for example, it affected the before
condition of benzyl alcohol more than the after condition, then the *measured* affect of benzyl alcohol on focal adhesion size would be markedly less the *actual* effect. Lastly, the cells were not under any flow conditions like they would be in-vivo. This may have affected the response of the focal adhesions to the non-lipid amphiphiles.

**Future Work**

A future study could be conducted to eliminate some of these limitations and increase the accuracy of the study. This future study would include human cells and more replicates of each condition to confirm the results of this study. The human cells would increase the validity of the study and the additional replicates would increase the precision of the study. To elucidate the time dependent nature of focal adhesions and membrane microdomains, a time-dependent flow chamber could be used. This would place the cells under parallel flow, which is closer to their in-vivo conditions, and it would allow us to define the time dependence of delivery of a bioactive drug to a cell, using non-lipid amphiphiles: VE, TX, and BA. To accomplish this, a pressure dependent fluidic device would be used with an input and output pressure, causing the fluid to flow across the chamber. The control fluid would be CO$_2$ independent media (optimal for imaging) and the experimental fluid would be CO$_2$ independent media containing an additive in the micromolar range. The main component of this device would be the Focht Chamber System 2 (FCS2), made by Bioptechs, which allows for precise temperature control of cells and fluid flow across the cells.

To conduct this study, the pressure in the chamber would need to be held constant as the media would switch from CO$_2$ independent media to the media containing the additive, because
pressure changes could compromise the quality of imaging of the cells. The pressure would be held constant by keeping the two respective fluid reservoirs at the same height during experimentation. These reservoirs would be connected to a computer-controlled valve that could open and close the valves for each reservoir. This would allow us to precisely define when the fluid was being switched to the additive-containing fluid. To verify that pressure would remain constant when the valve would switch, a differential pressure sensor would be set up in parallel with the flow chamber. See Figure 20 for the experimental setup of this device.

Figure 20: Experimental Setup for Time-dependent Flow Chamber

A LabView program would need to be designed to trigger the valve to switch between open and closed positions of the two reservoirs. The device would be tested initially with a
fluorescent dye solution to quantify the concentration change in the middle of the chamber as a function of time. From this, it would be determined if the concentration will change as a step function or more gradually. After this, changes could be made to the length or shape of fluid flow path and experiments run again to determine the changes to the concentration gradient. Additionally, a finite element model in COMSOL could be created to model the transport of the media across the chamber. This model, in conjunction with the experiment would give us the exact time point in which the fluid was delivered to the cells and what type of concentration gradient it would be.

The last component of this experiment would be a camera with an automatic trigger. This camera could capture images every 1, 5, or 10 seconds and would help us understand the dynamic nature of focal adhesions. It would also help us calculate the time scale upon which these additives assemble into the cell membrane. Lastly, a camera with an automatic trigger would reduce the time that the cells are exposed to the light and thus reduce the photobleaching occurring in the cells, and improve the accuracy of the data.

The results gathered from these additional experiments would help confirm the results documented in this paper about the effect of each of the additives. The results would also help us understand the dynamic nature of focal adhesions and the time scale upon which these non-lipid amphiphiles assemble into the cell membrane. The dynamic nature of modeling and remodeling could be quantified using the assembly and disassembly rates from the FAAS, if the time scale is small enough. The results could again be analyzed by FAAS or in ImageJ to confirm their accuracy regarding the identification of focal adhesions.
Membrane microdomains play a key role in regulating focal adhesion formation, as evidenced by the results of this paper. Focal adhesion assembly and disassembly are the ways that a cell adheres to a substrate, crawls around, and can migrate to wounds. Therefore, understanding this process and different additives which can regulate focal adhesion formation is a very important step in medicine. Additionally, understanding how membrane microdomains can regulate focal adhesion formation is also important in understanding the mechanotransduction cascade.
Appendix A

Images of whole cells at critical time points

The following are pictures of the cells, corresponding to the initial time, the time that the additive was added, and the final time point. Each figure represents a different cell under different conditions; however, each of these pictures are from the same cells used to gather the data. The pictures are shown large for clarity, due to the relatively small size change of focal adhesion area. Important things to note, are the changes in morphology from one time point to another, the changes in focal adhesion size and distribution between cells and the effect of photobleaching.
Figure 21: Vitamin E Condition. This is one image containing the bovine aortic endothelial cell at three different time points. The pictures on the left are ones captured by the camera while the ones on the right are the ones analyzed by the FAAS. The first row shows the initial time point, the second row shows the time point right before vitamin E was added, and the last time point shows the final image taken (with additive). Note the photobleaching effect.
Figure 22: Triton X Condition. This is one image containing the bovine aortic endothelial cell at three different time points. The pictures on the left are ones captured by the camera while the ones on the right are the ones analyzed by the FAAS. The first row shows the initial time point, the second row shows the time point right before Triton X was added, and the last time point shows the final image taken (with additive). Note the photobleaching effect.
Figure 23: Benzyl Alcohol Condition. This is one image containing the bovine aortic endothelial cell at three different time points. The pictures on the left are ones captured by the camera while the ones on the right are the ones analyzed by the FAAS. The first row shows the initial time point, the second row shows the time point right before benzyl alcohol was added, and the last time point shows the final image taken (with additive). Note the photobleaching effect.


Academic Vita
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Education
The Pennsylvania State University, Schreyer Honors College  May 2016
Bachelor of Science in Biomedical Engineering–Chemical Option
Universidad Carlos Tercero, Madrid, Spain  Fall 2015
Biomedical Engineering Program

Job Experience
Cellular Mechanobiology Laboratory  June 2014-Current
NSF Research Undergraduate Experience
• Cultured and maintained live bovine and human aortic endothelial cells
• Studied focal adhesion dynamics through fluorescently labeled focal adhesion kinase
• Modified cell membrane by addition of chemicals to quantitatively analyze effect of additives in focal adhesion size and stability

Research Assistant  May 2013-December 2013
• Explored ability of light to trap a nanoparticle using an optical trapping microscope
• Developed a thorough protocol for calibrating and using optical trapping microscope

Summer Associate
• Investigated effects of different test conditions on radial force of aortic endovascular devices to support design verification testing
• Completed thermal profiling of aortic devices using multiple testing techniques
• Performed DOE on aortic devices to determine acceptable operating window

Leadership Experience
Women In Engineering Program  September 2014-December 2014
Facilitated Group Study Leader
• Conducted weekly study sessions for 15 women engineers in a first year physics course
• Mentored underclassmen regarding class scheduling and time management

Freelance Tutor  June 2014-July 2014
• Tutored two different students in algebra and calculus on a weekly basis

Centre LifeLink EMS  May 2012-January 2014
Emergency Medical Technician
• Responded to over one-hundred-fifty 911 calls to deliver life-saving and appropriate care
• Helped train new EMTs while caring for patients and communicating with the dispatcher

Skills
Laboratory: Cell culture, transfection, NMR, mass spectrometry, UV/Vis, IR
Microscopy: DIC/Phase contrast microscopy, fluorescence microscopy, and total internal reflection microscopy (TIRFM)
Programming: SolidWorks, MATLAB, COMSOL

Scholarships and Extracurricular Activities
Schreyer Honors College Academic Excellence Scholarship, Women in Engineering Scholarship, Semester-long study abroad participant, IES Abroad Ambassador, member of Navigators